

**COMPOSITIONS AND METHODS FOR
MODIFYING ANTIBODY EFFECTOR FUNCTIONS**

FIELD OF THE DISCLOSURE

[0001] The present application relates to feline, canine, and equine antibody variants and uses thereof, as well as compositions and methods for modifying therapeutic veterinary monoclonal antibodies by modulating immune effector functions and antibody variants using those methods and compositions.

BACKGROUND OF THE INVENTION:

[0002] Feline, canine, and equine IgG monoclonal antibodies (mAbs) and modified antibodies can be effective therapeutics in veterinary medicine. However, there has been limited work done to improve the characteristics of feline, canine, and equine IgGs.

[0003] Feline IgG monoclonal antibodies (mAbs) are being developed as effective therapeutics in veterinary medicine. Several years ago, feline IgG subclasses were identified and characterized (Strietzel et al., 2014, Vet Immunol Immunopathol., vol. 158(3-4), pages 214-223). Canine IgG monoclonal antibodies (mAbs) are also being developed as effective therapeutics in veterinary medicine. Several years ago, four canine IgG subclasses were identified and characterized (Bergeron et al., 2014, Vet Immunol Immunopathol., vol. 157(1-2), pages 31-41). The seven equine IgG subclasses are well known in the art and are fully described in, for example, Wagner et al., 2004, J. Immunol., vol. 173, pages 3230-3242; Wagner, 2006, Dev. Comp. Immunol., vol. 30, pages 155-164; Sheoran et al., 2000, Am. J. Vet. Res., vol 61, pages 1099-1105; and Wagner et al., 1998, Immunobiology, vol. 199 (1), pages 105-118

[0004] Through a recycling mechanism, the neonatal Fc receptor (FcRn) prolongs the half-life of an IgG in a pH-dependent interaction with its fragment crystallizable (Fc) region.

Specifically, the Fc region spanning the interface of CH2 and CH3 domains interacts with the FcRn on the surface of cells to regulate IgG homeostasis. This interaction is favoured by an acidic interaction after IgG pinocytosis and thus IgG is protected from degradation. The endocytosed IgG is then recycled back to the cell surface and released into the blood stream at a slightly alkaline pH thereby maintaining sufficient serum IgG for proper function. Accordingly, the pharmacokinetic profile of IgGs depend on the structural and functional properties of their Fc regions.

[0005] Fc regions are also responsible for antibody immune effector functions, such as complement dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP). These effector functions rely on the interactions of antibody Fc regions with FcγRs. Therefore, engineering Fc regions to tune their interactions with FcγRs and C1q has emerged as a promising approach for enhancing the activity of therapeutic antibodies.

[0006] Accordingly, there exists a need for novel IgG Fc region mutations to improve various characteristics of feline, canine, and equine IgGs for use as safe therapeutic mAbs, specifically mutations that reduce, abolish or enhance immune effector function in cell-based CDC, ADCC, and ADCP assays.

SUMMARY OF THE INVENTION:

[0007] The ability to mediate cytotoxic and phagocytic effector functions are potent mechanisms by which antibodies destroy targeted cells. The Fc region links the recognition domain of antibodies to these effector functions through an interaction with Fc receptors and ligands. Manipulation of these effector functions by alteration of the Fc region has important

implications in the treatment of numerous medical conditions, for example cancer, autoimmune diseases and infectious diseases.

[0008] The Fc domain can be modified to obtain beneficial gain-of-function modifications, such as enhancing immune effector function on therapeutic mAbs targeting cancer cells, bacteria or viruses in order to augment killing/clearing of the cells/virus particles. However, in some cases, it can be beneficial to reduce or abolish antibody Fc function. These situations include antibodies that are used as receptor agonists to crosslink receptors and induce signaling, (receptor antagonists to block receptor:ligand interactions to prevent signaling, or drug delivery vehicles to deliver drugs or a drug to antigen-expressing target cells. In these instances, Fc engagement of receptors on effector cells or engagement of C1q is not wanted, because it can lead to undesired killing of biologically-important cells expressing the receptor or recruitment of drug-conjugated antibodies to off-target cells. Effector function null IgG is thus important for a number of antibody mechanisms of action in a wide range of disease areas. In addition, this is also important in Fc-fusion proteins and alternative antibody formats such as bi- or multi-specific antibodies. Several strategies to manipulate FcγRs binding of human antibodies and complement protein C1q binding, including changes to Fc sequence and glycosylation, are described in Saunders, Front Immunol., Article 1296, Volume 10, 7 June 2019.

[0009] The invention provides modified canine, modified feline, and modified equine Fc regions (compared to wt) with advantageous properties. It is known that canine the IgG1 and IgG4 subclasses are effector function deficient, while IgG2 and IgG3 are effector function proficient. However, IgG2 is the predominant IgG currently being used in therapeutic antibodies due to its other characteristics such as protein A binding capacity and relatively long half-life compared to other canine IgGs. Therefore, generating effector function null canine IgG2 is

attractive for certain therapeutic areas. Similarly, feline IgG1 has been the preferred IgG subclass for the development of therapeutic mAbs, such that generating effector function null feline IgG1 is also attractive for certain therapeutic areas. There are seven reported equine IgG subclasses, IgG1- IgG7, each with different characterization Protein A binding, pharmacokinetics, and effector function profiles, although IgG4 and IgG7 are very similar. All seven equine IgG subclasses possess some capability of triggering effector function(s), with the possible exception of IgG2. Thus, the different subclasses may require different sets of mutations to reduce effector functions.

[0010] Accordingly, the invention relates to modified recombinant feline, canine, and equine IgGs that exhibit desired characteristics, relative to wild-type IgGs. Specifically, the inventors of the instant application have found that substituting an amino acid residue at positions described herein with another amino acid surprisingly and unexpectedly produced a desired effect. In an exemplary embodiment, the unexpected, desired effects include, but are not limited to, enhanced affinity to FcRn, reduced complement-dependent cytotoxicity (CDC); reduced antibody-dependent cellular cytotoxicity (ADCC); reduced antibody-dependent cellular phagocytosis (ADCP); reduced or enhanced binding to Fc gamma receptor (bFcγR); or a combination thereof.

[0011] In one aspect, an Fc region of feline IgG1a may comprise one or more substitutions at positions 234, 235, 235, 236, 237, 237, 239, 329, 330, 331, 345, 265, 267, 268, 270, 270, 297, 298, 322, 324, 329, 330, 331, 332, 333, and/or 334 using EU Index numbering as in Kabat.

[0012] In another aspect, an Fc region of feline IgG1a may comprise one or more substitutions selected from among M234A, L235A, L235Y, G236A, G237A, G237W, S239D, P329G, P329S, S330A, P331A, P331S, E345R, D265A, G267E, P268F, D270G, D270S,

N297G, S298A, K322A, N324T, P329G, S330A, S330L, P331A, P331S, I332E, E333A, and R334A using EU Index numbering.

[0013] In another aspect, various combinations of substitutions in a Fc region of feline IgG1a can include one or more of the following, referred to herein by short designations: WIN (M234A, L235A, G237A), GSP (P329G), PSS (P331S), GAP (P329G, S330A), DANG (D265A, N297G), KA (K322A), PA (P331A), KAPA (K322A, P331A), WIN_PA (M234A, L235A, G237A, P331A), WIN_KA (M234A, L235A, G237A, K322A), WIN_KAPA (M234A, L235A, G237A, K322A, P331A), WIN_GAP (M234A, L235A, G237A, P329G, S330A), DANG-GAP (D265A, N297G, P329G, S330A), WIN_DANG (M234A, L235A, G237A, D265A, N297G), WIN_DANG_GAP (M234A, L235A, G237A, D265A, N297G, P329G, S330A), DANG_PSS (D265A, N297G, P331S), WIN_PSA (M234A, L235A, G237A, P331A), WIN_DANG_PSS (M234A, L235A, G237A, D265A, N297G, P331S), WIN_PSS (M234A, L235A, G237A, P331S), WIN_GSA (M234A, L235A, G237A, P329G, P331A), WIN_GSP (M234A, L235A, G237A, P329G), WIN_GSS (M234A, L235A, G237A, P329G, P331S), WIN_SAS (M234A, L235A, G237A, P329S, S330A, P331S), AAA (S298A, E333A, R334A), DLE (S239D, S330L, I332E), DE (S239D, I332E), DAE (G236A, S239D, I332E), YWA (L235Y, G237W, S298A), EFT (G267E, P268F, N324T), D270G (D270G), D270S (D270S), and E345R (E345R).

[0014] In another aspect, an Fc region of feline IgG1b may also comprise one or more substitutions or combinations of substitutions in positions corresponding to the positions in IgG1a described above. Of course, one will also recognize that canine and equine IgG of any subclass could contain one or more corresponding substitutions or combinations of substitutions in positions corresponding to the positions in feline IgG1a described above and that the short designations used above may be used in identifying those corresponding substitutions.

[0015] In another aspect, an Fc region of canine IgG2 may comprise one or more mutations, such at locations including 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 294, 295, 296, 297, 298, 299, 300, 301, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 422 423, and/or 441, using EU Index numbering as in Kabat.

[0016] In another aspect, an Fc region of canine IgG2 may comprise one or more substitutions listed in Table 5, and/or substitutions selected from among M234A, L235A, L235Y, G236A, G237A, G237W, S239D, D265A, D270G, D270S, N297G, G298A, K322A, P329G, S330A, S330L, P331A, I332E, E333A, R334A, using EU Index numbering as in Kabat.

[0017] In another aspect, an Fc region of canine IgG2 may comprise various combinations of substitutions in a Fc region of canine IgG2, which can include one or more of the following, referred to herein by short designations: GSP (P329G), GAP (P329G, S330A), DANG (D265A, N297G), DANG-GAP (D265A, N297G; P329G, S330A), WIN (M234A, L235A, G237A), KA (K322A), PA (P331A), KAPA (K322A, P331A), WIN_PA (M234A, L235A, G237A, P331A), WIN_KA (M234A, L235A, G237A, K322A), WIN_KAPA (M234A, L235A, G237A, K322A, P331A), WIN_DANG_GAP (M234A, L235A, G237A; D265A, N297G; P329G, S330A), AAA (G298A, E333A, R334A), DE (S239D, I332E), DLE (S239D, S330L, I332E), DAE (G236A, S239D, I332E), YWA (L235Y, G237W, G298A), EFT (D267E, P268F, N324T), Q345R (Q345R), D270G (D270G), and D270S (D270S).

[0018] In another aspect, an Fc region of equine IgG1 may comprise substitutions at one or more positions, including 229, 234, 235, 237, 329, 330, and/or 331, using EU Index numbering as in Kabat.

[0019] In another aspect, an Fc region of equine IgG1 may comprise one or more substitutions selected from among P229S, L234A, L235A, G237A, P329S, Q330A, Q330S, and P331S, using EU Index numbering as in Kabat.

[0020] In another aspect, an Fc region of equine IgG1 may comprise, various combinations of substitutions in a Fc region, including one or more of the following, referred to herein by short designations: SAP (P329S, Q330A), PAP (Q330A), SQP (P229S), WIN (L234A, L235A, G237A), KA (K322A), PA (P331A), KAPA (K322A, P331A), WIN_PA (M234A, L235A, G237A, P331A), WIN_KA (M234A, L235A, G237A, K322A), WIN_KAPA (M234A, L235A, G237A, K322A, P331A), PSS (Q330S, P331S), WIN_SAP (L234A, L235A, G237A; P329S, Q330A), and WIN_PSS (L234A, L235A, G237A; Q330S, P331S).

[0021] In another aspect, an Fc region of equine IgG4 may comprise one or more mutations and/or substitutions at positions selected from among 234, 235, 237, 239, 330, and/or 331.

[0022] In another aspect, an Fc region of equine IgG4 may comprise one or more substitutions selected from among L234A, Q235A, G237A, P239S, A330Q, A330S, P331S, using EU Index numbering as in Kabat.

[0023] In another aspect, an Fc region of equine IgG4 may comprise various combinations of substitutions in a Fc region of equine IgG4, which can include one or more of the following, referred to herein by short designations: PSS (A330S, P331S), SQP (P239S, A330Q), Win (L234A, Q235A, G237A), WinPSS (L234A, Q235A, G237A; A330S, P331S), SAP (P329S), and WinSAP (L234A, Q235A, G237A; P329S).

[0024] In another aspect, an Fc region of equine IgG7 may comprise one or more mutations and/or substitutions at positions selected from among 234, 235, 236, 237, 239, 330, and/or 331 , using EU Index numbering as in Kabat.

[0025] In another aspect, an Fc region of equine IgG7 may comprise one or more substitutions selected from among L234A, S235A, V236G, G237A, P239S, P329S, A330S, A330Q, P331S, using EU Index numbering as in Kabat.

[0026] In another aspect, an Fc region of equine IgG7 may comprise combinations of substitutions in a Fc region of equine IgG7, which can include one or more of the following, referred to herein by short designations: PSS (A330S, P331S), SAP (P329S), SQP (P239S, A330Q), WIN (L234A, S235A, V236G, G237A), WIN_PSS (L234A, S235A, V236G, G237A; A330S, P331S), and WIN_SAP (L234A, S235A, V236G, G237A; P329S).

[0027] In another aspect, the invention provides a recombinant polypeptide comprising a modified recombinant feline, canine, or equine IgG Fc region as described herein comprising one or more amino acid substitutions described herein.

[0028] In another aspect, the invention provides a recombinant antibody or a molecule comprising a feline, canine, or equine IgG Fc region as described herein comprising one or more amino acid substitutions described herein.

[0029] In another aspect, the invention provides a method for producing or manufacturing an antibody or a molecule, the method comprising: providing a vector or a host cell having a nucleic acid sequence that encodes an antibody, wherein said antibody comprises a feline, canine, or equine IgG constant domain comprising one or more amino acid substitutions described herein.

[0030] Other features and advantages of the present invention will become apparent from the following detailed description examples and figures. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within

the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0031] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[0032] **FIG. 1:** Cell-based complement-dependent cytotoxicity (CDC) activity of feline IgG1a, IgG1b and IgG2 Fc subclass CTLA4 fusion proteins.

[0033] **FIGS. 2A & 2B – 4:** Cell-based CDC activity of feline wild type Fc IgG1a subclass CTLA4 fusion proteins and Fc mutants of that subclass

[0034] **FIGS. 5-9:** Cell-based antibody-dependent cellular phagocytosis (ADCP) activity of feline wild type IgG1a Fc subclass CTLA4 fusion protein and Fc mutants of that subclass.

[0035] **FIGS. 10-12:** Cell-based antibody-dependent cellular cytotoxicity (ADCC) activity of feline wild type IgG1a Fc subclass CTLA4 fusion protein and Fc mutants of that subclass.

[0036] **FIG. 13:** Feline IgG1a Fc mutations investigated by the ADCC assay.

[0037] **FIGS. 14A – 14C:** Protein modeling of Feline IgG1a and IgG1b Fc regions. Fig. 14A shows an overlay of protein models of variations with a zoomed-in sub-panel showing amino acid residue positions in ball-and-stick form. Fig 14B shows root mean square deviation (RMSD) comparisons of wild-type constructs of feline IgG1a and IgG1b. Fig. 14C shows an overlay of protein models with G236, G267, P268, S298, N324, E333, R334 and E345 variations with a zoomed-in sub-panel showing the amino acid residue positions in ball-and-stick form.

[0038] **FIG. 15:** Cell-based CDC activity of canine IgG1 and IgG2 Fc subclass CTLA4 fusion proteins.

- [0039] **FIG. 16:** Cell-based CDC activity of canine wild type Fc IgG2 subclass CTLA4 fusion proteins and Fc mutants of that subclass.
- [0040] **FIGS. 17-21:** Cell-based ADCP activity of canine wild type IgG2 Fc subclass CTLA4 fusion protein and Fc mutants of that subclass.
- [0041] **FIGS. 22-24:** Cell-based antibody-dependent cellular cytotoxicity (ADCC) activity of canine wild type IgG2 Fc subclass CTLA4 fusion protein and Fc mutants of that subclass.
- [0042] **FIG. 25:** Canine IgG2 Fc mutations investigated by the ADCC assay.
- [0043] **FIG. 26:** Cell-based CDC activity of equine wild type Fc subclass CTLA4 fusion proteins.
- [0044] **FIG. 27:** Cell-based CDC activity of equine wild type Fc IgG1 subclass CTLA4 fusion proteins and Fc mutants of that subclass.
- [0045] **FIG. 28:** Cell-based CDC activity of equine wild type Fc IgG4 subclass CTLA4 fusion proteins and Fc mutants of that subclass.
- [0046] **FIG. 29:** Cell-based CDC activity of equine wild type Fc IgG7 subclass CTLA4 fusion proteins and Fc mutants of that subclass.
- [0047] **FIG. 30:** Cell-based ADCP activity of equine wild type Fc IgG1 subclass CTLA4 fusion proteins and Fc mutants of that subclass.
- [0048] **FIGS. 31-32:** Cell-based ADCP activity of equine wild type Fc IgG4 subclass CTLA4 fusion proteins and Fc mutants of that subclass.
- [0049] **FIGS. 33-34:** Cell-based ADCP activity of equine wild type Fc IgG7 subclass CTLA4 fusion proteins and Fc mutants of that subclass.
- [0050] **FIG 35:** Cell-based antibody-dependent cell-mediated cytotoxicity activity of equine wild type Fc subclass CTLA4 fusion proteins.

