The present invention provides caninized murine anti-human PD-1 antibodies that have specific sequences and a high binding affinity for canine PD-1. The invention also relates to use of these antibodies in the treatment of dogs.
CANINIZED MURINE ANTIBODIES TO HUMAN PD-1

CROSS-REFERENCE TO RELATED APPLICATIONS
This application claims benefit of U.S. Provisional Application Serial No. 61/918,847, filed on December 20, 2013, the contents of which are herein incorporated by reference in their entirety.

FIELD OF THE INVENTION
The present invention relates to caninized murine antibodies to human PD-1 that have specific sequences and a high binding affinity for canine PD-1. The invention also relates to use of the antibodies of the present invention in the treatment of cancer in dogs.

BACKGROUND OF THE INVENTION
An immunoinhibitory receptor that is primarily expressed on activated T and B cells, Programmed Cell Death Receptor 1, also referred to as Programmed Death Receptor (PD-1), is a member of the immunoglobulin superfamily related to CD28 and CTLA-4. PD-1 and like family members are type I transmembrane glycoproteins containing an extracellular Ig Variable-type (V-type) domain that binds its ligands and a cytoplasmic tail that binds 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).

PD-1 attenuates T-cell responses when bound to Programmed Cell Death Ligand 1, also referred to as Programmed Death Ligand 1 (PD-L1), and/or Programmed Cell Death Ligand 2, also referred to as Programmed Death Ligand 2 (PD-L2). The binding of either of these ligands to PD-1 negatively regulates antigen receptor signaling. Blocking the binding of PD-L1 to PD-1 enhances tumor-specific CD8⁺ T-cell immunity, while aiding the clearance of tumor cells by the immune system. The three-dimensional structure of murine PD-1, as well as the co-crystal structure of mouse PD-1 with human PD-L1 have been reported [Zhang et al., *Immunity* 20: 337-347 (2004); Lin et al., *Proc. Natl. Acad. Sci. USA* 105: 3011-3016 (2008)].

PD-L1 and PD-L2 are type I transmembrane ligands that contain both IgV- and IgC-like domains in the extracellular region along with short cytoplasmic regions with no known
signaling motifs. Both PD-L1 and PD-L2 are either constitutively expressed or can be induced in a variety of cell types, including non-hematopoietic tissues as well as various tumor types. PD-L1 is not only expressed on B, T, myeloid and dendritic cells (DCs), but also on peripheral cells, such as microvascular endothelial cells and non-lymphoid organs e.g., heart or lung. In contrast, PD-L2 is only found on macrophages and DCs. The expression pattern of PD-1 ligands suggests that PD-1 plays a role in maintaining peripheral tolerance and may further serve to regulate self-reactive T- and B-cell responses in the periphery.

In any case, it is now abundantly clear that PD-1 plays a critical role in at least certain human cancers, presumably by mediating immune evasion. Accordingly, PD-L1 has been shown to be expressed on a number of mouse and human tumors and is inducible by IFN gamma in the majority of PD-L1 negative tumor cell lines [Iwai et al., Proc. Natl. Acad. Sci. U.S.A. 99: 12293-12297 (2002); Strome et al., Cancer Res., 63: 6501-6505 (2003)]. Furthermore, the expression of PD-1 on tumor infiltrating lymphocytes and/or PD-L1 on tumor cells has been identified in a number of primary human tumor biopsies. Such tumor tissues include cancers of the lung, liver, ovary, cervix, skin, colon, glioma, bladder, breast, kidney, esophagus, stomach, oral squamous cell, urothelial cell, and pancreas, as well as tumors of the head and neck [Brown et al., J. Immunol. 170: 1257-1266 (2003); Dong et al., Nat. Med. 8: 793-800 (2002); Wintterle et al., Cancer Res. 63: 7462-7467 (2003); Strome et al., Cancer Res., 63: 6501-6505 (2003); Thompson et al., Cancer Res. 66: 3381-5 (2006); Thompson et al., Clin. Cancer Res. 13: 1757-1761 (2007); Nomi et al., Clin.Cancer Res. 13: 2151-2157. (2007)]. More strikingly, PD-ligand expression on tumor cells has been correlated to poor prognosis of human cancer patients across multiple tumor types [reviewed in Okazaki and Honjo, Int. Immunol. 19: 813-824 (2007)].

Moreover, Nomi et al. [Clin. Cancer Res. 13: 2151-2157 (2007)] demonstrated the therapeutic efficacy of blocking the binding of PD-L1 to PD-1 in a murine model of aggressive pancreatic cancer through administering either PD-1 or PD-L1 directed antibody. These antibodies effectively promoted tumor reactive CD8+ T cell infiltration into the tumor resulting in the upregulation of anti-tumor effectors including IFN gamma, granzyme B, and perforin. Similarly, the use of antibodies to block the binding of PD-L1 and PD-1 significantly inhibited tumor growth in a model of mouse squamous cell carcinoma [Tsushima et al., Oral Oncol. 42: 268-274 (2006)].
In other studies, transfection of a murine mastocytoma line with PD-L1 led to decreased lysis of the tumor cells when co-cultured with a tumor-specific CTL clone. Lysis was restored when anti-PD-L1 monoclonal antibody was added [Iwai et al., Proc. Natl. Acad. Sci. U.S.A. 99: 12293-12297 (2002)]. In vivo, blocking the PD1/PD-L1 interaction was shown to increase the efficacy of adoptive T cell transfer therapy in a mouse tumor model [Strome et al., Cancer Res. 63: 6501-6505 (2003)]. Further evidence for the role of PD-1 in cancer treatment comes from experiments performed with PD-1 knockout mice in which PD-L1 expressing myeloma cells grew only in wild-type animals (resulting in tumor growth and associated animal death), but not in PD-1 deficient mice [Iwai Y. et al., Proc. Natl. Acad. Sci. U.S.A. 99: 12293-12297 (2002)]. More recently, antibodies against PD-1 (including humanized murine monoclonal antibodies against human PD-1) have shown at least initial success in cancer therapy in humans [see e.g., US 8,354,509 B2, US 8,008,449 B2, and US 7,595,048 B2].

Anti-PD-1 antibodies may also be useful in chronic viral infection. Memory CD8+ T cells generated after an acute viral infection are highly functional and constitute an important component of protective immunity. In contrast, chronic infections are often characterized by varying degrees of functional impairment (exhaustion) of virus-specific T-cell responses, and this defect is a principal reason for the inability of the host to eliminate the persisting pathogen. Although functional effector T cells are initially generated during the early stages of infection, they gradually lose function during the course of a chronic infection. Barber et al. [Nature 439: 682-687 (2006)] showed that mice infected with a laboratory strain of LCMV developed chronic infection resulted in high levels of virus in the blood and other tissues. These mice initially developed a robust T cell response, but eventually succumbed to the infection upon T cell exhaustion. Barber et al. found that the decline in number and function of the effector T cells in chronically infected mice could be reversed by injecting an antibody that blocked the interaction between PD-1 and PD-L1.

The citation of any reference herein should not be construed as an admission that such reference is available as "prior art" to the instant application.
SUMMARY OF THE INVENTION

The present invention relates to caninized murine anti-human PD-1 antibodies that have a high binding affinity to canine PD-1, as well as having the ability to block the binding of canine PD-1 to canine PD-L1. The present invention also relates to use of such antibodies in the treatment of disease such as cancer and/or those due to infections.

Accordingly, the present invention provides an isolated caninized antibody or antigen binding fragment thereof that specifically binds Programmed Death Receptor 1 (PD-1) comprising a canine IgG heavy chain and a canine kappa or lambda light chain. In particular embodiments of this type, the canine kappa or lambda light chain that comprises three light chain complementary determining regions (CDRs): CDR light 1 (CDRL1), CDR light 2 (CDRL2), and CDR light 3 (CDRL3); and the canine IgG heavy chain comprises three heavy chain CDRs: CDR heavy 1 (CDRH1), CDR heavy 2 (CDRH2) and CDR heavy 3 (CDRH3) obtained from a mammalian PD-1 antibody. Particular embodiments of the caninized antibodies and fragments thereof of the present invention bind canine PD-1 and/or block the binding of canine PD-1 to canine Programmed Death Ligand 1 (PD-L1).

In certain embodiments, canine light chain is a kappa chain. In particular embodiments of this type, the CDRL1 comprises the amino acid sequence of SEQ ID NO: 20. In related embodiments the CDRL1 comprises a conservatively modified variant of SEQ ID NO: 20. In other embodiments, the CDRL2 comprises the amino acid sequence comprising SEQ ID NO: 22. In related embodiments the CDRL2 comprises a conservatively modified variant of SEQ ID NO: 22. In still other embodiments the CDRL3 comprises the amino acid sequence of SEQ ID NO: 24. In related embodiments the CDRL3 comprises a conservatively modified variant of SEQ ID NO: 24. In yet other embodiments the CDRH1 comprises the amino acid sequence of SEQ ID NO: 14. In related embodiments the CDRH1 comprises a conservatively modified variant of of SEQ ID NO: 14. In still other embodiments the CDRH2 comprises the amino acid sequence of SEQ ID NO: 16. In related embodiments the CDRH2 comprises a conservatively modified variant of SEQ ID NO: 16. In yet other embodiments the CDRH3 comprises the amino acid sequence of SEQ ID NO: 18. In related embodiments the CDRH3 comprises a conservatively modified variant of SEQ ID NO: 18.
In specific embodiments the CDRL1 comprises the amino acid sequence of SEQ ID NO: 20 or a conservatively modified variant of SEQ ID NO: 20, the CDRL2 comprises the amino acid sequence comprising SEQ ID NO: 22 or a conservatively modified variant of SEQ ID NO: 22, and the CDRL3 comprises the amino acid sequence of SEQ ID NO: 24 or a conservatively modified variant of SEQ ID NO: 24.

In other specific embodiments the CDRH1 comprises the amino acid sequence of SEQ ID NO: 14 or a conservatively modified variant of SEQ ID NO: 14, the CDRH2 comprises the amino acid sequence comprising SEQ ID NO: 16 or a conservatively modified variant of SEQ ID NO: 16, and the CDRH3 comprises the amino acid sequence of SEQ ID NO: 18 or a conservatively modified variant of SEQ ID NO: 18.

In a more specific embodiment the CDRL1 comprises the amino acid sequence of SEQ ID NO: 20 or a conservatively modified variant of SEQ ID NO: 20, the CDRL2 comprises the amino acid sequence comprising SEQ ID NO: 22 or a conservatively modified variant of SEQ ID NO: 22, and the CDRL3 comprises the amino acid sequence of SEQ ID NO: 24 or a conservatively modified variant of SEQ ID NO: 24, and the CDRH1 comprises the amino acid sequence of SEQ ID NO: 14 or a conservatively modified variant of SEQ ID NO: 14, the CDRH2 comprises the amino acid sequence comprising SEQ ID NO: 16 or a conservatively modified variant of SEQ ID NO: 16, and the CDRH3 comprises the amino acid sequence of SEQ ID NO: 18 or a conservatively modified variant of SEQ ID NO: 18.

In an even more specific embodiment the CDRL1 comprises the amino acid sequence of SEQ ID NO: 20, the CDRL2 comprises the amino acid sequence comprising SEQ ID NO: 22, and the CDRL3 comprises the amino acid sequence of SEQ ID NO: 24, the CDRH1 comprises the amino acid sequence of SEQ ID NO: 14, the CDRH2 comprises the amino acid sequence comprising SEQ ID NO: 16, and the CDRH3 comprises the amino acid sequence of SEQ ID NO: 18.

For embodiments of the present invention, the IgG heavy chain comprises an amino acid sequence of SEQ ID NO: 26. In related embodiments the IgG heavy chain comprises a conservatively modified variant of SEQ ID NO: 26. In other embodiments the IgG heavy chain comprises an amino acid sequence of SEQ ID NO: 28. In related embodiments the IgG
heavy chain comprises a conservatively modified variant of SEQ ID NO: 28. In still other embodiments the IgG heavy chain comprises an amino acid sequence of SEQ ID NO: 30. In related embodiments the IgG heavy chain comprises a conservatively modified variant of SEQ ID NO: 30.

In certain embodiments the kappa light chain comprises an amino acid sequence of SEQ ID NO: 32. In related embodiments, the kappa light chain comprises conservatively modified variant of SEQ ID NO: 32. In particular embodiments the kappa light chain comprises an amino acid sequence of SEQ ID NO: 34. In related embodiments, the kappa light chain comprises conservatively modified variant of SEQ ID NO: 34.

In a more particular embodiment, an isolated caninized antibody comprises the amino acid sequence of SEQ ID NO: 28 and of SEQ ID NO: 34. In related embodiments the isolated caninized antibody comprises a conservatively modified variant of SEQ ID NO: 28 and a conservatively modified variant of SEQ ID NO: 34. In still other related embodiment the isolated caninized antibody comprises the amino acid sequence of SEQ ID NO: 28 and a conservatively modified variant of SEQ ID NO: 34. In yet other related embodiment the isolated caninized antibody comprises a conservatively modified variant of SEQ ID NO: 28 and the amino acid sequence of SEQ ID NO: 34.

The present invention further provides isolated nucleic acids that encode any one of the light chains of the caninized antibody of the present invention. Similarly, the present invention further provides isolated nucleic acids that encode any one of the heavy chains of the caninized antibody of the present invention. The present invention further provides expression vectors that comprise one or more of the isolated nucleic acids of the present invention. The present invention further provides host cells that comprise one or more expression vectors of the present invention.

In particular embodiments, the antibody is a recombinant antibody or an antigen binding fragment thereof. In related embodiments, the variable heavy chain domain and variable light chain domain are connected by a flexible linker to form a single-chain antibody.

In particular embodiments, the antibody or antigen binding fragment is a Fab fragment.
In other embodiments, the antibody or antigen binding fragment is a Fab' fragment. In other embodiments, the antibody or antigen binding fragment is a (Fab')₂ fragment. In still other embodiments, the antibody or antigen binding fragment is a diabody. In particular embodiments, the antibody or antigen binding fragment is a domain antibody. In particular embodiments, the antibody or antigen binding fragment is a camelized single domain antibody.

In particular embodiments, the caninized murine anti-human PD-1 antibody or antigen binding fragment increases the immune response of the canine subject being treated.

The present invention further provides isolated nucleic acids that encode the caninized murine anti-human PD-1 antibodies or antigen binding fragments as disclosed herein. In related embodiments such antibodies or antigen binding fragments can be used for the preparation of a medicament to treat cancer in a canine subject. Alternatively, or in conjunction, the present invention provides for the use of any of the antibodies or antibody fragments of the present invention for diagnostic use. In yet additional embodiments, a kit is provided comprising any of the caninized antibodies or antigen binding fragments disclosed herein.

In yet additional embodiments, an expression vector is provided comprising an isolated nucleic acid encoding any of the caninized murine anti-human PD-1 antibodies or antigen binding fragments of the invention. The invention also relates to a host cell comprising any of the expression vectors described herein. In particular embodiments, these nucleic acids, expression vectors or polypeptides of the invention are useful in methods of making an antibody.

The present invention further includes pharmaceutical compositions comprising an antibody or antigen binding fragment thereof together with a pharmaceutically acceptable carrier or diluent. In addition, the present invention provides methods of increasing the activity of an immune cell, comprising administering to a subject in need thereof a therapeutically effective amount of such pharmaceutical compositions. In certain embodiments the method is used for the treatment of cancer. In other embodiments, the method is used in the treatment of an infection or infectious disease. In still other embodiments, a caninized antibody of the present invention or antigen binding fragment thereof is used as a vaccine adjuvant.
These and other aspects of the present invention will be better appreciated by reference to the following Brief Description of the Drawings and the Detailed Description.

**BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 shows the reactivity of murine anti-human PD-1 monoclonal antibody 08A [mAb 08A; as first described in US 8,354,509 B2 in relation to human PD-1] against the His-tagged extracellular domain of canine PD-1.

Figure 2 shows the reactivity of murine anti-human PD-1 monoclonal antibody 08A (see above) against canine PD-1 proteins expressed on CHO cells using CELISA. Murine anti-human PD-1 monoclonal antibody 08A and its caninized variants were found to react with canine PD-1 in a dose dependent manner.

Figure 3 depicts the ligand blockade by murine and caninized monoclonal antibodies. Murine anti-human PD-1 monoclonal antibody 08A (see above) and its caninized variants blocked the binding of canine PD-L1 to PD-1 expressed on CHO cell surface.

Figure 4 provides the alignment of canine IgGB constant heavy chains (CHs) lacking ADCC function. The canine wild type IgB [cIgGB wt], Canine IgGB(+)A-hinge [cIgGB(+)-A-hinge], Canine IgGB(+) D-hinge [cIgGB(+)-D-hinge], and Canine IgGB (-)ADCC [cIgGB(-) ADCC] are depicted. The (+) A-hinge is the replacement with IgG-A hinge plus a lysine and asparagine amino acid replacement as shown; the (+) D-hinge is the replacement with IgG-D hinge plus a lysine and the asparagine amino acid replacement as shown. The (-)ADCC is the lysine and asparagine amino acid replacement.
DETAILED DESCRIPTION

Abbreviations
Throughout the detailed description and examples of the invention the following abbreviations will be used:

ADCC  Antibody-dependent cellular cytotoxicity
CDC   Complement-dependent cytotoxicity
CDR   Complementarity determining region in the immunoglobulin variable regions, defined using the Kabat numbering system
CHO   Chinese hamster ovary
EC50  concentration resulting in 50% efficacy or binding
ELISA Enzyme-linked immunosorbent assay
FR    Antibody framework region: the immunoglobulin variable regions excluding the CDR regions.
HRP   Horseradish peroxidase
IFN   interferon
IC50  concentration resulting in 50% inhibition
IgG   Immunoglobulin G
mAb   Monoclonal antibody (also Mab or MAb)
MES   2-(N-morpholino)ethanesulfonic acid
MOA   Mechanism of action
NHS   Normal human serum
PCR   Polymerase chain reaction
PK    Pharmacokinetics
SEB   Staphylococcus Enterotoxin B
TT    Tetanus toxoid
V region The segment of IgG chains which is variable in sequence between different antibodies. It extends to Kabat residue 109 in the light chain and 113 in the heavy chain.
VH    Immunoglobulin heavy chain variable region
DEFINITIONS

So that the invention may be more readily understood, certain technical and scientific terms are specifically defined below. Unless specifically defined elsewhere in this document, all other technical and scientific terms used herein have the meaning commonly understood by one of ordinary skill in the art to which this invention belongs.

As used herein, including the appended claims, the singular forms of words such as "a," "an," and "the," include their corresponding plural references unless the context clearly dictates otherwise.

"Activation" as it applies to cells or to receptors refers to the activation or treatment of a cell or receptor with a ligand, unless indicated otherwise by the context or explicitly. "Ligand" encompasses natural and synthetic ligands, e.g., cytokines, cytokine variants, analogues, muteins, and binding compounds derived from antibodies. "Ligand" also encompasses small molecules, e.g., peptide mimetics of cytokines and peptide mimetics of antibodies.

"Activation" can refer to cell activation as regulated by internal mechanisms as well as by external or environmental factors.

"Activity" of a molecule may describe or refer to the binding of the molecule to a ligand or to a receptor, to catalytic activity; to the ability to stimulate gene expression or cell signaling, differentiation, or maturation; to antigenic activity, to the modulation of activities of other molecules, and the like. "Activity" of a molecule may also refer to activity in modulating or maintaining cell-to-cell interactions, e.g., adhesion, or activity in maintaining a structure of a cell, e.g., cell membranes or cytoskeleton. "Activity" can also mean specific activity, e.g., [catalytic activity]/[mg protein], or [immunological activity]/[mg protein], concentration in a biological compartment, or the like. "Activity" may refer to modulation of components of the innate or the adaptive immune systems.

"Administration" and "treatment," as it applies to an animal, e.g., a canine experimental subject, cell, tissue, organ, or biological fluid, refers to contact of an exogenous pharmaceutical, therapeutic, diagnostic agent, or composition to the animal e.g., a canine
subject, cell, tissue, organ, or biological fluid. Treatment of a cell encompasses contact of a reagent to the cell, as well as contact of a reagent to a fluid, where the fluid is in contact with the cell. "Administration" and "treatment" also means *in vitro* and *ex vivo* treatments, e.g., of a cell, by a reagent, diagnostic, binding compound, or by another cell. The term "subject" includes any organism, preferably an animal, more preferably a mammal (e.g., canine, feline, or human) and most preferably a canine.

"Treat" or "treating" means to administer a therapeutic agent, such as a composition containing any of the antibodies or antigen binding fragments of the present invention, internally or externally to a canine subject or patient having one or more disease symptoms, or being suspected of having a disease, for which the agent has therapeutic activity. Typically, the agent is administered in an amount effective to alleviate and/or ameliorate one or more disease symptoms in the treated subject or population, whether by inducing the regression of or inhibiting the progression of such symptom(s) by any clinically measurable degree. The amount of a therapeutic agent that is effective to alleviate any particular disease symptom (also referred to as the "therapeutically effective amount") may vary according to factors such as the disease state, age, and weight of the patient (e.g., canine), and the ability of the pharmaceutical composition to elicit a desired response in the subject. Whether a disease symptom has been alleviated or ameliorated can be assessed by any clinical measurement typically used by veterinarians or other skilled healthcare providers to assess the severity or progression status of that symptom. While an embodiment of the present invention (e.g., a treatment method or article of manufacture) may not be effective in alleviating the target disease symptom(s) in every subject, it should alleviate the target disease symptom(s) in a statistically significant number of subjects as determined by any statistical test known in the art such as the Student’s t-test, the chi²-test, the U-test according to Mann and Whitney, the Kruskal-Wallis test (H-test), Jonckheere-Terpstra-test and the Wilcoxon-test.

"Treatment," as it applies to a human, veterinary (e.g., canine) or research subject, refers to therapeutic treatment, as well as research and diagnostic applications. "Treatment" as it applies to a human, veterinary (e.g., canine), or research subject, or cell, tissue, or organ, encompasses contact of the antibodies or antigen binding fragments of the present invention to a canine or other animal subject, a cell, tissue, physiological compartment, or physiological fluid.
Canine PD-1 has been found to comprise the amino acid sequence of SEQ ID NO: 2. In a specific embodiment canine PD-1 is encoded by a nucleic acid that comprises the nucleotide sequence of SEQ ID NO: 1. Canine PD-1 sequences may differ by having, for example, conserved variations in non-conserved regions, but the canine PD-1 will have substantially the same biological function as the canine PD-1 comprising the amino acid sequence of SEQ ID NO: 2. For example, a biological function of PD-1 is to attenuate T-cell responses when bound to PD-L1 and/or PD-L2. That is, PD-1 may be considered a negative regulator. Notably, 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). In addition, a biological function of canine PD-1 may be having, for example, an epitope in the extracellular domain that is specifically bound by an antibody of the instant disclosure.

Canine PD-L1 has been found to comprise the amino acid sequence of SEQ ID NO: 8. In a specific embodiment canine PD-L1 is encoded by a nucleotide sequence comprising SEQ ID NO: 7. Canine PD-L1 sequences may differ by having, for example, conserved variations in non-conserved regions, but the canine PD-L1 will have substantially the same biological function as the canine PD-L1 comprising the amino acid sequence of SEQ ID NO: 8. For example, one biological function of PD-L1 is to attenuate T-cell responses when bound to PD-1.

A particular canine PD-1 or PD-L1 amino acid sequence respectively, will generally be at least 90% identical to the canine PD-1 comprising the amino acid sequence of SEQ ID NO: 2, or canine PD-L1 comprising the amino acid sequence of SEQ ID NO: 8, respectively. In certain cases, a canine PD-1 or PD-L1 respectively, may be at least 95%, or even at least 96%, 97%, 98% or 99% identical to the canine PD-1 comprising the amino acid sequence of SEQ ID NO: 2, or the canine PD-L1 comprising the amino acid sequence of SEQ ID NO: 8, respectively. In certain embodiments, a canine PD-1 or a PD-L1 amino acid sequence will display no more than 10 amino acid differences from the canine PD-1 comprising the amino acid sequence of SEQ ID NO: 2, or the canine PD-L1 comprising the amino acid sequence of SEQ ID NO: 8, respectively. In certain embodiments, the canine PD-1 or the PD-L1 amino acid sequence respectively, may display no more than 5, or even no more than 4, 3, 2, or 1
amino acid difference from the canine PD-1 comprising the amino acid sequence of SEQ ID NO: 2, or the canine PD-L1 comprising the amino acid sequence of SEQ ID NO: 8, respectively. Percent identity can be determined as described herein below.

The term "immune response" refers to the action of, for example, lymphocytes, antigen presenting cells, phagocytic cells, granulocytes, and soluble macromolecules produced by the above cells or the liver (including antibodies, cytokines, and complement) that results in selective damage to, destruction of, or elimination from the mammalian body (e.g., canine body) of cancerous cells, cells or tissues infected with pathogens, or invading pathogens.

**Caninized Anti-Human PD-1 Antibodies**

The present invention provides isolated caninized murine anti-human PD-1 antibodies or antigen binding fragments thereof that bind canine PD-1 and uses of such antibodies or fragments.