- [0051] **FIGS. 36-37:** Cell-based antibody-dependent cell-mediated cytotoxicity activity of equine wild type Fc IgG1 subclass CTLA4 fusion protein and Fc mutants of that subclass.
- [0052] **FIG. 38:** Cell-based antibody-dependent cell-mediated cytotoxicity activity of equine wild type Fc IgG4 subclass CTLA4 fusion protein and mutations of that Fc subclass.
- [0053] **FIG. 39:** Cell-based antibody-dependent cell-mediated cytotoxicity activity of equine wild type Fc IgG7 subclass CTLA4 fusion protein and mutations of that Fc subclass.
- [0054] **FIG. 40:** Protein modeling of equine FcRn and IgG1 Fc regions with a zoomed-in sub-panel showing amino acid residue positions are shown in ball-and-stick form.
- [0055] **FIG 41:** Protein modeling of equine FcRn, IgG4a-Fc and IgG4b-Fc regions with a zoomed-in sub-panel showing amino acid residue positions are shown in ball-and-stick form.
- [0056] **FIG. 42:** Protein modeling of Equine FcRn, IgG7a-Fc and IgG7b-Fc regions with a zoomed-in sub-panel showing amino acid residue positions are shown in ball-and-stick form.
- [0057] **FIG 43:** Root Mean Square Deviation (RMSD) comparisons of wild-type constructs of equine IgG1, IgG4a, IgG4b, IgG7a and IgG7b.
- [0058] **FIG. 44:** Cell-based CDC activity of feline wild type Fc IgG1a subclass and Fc mutants of that subclass.
- [0059] **FIGS. 45 and 46:** Cell-based CDC activity of feline wild type Fc IgG1b subclass and Fc mutants of that subclass.
- [0060] **FIG. 47:** Cell-based CDC activity of feline wild type Fc IgG2 subclass and Fc mutants of that subclass.
- [0061] **FIGS. 48 and 49:** Cell-based CDC activity of feline wild type Fc IgG3 subclass and Fc mutants of that subclass.
- [0062] **FIG. 50:** Cell-based CDC activity of feline IgG1a variants.

[0063] **FIGS. 51-61:** Cell-based CDC activity of canine wild type Fc IgG2 subclass and Fc mutants of that subclass.

[0064] **FIGS. 62 and 63:** Cell-based CDC activity of canine wild type Fc IgG3 subclass and Fc mutants of that subclass.

[0065] **FIG. 64:** Cell-based ADCP activity of canine wild type anti canine CD20 chimeric IgG mAb and Fc mutants of that subclass.

[0066] **FIGS. 65-92:** Cell-based ADCP activity of canine wild type IgG2 Fc subclass CTLA4 fusion protein and Fc mutants of that subclass.

[0067] **FIG. 93:** Cell-based ADCP activity of canine wild type anti canine CD20 chimeric IgG mAb and Fc mutants of that subclass.

[0068] **FIG. 94:** A schema for an ADCC assay is illustrated.

[0069] **FIGS. 95-98:** Cell-based canine FcRIII binding of canine wild type IgG2 Fc subclass antibody and Fc mutants of that subclass.

BRIEF DESCRIPTION OF THE SEQUENCES

[0070] The contents of the electronic sequence listing (sequencelisting_ST26.xml; Size: 82,432 bytes; and Date of Creation: February 1, 2024) is herein incorporated by reference in its entirety.

[0071] SEQ ID NO:1 is a sequence for a feline IgG1a heavy chain comprising a rituximab variable region and a GSP (P329G) mutation in the Fc region.

[0072] SEQ ID NO:2 is a sequence for a feline IgG1a heavy chain comprising a rituximab variable region and a GSP (P329G) mutation.

[0073] SEQ ID NO:3 is a sequence for feline IgG1a heavy chain comprising a rituximab variable region and a GAP (P329G,S330A) mutation in the Fc region.

[0074] SEQ ID NO:4 is a sequence for a feline IgG1a heavy chain comprising a rituximab variable region and a DANG-GAP (D265A, N297G; P329G, S330A) mutation in the Fc region.

[0075] SEQ ID NO:5 is a sequence for a feline IgG1a heavy chain comprising a rituximab variable region and a WIN_DANG_GAP (M234A, L235A, G237A; D265A, N297G; P329G, S330A) mutation in the Fc region.

[0076] SEQ ID NO:6 is a sequence for a feline IgG1a heavy chain comprising a rituximab variable region and a DANG (D265A, N297G) mutation in the Fc region.

[0077] SEQ ID NO:7 is a sequence for a feline IgG1a heavy chain comprising a rituximab variable region and a AAA (S298A, E333A, R334A) mutation in the Fc region.

[0078] SEQ ID NO:8 is a sequence for a feline IgG1a heavy chain comprising a rituximab variable region and a DLE (S239D, S330L, I332E) mutation in the Fc region.

[0079] SEQ ID NO:9 is a sequence for a feline IgG1a heavy chain comprising a rituximab variable region and a DE (S239D, I332E) mutation in the Fc region.

[0080] SEQ ID NO:10 is a sequence for a feline IgG1a heavy chain comprising a rituximab variable region and a DAE (G236A, S239D, I332E) mutation in the Fc region.

[0081] SEQ ID NO:11 is a sequence for a feline IgG1a heavy chain comprising a rituximab variable region and a YWA (L235Y, G237W, S298A) mutation in the Fc region.

[0082] SEQ ID NO:12 is a sequence for a feline IgG1a heavy chain comprising a rituximab variable region and a EFT (G267E, P268F, N324T) mutation in the Fc region.

[0083] SEQ ID NO:13 is a sequence for a feline IgG1a heavy chain comprising a rituximab variable region and a E345R (E345R) mutation in the Fc region.

[0084] SEQ ID NO:14 is a sequence for a feline IgG1a heavy chain comprising a canine CTLA-4 peptide fused to a feline Fc region having a WIN_DANG (M234A, L235A, G237A; D265A, N297G) mutation.

[0085] SEQ ID NO:15 is a sequence for a feline IgG1a heavy chain comprising a canine CTLA-4 peptide fused to a feline Fc region having a WIN_DANG_PSS (M234A, L235A, G237A; D265A, N297G; P331S) mutation.

[0086] SEQ ID NO:16 is a sequence for a feline IgG1a heavy chain comprising a canine CTLA-4 peptide fused to a feline Fc region having a DANG_PSS (D265A, N297G; P331S) mutation.

[0087] SEQ ID NO:17 is a sequence for a feline IgG1a heavy chain comprising a canine CTLA-4 peptide fused to a feline Fc region having a WIN (M234A, L235A, G237A) mutation.

[0088] SEQ ID NO:18 is a sequence for a feline IgG1a heavy chain comprising a canine CTLA-4 peptide fused to a feline Fc region having a D270G mutation.

[0089] SEQ ID NO:19 is a sequence for a feline IgG1a heavy chain comprising a canine CTLA-4 peptide fused to a feline Fc region having a D270S mutation.

[0090] SEQ ID NO:20 is a sequence for a feline IgG1a heavy chain comprising a canine CTLA-4 peptide fused to a feline Fc region having a WIN_PSA (M234A, L235A, G237A; P331A) mutation.

[0091] SEQ ID NO:21 is a sequence for a feline IgG1a heavy chain comprising a canine CTLA-4 peptide fused to a feline Fc region having a WIN_GSA (M234A, L235A, G237A; P329G, P331A) mutation.

- [0092] SEQ ID NO:22 is a sequence for a feline IgG1a heavy chain comprising a canine CTLA-4 peptide fused to a feline Fc region having a WIN_GSP (M234A, L235A, G237A; P329G) mutation.
- [0093] SEQ ID NO:23 is a sequence for a feline IgG1a heavy chain comprising a canine CTLA-4 peptide fused to a feline Fc region having a WIN_PSS (M234A, L235A, G237A; P331S) mutation.
- [0094] SEQ ID NO:24 is a sequence for a feline IgG1a heavy chain comprising a canine CTLA-4 peptide fused to a feline Fc region having a PSS (P331S) mutation.
- [0095] SEQ ID NO:25 is a sequence for a feline IgG1a heavy chain comprising a canine CTLA-4 peptide fused to a feline Fc region having a WIN_GSS (M234A, L235A, G237A; P329G, P331S) mutation.
- [0096] SEQ ID NO:26 is a sequence for a feline IgG1a heavy chain comprising a canine CTLA-4 peptide fused to a feline Fc region having a WIN_GAP (M234A,L235A,G237A; P329G, S330A) mutation.
- [0097] SEQ ID NO:27 is a sequence for a feline IgG1a heavy chain comprising a canine CTLA-4 peptide fused to a feline Fc region having a WIN_GAP (M234A,L235A,G237A; P329G, S330A) mutation.
- [0098] SEQ ID NO:28 is a sequence for a canine IgG2 heavy chain comprising a rituximab variable region and a GSP (P329G) mutation in the Fc region.
- [0099] SEQ ID NO:29 is a sequence for a canine IgG2 heavy chain comprising a rituximab variable region and a GAP (P329G, S330A) mutation in the Fc region.
- [00100] SEQ ID NO:30 is a sequence for a canine IgG2 heavy chain comprising a rituximab variable region and a DANG-GAP (D265A, N297G; P329G, S330A) mutation in the Fc region.

[00101] SEQ ID NO:31 is a sequence for a canine IgG2 heavy chain comprising a rituximab variable region and a KAPA (K322A, P331A) mutation in the Fc region.

[00102] SEQ ID NO:32 is a sequence for a canine IgG2 heavy chain comprising a rituximab variable region and a WIN_DANG_GAP (M234A, L235A, G237A; D265A, N297G; P329G, S330A) mutation in the Fc region.

[00103] SEQ ID NO:33 is a sequence for a canine IgG2 heavy chain comprising a rituximab variable region and a DANG (D265A, N297G) mutation in the Fc region.

[00104] SEQ ID NO:34 is a sequence for a canine IgG2 heavy chain comprising a rituximab variable region and a AAA (G298A, E333A, R334A) mutation in the Fc region.

[00105] SEQ ID NO:35 is a sequence for a canine IgG2 heavy chain comprising a rituximab variable region and a DLE (S239D, S330L, I332E) mutation in the Fc region.

[00106] SEQ ID NO:36 is a sequence for a canine IgG2 heavy chain comprising a rituximab variable region and a DE (S239D, I332E) mutation in the Fc region.

[00107] SEQ ID NO:37 is a sequence for a canine IgG2 heavy chain comprising a rituximab variable region and a DAE (G236A, S239D, I332E) mutation in the Fc region.

[00108] SEQ ID NO:38 is a sequence for a canine IgG2 heavy chain comprising a rituximab variable region and a YWA (L235Y, G237W, G298A) mutation in the Fc region.

[00109] SEQ ID NO:39 is a sequence for a canine IgG2 heavy chain comprising a rituximab variable region and a EFT (D267E, P268F, N324T) mutation in the Fc region.

[00110] SEQ ID NO:40 is a sequence for a canine IgG2 heavy chain comprising a rituximab variable region and a Q345R mutation in the Fc region.

[00111] SEQ ID NO:41 is a sequence for a canine IgG2 heavy chain comprising a canine CTLA-4 peptide fused to a canine Fc region having a D270G mutation.

- [00112] SEQ ID NO:42 is a sequence for a canine IgG2 heavy chain comprising a canine CTLA-4 peptide fused to a canine Fc region having a D270S mutation.
- [00113] SEQ ID NO:43 is a sequence for a canine IgG2 heavy chain comprising a canine CTLA-4 peptide fused to a canine Fc region having a WIN (M234A, L235A, G237A) mutation.
- [00114] SEQ ID NO:44 is a sequence for a canine IgG2 heavy chain comprising a canine CTLA-4 peptide fused to a canine Fc region having a WIN_DANG_GAP (M234A, L235A, G237A; D265A, N297G; P329G, S330A) mutation.
- [00115] SEQ ID NO:45 is a sequence for a equine IgG1 heavy chain comprising a rituximab variable region and a wild type Fc region.
- [00116] SEQ ID NO:46 is a sequence for a equine IgG1 heavy chain comprising a rituximab variable region and a SAP (P329S, Q330A) mutation in the Fc region.
- [00117] SEQ ID NO:47 is a sequence for a equine IgG1 heavy chain comprising a rituximab variable region and a WIN (L234A, L235A, G237A) mutation in the Fc region.
- [00118] SEQ ID NO:48 is a sequence for a equine IgG1 heavy chain comprising a rituximab variable region and a PSS (Q330S, P331S) mutation in the Fc region.
- [00119] SEQ ID NO:49 is a sequence for a equine IgG1 heavy chain comprising a rituximab variable region and a WIN_SAP (L234A, L235A, G237A; P329S, Q330A) mutation in the Fc region.
- [00120] SEQ ID NO:50 is a sequence for a equine IgG1 heavy chain comprising a rituximab variable region and a WIN_PSS (L234A, 235A, G237A; Q330S, P331S) mutation in the Fc region.
- [00121] SEQ ID NO:51 is a sequence for an equine IgG1 heavy chain comprising a canine CTLA-4 peptide fused to an equine Fc region having a PAP (Q330A) mutation.

[00122] SEQ ID NO:52 is a sequence for an equine IgG1 heavy chain comprising a canine CTLA-4 peptide fused to an equine Fc region having a SQP (P229S) mutation.

[00123] SEQ ID NO:53 is a sequence for an equine IgG4 heavy chain comprising a canine CTLA-4 peptide fused to an equine Fc region having a PSS (A330S, P331S) mutation.

[00124] SEQ ID NO:54 is a sequence for an equine IgG4 heavy chain comprising a canine CTLA-4 peptide fused to an equine Fc region having a SQP (P239S, A330Q) mutation.

[00125] SEQ ID NO:55 is a sequence for an equine IgG4 heavy chain comprising a canine CTLA-4 peptide fused to an equine Fc region having a WIN_PSS (L234A, Q235A, G237A; A330S, P331S) mutation.

[00126] SEQ ID NO:56 is a sequence for an equine IgG4 heavy chain comprising a canine CTLA-4 peptide fused to an equine Fc region having a WIN_SAP (L234A, Q235A, G237A; P329S) mutation.

[00127] SEQ ID NO:57 is a sequence for an equine IgG4 heavy chain comprising a canine CTLA-4 peptide fused to an equine Fc region having a SAP (P329S) mutation.

[00128] SEQ ID NO:58 is a sequence for an equine IgG4 heavy chain comprising a canine CTLA-4 peptide fused to an equine Fc region having a WIN (L234A, Q235A, G237A) mutation.

[00129] SEQ ID NO:59 is a sequence for an equine IgG7 heavy chain comprising a canine CTLA-4 peptide fused to an equine Fc region having a PSS (A330S, P331S) mutation.

[00130] SEQ ID NO:60 is a sequence for an equine IgG7 heavy chain comprising a canine CTLA-4 peptide fused to an equine Fc region having a SAP (P329S) mutation.

[00131] SEQ ID NO:61 is a sequence for an equine IgG7 heavy chain comprising a canine CTLA-4 peptide fused to an equine Fc region having a SQP (P239S, A330Q) mutation.

[00132] SEQ ID NO:62 is a sequence for an equine IgG7 heavy chain comprising a canine CTLA-4 peptide fused to an equine Fc region having a WIN (L234A, S235A, V236G, G237A) mutation.

[00133] SEQ ID NO:63 is a sequence for an equine IgG7 heavy chain comprising a canine CTLA-4 peptide fused to an equine Fc region having a WIN_PSS (L234A, S235A, V236G, G237A; A330S, P331S) mutation.

[00134] SEQ ID NO:64 is a sequence for an equine IgG7 heavy chain comprising a canine CTLA-4 peptide fused to an equine Fc region having a WIN_SAP (L234A, S235A, V236G, G237A; P329S) mutation.

DETAILED DESCRIPTION:

[00135] The present subject matter may be understood more readily by reference to the following detailed description which forms a part of this disclosure. It is to be understood that this invention is not limited to the specific products, methods, conditions, or parameters described and/or shown herein, and that the terminology used herein is for the purpose of describing particular embodiments by way of example only and is not intended to be limiting of the claimed invention.

[00136] Unless otherwise defined herein, scientific and technical terms used in connection with the present application shall have meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular.

[00137] In the present disclosure the singular forms “a,” “an,” and “the” include the plural reference, and reference to a particular numerical value includes at least that particular value, unless the context clearly indicates otherwise. Thus, for example, a reference to “a molecule” or “a compound” is a reference to one or more of such molecules or compounds and equivalents thereof known to those skilled in the art, and so forth. The term “plurality”, as used herein, means more than one. When a range of values is expressed, another embodiment includes from the one particular and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent “about,” it is understood that the particular value forms another embodiment. All ranges are inclusive and combinable.

[00138] In the specification and claims, the numbering of the amino acid residues in an immunoglobulin heavy chain is that of the Eu index as in Kabat *et al.*, Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991). The “Eu index as in Kabat” refers to the residue numbering of the IgG antibody.

[00139] The term “isolated” when used in relation to a nucleic acid is a nucleic acid that is identified and separated from at least one contaminant nucleic acid with which it is ordinarily associated in its natural source. Isolated nucleic acid is in a form or setting different from that in which it is found in nature. Isolated nucleic acid molecules therefore are distinguished from the nucleic acid molecule as it exists in natural cells. An isolated nucleic acid molecule includes a nucleic acid molecule contained in cells that ordinarily express the polypeptide encoded therein where, for example, the nucleic acid molecule is in a plasmid or a chromosomal location different from that of natural cells. The isolated nucleic acid may be present in single-stranded or double-stranded form. When an isolated nucleic acid molecule is to be utilized to express a protein, the oligonucleotide or polynucleotide will contain at a minimum the sense or coding strand, but may contain both the sense and anti-sense strands (*i.e.*, may be double-stranded).

[00140] A nucleic acid molecule is “operably linked” or “operably attached” when it is placed into a functional relationship with another nucleic acid molecule. For example, a promoter or enhancer is operably linked to a coding sequence of nucleic acid if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence of nucleic acid if it is positioned so as to facilitate translation. A

nucleic acid molecule encoding a variant Fc region is operably linked to a nucleic acid molecule encoding a heterologous protein (*i.e.*, a protein or functional fragment thereof which does not, as it exists in nature, comprise an Fc region) if it is positioned such that the expressed fusion protein comprises the heterologous protein or functional fragment thereof adjoined either upstream or downstream to the variant Fc region polypeptide; the heterologous protein may be immediately adjacent to the variant Fc region polypeptide or may be separated therefrom by a linker sequence of any length and composition. Likewise, a polypeptide (used synonymously herein with “protein”) molecule is “operably linked” or “operably attached” when it is placed into a functional relationship with another polypeptide.

[00141] As used herein the term “functional fragment” when in reference to a polypeptide or protein (*e.g.*, a variant Fc region, or a monoclonal antibody) refers to fragments of that protein which retain at least one function of the full-length polypeptide. The fragments may range in size from six amino acids to the entire amino acid sequence of the full-length polypeptide minus one amino acid. A functional fragment of a variant Fc region polypeptide of the present invention retains at least one “amino acid substitution” as herein defined. A functional fragment of a variant Fc region polypeptide retains at least one function known in the art to be associated with the Fc region (*e.g.*, ADCC, CDC, Fc receptor binding, C1q binding, down regulation of cell surface receptors or may, *e.g.*, increase the *in vivo* or *in vitro* half-life of a polypeptide to which it is operably attached).

[00142] The term “purified” or “purify” refers to the substantial removal of at least one contaminant from a sample. For example, an antigen-specific antibody may be

purified by complete or substantial removal (at least 90%, 91%, 92%, 93%, 94%, 95%, or more preferably at least 96%, 97%, 98% or 99%) of at least one contaminating non-immunoglobulin protein; it may also be purified by the removal of immunoglobulin protein that does not bind to the same antigen. The removal of non-immunoglobulin proteins and/or the removal of immunoglobulins that do not bind a particular antigen results in an increase in the percent of antigen-specific immunoglobulins in the sample. In another example, a polypeptide (*e.g.*, an immunoglobulin) expressed in bacterial host cells is purified by the complete or substantial removal of host cell proteins; the percent of the polypeptide is thereby increased in the sample.

[00143] The term “native” as it refers to a polypeptide (*e.g.*, Fc region) is used herein to indicate that the polypeptide has an amino acid sequence consisting of the amino acid sequence of the polypeptide as it commonly occurs in nature or a naturally occurring polymorphism thereof. A native polypeptide (*e.g.*, native Fc region) may be produced by recombinant means or may be isolated from a naturally occurring source.

[00144] The term “expression vector” as used herein refers to a recombinant DNA molecule containing a desired coding sequence and appropriate nucleic acid sequences necessary for the expression of the operably linked coding sequence in a particular host organism.

[00145] As used herein, the term “host cell” refers to any eukaryotic or prokaryotic cell (*e.g.*, bacterial cells such as *E.coli*, CHO cells, yeast cells, mammalian cells, avian cells, amphibian cells, plant cells, fish cells, and insect cells), whether located in vitro or in situ, or in vivo.

[00146] As used herein, the term “Fc region” refers to a C-terminal region of an immunoglobulin heavy chain. The “Fc region” may be a native sequence Fc region or a variant Fc region. Although the generally accepted boundaries of the Fc region of an immunoglobulin heavy chain might vary, the IgG heavy chain Fc region is usually defined to stretch, for example, from an amino acid residue at about position 231, to the carboxyl-terminus thereof.

[00147] In some embodiments, variants comprise only portions of the Fc region and can include or not include the carboxy-terminus. The Fc region of an immunoglobulin generally comprises two constant domains, CH2 and CH3. In some embodiments, variants having one or more of the constant domains are contemplated. In other embodiments, variants without such constant domains (or with only portions of such constant domains) are contemplated.

[00148] The “CH2 domain” of an IgG Fc region usually extends, for example, from about amino acid 231 to about amino acid 340. The CH2 domain is unique in that it is not closely paired with another domain. Two N-linked branched carbohydrate chains are interposed between the two CH2 domains of an intact native IgG molecule.

[00149] The “CH3 domain” of a feline IgG Fc region generally is the stretch of residues C-terminal to a CH2 domain in an Fc region extending, for example, from about amino acid residue 341 to about amino acid residue 447.

[00150] A “functional Fc region” possesses an “effector function” of a native sequence Fc region. At least one effector function of a polypeptide comprising a variant Fc region of the present invention may be enhanced or diminished with respect to a polypeptide comprising a native Fc region or the parent Fc region of the variant. Examples of

effector functions include, but are not limited to: C1q binding; complement dependent cytotoxicity (CDC); Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (*e.g.*, B cell receptor; BCR), etc. Such effector functions may require the Fc region to be operably linked to a binding domain (*e.g.*, an antibody variable domain) and can be assessed using various assays (*e.g.*, Fc binding assay, ADCC assays, CDC assays, target cell depletion from whole or fractionated blood samples, etc.).

[00151] A “native sequence Fc region” or “wild type Fc region” refers to an amino acid sequence that is identical to the amino acid sequence of an Fc region commonly found in nature.

[00152] A “variant Fc region” comprises an amino acid sequence that differs from that of a native sequence Fc region (or fragment thereof) by virtue of at least one “amino acid substitution” as defined herein. In preferred embodiments, the variant Fc region has at least one amino acid substitution compared to a native sequence Fc region or in the Fc region of a parent polypeptide, preferably 1, 2, 3, 4 or 5 amino acid substitutions in a native sequence Fc region or in the Fc region of the parent polypeptide. In an alternative embodiment, a variant Fc region may be generated according to the methods herein disclosed and this variant Fc region can be fused to a heterologous polypeptide of choice, such as an antibody variable domain or a non- antibody polypeptide, *e.g.*, binding domain of a receptor or ligand.

[00153] As used herein, the term “derivative” in the context of polypeptides refers to a polypeptide that comprises an amino acid sequence which has been altered by introduction of an amino acid residue substitution. The term “derivative” as used

herein also refers to a polypeptide which has been modified by the covalent attachment of any type of molecule to the polypeptide. For example, but not by way of limitation, an antibody may be modified, *e.g.*, by glycosylation, acetylation, pegylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, etc. A derivative polypeptide may be produced by chemical modifications using techniques known to those of skill in the art, including, but not limited to specific chemical cleavage, acetylation, formylation, metabolic synthesis of tunicamycin, etc. Further, a derivative polypeptide possesses a similar or identical function as the polypeptide from which it was derived. It is understood that a polypeptide comprising a variant Fc region of the present invention may be a derivative as defined herein, preferably the derivatization occurs within the Fc region.

[00154] “Substantially of feline origin” as used herein in reference to a polypeptide (*e.g.*, an Fc region or a monoclonal antibody), indicates the polypeptide has an amino acid sequence at least 80%, at least 85%, more preferably at least 90%, 91%, 92%, 93%, 94% or even more preferably at least 95%, 95%, 97%, 98% or 99% homologous to that of a native feline amino polypeptide. “Substantially of canine origin” and “substantially of equine origin” are similarly defined with respect to a native canine or equine polypeptide, respectively.

[00155] The terms “Fc receptor” or “FcR” are used to describe a receptor that binds to an Fc region (*e.g.*, the Fc region of an antibody). The preferred FcR is a native sequence FcR. Moreover, a preferred FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the Fc gamma RI, Fc gamma RII, Fc gamma RIi subclasses,

including allelic variants and alternatively spliced forms of these receptors. Another preferred FcR includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer et al., J. Immunol. 117:587 (1976) and Kim et al., J. Immunol. 24:249 (1994)). Other FcRs, including those to be identified in the future, are encompassed by the term “FcR” herein.

[00156] The phrases “complement-dependent cytotoxicity” and “CDC” refer to an effector function of IgG and IgM antibodies. When IgG are bound to surface antigen on target cell (*e.g.*, bacterial or viral infected cell), the classical complement pathway is triggered by bonding protein C1q to these antibodies, resulting in formation of a membrane attack complex (MAC) and target cell lysis.

[00157] “C1q” is a polypeptide that includes a binding site for the Fc region of an immunoglobulin. C1q together with two serine proteases, C1r and C1s, forms the complex C1, the first component of the CDC pathway.

[00158] The phrase “antibody-dependent cell-mediated cytotoxicity” and “ADCC” refer to a cell-mediated reaction in which nonspecific cytotoxic cells (*e.g.*, nonspecific) that express FcRs (*e.g.*, Natural Killer (“NK”) cells, neutrophils, and macrophages) recognize bound antibody on a target cell and subsequently cause lysis of the target cells. The primary cells for mediating ADCC, NK cells, express Fc gamma RIII only, whereas monocytes express Fc gamma RI, Fc gamma RII and Fc gamma RIII.

[00159] As used herein, the phrase “effector cells” refers to leukocytes which express one or more FcRs and perform effector functions. Preferably, the cells express at least Fc gamma RIi and perform ADCC effector function. Examples of leukocytes which

mediate ADCC include PBMC, NK cells, monocytes, cytotoxic T cells and neutrophils.

The effector cells may be isolated from a native source (*e.g.*, from blood or PBMCs).

[00160] A variant polypeptide with “altered” FcRn binding affinity is one which has either enhanced (*i.e.*, increased, greater or higher) or diminished (*i.e.*, reduced, decreased or lesser) FcRn binding affinity compared to the variant’s parent polypeptide or to a polypeptide comprising a native Fc region when measured at pH 6.0. A variant polypeptide which displays increased binding or increased binding affinity to an FcRn binds FcRn with greater affinity than the parent polypeptide. A variant polypeptide which displays decreased binding or decreased binding affinity to an FcRn, binds FcRn with lower affinity than its parent polypeptide. Such variants which display decreased binding to an FcRn may possess little or no appreciable binding to an FcRn, *e.g.*, 0-20% binding to the FcRn compared to a parent polypeptide. A variant polypeptide which binds an FcRn with "enhanced affinity" as compared to its parent polypeptide, is one which binds FcRn with higher binding affinity than the parent polypeptide, when the amounts of variant polypeptide and parent polypeptide in a binding assay are essentially the same, and all other conditions are identical. For example, a variant polypeptide with enhanced FcRn binding affinity may display from about 1.10 fold to about 100 fold (more typically from about 1.2 fold to about 50 fold) increase in FcRn binding affinity compared to the parent polypeptide, where FcRn binding affinity is determined, for example, in an ELISA assay or other method available to one of ordinary skill in the art.

[00161] As used herein, an “amino acid substitution” refers to the replacement of at least one existing amino acid residue in a given amino acid sequence with another

different “replacement” amino acid residue. The replacement residue or residues may be “naturally occurring amino acid residues” (*i.e.*, encoded by the genetic code) and selected from: alanine (Ala); arginine (Arg); asparagine (Asn); aspartic acid (Asp); cysteine (Cys); glutamine (Gln); glutamic acid (Glu); glycine (Gly); histidine (His); isoleucine (Ile); leucine (Leu); lysine (Lys); methionine (Met); phenylalanine (Phe); proline (Pro); serine (Ser); threonine (Thr); tryptophan (Trp); tyrosine (Tyr); and valine (Val). Substitution with one or more non-naturally occurring amino acid residues is also encompassed by the definition of an amino acid substitution herein. A “non-naturally occurring amino acid residue” refers to a residue, other than those naturally occurring amino acid residues listed above, which is able to covalently bind adjacent amino acid residues (s) in a polypeptide chain. Examples of non-naturally occurring amino acid residues include norleucine, ornithine, norvaline, homoserine and other amino acid residue analogues such as those described in Ellman et al. *Meth. Enzym.* 202: 301-336 (1991).

[00162] The term “assay signal” refers to the output from any method of detecting protein-protein interactions, including but not limited to, SPR assays, absorbance measurements from colorimetric assays, fluorescent intensity, or disintegrations per minute. Assay formats could include ELISA, FACS, SPR, or other methods. A change in the “assay signal” may reflect a change in cell viability and/or a change in the kinetic off-rate, the kinetic on-rate, or both. A “higher assay signal” refers to the measured output number being larger than another number (*e.g.*, a variant may have a higher (larger) measured number in an ELISA assay as compared to the parent polypeptide). A “lower” assay signal refers to the measured output number being

smaller than another number (*e.g.*, a variant may have a lower (smaller) measured number in an ELISA assay as compared to the parent polypeptide).

[00163] The term “binding affinity” refers to the equilibrium dissociation constant (expressed in units of concentration) associated with each Fc receptor-Fc binding interaction. The binding affinity is directly related to the ratio of the kinetic off-rate (generally reported in units of inverse time, *e.g.*, seconds⁻¹) divided by the kinetic on-rate (generally reported in units of concentration per unit time, *e.g.*, molar/second). In general it is not possible to unequivocally state whether changes in equilibrium dissociation constants are due to differences in on-rates, off-rates or both unless each of these parameters are experimentally determined (*e.g.*, by BIACORE or SAPIDYNE measurements).

[00164] As used herein, the term “hinge region” refers to the stretch of amino acids, for example, in feline IgG1a (*e.g.*, stretching from position 216 to position 230 of feline IgG1a). Hinge regions of other IgG isotypes may be aligned with the IgG sequence by placing the first and last cysteine residues forming inter-heavy chain disulfide (S-S) bonds in the same positions.

[00165] As used herein, the term “antibody” is used interchangeably with “immunoglobulin” or “Ig,” and is used in the broadest sense and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (*e.g.*, bispecific antibodies), and antibody fragments so long as they exhibit the desired biological activity or functional activity. Single chain antibodies, and chimeric, feline, or felinized antibodies, canine or caninized, or equine or equinized, as well as chimeric or CDR-grafted single chain

antibodies, and the like, comprising portions derived from different species, are also encompassed by the present invention and the term “antibody”. The various portions of these antibodies can be joined together chemically by conventional techniques, synthetically, or can be prepared as a contiguous protein using genetic engineering techniques. For example, nucleic acids encoding a chimeric or felinized chain can be expressed to produce a contiguous protein. *See, e.g.*, U.S. Pat. No. 4,816,567; U.S. Pat. No. 4,816,397; WO 86/01533; U.S. Pat. No. 5,225,539; and U.S. Pat. Nos. 5,585,089 and 5,698,762. See also, Newman, R. et al. *BioTechnology*, 10: 1455-1460, 1993, regarding primatized antibody, and Ladner et al., U.S. Pat. No. 4,946,778 and Bird, R. E. et al., *Science*, 242:423-426, 1988, regarding single chain antibodies. It is understood that all forms of the antibodies comprising an Fc region (or portion thereof) are encompassed herein within the term “antibody.” Furthermore, the antibody may be labeled with a detectable label, immobilized on a solid phase and/or conjugated with a heterologous compound (*e.g.*, an enzyme or toxin) according to methods known in the art.