As used herein, a caninized murine anti-human PD-1 antibody refers to a caninized antibody that specifically binds to mammalian PD-1. An antibody that specifically binds to mammalian PD-1, and in particular canine PD-1, is an antibody that exhibits preferential binding to mammalian PD-1 as compared to other antigens, but this specificity does not require absolute binding specificity. A caninized murine anti-human PD-1 antibody is considered "specific" for canine PD-1 if its binding is determinative of the presence of canine PD-1 in a biological sample obtained from a canine, or if it is capable of altering the activity of canine PD-1 without unduly interfering with the activity of other canine proteins in a canine sample, e.g. without producing undesired results such as false positives in a diagnostic context or side effects in a therapeutic context. The degree of specificity necessary for a caninized murine anti-human PD-1 antibody may depend on the intended use of the antibody, and at any rate is defined by its suitability for use for an intended purpose. The antibody, or binding compound derived from the antigen-binding site of an antibody, of the contemplated method binds to its antigen, or a variant or mutein thereof, with an affinity that is at least two-fold greater, preferably at least ten-times greater, more preferably at least 20-times greater, and most preferably at least 100-times greater than the affinity with any other canine antigen.
As used herein, an antibody is said to bind specifically to a polypeptide comprising a given sequence (in this case canine PD-1) if it binds to polypeptides comprising the sequence of canine PD-1, but does not bind to other canine proteins lacking the amino acid sequence of canine PD-1. For example, an antibody that specifically binds to a polypeptide comprising canine PD-1 may bind to a FLAG®-tagged form of canine PD-1, but will not bind to other FLAG®-tagged canine proteins.

As used herein, unless otherwise indicated, "antibody fragment" or "antigen binding fragment" refers to antigen binding fragments of antibodies, i.e. antibody fragments that retain the ability to bind specifically to the antigen (e.g., canine PD-1) bound by the full-length antibody, e.g. fragments that retain one or more CDR regions. Examples of antigen binding fragments include, but are not limited to, Fab, Fab’, F(abs')₂, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules, e.g., sc-Fv; nanobodies and multispecific antibodies formed from antibody fragments.

Typically, a caninized antibody or antigen binding fragment thereof of the invention retains at least 10% of its canine PD-1 binding activity (when compared to the corresponding parental antibody) when that activity is expressed on a molar basis. Preferably, an antibody or antigen binding fragment of the invention retains at least 20%, 50%, 70%, 80%, 90%, 95% or 100% or more of the canine PD-1 binding affinity as the parental antibody. It is also intended that an an antibody or antigen binding fragment of the invention can include conservative or non-conservative amino acid substitutions (referred to as "conservative variants" or "function conserved variants" of the antibody) that do not substantially alter its biologic activity.

"Isolated antibody" refers to the purification status and in such context means the molecule is substantially free of other biological molecules such as nucleic acids, proteins, lipids, carbohydrates, or other material such as cellular debris and growth media. Generally, the term "isolated" is not intended to refer to a complete absence of such material or to an absence of water, buffers, or salts, unless they are present in amounts that substantially interfere with experimental or therapeutic use of the binding compound as described herein.
The variable regions of each light/heavy chain pair form the antigen binding site of the antibody. Thus, in general, an intact antibody has two binding sites. Except in bifunctional or bispecific antibodies, the two binding sites are, in general, the same.


As used herein, the term "hypervariable region" refers to the amino acid residues of an antibody that are responsible for antigen-binding. The hypervariable region comprises amino acid residues from a "complementarity determining region" or "CDR" (i.e. CDRL1, CDRL2 and CDRL3 in the light chain variable domain and CDRH1, CDRH2 and CDRH3 in the heavy chain variable domain). [See Kabat et al. *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991), defining the CDR regions of an antibody by sequence; see also Chothia and Lesk, *J. Mol. Biol.* 196: 901-917 (1987) defining the CDR regions of an antibody by structure]. As used herein, the term "framework" or "FR" residues refers to those variable domain residues other than the hypervariable region residues defined herein as CDR residues.

As used herein, the term "canine" includes all domestic dogs, Canis lupus familiaris or Canis familiaris, unless otherwise indicated.

As used herein the term “canine frame” refers to the amino acid sequence of the heavy chain and light chain of a canine antibody other than the hypervariable region residues defined herein as CDR residues. With regard to a caninized antibody, in the majority of embodiments the
amino acid sequences of the native canine CDRs are replaced with the corresponding foreign
CDRs (e.g., those from a mouse antibody) in both chains. Optionally the heavy and/or light
chains of the canine antibody may contain some foreign non-CDR residues, e.g., so as to
preserve the conformation of the foreign CDRs within the canine antibody, and/or to modify
the Fc function, as discussed below.

There are four known IgG heavy chain subtypes of dog IgG and they are referred to as IgG-A,
IgG-B, IgG-C, and IgG-D. The two known light chain subtypes are referred to as lambda and
kappa.

Besides binding and activating of canine immune cells, a canine or caninized antibody against
PD-1 optimally has two attributes:

1. Lack of effector functions such as antibody-dependent cytotoxicity (ADCC) and
complement-dependent cytotoxicity (CDC), and
2. be readily purified on a large scale using industry standard technologies such as that
based on protein A chromatography.

None of the naturally occurring canine IgG isotypes satisfy both criteria. For example, IgG-B
can be purified using protein A, but has high level of ADCC activity. On the other hand, IgG-
A binds weakly to protein A, but displays undesirable ADCC activity. Moreover, neither
IgG-C nor IgG-D can be purified on protein A columns, although IgG-D display no ADCC
activity. (IgG-C has considerable ADCC activity). The present invention overcomes this
difficulty by providing mutant canine IgG-B antibodies specific to PD-1; such antibodies lack
effector functions such as ADCC and can be easily of purified using industry standard protein
A chromatography.

As used herein, the term "caninized antibody" refers to an antibody that comprises the three
heavy chain CDRs and the three light chain CDRS from a murine anti-human PD-1 antibody
together with a canine frame or a modified canine frame. A modified canine frame comprises
one or more amino acids changes as exemplified herein that further optimize the effectiveness
of the caninized antibody, e.g., to increase its binding to canine PD-1 and/or its ability to block
the binding of canine PD-1 to canine PD-L1.
"Homology" refers to sequence similarity between two polynucleotide sequences or between two polypeptide sequences when they are optimally aligned. When a position in both of the two compared sequences is occupied by the same base or amino acid monomer subunit, e.g., if a position in each of two DNA molecules is occupied by adenine, then the molecules are homologous at that position. The percent of homology is the number of homologous positions shared by the two sequences divided by the total number of positions compared \( \times 100 \). For example, if 6 of 10 of the positions in two sequences are matched or homologous when the sequences are optimally aligned then the two sequences are 60% homologous. Generally, the comparison is made when two sequences are aligned to give maximum percent homology.

"Isolated nucleic acid molecule" means a DNA or RNA of genomic, mRNA, cDNA, or synthetic origin or some combination thereof which is not associated with all or a portion of a polynucleotide in which the isolated polynucleotide is found in nature, or is linked to a polynucleotide to which it is not linked in nature. For purposes of this disclosure, it should be understood that "a nucleic acid molecule comprising" a particular nucleotide sequence does not encompass intact chromosomes. Isolated nucleic acid molecules "comprising" specified nucleic acid sequences may include, in addition to the specified sequences, coding sequences for up to ten or even up to twenty or more other proteins or portions or fragments thereof, or may include operably linked regulatory sequences that control expression of the coding region of the recited nucleic acid sequences, and/or may include vector sequences.

The phrase "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to use promoters, polyadenylation signals, and enhancers.

A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked"
means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice. It should also be readily understood that when a nucleic acid sequence is provided herein, it may include a stop codon. However, as stop codons are interchangeable the inclusion of a specific stop codon in a sequence should not be viewed as a necessary portion of that sequence.

As used herein, the expressions "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that not all progeny will have precisely identical DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

As used herein, "germline sequence" refers to a sequence of unrearranged immunoglobulin DNA sequences. Any suitable source of unrearranged immunoglobulin sequences may be used. Human germline sequences may be obtained, for example, from JOINSOLVER® germline databases on the website for the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the United States National Institutes of Health. Mouse germline sequences may be obtained, for example, as described in Giudicelli et al. [Nucleic Acids Res. 33:D256-D261 (2005)].

Properties of the Exemplary Caninized Murine Anti-human PD-1 Antibodies
The present invention provides isolated caninized murine anti-human PD-1 antibodies and methods of use of the antibodies or antigen binding fragments thereof in the treatment of disease e.g., the treatment of cancer in canines. Examples of caninized murine anti-human PD-1 antibodies that bind canine PD-1 include, but are not limited to: antibodies that comprise canine IgG-A, IgG-B, and IgG-D heavy chains and/or canine kappa light chains together with murine anti-human PD-1 CDRs. Accordingly, the present invention provides isolated
caninized murine anti-human PD-1 antibodies or antigen binding fragments thereof that bind to 
canine PD-1 and block the binding of canine PD-1 to canine PD-L1.

The isolated antibody or antigen binding fragment thereof that binds canine PD-1 can comprise 
one, two, three, four, five, or six of the complementarity determining regions (CDRs) of the 
murine anti-human antibody as described herein. The one, two, three, four, five, or six CDRs 
may be independently selected from the CDR sequences of those provided in Table 2 in the 
Examples below. In certain embodiments, one, two or three CDRs are selected from the $V_L$ 
CDRs (amino acid SEQ ID NOs: 20, 22, and/or 24) and/or one, two or three CDRs selected 
from the $V_H$ CDRs (SEQ ID NOs: 14, 16, and/or 18), and/or conservatively modified variants 
of the one, two or three of these $V_L$ CDRs and/or conservatively modified variants of the one, 
two or three of these $V_H$ CDRs.

In a further embodiment, the isolated antibody or antigen-binding fragment thereof that binds 
canine PD-1 comprises a canine antibody kappa light chain comprising a murine light chain 
CDR-1, CDR-2 and/or CDR-3 and a canine antibody heavy chain IgG comprising a murine 
heavy chain CDR-1, CDR-2 and/or CDR-3.

In other embodiments, the invention provides antibodies or antigen binding fragments thereof 
that specifically binds PD-1 and have canine antibody kappa light chains comprising CDRs 
comprising at least 80%, 85%, 90%, 95%, 98% or 99% sequence identity with SEQ ID NOs: 
20, 22, and/or 24 and canine antibody heavy chain IgG with CDRs comprising at least 80%, 
85%, 90%, 95%, 98% or 99% sequence identity with SEQ ID NOs: 14, 16, and/or 18, while 
still exhibiting the desired binding and functional properties. In another embodiment the 
antibody or antigen binding fragment of the present invention comprises a canine frame 
comprising of a combination of IgG heavy chain sequence (comprising an amino acid 
sequence of SEQ ID NO: 26, 28, or 30 with and without signal sequence) with a kappa light 
chain (comprising an amino acid sequence of SEQ ID NO: 32, or 34 with and without signal 
sequence) having up to 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more conservative or non-conservative 
amino acid substitutions, while still exhibiting the desired binding and functional properties. 
In a particular embodiment of this type, the number of conservative amino acid substitutions is 
between 0 to 5 for the IgG heavy chain and 0 to 5 for the kappa light chain.
"Conservatively modified variants" or "conservative substitution" refers to substitutions of amino acids in a protein with other amino acids having similar characteristics (e.g. charge, side-chain size, hydrophobicity/hydrophilicity, backbone conformation and rigidity, etc.), such that the changes can frequently be made without altering the biological activity of the protein. Those of skill in this art recognize that, in general, single amino acid substitutions in non-essential regions of a polypeptide do not substantially alter biological activity [see, e.g., Watson et al., Molecular Biology of the Gene, The Benjamin/Cummings Pub. Co., p. 224 (4th Ed.; 1987)]. In addition, substitutions of structurally or functionally similar amino acids are less likely to disrupt biological activity. Various embodiments of the antibody or antigen binding fragment of the present invention comprise polypeptide chains with the sequences disclosed herein, e.g., SEQ ID NOs: 26, 28, 30, 32, and/or 34, or polypeptide chains comprising up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 20 or more conservative amino acid substitutions. Exemplary conservative substitutions are set forth in Table I.