[00166] As used herein, the term “antibody fragments” refers to a portion of an intact antibody. Examples of antibody fragments include, but are not limited to, linear antibodies; single-chain antibody molecules; Fc or Fc’ peptides, Fab and Fab fragments, and multispecific antibodies formed from antibody fragments. The antibody fragments preferably retain at least part of the hinge and optionally the CHI region of an IgG heavy chain. In other preferred embodiments, the antibody fragments comprise at least a portion of the CH2 region or the entire CH2 region.

[00167] As used herein, the term “functional fragment”, when used in reference to a monoclonal antibody, is intended to refer to a portion of the monoclonal antibody that still retains a functional activity. A functional activity can be, for example, antigen binding activity or specificity, receptor binding activity or specificity, effector function activity and the like. Monoclonal antibody functional fragments include, for example, individual heavy or light chains and fragments thereof, such as VL, VH and Fd; monovalent fragments, such as Fv, Fab, and Fab’; bivalent fragments such as F(ab’)₂; single chain Fv (scFv); and Fc fragments. Such terms are described in, for example, Harlowe and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York (1989); Molec. Biology and Biotechnology: A Comprehensive Desk Reference (Myers, R. A. (ed.), New York: VCH Publisher, Inc.); Huston et al., *Cell Biophysics*, 22:189-224 (1993); Pluckthun and Skerra, *Meth. Enzymol.*, 178:497-515 (1989) and in Day, E. D., *Advanced Immunochemistry*, Second Ed., Wiley-Liss, Inc., New York, N.Y. (1990). The term functional fragment is intended to include, for example, fragments produced by protease digestion or reduction of a monoclonal antibody and by recombinant DNA methods known to those skilled in the art.

[00168] As used herein, the term “fragment” refers to a polypeptide comprising an amino acid sequence of at least 5, 15, 20, 25, 40, 50, 70, 90, 100 or more contiguous amino acid residues of the amino acid sequence of another polypeptide. In a preferred embodiment, a fragment of a polypeptide retains at least one function of the full-length polypeptide.

[00169] As used herein, the term “chimeric antibody” includes monovalent, divalent or polyvalent immunoglobulins. A monovalent chimeric antibody is a dimer formed by

a chimeric heavy chain associated through disulfide bridges with a chimeric light chain. A divalent chimeric antibody is a tetramer formed by two heavy chain-light chain dimers associated through at least one disulfide bridge. A chimeric feline heavy chain of an antibody comprises an antigen-binding region derived from the heavy chain of a non-feline antibody, which is linked to at least a portion of a feline heavy chain constant region, such as CH1 or CH2. A chimeric light chain of a feline antibody comprises an antigen binding region derived from the light chain of a non-feline antibody, linked to at least a portion of a feline light chain constant region (CL). Chimeric canine and equine antibodies are similarly defined.

[00170] Antibodies, fragments or derivatives having chimeric heavy chains and light chains of the same or different variable region binding specificity, can also be prepared by appropriate association of the individual polypeptide chains, according to known method steps. With this approach, hosts expressing chimeric heavy chains are separately cultured from hosts expressing chimeric light chains, and the immunoglobulin chains are separately recovered and then associated. Alternatively, the hosts can be co-cultured and the chains allowed to associate spontaneously in the culture medium, followed by recovery of the assembled immunoglobulin or fragment or both the heavy and light chains can be expressed in the same host cell. Methods for producing chimeric antibodies are well known in the art (*see, e.g.*, U.S. Pat. Nos. 6,284,471; 5,807,715; 4,816,567; and 4,816,397).

[00171] As used herein, “felinized” forms of non-feline (*e.g.*, murine) antibodies (*i.e.*, felinized antibodies) are antibodies that contain minimal sequence, or no sequence, derived from non-feline immunoglobulin. For the most part, felinized antibodies are

feline immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-feline species (donor antibody) such as mouse, rat, rabbit, human or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the feline immunoglobulin are replaced by corresponding non-feline residues. Furthermore, felinized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are generally made to further refine antibody performance. In general, the felinized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops (CDRs) correspond to those of a non-feline immunoglobulin and all or substantially all of the FR residues are those of a feline immunoglobulin sequence. The felinized antibody may also comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a feline immunoglobulin.

[00172] As used herein, “caninized” forms of non-canine (*e.g.*, murine) antibodies (*i.e.*, caninized antibodies) are antibodies that contain minimal sequence, or no sequence, derived from non-canine immunoglobulin. For the most part, caninized antibodies are canine immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-canine species (donor antibody) such as mouse, rat, rabbit, human or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the canine immunoglobulin are replaced by corresponding non-canine residues. Furthermore, caninized antibodies may comprise

residues that are not found in the recipient antibody or in the donor antibody. These modifications are generally made to further refine antibody performance. In general, the caninized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops (CDRs) correspond to those of a non-canine immunoglobulin and all or substantially all of the FR residues are those of a canine immunoglobulin sequence. The caninized antibody may also comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a canine immunoglobulin.

[00173] As used herein, “equinized” forms of non-equine (*e.g.*, murine) antibodies (*i.e.*, equinized antibodies) are antibodies that contain minimal sequence, or no sequence, derived from non-equine immunoglobulin. For the most part, equinized antibodies are equine immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-equine species (donor antibody) such as mouse, rat, rabbit, human or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the equine immunoglobulin are replaced by corresponding non-equine residues. Furthermore, equinized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are generally made to further refine antibody performance. In general, the equinized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops (CDRs) correspond to those of a non-equine immunoglobulin and all or substantially all of the FR residues are those of a equine immunoglobulin sequence. The equinized antibody

may also comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a equine immunoglobulin.

[00174] As used herein, the term “immunoadhesin” designates antibody-like molecules which combine the binding domain of a heterologous “adhesin” protein (*e.g.*, a receptor, ligand or enzyme) with an immunoglobulin constant domain. Structurally, immunoadhesins comprise a fusion of the adhesin amino acid sequence with the desired binding specificity which is other than the antigen recognition and binding site (antigen combining site) of an antibody (*i.e.*, is “heterologous”) with an immunoglobulin constant domain sequence.

[00175] As used herein, the term “ligand binding domain” refers to any native receptor or any region or derivative thereof retaining at least a qualitative ligand binding ability of a corresponding native receptor. In certain embodiments, the receptor is from a cell-surface polypeptide having an extracellular domain that is homologous to a member of the immunoglobulin supergenefamily. Other receptors, which are not members of the immunoglobulin supergenefamily but are nonetheless specifically covered by this definition, are receptors for cytokines, and in particular receptors with tyrosine kinase activity (receptor tyrosine kinases), members of the hematopoietin and nerve growth factor receptor superfamilies, and cell adhesion molecules (*e.g.*, E-, L-, and P-selectins).

[00176] As used herein, the term “receptor binding domain” refers to any native ligand for a receptor, including, *e.g.*, cell adhesion molecules, or any region or derivative of such native ligand retaining at least a qualitative receptor binding ability of a corresponding native ligand.

[00177] As used herein, an “isolated” polypeptide is one that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In certain embodiments, the isolated polypeptide is purified (1) to greater than 95% by weight of polypeptides as determined by the Lowry method, and preferably, more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-page under reducing or nonreducing conditions using Coomassie blue or silver stain. Isolated polypeptide includes the polypeptide in situ within recombinant cells since at least one component of the polypeptide’s natural environment will not be present. Ordinarily, however, isolated polypeptide will be prepared by at least one purification step.

[00178] As used herein, the term “disorder” and “disease” are used interchangeably to refer to any condition that would benefit from treatment with a variant polypeptide (a polypeptide comprising a variant Fc region of the invention), including chronic and acute disorders or diseases (*e.g.*, pathological conditions that predispose a patient to a particular disorder).

[00179] As used herein, the term “receptor” refers to a polypeptide capable of binding at least one ligand. The preferred receptor is a cell-surface or soluble receptor having an extracellular ligand-binding domain and, optionally, other domains (*e.g.*, transmembrane domain, intracellular domain and/or membrane anchor). A receptor to be evaluated in an assay described herein may be an intact receptor or a fragment or

derivative thereof (*e.g.*, a fusion protein comprising the binding domain of the receptor fused to one or more heterologous polypeptides). Moreover, the receptor to be evaluated for its binding properties may be present in a cell or isolated and optionally coated on an assay plate or some other solid phase or labeled directly and used as a probe.

Feline IgGs

[00180] The inventors of the instant application have found that by substituting one or more amino acid residues in the Fc region of feline IgG1, advantageous characteristics may be introduced into feline IgGs, for example reducing or completely knocking out CDC, ADCC and ADCP activities.

[00181] Accordingly, an Fc region of feline IgG1a may comprise one or more mutations or substitutions at positions including amino acid 234, 235, 236, 237, 239, 329, 265, 267, 268, 270, 297, 298, 330, 331, 332, 333, 334, and/or 345, referring to positions numbered according to the EU index as in Kabat (Kabat *et al.*, Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)).

[00182] In various embodiments, an Fc region of feline IgG1a may comprise one or more substitutions selected from among M234A, L235A, L235Y, G236A, G237A, G237W, S239D, D265A, G267E, P268F, D270G, D270S, N297G, S298A, K322A, N324T, P329G, P329S, S330A, S330L, P331A, P331S, P331A, I332E, E333A, R334A, and E345R using EU Index numbering. For example, various combinations of substitutions in a Fc region of feline IgG1a can include one or more of the following, which are referred to herein by short designations: WIN (M234A, L235A, G237A), GSP (P329G), PSS (P331S), GAP (P329G, S330A), DANG (D265A, N297G), KA (K322A), PA (P331A), KAPA (K322A, P331A), WIN_KA (M234A,

L235A, G237A, K322A), WIN_PA (M234A, L235A, G237A, P331A), WIN_KAPA (M234A, L235A, G237A, K322A, P331A), WIN_GAP (M234A, L235A, G237A, P329G, S330A), DANG-GAP (D265A, N297G, P329G, S330A), WIN_DANG (M234A, L235A, G237A, D265A, N297G), WIN_DANG_GAP (M234A, L235A, G237A, D265A, N297G, P329G, S330A), DANG_PSS (D265A, N297G, P331S), WIN_PSA (M234A, L235A, G237A, P331A), WIN_DANG_PSS (M234A, L235A, G237A, D265A, N297G, P331S), WIN_PSS (M234A, L235A, G237A, P331S), WIN_GSA (M234A, L235A, G237A, P329G, P331A), WIN_GSP (M234A, L235A, G237A, P329G), WIN_GSS (M234A, L235A, G237A, P329G, P331S), WIN_SAS (M234A, L235A, G237A, P329S, S330A, P331S), AAA (S298A, E333A, R334A), DLE (S239D, S330L, I332E), DE (S239D, I332E), DAE (G236A, S239D, I332E), YWA (L235Y, G237W, S298A), EFT (G267E, P268F, N324T), D270G (D270G), D270S (D270S), and E345R (E345R). Due to apparent sequence and structural similarities, an Fc region of feline IgG1b may also comprise one or more corresponding substitutions or combinations of substitutions in positions corresponding to the positions in IgG1a described above.

[00183] As further examples, feline IgG1a Fc can be comprised within a recombinantly modified protein, *e.g.*, a feline IgG heavy chain comprising a rituximab variable region and a mutated constant region, or a feline IgG1a Fc fused to another functional peptide such as a CTLA-4 polypeptide fragment. Examples of such proteins include the following sequences:

Rituximab Feline IgG1a GSP (P329G) SEQ ID NO: 1

```

QVQLQQPGAE LVKPGASVKM SCKASGYTFT SYNMHWVKQT PGRGLEWIGA IYPGNGDTSY 60
NQKFKGKATL TADKSSSTAY MQLSSLTSED SAVYYCARST YYGGDWYFNV WGAGTTVTVS 120
SVSSASTTAP SVFPLAPSCG TTSGATVALA CLVLGYFPEP VTVSWNSGAL TSGVHTFPAV 180
LQASGLYSLS SMVTVPSSRW LSDTFTCNVA HPPSNTKVVDK TVRKTDHPPG PKPCDCPKCP 240
PPEMLGGPSI FIFPPKPKDT LSISRTPEVT CLVVDLGPDD SDVQITWFVD NTQVYTAKTS 300

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PREEQFNSTY RVSVLPILH QDWLKGKEFK CKVNSKSLGS PIERTISKAK GQPHEPQVYV 360
 LPPAQEELSR NKVSVTCLIK SFHPPDIAVE WEITGQPEPE NNYRTTPPQL DSDGTYFVYS 420
 KLSVDRSHWQ RGNTYTCSVS HEALHSHHTQ KSLTQSPGK 459

where amino acids 1-116 of this sequence comprise a rituximab heavy chain fragment and amino acids 117-459 comprise a heavy chain fragment containing the constant domain of feline IgG1a.

Rituximab Feline IgG1a GAP (P329G, S330A) SEQ ID NO: 2

QVQLQQPGAE LVKPGASVKM SCKASGYTFT SYNMHWVKQT PGRGLEWIGA IYPGNGDTSY 60
 NQKFKGKATL TADKSSSTAY MQLSSLTSED SAVYYCARST YYGGDWYFNV WGAGTTVTVS 120
 SVSSASTTAP SVFPLAPSCG TTSGATVALA CLVLGYFPEP VTVSWNSGAL TSGVHTFPAV 180
 LQASGLYSLs SMVTVPSRW LSDTFTCNVA HPPSNTKVDK TVRKTDHPPG PKPCDCPKCP 240
 PPEMLGGPSI FIFPPKPKDT LSISRTPEVT CLVVDLGPDD SDVQITWFVD NTQVYTAKTS 300
 PREEQFNSTY RVSVLPILH QDWLKGKEFK CKVNSKSLGA PIERTISKAK GQPHEPQVYV 360
 LPPAQEELSR NKVSVTCLIK SFHPPDIAVE WEITGQPEPE NNYRTTPPQL DSDGTYFVYS 420
 KLSVDRSHWQ RGNTYTCSVS HEALHSHHTQ KSLTQSPGK 459

where amino acids 1-116 of this sequence comprise a rituximab heavy chain fragment and amino acids 117-459 comprise a heavy chain fragment containing the constant domain of feline IgG1a.

Rituximab Feline IgG1a DANG-GAP (D265A, N297G; P329G, S330A) SEQ ID NO: 3

QVQLQQPGAE LVKPGASVKM SCKASGYTFT SYNMHWVKQT PGRGLEWIGA IYPGNGDTSY 60
 NQKFKGKATL TADKSSSTAY MQLSSLTSED SAVYYCARST YYGGDWYFNV WGAGTTVTVS 120
 SVSSASTTAP SVFPLAPSCG TTSGATVALA CLVLGYFPEP VTVSWNSGAL TSGVHTFPAV 180
 LQASGLYSLs SMVTVPSRW LSDTFTCNVA HPPSNTKVDK TVRKTDHPPG PKPCDCPKCP 240
 PPEMLGGPSI FIFPPKPKDT LSISRTPEVT CLVVALGPDD SDVQITWFVD NTQVYTAKTS 300
 PREEQFGSTY RVSVLPILH QDWLKGKEFK CKVNSKSLGA PIERTISKAK GQPHEPQVYV 360
 LPPAQEELSR NKVSVTCLIK SFHPPDIAVE WEITGQPEPE NNYRTTPPQL DSDGTYFVYS 420

KLSVDRSHWQ RGNTYTCSVS HEALHSHHTQ KSLTQSPGK

459

where amino acids 1-116 of this sequence comprise a rituximab heavy chain fragment and amino acids 117-459 comprise a heavy chain fragment containing the constant domain of feline IgG1a.

Rituximab Feline IgG1a KAPA (K322A, P331A) SEQ ID NO: 4

QVQLQQPGAE LVKPGASVKM SCKASGYTFT SYNMHWVKQT PGRGLEWIGA IYPGNGDTSY 60
 NQKFKGKATL TADKSSSTAY MQLSSLTSED SAVYYCARST YYGGDWYFNV WGAGTTVTVS 120
 SVSSASTTAP SVFPLAPSCG TTSGATVALA CLVLGYFPEP VTVSWNSGAL TSGVHTFPAV 180
 LQASGLYSLS SMVTPSSRW LSDTFTCNVA HPPSNTKVDK TVRKTDHPPG PKPCDCPKCP 240
 PPEMLGGPSI FIFPPKPKDT LSISRTPEVT CLVVDLGPDD SDVQITWFVD NTQVYTAKTS 300
 PREEQFNSTY RVVSVLPILH QDWLKGKEFK **CAV**NSKSLPS **AI**ERTISKAK GQPHEPQVYV 360
 LPPAQEELSR NKVSVTCLIK SFHPPDIAVE WEITGQPEPE NNYRTTPPQL DSDGTYFVYS 420
 KLSVDRSHWQ RGNTYTCSVS HEALHSHHTQ KSLTQSPGK 459

where amino acids 1-116 of this sequence comprise a rituximab heavy chain fragment and amino acids 117-459 comprise a heavy chain fragment containing the constant domain of feline IgG1a.

Rituximab Feline IgG1a WIN_DANG_GAP (M234A, L235A, G237A; D265A, N297G; P329G, S330A) SEQ ID NO: 5

QVQLQQPGAE LVKPGASVKM SCKASGYTFT SYNMHWVKQT PGRGLEWIGA IYPGNGDTSY 60
 NQKFKGKATL TADKSSSTAY MQLSSLTSED SAVYYCARST YYGGDWYFNV WGAGTTVTVS 120
 SVSSASTTAP SVFPLAPSCG TTSGATVALA CLVLGYFPEP VTVSWNSGAL TSGVHTFPAV 180
 LQASGLYSLS SMVTPSSRW LSDTFTCNVA HPPSNTKVDK TVRKTDHPPG PKPCDCPKCP 240
 PPE**AAG**APSI FIFPPKPKDT LSISRTPEVT CLVV**AL**GPDD SDVQITWFVD NTQVYTAKTS 300
 PREEQ**FG**STY RVVSVLPILH QDWLKGKEFK CKVNSKSL**GA** PIERTISKAK GQPHEPQVYV 360
 LPPAQEELSR NKVSVTCLIK SFHPPDIAVE WEITGQPEPE NNYRTTPPQL DSDGTYFVYS 420
 KLSVDRSHWQ RGNTYTCSVS HEALHSHHTQ KSLTQSPGK 459

where amino acids 1-116 of this sequence comprise a rituximab heavy chain fragment and amino acids 117-459 comprise a heavy chain fragment containing the constant domain of feline IgG1a.

Rituximab Feline IgG1a DANG (D265A, N297G) SEQ ID NO: 6

QVQLQQPGAE LVKPGASVKM SCKASGYTFT SYNMHWVKQT PGRGLEWIGA IYPGNGDTSY 60
 NQKFKGKATL TADKSSSTAY MQLSSLTSED SAVYYCARST YYGGDWYFNV WGAGTTVTVS 120
 SVSSASTTAP SVFPLAPSCG TTSGATVALA CLVLGYFPEP VTVSWNSGAL TSGVHTFPVAV 180
 LQASGLYSLS SMVTPSSRW LSDTFTCNVA HPPSNTKVDK TVRKTDHPPG PKPCDCPKCP 240
 PPEMLGGPSI FIFPPKPKDT LSISRTPEVT CLVVAALGPDD SDVQITWFVD NTQVYTAKTS 300
 PREEQFGSTY RVVSVLPILH QDWLKGKEFK CKVNSKSLPS PIERTISKAK GQPHEPQVYV 360
 LPPAQEELSR NKVSVTCLIK SFHPPDIAVE WEITGQPEPE NNYRTTPPQL DSDGTYFVYS 420
 KLSVDRSHWQ RGNTYTCSVS HEALSHHTQ KSLTQSPGK 459

where amino acids 1-116 of this sequence comprise a rituximab heavy chain fragment and amino acids 117-459 comprise a heavy chain fragment containing the constant domain of feline IgG1a.

Rituximab Feline IgG1a AAA (S298A, E333A, R334A) SEQ ID NO: 7

QVQLQQPGAE LVKPGASVKM SCKASGYTFT SYNMHWVKQT PGRGLEWIGA IYPGNGDTSY 60
 NQKFKGKATL TADKSSSTAY MQLSSLTSED SAVYYCARST YYGGDWYFNV WGAGTTVTVS 120
 SVSSASTTAP SVFPLAPSCG TTSGATVALA CLVLGYFPEP VTVSWNSGAL TSGVHTFPVAV 180
 LQASGLYSLS SMVTPSSRW LSDTFTCNVA HPPSNTKVDK TVRKTDHPPG PKPCDCPKCP 240
 PPEMLGGPSI FIFPPKPKDT LSISRTPEVT CLVVDLGPDD SDVQITWFVD NTQVYTAKTS 300
 PREEQFNATY RVVSVLPILH QDWLKGKEFK CKVNSKSLPS PIAATISKAK GQPHEPQVYV 360
 LPPAQEELSR NKVSVTCLIK SFHPPDIAVE WEITGQPEPE NNYRTTPPQL DSDGTYFVYS 420
 KLSVDRSHWQ RGNTYTCSVS HEALSHHTQ KSLTQSPGK 459

where amino acids 1-116 of this sequence comprise a rituximab heavy chain fragment and amino acids 117-459 comprise a heavy chain fragment containing the constant domain of feline IgG1a.

Rituximab Feline IgG1a DLE (S239D, S330L, I332E) SEQ ID NO: 8

QVQLQQPGAE LVKPGASVKM SCKASGYTFT SYNMHWVKQT PGRGLEWIGA IYPGNGDTSY 60
 NQKFKGKATL TADKSSSTAY MQLSSLTSED SAVYYCARST YYGGDWYFNV WGAGTTTVTS 120
 SVSSASTTAP SVFPLAPSCG TTSGATVALA CLVLGYFPEP VTVSWNSGAL TSGVHTFPAV 180
 LQASGLYSLS SMVTVPSSRW LSDTFTCNVA HPPSNTKVDK TVRKTDHPPG PKPCDCPKCP 240
 PPEMLGGEDI FIFPPKPKDT LSISRTPEVT CLVVDLGPDD SDVQITWFVD NTQVYTAKTS 300
 PREEQFNSTY RVVSVLPILH QDWLKGKEFK CKVNSKSLPL PEERTISKAK GQPHEPQVYV 360
 LPPAQEELSR NKVSVTCLIK SFHPPDIAVE WEITGQPEPE NNYRTTPPQL DSDGTYFVYS 420
 KLSVDRSHWQ RGNTYTCVS HEALSHHTQ KSLTQSPGK 459

where amino acids 1-116 of this sequence comprise a rituximab heavy chain fragment and amino acids 117-459 comprise a heavy chain fragment containing the constant domain of feline IgG1a.

Rituximab Feline IgG1a DE (S239D, I332E) SEQ ID NO: 9

QVQLQQPGAE LVKPGASVKM SCKASGYTFT SYNMHWVKQT PGRGLEWIGA IYPGNGDTSY 60
 NQKFKGKATL TADKSSSTAY MQLSSLTSED SAVYYCARST YYGGDWYFNV WGAGTTTVTS 120
 SVSSASTTAP SVFPLAPSCG TTSGATVALA CLVLGYFPEP VTVSWNSGAL TSGVHTFPAV 180
 LQASGLYSLS SMVTVPSSRW LSDTFTCNVA HPPSNTKVDK TVRKTDHPPG PKPCDCPKCP 240
 PPEMLGGEDI FIFPPKPKDT LSISRTPEVT CLVVDLGPDD SDVQITWFVD NTQVYTAKTS 300
 PREEQFNSTY RVVSVLPILH QDWLKGKEFK CKVNSKSLPS PEERTISKAK GQPHEPQVYV 360
 LPPAQEELSR NKVSVTCLIK SFHPPDIAVE WEITGQPEPE NNYRTTPPQL DSDGTYFVYS 420
 KLSVDRSHWQ RGNTYTCVS HEALSHHTQ KSLTQSPGK 459

where amino acids 1-116 of this sequence comprise a rituximab heavy chain fragment and amino acids 117-459 comprise a heavy chain fragment containing the constant domain of feline IgG1a.

Rituximab Feline IgG1a DAE (G236A, S239D, I332E) SEQ ID NO: 10

QVQLQQPGAE LVKPGASVKM SCKASGYTFT SYNMHWVKQT PGRGLEWIGA IYPGNGDTSY 60
 NQKFKGKATL TADKSSSTAY MQLSSLTSED SAVYYCARST YYGGDWYFNV WGAGTTVTVS 120
 SVSSASTTAP SVFPLAPSCG TTSGATVALA CLVLGYFPEP VTVSWNSGAL TSGVHTFPAV 180
 LQASGLYSLs SMVTVPSSRW LSDTFTCNVA HPPSNTKVDK TVRKTDHPPG PKPCDCPKCP 240
 PPEML**LAG**DI FIFPPKPKDT LSISRTPEVT CLVVDLGPDD SDVQITWFVD NTQVYTAKTS 300
 PREEQFNSTY RVVSVLPILH QDWLKGKEFK CKVNSKSLPS **PE**ERTISKAK GQPHEPQVYV 360
 LPPAQEELSR NKVSVTCLIK SFHPPDIAVE WEITGQPEPE NNYRTTPPQL DSDGTYFVYS 420
 KLSVDRSHWQ RGNTYTCSVS HEALSHHTQ KSLTQSPGK 459

where amino acids 1-116 of this sequence comprise a rituximab heavy chain fragment and amino acids 117-459 comprise a heavy chain fragment containing the constant domain of feline IgG1a.

Rituximab Feline IgG1a YWA (L235Y, G237W, S298A) SEQ ID NO: 11

QVQLQQPGAE LVKPGASVKM SCKASGYTFT SYNMHWVKQT PGRGLEWIGA IYPGNGDTSY 60
 NQKFKGKATL TADKSSSTAY MQLSSLTSED SAVYYCARST YYGGDWYFNV WGAGTTVTVS 120
 SVSSASTTAP SVFPLAPSCG TTSGATVALA CLVLGYFPEP VTVSWNSGAL TSGVHTFPAV 180
 LQASGLYSLs SMVTVPSSRW LSDTFTCNVA HPPSNTKVDK TVRKTDHPPG PKPCDCPKCP 240
 PPE**MY**GWPSI FIFPPKPKDT LSISRTPEVT CLVVDLGPDD SDVQITWFVD NTQVYTAKTS 300
 PREEQFN**A**TY RVVSVLPILH QDWLKGKEFK CKVNSKSLPS **PI**ERTISKAK GQPHEPQVYV 360
 LPPAQEELSR NKVSVTCLIK SFHPPDIAVE WEITGQPEPE NNYRTTPPQL DSDGTYFVYS 420
 KLSVDRSHWQ RGNTYTCSVS HEALSHHTQ KSLTQSPGK 459

where amino acids 1-116 of this sequence comprise a rituximab heavy chain fragment and amino acids 117-459 comprise a heavy chain fragment containing the constant domain of feline IgG1a.

Rituximab Feline IgG1a EFT (G267E, P268F, N324T) SEQ ID NO: 12

QVQLQQPGAE LVKPGASVKM SCKASGYTFT SYNMHWVKQT PGRGLEWIGA IYPGNGDTSY 60
 NQKFKGKATL TADKSSSTAY MQLSSLTSED SAVYYCARST YYGGDWYFNV WGAGTTVTVS 120
 SVSSASTTAP SVFPLAPSCG TTSGATVALA CLVLGYFPEP VTVSWNSGAL TSGVHTFPAV 180
 LQASGLYSL SMTVPSSRW LSDTFTCNVA HPPSNTKVDK TVRKTDHPPG PKPCDCPKCP 240
 PPEMLGGPSI FIFPPKPKDT LSISRTPEVT CLVVDLEFDD SDVQITWFVD NTQVYTAKTS 300
 PREEQFNSTY RVSVLPILH QDWLKGKEFK CKVTSKSLPS PIERTISKAK GQPHEPQVYV 360
 LPPAQEELSR NKSVTCLIK SFHPPDIAVE WEITGQPEPE NNYRTTPPQL DSDGTYFVYS 420
 KLSVDRSHWQ RGNTYTCSVS HEALSHHTQ KSLTQSPGK 459

where amino acids 1-116 of this sequence comprise a rituximab heavy chain fragment and amino acids 117-459 comprise a heavy chain fragment containing the constant domain of feline IgG1a.

Rituximab Feline IgG1a E345R (E345R) SEQ ID NO: 13

MEWSWVFLFF LSVTGVHSQ VQLQQGAEL VKPGASVKMS CKASGYFTS YNMHWVKQTP 60
 GRGLEWIGAI YPGNGDTSYN QKFKGKATLT ADKSSSTAYM QLSSLTSEDS AVYYCARSTY 120
 YGGDWYFNVW GAGTTVTVSS VSSASTTAPS VFPLAPSCGT TSGATVALAC LVLGYFPEPV 180
 TVSWNSGALT SGVHTFPAVL QASGLYSLSS MVTVPSSRWL SDTFTCNVAH PPSNTKVDKT 240
 VRKTDHPPGP KPCDCPKCPP PPEMLGGPSIF IFPPKPKDTL SISRTPEVTC LVVDLGPDDS 300
 DVQITWFVDN TQVYTAKTSP REEQFNSTYR VSVLPILHQ DWLKGKEFKC KVNSKSLPSP 360
 IERTISKAKG QPHRPQVYVL PPAQEELSRN KSVTCLIKS FHPPDIAVEW EITGQPEPEN 420
 NYRTTPPQLD SDGTYFVYSK LSVDRSHWQR GNTYTCSVSH EALSHHTQK SLTQSPGK 478

where amino acids 1-116 of this sequence comprise a rituximab heavy chain fragment and amino acids 117-459 comprise a heavy chain fragment containing the constant domain of feline IgG1a.

Canine CTLA-4 Feline IgG1a fusion WIN_DANG (M234A, L235A, G237A;

D265A, N297G) SEQ ID NO: 14

```
METDTLLLWV LLLWVPGSTG DMHVAQPAVV LASSRGVASF VCEYGSSGNA AEVRVTVLRQ 60
AGSQMTEVCA ATYTVEDELA FLDDSTCTGT SSGNKVNLT I QGLRAMDTGL YICKVELMYP 120
PPYYVGMGNG TQIYVIDPEP CPDSDRKT DH PPGPKPCDCP KCPPPEAAGA PSIFIFPPKP 180
KDTLSISRTP EVTCLVVALG PDDSDVQITW FVDNTQVYTA KTSPREEQFG STYRVVSVLP 240
ILHQDWLKGK EFKCKVNSKS LPSPIERTIS KAKGQPHEPQ VYVLPPAQEE LSRNKVSVTC 300
LIKSFHPPDI AVEWEITGQP EPENNYRTTP PQLDSDGTYF VYSKLSVDRS HWQRGNTYTC 360
SVSHEALHSH HTQKSLTQSP GK 382
```

where amino acids 21-145 comprise a canine CTLA-4 sequence and amino acids 146-382
comprise a fragment of feline IgG1a heavy chain containing the Fc region.

Canine CTLA-4 Feline IgG1a fusion WIN_DANG_PSS (M234A, L235A, G237A;

D265A, N297G; P331S) SEQ ID NO: 15

```
METDTLLLWV LLLWVPGSTG DMHVAQPAVV LASSRGVASF VCEYGSSGNA AEVRVTVLRQ 60
AGSQMTEVCA ATYTVEDELA FLDDSTCTGT SSGNKVNLT I QGLRAMDTGL YICKVELMYP 120
PPYYVGMGNG TQIYVIDPEP CPDSDRKT DH PPGPKPCDCP KCPPPEAAGA PSIFIFPPKP 180
KDTLSISRTP EVTCLVVALG PDDSDVQITW FVDNTQVYTA KTSPREEQFG STYRVVSVLP 240
ILHQDWLKGK EFKCKVNSKS LPSSSIERTIS KAKGQPHEPQ VYVLPPAQEE LSRNKVSVTC 300
LIKSFHPPDI AVEWEITGQP EPENNYRTTP PQLDSDGTYF VYSKLSVDRS HWQRGNTYTC 360
SVSHEALHSH HTQKSLTQSP GK 382
```

where amino acids 21-145 comprise a canine CTLA-4 sequence and amino acids 146-382
comprise a fragment of feline IgG1a heavy chain containing the Fc region.

Canine CTLA-4 Feline IgG1a fusion DANG_PSS (D265A, N297G; P331S) SEQ**ID NO: 16**

```

METDTLLLWV LLLWVPGSTG DMHVAQPAVV LASSRGVASF VCEYGSSGNA AEVRVTVLRQ 60
AGSQMTEVCA ATYTVEDELA FLDDSTCTGT SSGNKVNLTl QGLRAMDTGL YICKVELMYP 120
PPYYVGMGNG TQIYVIDPEP CPDSDRKTdH PPGPKPCDCP KCPPPEMLGG PSIFIFPPKP 180
KDTLSISRTP EVTCLVVALG PDDSDVQITW FVDNTQVYTA KTSPREEQFG STYRVVSVLP 240
ILHQDWLKGK EFKCKVNSKS LPSSSIERTIS KAKGQPHEPQ VYVLPPAQEE LSRNKVSVTC 300
LIKSFHPPDI AVEWEITGQP EPENNYRTTP PQLDSDGTYF VYSKLSVDRS HWQRGNTYTC 360
SVSHEALHSH HTQKSLTQSP GK 382

```

where amino acids 21-145 comprise a canine CTLA-4 sequence and amino acids 146-382 comprise a fragment of feline IgG1a heavy chain containing the Fc region.

Canine CTLA-4 Feline IgG1a fusion WIN (M234A, L235A, G237A) SEQ ID**NO: 17**