**TABLE I.**

<table>
<thead>
<tr>
<th>Original residue</th>
<th>Conservative substitution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala (A)</td>
<td>Gly; Ser;</td>
</tr>
<tr>
<td>Arg (R)</td>
<td>Lys; His</td>
</tr>
<tr>
<td>Asn (N)</td>
<td>Gln; His</td>
</tr>
<tr>
<td>Asp (D)</td>
<td>Glu; Asn</td>
</tr>
<tr>
<td>Cys (C)</td>
<td>Ser; Ala</td>
</tr>
<tr>
<td>Gln (Q)</td>
<td>Asn</td>
</tr>
<tr>
<td>Glu (E)</td>
<td>Asp; Gln</td>
</tr>
<tr>
<td>Gly (G)</td>
<td>Ala</td>
</tr>
<tr>
<td>His (H)</td>
<td>Asn; Gln</td>
</tr>
<tr>
<td>Ile (I)</td>
<td>Leu; Val</td>
</tr>
<tr>
<td>Leu (L)</td>
<td>Ile; Val</td>
</tr>
<tr>
<td>Lys (K)</td>
<td>Arg; His</td>
</tr>
<tr>
<td>Met (M)</td>
<td>Leu; Ile; Tyr</td>
</tr>
<tr>
<td>Phe (F)</td>
<td>Tyr; Met; Leu</td>
</tr>
<tr>
<td>Pro (P)</td>
<td>Ala</td>
</tr>
</tbody>
</table>
Function-conservative variants of the antibodies of the invention are also contemplated by the present invention. "Function-conservative variants," as used herein, refers to antibodies or fragments in which one or more amino acid residues have been changed without altering a desired property, such as antigen affinity and/or specificity. Such variants include, but are not limited to, replacement of an amino acid with one having similar properties, such as the conservative amino acid substitutions of Table I.

### Nucleic Acids

The present invention further comprises the nucleic acids encoding the immunoglobulin chains of caninized murine anti-human PD-1 antibodies and antigen binding fragments thereof disclosed herein. For example, the present invention includes the nucleic acids listed in Tables 2 and 3 and the Sequence Listing Table below.

Also included in the present invention are nucleic acids that encode immunoglobulin polypeptides comprising amino acid sequences that are at least about 70% identical, preferably at least about 80% identical, more preferably at least about 90% identical and most preferably at least about 95% identical (e.g., 95%, 96%, 97%, 98%, 99%, 100%) to the amino acid sequences of the antibodies provided herein when the comparison is performed with a BLAST algorithm wherein the parameters of the algorithm are selected to give the largest match between the respective sequences over the entire length of the respective reference sequences. The present invention further provides nucleic acids that encode immunoglobulin polypeptides comprising amino acid sequences that are at least about 70% similar, preferably at least about 80% similar, more preferably at least about 90% similar and most preferably at least about 95% similar (e.g., 95%, 96%, 97%, 98%, 99%, 100%) to any of the reference amino acid sequences when the comparison is performed with a BLAST algorithm, wherein the parameters of the algorithm are selected to give the largest match between the respective
sequences over the entire length of the respective reference sequences, are also included in the present invention.

Sequence identity refers to the degree to which the amino acids of two polypeptides are the same at equivalent positions when the two sequences are optimally aligned. Sequence similarity includes identical residues and nonidentical, biochemically related amino acids. Biochemically related amino acids that share similar properties and may be interchangeable are discussed above.

The following references relate to BLAST algorithms often used for sequence analysis:


This present invention also provides expression vectors comprising the isolated nucleic acids of the invention, wherein the nucleic acid is operably linked to control sequences that are recognized by a host cell when the host cell is transfected with the vector. Also provided are host cells comprising an expression vector of the present invention and methods for producing the antibody or antigen binding fragment thereof disclosed herein comprising culturing a host
cell harboring an expression vector encoding the antibody or antigen binding fragment in
culture medium, and isolating the antigen or antigen binding fragment thereof from the host
cell or culture medium.

**Epitope Binding and Binding Affinity**
The present invention further provides antibodies or antigen binding fragments thereof that
bind to the same epitope on canine PD-1 as the caninized murine anti-human PD-1 antibody
comprising the amino acid sequence of SEQ ID NO: 28 and/or of SEQ ID NO: 32, or the
caninized murine anti-human PD-1 antibody comprising the amino acid sequence of SEQ ID
NO: 28 and/or of SEQ ID NO: 34. The caninized murine anti-human PD-1 antibodies or
antigen binding fragments thereof are capable of inhibiting the binding of canine PD-1 to
canine PD-L1.

The caninized murine anti-human PD-1 antibody can be produced recombinantly as described
below in the examples. Mammalian cell lines available as hosts for expression of the
antibodies or fragments disclosed herein are well known in the art and include many
immortalized cell lines available from the American Type Culture Collection (ATCC). These
include, *inter alia*, Chinese hamster ovary (CHO) cells, NSO, SP2 cells, HeLa cells, baby
hamster kidney (BHK) cells, monkey kidney cells (COS), human hepatocellular carcinoma
cells (e.g., Hep G2), A549 cells, 3T3 cells, HEK-293 cells and a number of other cell lines.
Mammalian host cells include human, mouse, rat, dog, monkey, pig, goat, bovine, horse and
hamster cells. Cell lines of particular preference are selected through determining which cell
lines have high expression levels. Other cell lines that may be used are insect cell lines, such
as Sf9 cells, amphibian cells, bacterial cells, plant cells and fungal cells. When recombinant
expression vectors encoding the heavy chain or antigen-binding portion or fragment thereof,
the light chain and/or antigen-binding fragment thereof 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. Further, expression of antibodies of the invention (or other moieties therefrom) from
production cell lines can be enhanced using a number of known techniques. For example, the
glutamine synthetase gene expression system (the GS system) is a common approach for enhancing expression under certain conditions. The GS system is discussed in whole or part in connection with European Patent Nos. 0 216 846, 0 256 055, and 0 323 997 and European Patent Application No. 89303964.4.

In general, glycoproteins produced in a particular cell line or transgenic animal will have a glycosylation pattern that is characteristic for glycoproteins produced in the cell line or transgenic animal. Therefore, the particular glycosylation pattern of an antibody will depend on the particular cell line or transgenic animal used to produce the antibody. However, all antibodies encoded by the nucleic acid molecules provided herein, or comprising the amino acid sequences provided herein, comprise the instant invention, independent of the glycosylation pattern that the antibodies may have. Similarly, in particular embodiments, antibodies with a glycosylation pattern comprising only non-fucosylated N-glycans may be advantageous, because these antibodies have been shown to typically exhibit more potent efficacy than their fucosylated counterparts both in vitro and in vivo [See for example, Shinkawa et al., J. Biol. Chem. 278: 3466-3473 (2003); U.S. Patent Nos. 6,946,292 and 7,214,775].

The present invention further includes antibody fragments of the caninized murine anti-human PD-1 antibodies disclosed herein. The antibody fragments include F(ab)2 fragments, which may be produced by enzymatic cleavage of an IgG by, for example, pepsin. Fab fragments may be produced by, for example, reduction of F(ab)2 with dithiothreitol or mercaptoethylamine. A Fab fragment is a V_L-C_L chain appended to a V_H-C_H1 chain by a disulfide bridge. A F(ab)2 fragment is two Fab fragments which, in turn, are appended by two disulfide bridges. The Fab portion of an F(ab)2 molecule includes a portion of the Fc region between which disulfide bridges are located. An FV fragment is a V_L or V_H region.

In one embodiment, the antibody or antigen binding fragment comprises a heavy chain constant region, e.g., a canine constant region, such as IgG-A, IgG-B, IgG-C and IgG-D canine heavy chain constant region or a variant thereof. In another embodiment, the antibody or antigen binding fragment comprises a light chain constant region, e.g., a canine light chain constant region, such as lambda or kappa canine light chain region or variant thereof. By way
of example, and not limitation the canine heavy chain constant region can be from IgG-D and the canine light chain constant region can be from kappa.

**Antibody Engineering**

The caninized murine anti-human PD-1 antibodies of the present invention have been engineered to include modifications to framework residues within the variable domains of a parental (i.e., canine) monoclonal antibody, e.g. to improve the properties of the antibody.

**Experimental and diagnostic uses**

Caninized murine anti-human PD-1 antibodies or antigen-binding fragments thereof of the present invention may also be useful in diagnostic assays for canine PD-1 protein, e.g., detecting its expression in specific tumor cells, tissues, or serum. Such diagnostic methods may be useful in various disease diagnoses, particularly certain cancers in canines.

For example, such a method comprises the following steps:

1. (a) coat a substrate (e.g., surface of a microtiter plate well, e.g., a plastic plate) with caninized murine anti-human PD-1 antibody or an antigen-binding fragment thereof;
2. (b) apply a sample to be tested for the presence of canine PD-1 to the substrate;
3. (c) wash the plate, so that unbound material in the sample is removed;
4. (d) apply detectably labeled antibodies (e.g., enzyme-linked antibodies) which are also specific to the PD-1 antigen;
5. (e) wash the substrate, so that the unbound, labeled antibodies are removed;
6. (f) if the labeled antibodies are enzyme linked, apply a chemical which is converted by the enzyme into a fluorescent signal; and
7. (g) detect the presence of the labeled antibody.

In a further embodiment, the labeled antibody is labeled with peroxidase which react with ABTS [e.g., 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)] or 3,3',5,5'-Tetramethylbenzidine to produce a color change which is detectable. Alternatively, the labeled antibody is labeled with a detectable radioisotope (e.g., ³H) which can be detected by scintillation counter in the presence of a scintillant. Caninized murine anti-human PD-1 antibodies of the invention may be used in a Western blot or immuno protein blot procedure.
Such a procedure forms part of the present invention and includes for example:

(i) contacting a membrane or other solid substrate to be tested for the presence of bound canine PD-1 or a fragment thereof with a caninized murine anti-human PD-1 antibody or antigen-binding fragment thereof of the present invention. Such a membrane may take the form of a nitrocellulose or vinyl-based [e.g., polyvinylidene fluoride (PVDF)] membrane to which the proteins to be tested for the presence of canine PD-1 in a non-denaturing PAGE (polyacrylamide gel electrophoresis) gel or SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) gel have been transferred (e.g., following electrophoretic separation in the gel). Before contact of membrane with the caninized murine anti-human PD-1 antibody or antigen-binding fragment thereof, the membrane is optionally blocked, e.g., with non-fat dry milk or the like so as to bind non-specific protein binding sites on the membrane.

(ii) washing the membrane one or more times to remove unbound caninized murine anti-human PD-1 antibody or an antigen-binding fragment thereof and other unbound substances; and

(iii) detecting the bound caninized murine anti-human PD-1 antibody or antigen-binding fragment thereof.

Detection of the bound antibody or antigen-binding fragment may be by binding the antibody or antigen-binding fragment with a secondary antibody (an anti-immunoglobulin antibody) which is detectably labeled and, then, detecting the presence of the secondary antibody.

The caninized murine anti-human PD-1 antibodies and antigen-binding fragments thereof disclosed herein may also be used for immunohistochemistry. Such a method forms part of the present invention and comprises, e.g., (1) contacting a cell to be tested for the presence of canine PD-1 with a caninized murine anti-human PD-1 antibody or antigen-binding fragment thereof of the present invention; and (2) detecting the antibody or fragment on or in the cell.

If the antibody or antigen-binding fragment itself is detectably labeled, it can be detected directly. Alternatively, the antibody or antigen-binding fragment may be bound by a detectably labeled secondary antibody which is detected.

Certain caninized murine anti-human PD-1 antibodies and antigen-binding fragments thereof disclosed herein may also be used for in vivo tumor imaging. Such a method may include
injection of a radiolabeled caninized murine anti-human PD-1 antibodies or antigen-binding fragment thereof into the body of a canine to be tested for the presence of a tumor associated with canine PD-1 expression followed by nuclear imaging of the body of the patient to detect the presence of the labeled antibody or antigen-binding fragment e.g., at loci comprising a high concentration of the antibody or antigen-binding fragment which are bound to the tumor.

Imaging techniques include SPECT imaging (single photon emission computed tomography) or PET imaging (positron emission tomography). Labels include e.g., iodine-123 (\(^{123}\text{I}\)) and technetium-99m (\(^{99m}\text{Tc}\)), e.g., in conjunction with SPECT imaging or \(^{11}\text{C}\), \(^{13}\text{N}\), \(^{15}\text{O}\) or \(^{18}\text{F}\), e.g., in conjunction with PET imaging or Indium-111 [See e.g., Gordon et al., International Rev. Neurobiol. 67:385-440 (2005)].

Pharmaceutical Compositions and Administration

To prepare pharmaceutical or sterile compositions of the caninized murine anti-human PD-1 antibody or antigen binding fragment thereof is admixed with a pharmaceutically acceptable carrier or excipient. [See, e.g., Remington's Pharmaceutical Sciences and U.S. Pharmacopeia: National Formulary, Mack Publishing Company, Easton, PA (1984)].