```

METDTLLLWV LLLWVPGSTG DMHVAQPAVV LASSRGVASF VCEYGSSGNA AEVRVTVLRQ 60
AGSQMTEVCA ATYTVEDELA FLDDSTCTGT SSGNKVNLTl QGLRAMDTGL YICKVELMYP 120
PPYYVGMGNG TQIYVIDPEP CPDSDrktdh ppgpkpcdcp kcpppeAAgA psififppkp 180
kDtlsisrtp evtclvvdlg pddsdvqitw fvdntqvyta ktspreeqfn styrvvsvlp 240
ilhqdwlkgk efkckvnSks lpspiertis kakgqphepq vyvlppaqee lsrnkvsvtc 300
liksfhppdi aveweitqgp epennyrttp pqlsdsgtyf vysklsvdrs hwqrgntytc 360
svshealhsh htqksltqsp gk 382

```

where amino acids 21-145 comprise a canine CTLA-4 sequence and amino acids 146-382 comprise a fragment of feline IgG1a heavy chain containing the Fc region.

Canine CTLA-4 Feline IgG1a fusion D270G (D270G) SEQ ID NO: 18

METDTLLLWV LLLWVPGSTG DMHVAQPAVV LASSRGVASF VCEYGSSGNA AEVRVTVLRQ 60
 AGSQMTEVCA ATYTVEDELA FLDDSTCTGT SSGNKVNLTl QGLRAMDTGL YICKVELMYP 120
 PPYYVGMGNG TQIYVIDPEP CPDSDrkt dh ppgpkpcdcp kcpppemlgg psififppkp 180
 kDtIsisrtp evtclvvdlg pdGsdvqitw fvdntqvyta ktspreeqfn styrvvsvlp 240
 ilhqdwlgk efkckvnSks lpspierrtis kakgqphepq vyvlpaaqee lsrnkvsvtc 300
 liksfhppdi aveweitgqp epennyrttp pQLdsdgtyf vysklsvdrs hwqrgntytc 360
 svshealhsh htqksltqsp gk 382

where amino acids 21-145 comprise a canine CTLA-4 sequence and amino acids 146-382
 comprise a fragment of feline IgG1a heavy chain containing the Fc region.

Canine CTLA-4 Feline IgG1a fusion D270S (D270S) SEQ ID NO: 19

METDTLLLWV LLLWVPGSTG DMHVAQPAVV LASSRGVASF VCEYGSSGNA AEVRVTVLRQ 60
 AGSQMTEVCA ATYTVEDELA FLDDSTCTGT SSGNKVNLTl QGLRAMDTGL YICKVELMYP 120
 PPYYVGMGNG TQIYVIDPEP CPDSDrkt dh ppgpkpcdcp kcpppemlgg psififppkp 180
 kDtIsisrtp evtclvvdlg pdSsdvqitw fvdntqvyta ktspreeqfn styrvvsvlp 240
 ilhqdwlgk efkckvnSks lpspierrtis kakgqphepq vyvlpaaqee lsrnkvsvtc 300
 liksfhppdi aveweitgqp epennyrttp pQLdsdgtyf vysklsvdrs hwqrgntytc 360
 svshealhsh htqksltqsp gk 382

where amino acids 21-145 comprise a canine CTLA-4 sequence and amino acids 146-382
 comprise a fragment of feline IgG1a heavy chain containing the Fc region.

Canine CTLA-4 Feline IgG1a fusion WIN_PSA (M234A, L235A, G237A;

P331A) SEQ ID NO: 20

```
METDTLLLWV LLLWVPGSTG DMHVAQPAVV LASSRGVASF VCEYGSSGNA AEVRVTVLRQ 60
AGSQMTEVCA ATYTVEDELA FLDDSTCTGT SSGNKVNLTl QGLRAMDTGL YICKVELMYP 120
PPYYVGMGNG TQIYVIDPEP CPDSDrkt dh ppgpkpcdcp kcppeAAgA psififppkp 180
kDtlsisrtp evtclvvdlg pddsdvqitw fvdntqvyta ktspreeqfn styrvvsvlp 240
ilhqdwlkgk efkckvnSks lpsAiertis kakgqphep q vyvlp paqee lsrnkvs vtc 300
liksfh p p d i avew eit g q p epennyrtt p pql d s d g t y f v y s k l s v d r s h w q r g n t y t c 360
svshealhsh htqksltqsp gk 382
```

where amino acids 21-145 comprise a canine CTLA-4 sequence and amino acids 146-382 comprise a fragment of feline IgG1a heavy chain containing the Fc region.

Canine CTLA-4 Feline IgG1a fusion Win_GSA(M234A, L235A, G237A;

P329G, P331A) SEQ ID NO: 21

```
METDTLLLWV LLLWVPGSTG DMHVAQPAVV LASSRGVASF VCEYGSSGNA AEVRVTVLRQ 60
AGSQMTEVCA ATYTVEDELA FLDDSTCTGT SSGNKVNLTl QGLRAMDTGL YICKVELMYP 120
PPYYVGMGNG TQIYVIDPEP CPDSDrkt dh ppgpkpcdcp kcppeAAgA psififppkp 180
kDtlsisrtp evtclvvdlg pddsdvqitw fvdntqvyta ktspreeqfn styrvvsvlp 240
ilhqdwlkgk efkckvnSks lGsAiertis kakgqphep q vyvlp paqee lsrnkvs vtc 300
liksfh p p d i avew eit g q p epennyrtt p pql d s d g t y f v y s k l s v d r s h w q r g n t y t c 360
svshealhsh htqksltqsp gk 382
```

where amino acids 21-145 comprise a canine CTLA-4 sequence and amino acids 146-382 comprise a fragment of feline IgG1a heavy chain containing the Fc region.

Canine CTLA-4 Feline IgG1a fusion WIN_GSP (M234A, L235A, G237A;

P329G) SEQ ID NO: 22

```
METDTLLLWV LLLWVPGSTG DMHVAQPAVV LASSRGVASF VCEYGSSGNA AEVRVTVLRQ 60
AGSQMTEVCA ATYTVEDELA FLDDSTCTGT SSGNKVNLTl QGLRAMDTGL YICKVELMYP 120
PPYYVGMGNG TQIYVIDPEP CPDSDrkt dh ppgpkpcdcp kcppeAAgA psififppkp 180
kDtlsisrtp evtclvvdlg pddsdvqitw fvdntqvyta ktspreeqfn styrvvsvlp 240
ilhqdwlkgk efkckvnSks lGspiertis kakgqphepq vyvlppaqee lsrnkvsvtc 300
liksfhppdi aveweitgqp epennyrttp pqlsdsgtyf vysklsvdrs hwqrgntytc 360
svshealhsh htqksltqsp gk 382
```

where amino acids 21-145 comprise a canine CTLA-4 sequence and amino acids 146-382 comprise a fragment of feline IgG1a heavy chain containing the Fc region.

Canine CTLA-4 Feline IgG1a fusion Win_PSS (M234A, L235A, G237A;

P331S) SEQ ID NO: 23

```
METDTLLLWV LLLWVPGSTG DMHVAQPAVV LASSRGVASF VCEYGSSGNA AEVRVTVLRQ 60
AGSQMTEVCA ATYTVEDELA FLDDSTCTGT SSGNKVNLTl QGLRAMDTGL YICKVELMYP 120
PPYYVGMGNG TQIYVIDPEP CPDSDrkt dh ppgpkpcdcp kcppeAAgA psififppkp 180
kDtlsisrtp evtclvvdlg pddsdvqitw fvdntqvyta ktspreeqfn styrvvsvlp 240
ilhqdwlkgk efkckvnSks lpsSiertis kakgqphepq vyvlppaqee lsrnkvsvtc 300
liksfhppdi aveweitgqp epennyrttp pqlsdsgtyf vysklsvdrs hwqrgntytc 360
svshealhsh htqksltqsp gk 382
```

where amino acids 21-145 comprise a canine CTLA-4 sequence and amino acids 146-382 comprise a fragment of feline IgG1a heavy chain containing the Fc region.

Canine CTLA-4 Feline IgG1a fusion PSS (P331S) SEQ ID NO: 24

```

METDTLLLWV LLLWVPGSTG DMHVAQPAVV LASSRGVASF VCEYGSSGNA AEVRVTVLRQ 60
AGSQMTEVCA ATYTVEDELA FLDDSTCTGT SSGNKVNLTl QGLRAMDTGL YICKVELMYP 120
PPYYVGMGNG TQIYVIDPEP CPDSDrkt dh ppgpkpcdcp kcppp eml gg psififppkp 180
kDtlsisrtp evtclvvdlg pddsdvqitw fvdntqvyta ktspre eqfn styrvvsvlp 240
ilhqdwlkgk efkckvnSks lpsSiertis kakgqphepq vyvlppaqee lsrnkvsvtc 300
liksfhppdi aveweitgqp epennyrttp pqlsdsgtyf vysklsvdrs hwqrgntytc 360
svshealhsh htqksltqsp gk 382

```

where amino acids 21-145 comprise a canine CTLA-4 sequence and amino acids 146-382 comprise a fragment of feline IgG1a heavy chain containing the Fc region.

Canine CTLA-4 Feline IgG1a fusion Win_GSS (M234A, L235A, G237A; P329G, P331S) SEQ ID NO: 25

```

METDTLLLWV LLLWVPGSTG DMHVAQPAVV LASSRGVASF VCEYGSSGNA AEVRVTVLRQ 60
AGSQMTEVCA ATYTVEDELA FLDDSTCTGT SSGNKVNLTl QGLRAMDTGL YICKVELMYP 120
PPYYVGMGNG TQIYVIDPEP CPDSDrkt dh ppgpkpcdcp kcpppeAAgA psififppkp 180
kDtlsisrtp evtclvvdlg pddsdvqitw fvdntqvyta ktspre eqfn styrvvsvlp 240
ilhqdwlkgk efkckvnSks lGsSiertis kakgqphepq vyvlppaqee lsrnkvsvtc 300
liksfhppdi aveweitgqp epennyrttp pqlsdsgtyf vysklsvdrs hwqrgntytc 360
svshealhsh htqksltqsp gk 382

```

where amino acids 21-145 comprise a canine CTLA-4 sequence and amino acids 146-382 comprise a fragment of feline IgG1a heavy chain containing the Fc region.

Canine CTLA-4 Feline IgG1a fusion WIN_GAP (M234A,L235A,G237A;

P329G, S330A) SEQ ID NO: 26

```
METDTLLLWV LLLWVPGSTG DMHVAQPAVV LASSRGVASF VCEYGSSGNA AEVRVTVLRQ 60
AGSQMTEVCA ATYTVEDELA FLDDSTCTGT SSGNKVNLTl QGLRAMDTGL YICKVELMYP 120
PPYYVGMGNG TQIYVIDPEP CPDSDrkt dh ppgpkpcdcp kcppeAAgA psififppkp 180
kDtlsisrtp evtclvvdlg pddsdvqitw fvdntqvyta ktspreeqfn styrvvsvlp 240
ilhqdwlkgk efkckvnSks lGApiertis kakgqphepq vyvlppaqee lsrnkvsvtc 300
liksfhppdi aveweitgqp epennyrttp pqlsdgtyf vysklsvdrs hwqrgntytc 360
svshealhshh tqksltqsp gk 382
```

where amino acids 21-145 comprise a canine CTLA-4 sequence and amino acids 146-382 comprise a fragment of feline IgG1a heavy chain containing the Fc region.

Canine CTLA-4 Feline IgG1a fusion WIN_SAS

(M234A,L235A,G237A;P329S,S330A,P331S) SEQ ID NO: 27

```
METDTLLLWV LLLWVPGSTG DMHVAQPAVV LASSRGVASF VCEYGSSGNA AEVRVTVLRQ 60
AGSQMTEVCA ATYTVEDELA FLDDSTCTGT SSGNKVNLTl QGLRAMDTGL YICKVELMYP 120
PPYYVGMGNG TQIYVIDPEP CPDSDrkt dh ppgpkpcdcp kcppeAAgA psififppkp 180
kDtlsisrtp evtclvvdlg pddsdvqitw fvdntqvyta ktspreeqfn styrvvsvlp 240
ilhqdwlkgk efkckvnSks lSASiertis kakgqphepq vyvlppaqee lsrnkvsvtc 300
liksfhppdi aveweitgqp epennyrttp pqlsdgtyf vysklsvdrs hwqrgntytc 360
svshealhsh htqksltqsp gk 382
```

where amino acids 21-145 comprise a canine CTLA-4 sequence and amino acids 146-382 comprise a fragment of feline IgG1a heavy chain containing the Fc region.

Canine IgGs

[00184] The inventors of the instant application have surprisingly found that by substituting one or more amino acid residues in the Fc region of canine IgG2, advantageous characteristics may be introduced into canine IgGs, for example reducing or completely knocking out CDC, ADCC and ADCP activities or increasing or decreasing binding affinity to canine Fcγ receptor and C1q.

[00185] Accordingly, in various embodiments, an Fc region of canine IgG2 may comprise one or more mutations or substitutions, such as at locations listed in Table 4, for example the substitutions listed in Table 5, and/or substitutions selected from among M234A, L235A, L235Y, G236A, G237A, G237W, S239D, D265A, D270G, D270S, N297G, G298A, K322A, P329G, S330A, S330L, P331A, I332E, E333A, R334A, using EU Index numbering as in Kabat. For example, various combinations of substitutions in a Fc region of canine IgG2 can include one or more of the following, referred to herein by short designations: GSP (P329G), GAP (P329G, S330A), DANG (D265A, N297G), DANG-GAP (D265A, N297G; P329G, S330A), WIN (M234A, L235A, G237A), WIN_DANG_GAP (M234A, L235A, G237A; D265A, N297G; P329G, S330A), AAA (G298A, E333A, R334A), DE (S239D, I332E), DLE (S239D, S330L, I332E), DAE (G236A, S239D, I332E), YWA (L235Y, G237W, G298A), EFT (D267E, P268F, N324T), Q345R (Q345R), D270G (D270G), and D270S (D270S).

[00186] As further examples, mutated canine IgG2 Fc can be comprised within a recombinantly modified protein, *e.g.*, a canine IgG heavy chain comprising a rituximab variable region and a mutated constant region, or a fusion protein comprising another effector region (such as a CTLA-4 sequence) fused to a canine IgG heavy chain comprising a mutated constant region. Examples of such proteins include the following sequences:

Rituximab Canine IgG2 GSP (P329G) SEQ ID NO: 28

QVQLQQPGAE LVKPGASVKM SCKASGYTFT SYNMHWVKQT PGRGLEWIGA IYPGNGDTSY 60
 NQKFKGKATL TADKSSSTAY MQLSSLTSED SAVYYCARST YYGGDWYFNV WGAGTTVTVS 120
 AASTTAPSVF PLAPSCGSTS GSTVALACLV SGYFPEPVTV SWNSGSLTSG VHTFPSVLQS 180
 SGLYSLSSMV TVPSSRPSE TFTCNVAHPA SKTKVDKPVP KRENGRVPRP PDCPKCPAPE 240
 MLGGPSVFIF PPKPKDTLLI ARTPEVTCVV VDLDPEDPEV QISWFVDGKQ MQTAKTQPRE 300
 EQFNGTYRVV SVLPIGHQDW LKGKQFTCKV NNKALGSPIE RTISKARGQA HQPSVYVLPP 360
 SREELSKNTV SLTCLIKDFF PPDIDVEWQS NGQQEPESKY RTTPPQLDED GSYFLYSKLS 420
 VDKSRWQRGD TFICAVMHEA LHNHYTQESL SHSPGK 456

where amino acids 1-116 comprise a fragment of heavy chain variable region of rituximab and amino acids 117-456 comprise a mutated canine IgG2 heavy chain containing the constant region.

Rituximab Canine IgG2 GAP (P329G, S330A) SEQ ID NO: 29

QVQLQQPGAE LVKPGASVKM SCKASGYTFT SYNMHWVKQT PGRGLEWIGA IYPGNGDTSY 60
 NQKFKGKATL TADKSSSTAY MQLSSLTSED SAVYYCARST YYGGDWYFNV WGAGTTVTVS 120
 AASTTAPSVF PLAPSCGSTS GSTVALACLV SGYFPEPVTV SWNSGSLTSG VHTFPSVLQS 180
 SGLYSLSSMV TVPSSRPSE TFTCNVAHPA SKTKVDKPVP KRENGRVPRP PDCPKCPAPE 240
 MLGGPSVFIF PPKPKDTLLI ARTPEVTCVV VDLDPEDPEV QISWFVDGKQ MQTAKTQPRE 300
 EQFNGTYRVV SVLPIGHQDW LKGKQFTCKV NNKALGAPIE RTISKARGQA HQPSVYVLPP 360
 SREELSKNTV SLTCLIKDFF PPDIDVEWQS NGQQEPESKY RTTPPQLDED GSYFLYSKLS 420
 VDKSRWQRGD TFICAVMHEA LHNHYTQESL SHSPGK 456

where amino acids 1-116 comprise a fragment of heavy chain variable region of rituximab and amino acids 117-456 comprise a mutated canine IgG2 heavy chain containing the constant region.

Rituximab Canine IgG2 DANG-GAP (D265A, N297G; P329G, S330A) SEQ ID NO:

30

```

QVQLQQPGAE LVKPGASVKM SCKASGYTFT SYNMHWVKQT PGRGLEWIGA IYPGNGDTSY 60
NQKFKGKATL TADKSSSTAY MQLSSLTSED SAVYYCARST YYGGDWYFNV WGAGTTVTVS 120
AASTTAPSVF PLAPSCGSTS GSTVALACLV SGYFPEPVTV SWNSGSLTSG VHTFPSVLQS 180
SGLYSLSSMV TVPSSRPSE TFTCNVAHPA SKTKVDKPVP KRENGRVPRP PDCPKCPAPE 240
MLGGPSVFIF PPKPKDTLLI ARTPEVTCVV VALDPEDPEV QISWFVDGKQ MQTAKTQPRE 300
EQFGGTYRVV SVLPIGHQDW LKGKQFTCKV NNKALGAPIE RTISKARGQA HQPSVYVLPP 360
SREELSKNTV SLTCLIKDFF PPDIDVEWQS NGQQEPESKY RTTPPQLDED GSYFLYSKLS 420
VDKSRWQRGD TFICAVMHEA LHNHYTQESL SHSPGK 456

```

where amino acids 1-116 comprise a fragment of heavy chain variable region of rituximab and amino acids 117-456 comprise a mutated canine IgG2 heavy chain containing the constant region.

Rituximab Canine IgG2 KAPA (K322A, P331A) SEQ ID NO: 31

```

QVQLQQPGAE LVKPGASVKM SCKASGYTFT SYNMHWVKQT PGRGLEWIGA IYPGNGDTSY 60
NQKFKGKATL TADKSSSTAY MQLSSLTSED SAVYYCARST YYGGDWYFNV WGAGTTVTVS 120
AASTTAPSVF PLAPSCGSTS GSTVALACLV SGYFPEPVTV SWNSGSLTSG VHTFPSVLQS 180
SGLYSLSSMV TVPSSRPSE TFTCNVAHPA SKTKVDKPVP KRENGRVPRP PDCPKCPAPE 240
MLGGPSVFIF PPKPKDTLLI ARTPEVTCVV VDLDPEDPEV QISWFVDGKQ MQTAKTQPRE 300
EQFNGTYRVV SVLPIGHQDW LKGKQFTCAV NNKALPSAIE RTISKARGQA HQPSVYVLPP 360
SREELSKNTV SLTCLIKDFF PPDIDVEWQS NGQQEPESKY RTTPPQLDED GSYFLYSKLS 420
VDKSRWQRGD TFICAVMHEA LHNHYTQESL SHSPGK 456

```

where amino acids 1-116 comprise a fragment of heavy chain variable region of rituximab and amino acids 117-456 comprise a mutated canine IgG2 heavy chain containing the constant region.

Rituximab Canine IgG2 WIN_DANG_GAP (M234A, L235A, G237A; D265A, N297G; P329G, S330A) SEQ ID NO: 32

```

QVQLQQPGAE LVKPGASVKM SCKASGYTFT SYNMHWVKQT PGRGLEWIGA IYPGNGDTSY 60
NQKFKGKATL TADKSSSTAY MQLSSLTSED SAVYYCARST YYGGDWYFNV WGAGTTVTVS 120
AASTTAPSVF PLAPSCGSTS GSTVALACLV SGYFPEPVTV SWNSGSLTSG VHTFPSVLQS 180
SGLYSLSSMV TVPSSRPSE TFTCNVAHPA SKTKVDKPVP KRENGRVPRP PDCPKCPAPE 240
AAGAPSVFIF PPKPKDTLLI ARTPEVTCVV VALDPEDPEV QISWFVDGKQ MQTAKTQPRE 300
EQFGGTYRVV SVLPIGHQDW LKGKQFTCKV NNKALGAPIE RTISKARGQA HQPSVYVLPP 360
SREELSKNTV SLTCLIKDFF PPDIDVEWQS NGQQEPESKY RTTPPQLDED GSYFLYSKLS 420
VDKSRWQRGD TFICAVMHEA LHNHYTQESL SHSPGK 456

```

where amino acids 1-116 comprise a fragment of heavy chain variable region of rituximab and amino acids 117-456 comprise a mutated canine IgG2 heavy chain containing the constant region.

Rituximab Canine IgG2 DANG D265A, N297G) SEQ ID NO: 33

```

QVQLQQPGAE LVKPGASVKM SCKASGYTFT SYNMHWVKQT PGRGLEWIGA IYPGNGDTSY 60
NQKFKGKATL TADKSSSTAY MQLSSLTSED SAVYYCARST YYGGDWYFNV WGAGTTVTVS 120
AASTTAPSVF PLAPSCGSTS GSTVALACLV SGYFPEPVTV SWNSGSLTSG VHTFPSVLQS 180
SGLYSLSSMV TVPSSRPSE TFTCNVAHPA SKTKVDKPVP KRENGRVPRP PDCPKCPAPE 240
MLGGPSVFIF PPKPKDTLLI ARTPEVTCVV VALDPEDPEV QISWFVDGKQ MQTAKTQPRE 300
EQFGGTYRVV SVLPIGHQDW LKGKQFTCKV NNKALPSPIE RTISKARGQA HQPSVYVLPP 360
SREELSKNTV SLTCLIKDFF PPDIDVEWQS NGQQEPESKY RTTPPQLDED GSYFLYSKLS 420
VDKSRWQRGD TFICAVMHEA LHNHYTQESL SHSPGK 456

```

where amino acids 1-116 comprise a fragment of heavy chain variable region of rituximab and amino acids 117-456 comprise a mutated canine IgG2 heavy chain containing the constant region.

Rituximab Canine IgG2 AAA(G298A, E333A, R334A) SEQ ID NO: 34

QVQLQQPGAE LVKPGASVKM SCKASGYTFT SYNMHWVKQT PGRGLEWIGA IYPGNGDTSY 60
 NQKFKGKATL TADKSSSTAY MQLSSLTSED SAVYYCARST YYGGDWYFNV WGAGTTTVTS 120
 AASTTAPSVF PLAPSCGSTS GSTVALACLV SGYFPEPVTV SWNSGSLTSG VHTFPSVLQS 180
 SGLYSLSSMV TVPSSRPSE TFTCNVAHPA SKTKVDKPVP KRENGRVPRP PDCPKCPAPE 240
 MLGGPSVFIF PPKPKDTLLI ARTPEVTCVV VDLDPEDPEV QISWFVDGKQ MQTAKTQPRE 300
 EQFNATYRVV SVLPIGHQDW LKGKQFTCKV NNKALPSPIA ATISKARGQA HQPSVYVLPP 360
 SREELSKNTV SLTCLIKDFF PPDIDVEWQS NGQQEPESKY RTTPPQLDED GSYFLYSKLS 420
 VDKSRWQRGD TFICAVMHEA LHNHYTQESL SHSPGK 456

where amino acids 1-116 comprise a fragment of heavy chain variable region of rituximab and amino acids 117-456 comprise a mutated canine IgG2 heavy chain containing the constant region.

Rituximab Canine IgG2 DLE (S239D, S330L, I332E) SEQ ID NO: 35

QVQLQQPGAE LVKPGASVKM SCKASGYTFT SYNMHWVKQT PGRGLEWIGA IYPGNGDTSY 60
 NQKFKGKATL TADKSSSTAY MQLSSLTSED SAVYYCARST YYGGDWYFNV WGAGTTTVTS 120
 AASTTAPSVF PLAPSCGSTS GSTVALACLV SGYFPEPVTV SWNSGSLTSG VHTFPSVLQS 180
 SGLYSLSSMV TVPSSRPSE TFTCNVAHPA SKTKVDKPVP KRENGRVPRP PDCPKCPAPE 240
 MLGGPDVFIF PPKPKDTLLI ARTPEVTCVV VDLDPEDPEV QISWFVDGKQ MQTAKTQPRE 300
 EQFNGTYRVV SVLPIGHQDW LKGKQFTCKV NNKALPLPEE RTISKARGQA HQPSVYVLPP 360
 SREELSKNTV SLTCLIKDFF PPDIDVEWQS NGQQEPESKY RTTPPQLDED GSYFLYSKLS 420
 VDKSRWQRGD TFICAVMHEA LHNHYTQESL SHSPGK 456

where amino acids 1-116 comprise a fragment of heavy chain variable region of rituximab and amino acids 117-456 comprise a mutated canine IgG2 heavy chain containing the constant region.

Rituximab Canine IgG2 DE (S239D, I332E) SEQ ID NO: 36

QVQLQQPGAE LVKPGASVKM SCKASGYTFT SYNMHWVKQT PGRGLEWIGA IYPGNGDTSY 60
 NQKFKGKATL TADKSSSTAY MQLSSLTSED SAVYYCARST YYGGDWYFNV WGAGTTVTVS 120
 AASTTAPSVF PLAPSCGSTS GSTVALACLV SGYFPEPVTV SWNSGSLTSG VHTFPSVLQS 180
 SGLYSLSSMV TVPSSRPSE TFTCNVAHPA SKTKVDKPVP KRENGRVPRP PDCPKCPAPE 240
 MLGGPDVFIF PPKPKDTLLI ARTPEVTCVV VDLDPEDPEV QISWFVDGKQ MQTAKTQPRE 300
 EQFNGTYRVV SVLPIGHQDW LKGKQFTCKV NNKALPSP~~EE~~ RTISKARGQA HQPSVYVLPP 360
 SREELSKNTV SLTCLIKDFF PPDIDVEWQS NGQQEPESKY RTTPPQLDED GSYFLYSKLS 420
 VDKSRWQRGD TFICAVMHEA LHNHYTQESL SHSPGK 456

where amino acids 1-116 comprise a fragment of heavy chain variable region of rituximab and amino acids 117-456 comprise a mutated canine IgG2 heavy chain containing the constant region.

Rituximab Canine IgG2 DAE (G236A, S239D, I332E) SEQ ID NO: 37

QVQLQQPGAE LVKPGASVKM SCKASGYTFT SYNMHWVKQT PGRGLEWIGA IYPGNGDTSY 60
 NQKFKGKATL TADKSSSTAY MQLSSLTSED SAVYYCARST YYGGDWYFNV WGAGTTVTVS 120
 AASTTAPSVF PLAPSCGSTS GSTVALACLV SGYFPEPVTV SWNSGSLTSG VHTFPSVLQS 180
 SGLYSLSSMV TVPSSRPSE TFTCNVAHPA SKTKVDKPVP KRENGRVPRP PDCPKCPAPE 240
 ML~~AG~~PDVFIF PPKPKDTLLI ARTPEVTCVV VDLDPEDPEV QISWFVDGKQ MQTAKTQPRE 300
 EQFNGTYRVV SVLPIGHQDW LKGKQFTCKV NNKALPSP~~EE~~ RTISKARGQA HQPSVYVLPP 360
 SREELSKNTV SLTCLIKDFF PPDIDVEWQS NGQQEPESKY RTTPPQLDED GSYFLYSKLS 420
 VDKSRWQRGD TFICAVMHEA LHNHYTQESL SHSPGK 456

where amino acids 1-116 comprise a fragment of heavy chain variable region of rituximab and amino acids 117-456 comprise a mutated canine IgG2 heavy chain containing the constant region.

Rituximab Canine IgG2 YWA (L235Y, G237W, G298A) SEQ ID NO: 38

QVQLQQPGAE LVKPGASVKM SCKASGYTFT SYNMHWVKQT PGRGLEWIGA IYPGNGDTSY 60
 NQKFKGKATL TADKSSSTAY MQLSSLTSED SAVYYCARST YYGGDWYFNV WGAGTTTVTS 120
 AASTTAPSVF PLAPSCGSTS GSTVALACLV SGYFPEPVTV SWNSGSLTSG VHTFPSVLQS 180
 SGLYSLSSMV TVPSSRPSE TFTCNVAHPA SKTKVDKPVP KRENGRVPRP PDCPKCPAPE 240
 MYGWPSVFIF PPKPKDTLLI ARTPEVTCVV VDLDPEDPEV QISWFVDGKQ MQTAKTQPRE 300
 EQFNATYRVV SVLPIGHQDW LKGKQFTCKV NNKALPSPIE RTISKARGQA HQPSVYVLPP 360
 SREELSKNTV SLTCLIKDFF PPDIDVEWQS NGQQEPESKY RTTPPQLDED GSYFLYSKLS 420
 VDKSRWQRGD TFICAVMHEA LHNHYTQESL SHSPGK 456

where amino acids 1-116 comprise a fragment of heavy chain variable region of rituximab and amino acids 117-456 comprise a mutated canine IgG2 heavy chain containing the constant region.

Rituximab Canine IgG2 EFT (D267E, P268F, N324T) SEQ ID NO: 39

QVQLQQPGAE LVKPGASVKM SCKASGYTFT SYNMHWVKQT PGRGLEWIGA IYPGNGDTSY 60
 NQKFKGKATL TADKSSSTAY MQLSSLTSED SAVYYCARST YYGGDWYFNV WGAGTTTVTS 120
 AASTTAPSVF PLAPSCGSTS GSTVALACLV SGYFPEPVTV SWNSGSLTSG VHTFPSVLQS 180
 SGLYSLSSMV TVPSSRPSE TFTCNVAHPA SKTKVDKPVP KRENGRVPRP PDCPKCPAPE 240
 MLGGPSVFIF PPKPKDTLLI ARTPEVTCVV VDL~~EF~~EDPEV QISWFVDGKQ MQTAKTQPRE 300
 EQFNGTYRVV SVLPIGHQDW LKGKQFTCKV ~~T~~NKALPSPIE RTISKARGQA HQPSVYVLPP 360
 SREELSKNTV SLTCLIKDFF PPDIDVEWQS NGQQEPESKY RTTPPQLDED GSYFLYSKLS 420
 VDKSRWQRGD TFICAVMHEA LHNHYTQESL SHSPGK 456

where amino acids 1-116 comprise a fragment of heavy chain variable region of rituximab and amino acids 117-456 comprise a mutated canine IgG2 heavy chain containing the constant region.

Rituximab Canine IgG2 Q345R (Q345R) SEQ ID NO: 40

QVQLQQPGAE LVKPGASVKM SCKASGYTFT SYNMHWVKQT PGRGLEWIGA IYPGNGDTSY 60
 NQKFKGKATL TADKSSSTAY MQLSSLTSED SAVYYCARST YYGGDWYFNV WGAGTTTVTS 120
 AASTTAPSVF PLAPSCGSTS GSTVALACLV SGYFPEPVTV SWNSGSLTSG VHTFPSVLQS 180
 SGLYSLSSMV TVPSSRPSE TFTCNVAHPA SKTKVDKPVP KRENGRVPRP PDCPKCPAPE 240
 MLGGPSVFIF PPKPKDTLLI ARTPEVTCVV VLDLDPEDPEV QISWFVDGKQ MQTAKTQPRE 300
 EQFNGTYRVV SVLPIGHQDW LKGKQFTCKV NNKALPSPIE RTISKARGQA HRPSVYVLPP 360
 SREELSKNTV SLTCLIKDFF PPDIDVEWQS NGQQEPESKY RTTPPQLDED GSYFLYSKLS 420
 VDKSRWQRGD TFICAVMHEA LHNHYTQESL SHSPGK 456

where amino acids 1-116 comprise a fragment of heavy chain variable region of rituximab and
 amino acids 117-456 comprise a mutated canine IgG2 heavy chain containing the constant
 region.

Canine CTL4 - Canine IgG2 Fusion D270G (D270G) SEQ ID NO: 41

METDTLLLVV LLLWVPGSTG DMHVAQPAVV LASSRGVASF VCEYGSSGNA AEVRVTVLRQ 60
 AGSQMTEVCA ATYTVEDELA FLDDSTCTGT SSGNKVNLT I QGLRAMDTGL YICKVELMYP 120
 PPYYVGMGNG TQIYVIDPEP CPDSDPKREN GRVPRPPDCP KCPAPEMLGG PSVFIFPPKP 180
 KDTLLIARTP EVTCVVVDLD PEGPEVQISW FVDGKQMOTA KTQPREEQFN GTYRVVSVLP 240
 IGHQDWLKGK QFTCKVNNKA LPSP IERTIS KARGQAHQPS VYVLPPSREE LSKNTVSLTC 300
 LIKDFFPDI DVEWQSNQQ EPESKYRTTP PQLDEDGSYF LYSKLSVDKS RWQRGDTFIC 360
 AVMHEALHNNH YTQESLSHSP GK 382

where amino acids 1-145 comprise a fragment of canine CTLA-4 (Accession XP_038303218)
 and amino acids 146-382 comprise a fragment of canine IgG2 heavy chain containing the
 constant region.

Canine CTL4 - Canine IgG2 Fusion D270S (D270S) SEQ ID NO: 42

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METDTLLLWV LLLWVPGSTG DMHVAQPAVV LASSRGVASF VCEYGSSGNA AEVRVTVLRQ 60
AGSQMTEVCA ATYTVEDELA FLDDSTCTGT SSGNKVNLT I QGLRAMDTGL YICKVELMYP 120
PPYYVGMGNG TQIYVIDPEP CPDSDPKREN GRVPRPPDCP KCPAPEMLGG PSVFIFPPKP 180
KDTLLIARTP EVTCVVVDLD PESPEVQISW FVDGKQMOTA KTQPREEQFN GTYRVVSVLP 240
IGHQDWLKGK QFTCKVNNKA LPSPIERTIS KARGQAHQPS VYVLPPSREE LSKNTVSLTC 300
LIKDFFPDDI DVEWQSNQQ EPESKYRTTP PQLDEDGSYF LYSKLSVDKS RWQRGDTFIC 360
AVMHEALHNN YTQESLSHSP GK 382

```

where amino acids 1-145 comprise a fragment of canine CTLA-4 (Accession XP_038303218) and amino acids 146-382 comprise a fragment of canine IgG2 heavy chain containing the constant region.