Formulations of therapeutic and diagnostic agents may be prepared by mixing with acceptable carriers, excipients, or stabilizers in the form of, e.g., lyophilized powders, slurries, aqueous solutions or suspensions [see, e.g., Hardman, et al. (2001) Goodman and Gilman’s The Pharmacological Basis of Therapeutics, McGraw-Hill, New York, NY; Gennaro (2000) Remington: The Science and Practice of Pharmacy, Lippincott, Williams, and Wilkins, New York, NY; Avis, et al. (eds.) (1993) Pharmaceutical Dosage Forms: Parenteral Medications, Marcel Dekker, NY; Lieberman, et al. (eds.) (1990) Pharmaceutical Dosage Forms: Tablets, Marcel Dekker, NY; Lieberman, et al. (eds.) (1990) Pharmaceutical Dosage Forms: Disperse Systems, Marcel Dekker, NY; Weiner and Kotkoskie (2000) Excipient Toxicity and Safety, Marcel Dekker, Inc., New York, NY]. In one embodiment, anti-PD-1 antibodies of the present invention are diluted to an appropriate concentration in a sodium acetate solution pH 5-6, and NaCl or sucrose is added for tonicity. Additional agents, such as polysorbate 20 or polysorbate 80, may be added to enhance stability.
Toxicity and therapeutic efficacy of the antibody compositions, administered alone or in combination with another agent, can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD$_{50}$ (the dose lethal to 50% of the population) and the ED$_{50}$ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index (LD$_{50}$/ ED$_{50}$). In particular aspects, antibodies exhibiting high therapeutic indices are desirable. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in canines. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED$_{50}$ with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration.

The mode of administration can vary. Suitable routes of administration include oral, rectal, transmucosal, intestinal, parenteral; intramuscular, subcutaneous, intradermal, intramedullary, intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, intraocular, inhalation, insufflation, topical, cutaneous, transdermal, or intra-arterial.

In particular embodiments, the caninized murine anti-human PD-1 antibody or antigen binding fragment thereof can be administered by an invasive route such as by injection. In further embodiments of the invention, a caninized murine anti-human PD-1 antibody or antigen binding fragment thereof, or pharmaceutical composition thereof, is administered intravenously, subcutaneously, intramuscularly, intrarterially, intratumorally, or by inhalation, aerosol delivery. Administration by non-invasive routes (e.g., orally; for example, in a pill, capsule or tablet) is also within the scope of the present invention.

Compositions can be administered with medical devices known in the art. For example, a pharmaceutical composition of the invention can be administered by injection with a hypodermic needle, including, e.g., a prefilled syringe or autoinjector. The pharmaceutical compositions disclosed herein may also be administered with a needleless hypodermic injection device; such as the devices disclosed in U.S. Patent Nos. 6,620,135; 6,096,002; 5,399,163; 5,383,851; 5,312,335; 5,064,413; 4,941,880; 4,790,824 or 4,596,556.

The pharmaceutical compositions disclosed herein may also be administered by infusion. Examples of well-known implants and modules form administering pharmaceutical compositions include: U.S. Patent No. 4,487,603, which discloses an implantable micro-
infusion pump for dispensing medication at a controlled rate; U.S. Patent No. 4,447,233, which discloses a medication infusion pump for delivering medication at a precise infusion rate; U.S. Patent No. 4,447,224, which discloses a variable flow implantable infusion apparatus for continuous drug delivery; U.S. Patent. No. 4,439,196, which discloses an osmotic drug delivery system having multi-chamber compartments. Many other such implants, delivery systems, and modules are well known to those skilled in the art.

Alternately, one may administer the caninized murine anti-human PD-1 antibody in a local rather than systemic manner, for example, via injection of the antibody directly into an arthritic joint or pathogen-induced lesion characterized by immunopathology, often in a depot or sustained release formulation. Furthermore, one may administer the caninized murine anti-human PD-1 antibody in a targeted drug delivery system, for example, in a liposome coated with a tissue-specific antibody, targeting, for example, arthritic joint or pathogen-induced lesion characterized by immunopathology. The liposomes will be targeted to and taken up selectively by the afflicted tissue.

Determination of the appropriate dose is made by the veterinarian, e.g., using parameters or factors known or suspected in the art to affect treatment. Generally, the dose begins with an amount somewhat less than the optimum dose and it is increased by small increments thereafter until the desired or optimum effect is achieved relative to any negative side effects. Important diagnostic measures include those of symptoms of, e.g., the inflammation or level of inflammatory cytokines produced.

Antibodies or antigen binding fragments thereof disclosed herein may be provided by continuous infusion, or by doses administered, e.g., daily, 1-7 times per week, weekly, bi-weekly, monthly, bimonthly, quarterly, semiannually, annually etc. Doses may be provided, e.g., intravenously, subcutaneously, topically, orally, nasally, rectally, intramuscular, intracerebrally, intraspinally, or by inhalation. A total weekly dose is generally at least 0.05 μg/kg body weight, more generally at least 0.2 μg/kg, 0.5 μg/kg, 1 μg/kg, 10 μg/kg, 100 μg/kg, 0.25 mg/kg, 1.0 mg/kg, 2.0 mg/kg, 5.0 mg/ml, 10 mg/kg, 25 mg/kg, 50 mg/kg or more [see, e.g., Yang, et al. New Engl. J. Med. 349:427-434 (2003); Herold, et al. New Engl. J. Med. 346:1692-1698 (2002); Liu, et al. J. Neurol. Neurosurg. Psych. 67:451-456 (1999); Portielji, et al. Cancer Immunol. Immunother. 52:133-144 (2003)]. Doses may also be provided to achieve a pre-determined target concentration of the caninized murine anti-human PD-I antibody in the subject’s serum, such as 0.1, 0.3, 1, 3, 10, 30, 100, 300 μg/ml or more. In other embodiments, a caninized murine anti-human PD-I antibody of the present invention is administered subcutaneously or intravenously, on a weekly, biweekly, "every 4 weeks," monthly, bimonthly, or quarterly basis at 10, 20, 50, 80, 100, 200, 500, 1000 or 2500 mg/subject.

As used herein, "inhibit" or "treat" or "treatment" includes a postponement of development of the symptoms associated with a disorder and/or a reduction in the severity of the symptoms of such disorder. The terms further include ameliorating existing uncontrolled or unwanted symptoms, preventing additional symptoms, and ameliorating or preventing the underlying causes of such symptoms. Thus, the terms denote that a beneficial result has been conferred on a vertebrate subject with a disorder, disease or symptom, or with the potential to develop such a disorder, disease or symptom.

As used herein, the terms "therapeutically effective amount", "therapeutically effective dose" and "effective amount" refer to an amount of the caninized murine anti-human PD-I antibody
or antigen binding fragment thereof of the present invention that, when administered alone or in combination with an additional therapeutic agent to a cell, tissue, or subject, is effective to cause a measurable improvement in one or more symptoms of a disease or condition or the progression of such disease or condition. A therapeutically effective dose further refers to that amount of the binding compound sufficient to result in at least partial amelioration of symptoms, \textit{e.g.}, treatment, healing, prevention or amelioration of the relevant medical condition, or an increase in rate of treatment, healing, prevention or amelioration of such conditions. When applied to an individual active ingredient administered alone, a therapeutically effective dose refers to that ingredient alone. When applied to a combination, a therapeutically effective dose refers to combined amounts of the active ingredients that result in the therapeutic effect, whether administered in combination, serially or simultaneously. An effective amount of a therapeutic will result in an improvement of a diagnostic measure or parameter by at least 10%; usually by at least 20%; preferably at least about 30%; more preferably at least 40%, and most preferably by at least 50%. An effective amount can also result in an improvement in a subjective measure in cases where subjective measures are used to assess disease severity.

**Other Combination Therapies**

As previously described, the caninized murine anti-human PD-1 antibody or antigen binding fragment thereof may be coadministered with one or other more therapeutic agents (such as a chemotherapeutic agent). The antibody may be linked to the agent (as an immunocomplex) or can be administered separately from the agent. In the latter case (separate administration), the antibody can be administered before, after or concurrently with the agent or can be co-administered with other known therapies.

**Kits**

Further provided are kits comprising one or more components that include, but are not limited to, an antibody or antigen binding fragment, as discussed herein, which specifically binds PD-1 (\textit{e.g.}, a caninized murine anti-human PD-1 antibody or antigen binding fragment thereof of the present invention) in association with one or more additional components including, but not limited to a pharmaceutically acceptable carrier and/or a chemotherapeutic agent, as discussed herein. The binding composition and/or the chemotherapeutic agent can be formulated as a
pure composition or in combination with a pharmaceutically acceptable carrier, in a pharmaceutical composition.

In one embodiment, the kit includes a binding composition of the invention (the caninized murine anti-human PD-1 antibody comprising the amino acid sequence of SEQ ID NO: 28 and of SEQ ID NO: 32 or 34, or a pharmaceutical composition thereof in one container (e.g., in a sterile glass or plastic vial) and a pharmaceutical composition thereof and/or a chemotherapeutic agent in another container (e.g., in a sterile glass or plastic vial).

In another embodiment, the kit comprises a combination of the invention, including a binding composition component (e.g., the caninized murine anti-human PD-1 antibody comprising the amino acid sequence of SEQ ID NO: 28 and of SEQ ID NO: 32 or 34) along with a pharmaceutically acceptable carrier, optionally in combination with one or more therapeutic agent component formulated together, optionally, in a pharmaceutical composition, in a single, common container.

If the kit includes a pharmaceutical composition for parenteral administration to a subject, the kit can include a device for performing such administration. For example, the kit can include one or more hypodermic needles or other injection devices as discussed above. The kit can also include a package insert including information concerning the pharmaceutical compositions and dosage forms in the kit. Generally, such information aids pet owners and veterinarians in using the enclosed pharmaceutical compositions and dosage forms effectively and safely. For example, the following information regarding a combination of the invention may be supplied in the insert: pharmacokinetics, pharmacodynamics, clinical studies, efficacy parameters, indications and usage, contraindications, warnings, precautions, adverse reactions, overdosage, proper dosage and administration, how supplied, proper storage conditions, references, manufacturer/distributor information and patent information.

As a matter of convenience, an antibody or specific binding agent disclosed herein can be provided in a kit, i.e., a packaged combination of reagents in predetermined amounts with instructions for performing the diagnostic or detection assay. Where the antibody is labeled with an enzyme, the kit will include substrates and cofactors required by the enzyme (e.g., a substrate precursor which provides the detectable chromophore or fluorophore). In addition,
other additives may be included such as stabilizers, buffers (e.g., a block buffer or lysis buffer) and the like. The relative amounts of the various reagents may be varied widely to provide for concentrations in solution of the reagents which substantially optimize the sensitivity of the assay. Particularly, the reagents may be provided as dry powders, usually lyophilized, including excipients which on dissolution will provide a reagent solution having the appropriate concentration.

EXAMPLES

EXAMPLE 1
CANINE PD-1 AND PD-L1

Identification and Cloning of Canine PD-1:
A nucleic acid encoding a full length canine PD-1 (cPD-1) was identified through a search of the NCBI gene bank data bases (accession number XM_543338.4, SEQ ID NO: 1). The translated amino acid sequence SEQ ID NO: 2 (accession number XP-543338.3) corresponds to putative canine PD-1 protein which was further identified through searching the gene bank (NCBI) protein databases and aligning the identified amino acid sequence with murine, feline, and human PD-1 amino acid sequences. The DNA sequence corresponding to the full length canine PD-1 gene that was codon optimized for CHO cells was synthesized and cloned into a plasmid designated p96793. Comparison of DNA and protein sequences of predicted canine PD-1 with known PD-1 DNA and protein sequences led to the identification of the DNA sequences encoding the extra-cellular domain (ECD) of canine PD-1 (SEQ ID NO: 3) and the amino acid sequence of the ECD of canine PD-1 (SEQ ID NO: 4).

A DNA sequence encoding the ECD of canine PD-1 in addition to a GT linker and 8 histidine residues was synthesized and cloned into a plasmid designated LPD2726. A nucleic acid sequence (SEQ ID NO: 5) corresponding to the canine PD-1 ECD plus a GT linker and the Fc part of human IgG1 Fc gene was chemically synthesized and cloned into a plasmid designated LPD2727. Canine PD-1 ECD and the Fc part of human IgG1 Fc comprises the amino acid sequence of SEQ ID NO: 6.
Identification and Cloning of Canine PD-L1:

A nucleic acid encoding a full length canine PD-L1 was identified through a search of the NCBI gene bank databases (accession number XM_541302.4; SEQ ID NO: 7). The translated amino acid sequence (accession number XP-541302.4; SEQ ID NO: 8) corresponding to the putative canine PD-L1 protein was identified by searching the gene bank (NCBI) protein databases and alignment of the identified sequence with known PD-L1 mouse and human sequences.

Comparison of DNA encoding canine PD-L1 with known PD-L1 sequences identified the DNA sequence corresponding to the ECD domain of canine PD-L1 (SEQ ID NO: 9; which was codon optimized for CHO cells). The predicted amino acid sequence of the ECD of canine PD-L1 is SEQ ID NO: 10. DNA encoding PD-L1 ECD plus GT linker and 8 histidine residues was synthesized and cloned into a plasmid designated LPD2695.

A DNA sequence encoding the amino acid sequence of canine PD-L1 ECD plus GT linker and the Fc part of human IgG1 Fc (SEQ ID NO: 11) was chemically synthesized and cloned into a plasmid designated LPD2697. Canine PD-L1 ECD plus GT linker and the Fc part of human IgG1 comprises the amino acid sequence of SEQ ID NO: 12. Table 1 contains a description of the expression plasmids mentioned above.

<table>
<thead>
<tr>
<th>PLASMID NAME</th>
<th>EXPRESSED GENE</th>
</tr>
</thead>
<tbody>
<tr>
<td>P96793</td>
<td>Canine PD-1</td>
</tr>
<tr>
<td>LPD2726</td>
<td>Canine PD-1 ECD-8HIS</td>
</tr>
<tr>
<td>LPD2727</td>
<td>Canine PD-1 ECD-/Human IgG1 Fc</td>
</tr>
<tr>
<td>LPD2695</td>
<td>Canine PD-L1 ECD-8HIS</td>
</tr>
<tr>
<td>LPD2697</td>
<td>Canine PD-L1 ECD-/Human IgG1 Fc</td>
</tr>
</tbody>
</table>

Expression of PD-1 and PD-L1 proteins:

Expression plasmids encoding the PD-1ECD-HIS, PD-1ECD-Fc, PDL-1 ECD-HIS, and PD-L1ECD-Fc proteins were transfected into HEK 293 cells and the proteins were purified.
from the supernatant of transfected cells using Protein A for Fc fusion proteins or Nickel (Ni\textsuperscript{2+}) column chromatography for HIS-tagged proteins. Purified proteins were used for: ELISA or binding assays as detailed below. Expressed proteins were analyzed by SDS-PAGE gels.

**EXAMPLE 2**

**IDENTIFICATION OF MURINE ANTI-HUMAN MONOCLONAL ANTIBODIES THAT BIND CANINE PD-1**

*Confirmation of monoclonal antibodies reactivity against canine PD-1*

One of the mouse monoclonal antibodies that previously had been raised against human PD-1 [hPD-1.08A, identified in US 8,354,509 B2, hereby incorporated by reference in its entirety] also was found to strongly react with canine PD-1. Purified hPD-1.08A was tested for reactivity with the HIS-tagged ECD domain of canine PD-1 by ELISA as follows: HIS-tagged canine PD-1 ECD protein is diluted to 10\(\mu\)g/mL in coating buffer (Carbonate/Bicarbonate pH 9.0) and dispensed at 100 \(\mu\)L/well in 96-well flat bottomed ELISA plates (NUNC). The plates are incubated at 4\(^\circ\)C overnight. The plates are then washed three times with phosphate buffered saline containing 0.05% Tween-20 (PBST). Next, 200 \(\mu\)L of blocking buffer (5% skim milk in PBST) is added to each well and the plates are incubated at 37\(^\circ\)C for 60 minutes. The plates are then washed three times with PBST. Next, 100 \(\mu\)L of test monoclonal antibodies (mAbs) diluted in blocking buffer is added to the first wells of the appropriate columns. Test mAbs are then diluted two-fold to the appropriate plate position. Following incubation of the plates at 37\(^\circ\)C for 60 minutes, the plates are washed three times with PBST. Next, 100 \(\mu\)L per well of a 1:2,000 dilution of a horseradish peroxidase conjugated goat anti-mouse IgG (KPL) is added to the plates, which are then incubated at 37\(^\circ\)C for 60 minutes. Then the plates are washed three times with PBST, and 100 \(\mu\)L/well of 3,3',5,5' tetramethyl benzidine, (TMB) substrate (from KPL) is added to the plates. The color reaction is allowed to develop for 5-20 minutes at 37\(^\circ\)C prior to measuring absorbance at 650nm.

*CHO cells expressing canine PD-1 protein*

The full length canine PD-1 gene was cloned into plasmid p96793. In this plasmid the expression of the canine PD-1 protein is driven by an hCMV promoter. CHO DXB11 cells (dhfr-) were maintained in MEM-alpha (Gibco) supplemented with 10% fetal bovine serum.
Transfection of CHO cells with plasmid p96793 was carried out in 75 cm$^2$ flasks containing approximately 6 x 10$^6$ cells by liposome-mediated gene delivery using Lipofectamine (Invitrogen). After 48 hours, cells were passaged into MEM-alpha medium without nucleosides, supplemented with 10% FBS and 400µg/mL hygromycin B (selective medium). Limited-dilution cloning was performed on the pool of dhfr+, hygromycin resistant cells. Clones were assessed for expression of canine PD-1 by immunofluorescence assay. Briefly, cell monolayers were fixed in 96 well plates with 80% acetone. Fixed and dried cell monolayers were then incubated for 1 hour with a polyclonal goat anti-human PD-1 antibody (R&D Systems). Plates were washed with PBS, then incubated for 1 hour with a fluorescein-labeled rabbit anti-goat IgG antibody (KPL). Plates were washed with PBS. Clones exhibiting fluorescence were expanded and cell stocks were established.

**Reactivity of mouse mAbs against Canine PD-1 proteins expressed on CHO cells**

The reactivity of mouse anti-human PD-1 mAbs with canine PD-1 on CHO cells was determined by a cell-based assay using CHO cells that express PD-1. Briefly, the CHO cells expressing canine PD-1 were cultured to 80-100% confluency in 50 µl media (DMEM/HAM’s F12, 10% FBS; “CHO Media”). Next, 50 µl of media containing various concentrations of purified mAbs were added for 1 hour at 37°C. Following three washes with PBS-TWEEN, 100 µl of goat anti-mouse horse radish peroxidase (HRP) diluted 1:1000 in culture media was added for one hour at 37°C. After three additional washes with PBS-TWEEN, bound mAbs were visualized with a peroxidase substrate (TMB). The absorbance increase due to peroxidase activity at 450 nm was measured in a microplate reader. Color development is stopped by adding 50 µL per well of 1 M phosphoric acid.

**Ligand blockade by mouse and caninized anti-PD-1 mAbs**

For mouse anti-human PD-1 mAbs which react with canine PD-1, a cell-based ELISA (CELISA) assay based on the CHO cell line expressing canine PD-1 was used. Ligand blockade was confirmed using this assay in conjunction with biotinylated cPD-L1/Fc protein. Briefly, seed cPD-1 CHO cells in 96-well plates at 4 x 10$^4$ cells per well and incubate cells at 37°C for 18-24 hours till they are 95-100% confluent. Aspirate cell culture media, wash the plates 3 times with PBS + 0.05% Tween20 and 1 x CHO media. Make 3-fold serial dilutions of anti-cPD1 mAbs in CHO media, starting at 30 µg/mL, and add 50 µL/well of each antibody dilution to the plate. Incubate at 37°C, 5% CO$_2$ with shaking for 30 min. Add 50 µL/well of
cPD-L1-Fc–biotin (2 ug/ml in CHO media stock) and continue to incubate at 37°C, 5% CO₂ with shaking for 45 min. Wash the plates six times with PBS + 0.05% Tween 20. Add 100μl/well of 1:2000 Streptavidin-Horse Raddish Peroxidase (Streptavidin-HRP) in CHO media and incubate 30-60 min at 37°C/5% CO₂. Wash the plates five times with PBS + 0.05% Tween20. Add 100 μl/well of TMB color developing substrate. Stop color development by adding 50 μl/well of 1M phosphoric acid. Measure optical density (O.D.) at A450 – A620 using an ELISA plate reader.

Cloning and identification of DNA sequences corresponding to mouse Hpd-.08A mAb

The DNA sequence of mouse VH and VL chains and the DNA sequences encoding their CDRs are identified as described US 8,354,509 [see, Table IV of US 8,354,509; provided in Table 2 directly below].

**Table 2**

Mouse anti-Human PD-1 CDRs from hPD-1.08A of US 8,354,509

<table>
<thead>
<tr>
<th>CDR</th>
<th>Heavy Chain (SEQ ID NO:)</th>
<th>Light Chain (SEQ ID NO:)</th>
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</thead>
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<tr>
<td></td>
<td>N.A.</td>
<td>A.A.</td>
</tr>
<tr>
<td>CDR-1</td>
<td>13</td>
<td>14</td>
</tr>
<tr>
<td>CDR-2</td>
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<td>16</td>
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<tr>
<td>CDR-3</td>
<td>17</td>
<td>18</td>
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</tbody>
</table>

**EXAMPLE 3**

**CANINIZATION OF MOUSE ANTI-HUMAN PD-1 MONOCLONAL ANTIBODIES**

In order to execute the process of caninization, the DNA sequence that encodes the heavy and light chains of canine IgG were determined. The DNA and protein sequence of the canine heavy and light chains are known in the art and can be obtained by searching of the NCBI gene and protein databases. There are four known IgG subtypes of dog IgG and they are referred to as IgG-A, IgG-B, IgG-C, and IgG-D. There are two types of light chains in canine antibodies referred to as kappa and lambda. Table 3 lists both the amino and nucleic acid sequences of
modified canine heavy (IgG-A, IgG-B, IgG-D) and light (Kappa) antibody chains of the present invention that comprise the murine anti-human PD-1 CDRs of Table 2.

Table 3
MODIFIED CANINE HEAVY AND LIGHT CHAIN SEQUENCES

<table>
<thead>
<tr>
<th>Chain type</th>
<th>Subtype</th>
<th>Nucleic Acid SEQ ID NO:</th>
<th>Amino Acid SEQ ID NO:</th>
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<td>IgG-A</td>
<td>25</td>
<td>26</td>
</tr>
<tr>
<td>H</td>
<td>IgG-B</td>
<td>27</td>
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<tr>
<td>H</td>
<td>IgG-D</td>
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<td>30</td>
</tr>
<tr>
<td>L</td>
<td>Kappa (1011)</td>
<td>31</td>
<td>32</td>
</tr>
<tr>
<td>L</td>
<td>Kappa (1022)</td>
<td>33</td>
<td>34</td>
</tr>
</tbody>
</table>

# Sequences do not include the signal sequence.

Construction of Caninized anti PD-1 Antibodies

Without being bound by any specific approach, the process of producing variants of caninized anti-PD-1 mAbs with various contents of canine and mouse sequences involved the general following scheme:

i) Determine DNA sequence of VH and VL chains of mouse mabs

ii) Identify the H and L chain CDRs of mouse mabs

iii) Identify a suitable H and L chain of canine IgG

iv) Write down the DNA sequence of canine IgG H and L chains

v) Replace the DNA sequence encoding endogenous dog H and L chain CDRs with DNA sequences encoding the respective mouse CDRs. Also, optionally replace some canine frame residues with selected residues from the corresponding mouse frame regions.

vi) Synthesize the DNA from step (v) and clone it into a suitable expression plasmid

vii) Transfect plasmids into HEK 293 cells

viii) Purify expressed antibody from HEK 293 supernatant

ix) Test purified antibody for binding to canine PD-1

The above outlined steps resulted in a set of variant antibodies with various contents of canine and mouse sequences. The present invention identifies the caninized murine anti-human PD-1 antibodies comprising SEQ ID NO: 28 and of SEQ ID NO: 32 or 34 as having particularly tight binding with canine PD-1.
Full length canine PD-1 DNA sequence: signal sequence **underlined and in bold**

Nucleotide sequence **SEQ ID NO: 1** is without the signal sequence; and

Nucleotide sequence **SEQ ID NO: 35** includes the signal sequence.

```
ctggattcccccgacagaccctggagccctctcaccttctcccctgcccagctgaccgtccaggaaggcgagaatg
caccctgactcagctgctctgcagagctggtaaccgtctgcagagctggagaacgggcttggacggaatgcagttcaactggtacgtggacggcgtggaggtgcataatgccaagacaaagccgcgggaggagcagtacaacagcacgtacggtgtggtcagcgtcctcaccgtcctgcaccaggactggctgaatggcaaggagtacaagtgcaaggtctccaacaagccctcccagcccccatcgagaaaaccatctccaaagccaaagggcagccccgagaaccacaggtgtacaccctgcctccccatcccgggatgagctgaccaagaaccaggtcagcctgacctgcctggtcaaaggcttctatcccagcgacatcgccgtggagtgggagagcaatgggcagccggagaacaactacaagaccacgcctcccgtgctggactccgagc
```

Full length canine PD-1 Amino acid sequence: signal sequence **underlined and in bold**

Amino acid sequence **SEQ ID NO: 2** is without the signal sequence; and

Amino acid sequence **SEQ ID NO: 36** includes the signal sequence.

```
MGSRRGPWPLVWAVLQLGWWPGWLLDSPDRPWSPLTFSPAQLTVQEGENATFTCSLADIPDSFVLNWYRLSPRNQT
```

Canine PD-1 extracellular domain DNA sequence: **SEQ ID NO: 3** (Codon optimized for expression in CHO cells)

```
ctggattcccccgacagaccctggagccctctcaccttctcccctgcccagctgaccgtccaggaaggcgagaatg
caccctgactcagctgctctgcagagctggtaaccgtctgcagagctggagaacgggcttggacggaatgcagttcaactggtacgtggacggcgtggaggtgcataatgccaagacaaagccgcgggaggagcagtacaacagcacgtacggtgtggtcagcgtcctcaccgtcctgcaccaggactggctgaatggcaaggagtacaagtgcaaggtctccaacaagccctcccagcccccatcgagaaaaccatctccaaagccaaagggcagccccgagaaccacaggtgtacaccctgcctccccatcccgggatgagctgaccaagaaccaggtcagcctgacctgcctggtcaaaggcttctatcccagcgacatcgccgtggagtgggagagcaatgggcagccggagaacaactacaagaccacgcctcccgtgctggactccgagc
```

Canine PD-1 extracellular domain: **SEQ ID NO: 4**

```
ctgattcccccgacagacctcttgagccctctcaccttctcccctgcccagctgaccgtccaggaaggcgagaatg
caccctgactcagctgctctgcagagctggtaaccgtctgcagagctggagaacgggcttggacggaatgcagttcaactggtacgtggacggcgtggaggtgcataatgccaagacaaagccgcgggaggagcagtacaacagcacgtacggtgtggtcagcgtcctcaccgtcctgcaccaggactggctgaatggcaaggagtacaagtgcaaggtctccaacaagccctcccagcccccatcgagaaaaccatctccaaagccaaagggcagccccgagaaccacaggtgtacaccctgcctccccatcccgggatgagctgaccaagaaccaggtcagcctgacctgcctggtcaaaggcttctatcccagcgacatcgccgtggagtgggagagcaatgggcagccggagaacaactacaagaccacgcctcccgtgctggactccgagc
```

Canine PD-1 extracellular domain – human IgG1Fc DNA sequence: **SEQ ID NO: 5** (Codon optimized for expression in HEK-293 cells)

```
ctgattcccccgacagacctcttgagccctctcaccttctcccctgcccagctgaccgtccaggaaggcgagaatg
caccctgactcagctgctctgcagagctggtaaccgtctgcagagctggagaacgggcttggacggaatgcagttcaactggtacgtggacggcgtggaggtgcataatgccaagacaaagccgcgggaggagcagtacaacagcacgtacggtgtggtcagcgtcctcaccgtcctgcaccaggactggctgaatggcaaggagtacaagtgcaaggtctccaacaagccctcccagcccccatcgagaaaaccatctccaaagccaaagggcagccccgagaaccacaggtgtacaccctgcctccccatcccgggatgagctgaccaagaaccaggtcagcctgacctgcctggtcaaaggcttctatcccagcgacatcgccgtggagtgggagagcaatgggcagccggagaacaactacaagaccacgcctcccgtgctggactccgagc
```
Canine PD-I extracellular domain – human IgG1 Fc fusion protein: signal sequence underlined and in bold: SEQ ID NO: 6; SEQ ID NO: 53 includes the signal sequence.

MNFLLSWVHWSLALLLYLHHAKWSQALDSPDRPWSPLTFSPAQLTVQEGENATFTCSLADIPDSFVLNWYRLSPRNQHSSYSGRAAQRLKQDLFLGKAALQITIDVLRLGAVVCCLIIGIGGADYKRTILKMHAPYRINSIRISVDPRTSHELMQCAQGYPEAEVIWTSSDHRVLSKTTITNSNREEKLFNTSTMNINATANIFCYTFQRSGPEENNTAELVIPERLPVPFASERTHFMILGFLLLGLGVVLAVTFLKKHGRMMDVEKCTDRNSKRRNDIQFEEET

Full length canine PD-L1 DNA sequence: signal sequence underlined and in bold
Nucleotide sequence SEQ ID NO: 7 is without the signal sequence; and
Nucleotide sequence SEQ ID NO: 37 includes the signal sequence.

Canine PD-L1 extracellular domain DNA sequence: SEQ ID NO: 9 (Codon optimized for expression in CHO cells)

Canine PD-L1 extracellular domain protein: SEQ ID NO: 10

Canine PD-L1 extracellular domain – human IgG1 Fc DNA sequence: SEQ ID NO: 11 (Codon optimized for expression in HEK-293 cells)
Canine PD-L1 extracellular domain – human IgG1 Fc fusion protein: SEQ ID NO: 12

FTITVSKDLYVVEYGGNVTMECKFPVEKQLNLFALIVYWEMEDKKIQFVNGKEDLKVQHSSYSQRAQLLKDQLFL
GKAALQITDVRLQDAGVYCCLIGYGGADYKRITLKVHAPYRNISQRISVDPVTSEHLMCAEGYPEAEVIWTSSD
HRVLSGKTTINNSREELNVTSTLMINATANE1FTCFQRSGPEENNTALVIERLFPASERTTHGDKHTHC
FPFFPELLOGPSVLFPFPKPDTLIM1SRTEPVTCVVVDVSHEDPEVKFVNWFDGVHNAKTPFKREEQYINSTYR
VSLTVLHQLDWNLNGEKYCKVSNKALFAPF1ektIskAKQPTQEPFQVYLPFPRSRDLEITNQVLOC1VKGFYPSDIA
VEWESNGQPPNNYKVTTPFVLDGSFGFLYSKLTVDKSRWQGNGFSCSVNHEALNHHTQKSLSLSFGK

08A VH : CDR H1 DNA: SEQ ID NO: 13:
agttattatc tggatc

08A VH : CDR H1 protein: SEQ ID NO: 14:
Ser Tyr Tyr Leu Tyr

08A VH : CDR H2 DNA: SEQ ID NO: 15:
ggggttaatc ctagtaatgg tggtactaac ttcagtgaga agttcaag

08A VH : CDR H2 protein: SEQ ID NO: 16:
Gly Val Asn Pro Ser Asn Gly Val Asn Tyr Asp

08A VH : CDR H3 DNA: SEQ ID NO: 17:
agggattcta actacgacgg gggctttgac tac

08A VH : CDR H3 protein: SEQ ID NO: 18:
Arg Asp Ser Tyr Asp Gly Val Asn Phe Ser Tyr

08A VL : CDR L1 DNA: SEQ ID NO: 19:
agggccagca aaagttgact tagatctggc ttgtagttac ttgtagtga aagtaccc

08A VL : CDR L1 protein: SEQ ID NO: 20:
Arg Ala Ser Leu Ser Val Ser Thr Ser Gly Phe Ser Tyr Leu His

08A VL : CDR L2 DNA: SEQ ID NO: 21:
cttgcatcca acctagagtc

08A VL : CDR L2 protein: SEQ ID NO: 22:
Leu Ala Ser Asn Leu Glu Ser
Caninized Murine Anti-Human PD-1 Antibody 08A

canVH-canIgGA-Fc (12G8 signal sequence **underlined and in bold**): HEAVY CHAIN

Nucleotide sequence **SEQ ID NO: 27** is without the signal sequence; and

Nucleotide sequence **SEQ ID NO: 41** includes the signal sequence.

Amino acid sequence **SEQ ID NO: 28** is without the signal sequence; and

Amino acid sequence **SEQ ID NO: 42** includes the signal sequence.

canVH-canIgGB-Fc (12G8 signal sequence **underlined and in bold**): HEAVY CHAIN

Nucleotide sequence **SEQ ID NO: 25** is without the signal sequence; and

Nucleotide sequence **SEQ ID NO: 39** includes the signal sequence.
Amino acid sequence SEQ ID NO: 26 is without the signal sequence; and
Amino acid sequence SEQ ID NO: 40 includes the signal sequence.

can VH-can IgGD-Fc (12G8 signal sequence underlined and in bold):

HEAVY CHAIN

Nucleotide sequence SEQ ID NO: 29 is without the signal sequence; and
Nucleotide sequence SEQ ID NO: 43 includes the signal sequence.
canVL-canKappa (1022)xHGF signal sequence **underlined and in bold**: LIGHT CHAIN

Nucleotide sequence **SEQ ID NO: 33** is without the signal sequence; and
Nucleotide sequence **SEQ ID NO: 47** includes the signal sequence.

```plaintext
ATGGATATGAGATACCTGGACACACTCTGCTGGATTCTGCTGCTTCTTTGGCTGAGAGGGGCCCGCTGCGATATCGTCC
TGACAGCAGACGTCTGGCAGTACATCGTGGCTGAGAGGGGCCCGCTGCGATATCGTCC
```

Amino acid sequence **SEQ ID NO: 34** is without the signal sequence; and
Amino acid sequence **SEQ ID NO: 48** includes the signal sequence.

```plaintext
MDMRVPAQLLGLLALLGLGALCDIVLTQTPPSLSVSPGEPASICRAASKSVSTSGFSYLHWRYYRKGQPPQQLIIFL
```

EXAMPLE 4

**MUTANT CANINE IgG-B ANTIBODIES SPECIFIC TO PD-1**

There are four known IgG heavy chain subtypes of dog IgG and they are referred to as IgG-A, IgG-B, IgG-C, and IgG-D. The two known light chain subtypes are referred to as lambda and
kappa. However, besides binding and activating of canine immune cells, a canine or caninized antibody against PD-1 optimally has two attributes:

1. lack of effector functions such as antibody-dependent cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), and
2. be readily purified on a large scale using industry standard technologies such as that based on protein A chromatography.

None of the naturally occurring canine IgG isotypes satisfy both criteria. For example, IgG-B can be purified using protein A, but has a high level of ADCC activity. IgG-C also has considerable ADCC activity. On the other hand, IgG-A binds weakly to protein A, but displays undesirable ADCC activity. Moreover, neither IgG-C nor IgG-D can be purified on protein A columns, although IgG-D display no ADCC activity. The present invention overcomes this difficulty by providing mutant canine IgG-B antibodies specific to PD-1; such antibodies lack effector functions such as ADCC and can be easily of purified using industry standard protein A chromatography. The exact modifications are shown in Figure 4.

The IgG-B variants with reduced effector functions described encompass a first IgG-B variant in which a lysine (D 277) and an asparagine (N 325) residue is each mutated to an alanine residue [cIgGB(-) ADCC], a second variant in which the hinge region of IgG-B is replaced by the hinge region of IgG-D [cIgGB(+) D-hinge], and a third variant in which the hinge region of IgG-B is replaced with the hinge region of IgG-A [cIgGB(+) A-hinge]. Additionally, the second and third variants also include replacement of the same lysine and asparagine residues of the first variant with an alanine residue. The numbering of the lysine and asparagine residues mutated in this invention is based on the numbering scheme described for canine IgG heavy chains in Tang et al., [Vet Immunol and Immunopathol, 80:259-270 (2001)].

Canine IgGB wt
SASTTAPVFPLAPSCGSTSGSTVALACLVGYFEPVPVSVNWASLTSGVHTFPSVLQSGSYSLNSMVTVSSR
WPSETFTCNVAPASKKTVKDVPVFKNRGRVPFRPDPCKCPAPEMLGGPSVFIFPPHPKPLQQLPKIVTV
LDPEPDEVQISWFDVQGKMQTAKTQPREEQFNQTYVRVSVLPIGHQDWRGQKQFCTKVNKALPSPIERTIS
KARGQAHQPSYVLPPSREELSKNTVSLCLKDFPFPIDVEWQNSGQQEPESKRTTPQLDEGSYFLYSKS
VDKRSWQRGDTIFACMVHAHNYTQESLSSHSPGK  SEQ ID NO:49

Canine IgGB(+)A-hinge
SASTTAPVFPLAPSCGSTSGSTVALACLVGYFEPVPVSVNWASLTSGVHTFPSVLQSGSYSLNSMVTVSSR
WPSETFTCNVAPASKKTVKDVPVFKNRGRVPFRPDPCKCPAPEMLGGPSVFIFPPHPKPLQQLPKIVTV
LDPEPDEVQISWFDVQGKMQTAKTQPREEQFNQTYVRVSVLPIGHQDWRGQKQFCTKVNKALPSPIERTIS
KARGQAHQPSYVLPPSREELSKNTVSLCLKDFPFPIDVEWQNSGQQEPESKRTTPQLDEGSYFLYSKS
VDKRSWQRGDTIFACMVHAHNYTQESLSSHSPGK  SEQ ID NO:49
**Sequence Listing Table 1**

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All references cited herein are incorporated by reference to the same extent as if each individual publication, database entry (e.g. Genbank sequences or GeneID entries), patent application, or patent, was specifically and individually indicated to be incorporated by reference. This statement of incorporation by reference is intended by Applicants, pursuant to 37 C.F.R. §1.57(b)(1), to relate to each and every individual publication, database entry (e.g. Genbank sequences or GeneID entries), patent application, or patent, each of which is clearly identified in compliance with 37 C.F.R. §1.57(b)(2), even if such citation is not immediately adjacent to a dedicated statement of incorporation by reference. The inclusion of dedicated statements of incorporation by reference, if any, within the specification does not in any way weaken this general statement of incorporation by reference. Citation of the references herein is not intended as an admission that the reference is pertinent prior art, nor does it constitute any admission as to the contents or date of these publications or documents.

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

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.
We Claim:

1. An isolated caninized antibody or antigen binding fragment thereof that specifically binds Programmed Cell Death Receptor 1 (PD-1) comprising a canine IgG heavy chain and a canine kappa light chain; wherein the canine kappa light chain comprises three light chain complementary determining regions (CDRs): CDR light 1 (CDRL1), CDR light 2 (CDRL2), and CDR light 3 (CDRL3); and the canine IgG heavy chain comprises three heavy chain CDRs: CDR heavy 1 (CDRH1), CDR heavy 2 (CDRH2) and CDR heavy 3 (CDRH3): (a) wherein CDRL1 comprises the amino acid sequence of SEQ ID NO: 20, or a conservatively modified variant of SEQ ID NO: 20; (b) wherein CDRL2 comprises the amino acid sequence comprising SEQ ID NO: 22, or a conservatively modified variant of SEQ ID NO: 22; (c) wherein CDRL3 comprises the amino acid sequence of SEQ ID NO: 24, or a conservatively modified variant of SEQ ID NO: 24; (d) wherein CDRH1 comprises the amino acid sequence of SEQ ID NO: 14, or a conservatively modified variant of SEQ ID NO: 14; (e) wherein CDRH2 comprises the amino acid sequence of SEQ ID NO: 16, or a conservatively modified variant of SEQ ID NO: 16; and (f) wherein CDRH3 comprises the amino acid sequence of SEQ ID NO: 18, or a conservatively modified variant of SEQ ID NO: 18; wherein the antibody and fragment bind canine PD-1 and block the binding of canine PD-1 to canine Programmed Cell Death Ligand 1 (PD-L1).

2. The isolated caninized antibody of Claim 1 wherein the CDRL1 comprises the amino acid sequence of SEQ ID NO: 20.

3. The isolated caninized antibody of Claim 1 or 2 wherein CDRL2 comprises the amino acid sequence comprising SEQ ID NO: 22.

4. The isolated caninized antibody of Claim 1, 2, or 3 wherein CDRL3 comprises the amino acid sequence of SEQ ID NO: 24.
5. The isolated caninized antibody of Claim 1, 2, 3, or 4 wherein the CDRH1 comprises the amino acid sequence of SEQ ID NO: 14.

6. The isolated caninized antibody of Claim 1, 2, 3, 4, or 5 wherein CDRH2 comprises the amino acid sequence comprising SEQ ID NO: 16.

7. The isolated caninized antibody of Claim 1, 2, 3, 4, 5, or 6 wherein CDRH3 comprises the amino acid sequence of SEQ ID NO: 18.

8. The isolated caninized antibody of Claim 1, 2, 3, 4, 5, 6, or 7 wherein the IgG heavy chain comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 26, a conservatively modified variant of SEQ ID NO: 26, SEQ ID NO: 28, a conservatively modified variant of SEQ ID NO: 28, SEQ ID NO: 30, and a conservatively modified variant of SEQ ID NO: 30.

9. The isolated caninized antibody of Claim 1, 2, 3, 4, 5, 6, 7, or 8 wherein the kappa light chain comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 32, a conservatively modified variant of SEQ ID NO: 32, SEQ ID NO: 34, and a conservatively modified variant of SEQ ID NO: 34.

10. The isolated caninized antibody of Claim 9, comprising the amino acid sequence of SEQ ID NO: 28 and of SEQ ID NO: 34, or a conservatively modified variant of SEQ ID NO: 28 and of a conservatively modified variant of SEQ ID NO: 34.

11. An isolated nucleic acid that encodes the light chain of the caninized antibody of Claims 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

12. An isolated nucleic acid that encodes the heavy chain of the caninized antibody of Claims 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

13. The isolated nucleic acid of Claims 11 or 12 that comprises one or more nucleotide sequences selected from the group consisting of SEQ ID NOs: 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, or 33.
14. The isolated nucleic acid of Claim 13 that comprises the nucleotide sequence of SEQ ID NO: 27 or of SEQ ID NO: 33.

15. An expression vector comprising the isolated nucleic acid of Claims 11, 12, 13, or 14.

16. A host cell comprising one or more expression vectors of claim 15.

17. A pharmaceutical composition comprising the antibody or antigen binding fragment of Claims 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 and a pharmaceutically acceptable carrier or diluent.

18. A method of increasing the activity of an immune cell, comprising administering to a subject in need thereof a therapeutically effective amount of the pharmaceutical composition of Claim 17.

19. The method of claim 18, wherein said method is used for:
   a. the treatment of cancer;
   b. the treatment of an infection or infectious disease; or
   c. as a vaccine adjuvant.

20. A method of producing a caninized antibody or antigen binding fragment thereof that specifically binds PD-1 comprising:
   a. culturing the host cell of Claim 16 in culture medium under conditions wherein the nucleic acid is expressed, thereby producing a polypeptide comprising the light and heavy chain variable regions; and
   b. recovering the polypeptides from the host cell or culture medium.
Figure 1. Reactivity of mouse 08A mAb against His-tagged extracellular domain of canine PD-1.
Figure 2: Reactivity of mouse 08A mAbs against Canine PD-1 proteins expressed on CHO cells using CELISA. Murine 08A antibody and its caninized variants react with PD-1 in a dose dependent manner.
Figure 3. Ligand blockade by mouse and caninized antibodies. Murine 08A and its caninized variants block binding of canine PD-1 to PD-1 expressed on CHO cell surface.
Figure 4. Alignment of canine IgGB CHs lacking ADCC function

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cIgGB(+)-D-hinge
cIgGB(-)-ADCC
cIgGB

cIgGB(+)-A-hinge
cIgGB(+)-D-hinge
cIgGB(-)-ADCC

canIgGB
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cIgGB(+)-D-hinge
cIgGB(-)-ADCC

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cIgGB(+)-D-hinge

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Zhang, Yuanzheng
Bartels-Morozov, Denise
Erskine, Jason
Tarpey, Ian
Presta, Leonard G

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Pro Gly Arg Asp Arg Arg Phe Arg Val Met Arg Leu Pro Asn Gly Arg
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Tyr Leu Cys Gly Ala Ile Tyr Leu Pro Pro Asn Thr Gln Ile Asn Glu
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Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr 210 215 220 225
Asn Ser Thr Tyr Arg Val Ser Val Leu Thr Val Leu His Glu Asp 230 235 240
Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu 245 250 255
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Glu Pro Glu Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys 275 280 285

Page 5
Asp Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp
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Lys Leu Thr Val Asp Lys Ser Arg Trp Gin Gin Gin Asn Val Phe Ser
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Leu Ser Leu Ser Pro Gly Lys
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| TGGAGGATA AAAAATTAT ACAATTGCT TTGGAAAGG AAGACCTGAA AGTTCAAGC |
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| CAAAGAATTTC CTGTGGTATCC GTTCACCTCT GAAACATGAAC TAAATGCTCA GGTGAGGTT |
| TACCCTGAGG CTGAACTCAT CTGGCAAAGC AGTGACCCAC GAGTCCTGAG TGCCAAAACC |
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| AATGCAACAGCTATGAGAT TTCTACTGC ACTTTTTCAA GATCAGGTCG TGGAAAAAAC |
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Thr Ile Thr Asn Ser Asn Arg Glu Glu Lys Leu Phe Asn Val Thr Ser
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170                 175
Gln Arg Ser Gly Pro Glu Glu Asn Asn Thr Ala Glu Leu Val Ile Pro
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Glu Arg Leu Pro Val Pro Ala Ser Glu Arg Thr His Phe Met Ile Leu
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Page 8
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Leu Gln Ile Thr Asp Val Arg Leu Gln Asp Ala Gly Val Tyr Cys Cys 85
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His Ala Pro Tyr Arg Asn Ile Ser Gln Arg Ile Ser Val Asp Pro Val 115                 120                 125
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Gln His Ser Trp Glu Leu Pro Leu Thr
1 5

Artificial Sequence

caninized mouse antibody
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Gly Gly Val Asn Pro Ser Asn Gly Gly Thr Asn Phe Ser Gly Lys Phe 40
Lys Ser Arg Ala Thr Leu Ser Val Asp Lys Ala Lys Asn Thr Ala Tyr 50
Page 14
Pro Pro Ser Pro Lys Glu Leu Ser Ser Ser Asp Thr Val Ser Ile Thr
Cys Leu Ile Lys Asp Phe Tyr Pro Pro Asp Lle Asp Val Glu Trp Gln
Ser Asn Gly Gin Gin Gin Glu Pro Glu Arg Lys His Arg Met Thr Pro Pro
Gln Leu Asp Glu Asp Gly Ser Tyr Phe Leu Tyr Ser Lys Leu Ser Val
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Gly Gly Val Asn Pro Ser Asn Gly Gly Thr Asn Phe Ser Gly Lys Phe
Lys Ser Arg Ala Thr Leu Ser Val Asp Lys Ala Lys Asn Thr Ala Tyr
Met Glu Leu Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
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Ala Ser Lys Thr Lys Val Asp Lys Pro Val Pro Lys Arg Glu Asn Gly 210
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Gln Met Gln Thr Ala Lys Thr Gln Pro Arg Glu Glu Gln Phe Asn Gly 290
Thr Tyr Arg Val Val Ser Val Leu Pro Ile Gly His Gln Asp Trp Leu 305
Lys Gly Lys Gln Phe Thr Cys Lys Val Asn Asn Lys Ala Leu Pro Ser 320
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Artificial Sequence
caninized mouse antibody

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210 215 220
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**Artificial Sequence**

**caninized mouse antibody**

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Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Arg Ile Ser

Arg Val Glu Ala Asp Ala Gly Val Tyr Tyr Cys Gln His Ser Trp

Glu Leu Pro Leu Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg

Asn Asp Ala Gln Pro Ala Val Tyr Leu Phe Gln Pro Ser Pro Asp Gln

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Page 23
20  25  30
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Gln Leu Leu Ile Phe Leu Ala Ser Asn Leu Glu Ser Gly Val Pro Asp
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Arg Ile Ser
Arg Val Glu Ala Asp Asp Ala Gly Val Tyr Tyr Cys Gin His Ser Trp
Glu Leu Pro Leu Thr Phe Gly Gin Gly Thr Lys Val Glu Ile Lys Arg
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Thr Gin Ile Gin Gin Ser Val Thr Gin Asp Ser Lys Asp Ser Thr
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Tyr Gly Glu Leu Asp Phe Gln Trp Arg Glu Lys Thr Pro Glu Pro Pro

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Lys Ser Val Ser Thr Ser Gly Phe Ser Tyr Leu His Trp Tyr Arg Gln Page 38
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Glu Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp
Phe Thr Leu Arg Lle Ser Arg Val Glu Ala Asp Ala Gly Val Tyr
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Ser Lys Tyr Arg Thr Thr Pro Pro Gln Leu Asp Glu Asp Gly Ser Tyr

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Gly Glu Asn Ala Thr Phe Thr Cys Ser Leu Ala Asp Ile Pro Asp Ser

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Phe Val Leu Asn Trp Tyr Arg Leu Ser Pro Arg Asn Gln Thr Asp Lys

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Phe Arg Val Met Arg Leu Pro Asn Gly Arg Asp Phe His Met Ser Ile

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Val Ala Ala Arg Leu Asn Asp Ser Gly Ile Tyr Leu Cys Gly Ala Ile

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Val  Ser  Val  Leu  Thr  Val  Leu  His  Gin  Asp  Trp  Leu  Asn  Gly  Lys  Glu
260
265
270
Tyr  Lys  Cys  Lys  Val  Ser  Asn  Lys  Ala  Leu  Pro  Ala  Pro  Ile  Glu  Lys
275
280
285
Thr  Ile  Ser  Lys  Ala  Lys  Gly  Gin  Pro  Arg  Glu  Pro  Gin  Val  Tyr  Thr
290
295
300
Leu  Pro  Pro  Ser  Arg  Asp  Glu  Leu  Thr  Lys  Asn  Gin  Val  Ser  Leu  Thr
305
310
315
320
Cys  Leu  Val  Lys  Gly  Phe  Tyr  Pro  Ser  Asp  Ile  Ala  Val  Glu  Trp  Glu
325
330
335
Ser  Asn  Gly  Gin  Pro  Glu  Asn  Asn  Tyr  Lys  Thr  Thr  Pro  Pro  Val  Leu
340
345
350
Asp  Ser  Asp  Gly  Ser  Phe  Phe  Leu  Tyr  Ser  Lys  Leu  Thr  Val  Asp  Lys
355
360
365
Ser  Arg  Trp  Gin  Gin  Gly  Asn  Val  Phe  Ser  Cys  Ser  Val  Met  His  Glu
370
375
380
Ala  Leu  His  Asn  His  Tyr  Thr  Gin  Lys  Ser  Leu  Ser  Leu  Ser  Pro  Gly
385
390
395
400
Lys
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