Canine CTL4 - Canine IgG2 Fusion WIN (M234A, L235A, G237A) SEQ ID NO:**43**

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METDTLLLWV LLLWVPGSTG DMHVAQPAVV LASSRGVASF VCEYGSSGNA AEVRVTVLRQ 60
AGSQMTEVCA ATYTVEDELA FLDDSTCTGT SSGNKVNLT I QGLRAMDTGL YICKVELMYP 120
PPYYVGMGNG TQIYVIDPEP CPDSDPKREN GRVPRPPDCP KCPAPEAAGA PSVFIFPPKP 180
KDTLLIARTP EVTCVVVDLD PESPEVQISW FVDGKQMOTA KTQPREEQFN GTYRVVSVLP 240
IGHQDWLKGK QFTCKVNNKA LPSPIERTIS KARGQAHQPS VYVLPPSREE LSKNTVSLTC 300
LIKDFFPDDI DVEWQSNQQ EPESKYRTTP PQLDEDGSYF LYSKLSVDKS RWQRGDTFIC 360
AVMHEALHNN YTQESLSHSP GK 382

```

where amino acids 1-145 comprise a fragment of canine CTLA-4 (Accession XP_038303218) and amino acids 146-382 comprise a fragment of canine IgG2 heavy chain containing the constant region.

Canine CTL4 - Canine IgG2 Fusion WIN_DANG_GAP (M234A, L235A, G237A; D265A, N297G; P329G, S330A) SEQ ID NO: 44

```

METDTLLLVV LLLWVPGSTG DMHVAQPAVV LASSRGVASF VCEYGSSGNA AEVRVTVLRQ 60
AGSQMTEVCA ATYTVEDELA FLDDSTCTGT SSGNKVNLT I QGLRAMDTGL YICKVELMYP 120
PFYYVGMGNG TQIYVIDPEP CPDSDPKREN GRVPRPPDCP KCPAPEAAGA PSVFIFPPKP 180
KDTLLIARTP EVTCVVVALD PEDPEVQISW FVDGKQMOTA KTQPREEQFG GTYRVVSVLP 240
IGHQDWLKGK QFTCKVNNKA LGAPIERTIS KARGQAHQPS VYVLPPSREE LSKNTVSLTC 300
LIKDFFPDI DVEWQSNQQ EPESKYRTTP PQLEDEGSYF LYSKLSVDKS RWQRGDTFIC 360
AVMHEALHNH YTQESLSHSP GK 382

```

where amino acids 1-145 comprise a fragment of canine CTLA-4 (Accession XP_038303218) and amino acids 146-382 comprise a fragment of canine IgG2 heavy chain containing the constant region.

Equine IgG

[00187] The inventors of the instant application have surprisingly found that by substituting one or more amino acid residues in the Fc region of equine IgG1, advantageous characteristics may be introduced into equine IgGs, for example reducing or completely knocking out CDC, ADCC and ADCP activities or increasing or decreasing binding affinity to canine Fcγ receptor and C1q.

[00188] Accordingly, in various embodiments, an Fc region of equine IgG1 may comprise one or more mutations or substitutions, including at locations 229, 234, 235, 237, 329, 330, and/or 331. In various embodiments, an Fc region of equine IgG1 may comprise one or more mutations and/or substitutions selected from among P229S, L234A, L235A, G237A, P329S, Q330A, Q330S, and P331S, using EU Index numbering as in Kabat. For example, various combinations of substitutions in a Fc region of equine IgG1 can include one or more of the following, referred

to herein by short designations: SAP (P329S, Q330A), PAP (Q330A), SQP (P229S), WIN (L234A, L235A, G237A), PSS (Q330S, P331S), WIN_SAP (L234A, L235A, G237A; P329S, Q330A), and WIN_PSS (L234A, L235A, G237A; Q330S, P331S).

[00189] As further examples, mutated equine IgG1 Fc can be comprised within a recombinantly modified protein, *e.g.*, an equine IgG heavy chain comprising a rituximab variable region and a mutated constant region, or a fusion protein comprising an effector region (such as a CTLA-4 sequence) fused to an equine IgG heavy chain comprising a mutated constant region.

Examples of such proteins include the following sequences:

Rituximab Equine IgG1 wt SEQ ID NO: 45

```

QVQLQQPGAE LVKPGASVKM SCKASGYTFT SYNHWVVKQT PGRGLEWIGA IYPGNGDTSY 60
NQKFKGKATL TADKSSSTAY MQLSSLTSED SAVYYCARST YYGGDWYFNV WGAGTTVTVS 120
SASTTAPKVF ALAPGCGTTS DSTVALGCLV SGYFPEPVKV SWNSGSLTSG VHTFPSVLQS 180
SGFYSLSSMV TVPASTWTSE TYICNVVHAA SNFKVDKRIE PIPDNHQKVC DMSKCPKCPA 240
PELLGGPSVF IFPPNPKDTL MITRTPEVTC VVDVDSQENP DVKFNWYMDG VEVRTATTRP 300
KEEQFNSTYR VVSVLRIQHQ DWLSGKEFKC KVNQALPQP IERTITKTKG RSQEPQVYVL 360
APHPDELSKS KSVTCLVKD FYPPEINIEW QSNQPELET KYSTTQAQDD SDGSYFLYSK 420
LSVDRNRWQQ GTTFTCGVMH EALHNHYTQK NVSKNPGK 458

```

where amino acids 1-121 comprise a rituximab variable region and amino acids 122-458 comprise a wild type sequence of the equine IgG1 heavy chain constant region (*e.g.*, accession CAC44624).

Rituximab Equine IgG1 SAP (P329S, Q330A) SEQ ID NO: 46

QVQLQQPGAE LVKPGASVKM SCKASGYTFT SYNMHWVKQT PGRGLEWIGA IYPGNGDTSY 60
 NQKFKGKATL TADKSSSTAY MQLSSLTSED SAVYYCARST YYGGDWYFNV WGAGTTTVTS 120
 SASTTAPKVF ALAPGCGTTS DSTVALGCLV SGYFPEPVKV SWNSGSLTSG VHTFPSVLQS 180
 SGFYSLSSMV TVPASTWTSE TYICNVVHAA SNFKVDKRIE PIPDNHQKVC DMSKCPKCPA 240
 PELLGGPSVF IFPPNPKDTL MITRTPEVTC VVDVVSQENP DVKFNWYMDG VEVRTATTRP 300
 KEEQFNSTYR VVSVLRIQHQ DWLSGKEFKC KVNQAL**SAP** IERTITKTKG RSQEPQVYVL 360
 APHPDELSKS KSVTCLVKD FYPPEINIEW QSNQPELET KYSTTQAQQD SDGSYFLYSK 420
 LSVDRNRWQQ GTTFTCGVMH EALHNHYTQK NVSKNPGK 458

where amino acids 1-121 comprise a rituximab variable region and amino acids 122-458 comprise a mutated sequence of the equine IgG1 heavy chain constant region.

Rituximab Equine IgG1 WIN (L234A, L235A, G237A) SEQ ID NO:47

QVQLQQPGAE LVKPGASVKM SCKASGYTFT SYNMHWVKQT PGRGLEWIGA IYPGNGDTSY 60
 NQKFKGKATL TADKSSSTAY MQLSSLTSED SAVYYCARST YYGGDWYFNV WGAGTTTVTS 120
 SASTTAPKVF ALAPGCGTTS DSTVALGCLV SGYFPEPVKV SWNSGSLTSG VHTFPSVLQS 180
 SGFYSLSSMV TVPASTWTSE TYICNVVHAA SNFKVDKRIE PIPDNHQKVC DMSKCPKCPA 240
 PE**AAGA**PSVF IFPPNPKDTL MITRTPEVTC VVDVVSQENP DVKFNWYMDG VEVRTATTRP 300
 KEEQFNSTYR VVSVLRIQHQ DWLSGKEFKC KVNQALPQP IERTITKTKG RSQEPQVYVL 360
 APHPDELSKS KSVTCLVKD FYPPEINIEW QSNQPELET KYSTTQAQQD SDGSYFLYSK 420
 LSVDRNRWQQ GTTFTCGVMH EALHNHYTQK NVSKNPGK 458

where amino acids 1-121 comprise a rituximab variable region and amino acids 122-458 comprise a mutated sequence of the equine IgG1 heavy chain constant region.

Rituximab Equine IgG1 PSS (Q330S, P331S) SEQ ID NO:48

QVQLQQPGAE LVKPGASVKM SCKASGYTFT SYNMHWVKQT PGRGLEWIGA IYPGNGDTSY 60
 NQKFKGKATL TADKSSSTAY MQLSSLTSED SAVYYCARST YYGGDWYFNV WGAGTTVTVS 120
 SASTTAPKVF ALAPGCGTTS DSTVALGCLV SGYFPEPVKV SWNSGSLTSG VHTFPSVLQS 180
 SGFYSLSSMV TVPASTWTSE TYICNVVHAA SNFKVDKRIE PIPDNHQKVC DMSKCPKCPA 240
 PELLGGPSVF IFPPNPKDTL MITRTPEVTC VVDVVSQENP DVKFNWYMDG VEVRTATTRP 300
 KEEQFNSTYR VVSVLRIQHQ DWLSGKEFKC KVNQALPSS IERTITKTKG RSQEPQVYVL 360
 APHPDELSKS KSVTCLVKD FYPPEINIEW QSNGQPELET KYSTTQAQQD SDGSYFLYSK 420
 LSVDRNRWQQ GTTFTCGVMH EALHNHYTQK NVSKNPGK 458

where amino acids 1-121 comprise a rituximab variable region and amino acids 122-458
 comprise a mutated sequence of the equine IgG1 heavy chain constant region.

Rituximab Equine IgG1 WIN_SAP (L234A, L235A, G237A; P329S, Q330A) SEQ ID NO:49

QVQLQQPGAE LVKPGASVKM SCKASGYTFT SYNMHWVKQT PGRGLEWIGA IYPGNGDTSY 60
 NQKFKGKATL TADKSSSTAY MQLSSLTSED SAVYYCARST YYGGDWYFNV WGAGTTVTVS 120
 SASTTAPKVF ALAPGCGTTS DSTVALGCLV SGYFPEPVKV SWNSGSLTSG VHTFPSVLQS 180
 SGFYSLSSMV TVPASTWTSE TYICNVVHAA SNFKVDKRIE PIPDNHQKVC DMSKCPKCPA 240
 PEAAAGAPSVF IFPPNPKDTL MITRTPEVTC VVDVVSQENP DVKFNWYMDG VEVRTATTRP 300
 KEEQFNSTYR VVSVLRIQHQ DWLSGKEFKC KVNQALSAAP IERTITKTKG RSQEPQVYVL 360
 APHPDELSKS KSVTCLVKD FYPPEINIEW QSNGQPELET KYSTTQAQQD SDGSYFLYSK 420
 LSVDRNRWQQ GTTFTCGVMH EALHNHYTQK NVSKNPGK 458

where amino acids 1-121 comprise a rituximab variable region and amino acids 122-458
 comprise a mutated sequence of the equine IgG1 heavy chain constant region.

Rituximab Equine IgG1 WIN_PSS (L234A, L235A, G237A; Q330S, P331S) SEQ**ID NO:50**

QVQLQQPGAE LVKPGASVKM SCKASGYTFT SYNMHWVKQT PGRGLEWIGA IYPNGDTSY 60
 NQKFKGKATL TADKSSSTAY MQLSSLTSED SAVYYCARST YYGGDWYFNV WGAGTTVTVS 120
 SASTTAPKVF ALAPGCGTTS DSTVALGCLV SGYFPEPVKV SWNSGSLTSG VHTFPSVLQS 180
 SGFYSLSSMV TVPASTWTSE TYICNVVHAA SNFKVDKRIE PIPDNHQKVC DMSKCPKCPA 240
 PE**AAGA**PSVF IFPPNPKDTL MITRTPEVTC VVVDVSQENP DVKFNWYMDG VEVRTATTRP 300
 KEEQFNSTYR VVSVLRIQHQ DWLSGKEFKC KVNQALP**SS** IERTITKTKG RSQEPQVYVL 360
 APHPDELSKS KSVTCLVKD FYPPEINIEW QSNQPELET KYSTTQAQQD SDGSYFLYSK 420
 LSVDRNRWQQ GTTFTCGVMH EALHNHYTQK NVSKNPGK 458

where amino acids 1-121 comprise a rituximab variable region and amino acids 122-458 comprise a mutated sequence of the equine IgG1 heavy chain constant region.

Canine CTLA-4 Equine IgG1 fusion PAP (Q330A) SEQ ID NO: 51

METDTLLLVV LLLWVPGSTG DMHVAQPAVV LASSRGVASF VCEYGSSGNA AEVRVTVLRQ 60
 AGSQMTEVCA ATYTVEDELA FLDDSTCTGT SSGNKVNLT I QGLRAMDTGL YICKVELMYP 120
 PPYYVGMGNG TQIYVIDPEP CPDSDEPIP NHQKVCDMSK CPKCPAPELL GGPSVFIFPP 180
 NPKDTLMITR TPEVTCVVVD VSQENPDVKF NWYMDGVEVR TATTRPKEEQ FNSTYRVVSV 240
 LRIQHQDWLS GKEFKCKVNN QALP**API**ERT ITKTGRSQE PQVYVLAPHP DELSKSKVSV 300
 TCLVKDFYPP EINIEWQSNQ QPELETKYST TQAQQDSDGS YFLYSKLSVD RNRWQQGTTF 360
 TCGVMHEALH NHYTQKNVSK NPGK 384

where amino acids 22-145 comprise a fragment of canine CTLA-4 (Accession XP_038303218) and amino acids 146-384 comprise a fragment of equine IgG1 heavy chain containing mutated the constant region.

Canine CTLA-4 Equine IgG1 fusion SQP (P229S) SEQ ID NO: 52

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METDTLLLV LLLWVPGSTG DMHVAQPAVV LASSRGVASF VCEYGSSGNA AEVRVTVLRQ 60
AGSQMTEVCA ATYTVEDELA FLDDSTCTGT SSGNKVNLT I QGLRAMDTGL YICKVELMYP 120
PPYYVGMGNG TQIYVIDPEP CPDSDEPIPD NHQKVCDSK CPKCPAPELL GGPSVFIFPP 180
NPKDTLMITR TPEVTCVVVD VSQENPDVKF NWYMDGVEVR TATTRPKEEQ FNSTYRVVSV 240
LRIQHQDWLS GKEFKCKVNN QALSQPIERT ITKTGRSQE PQVYVLAPHP DELSKSKVSV 300
TCLVKDFYPP EINIEWQSNG QPELETKYST TQAQQSDSGS YFLYSKLSVD RNRWQQGTTF 360
TCGVMHEALH NHYTQKNVSK NPGK 384

```

where amino acids 22-145 comprise a fragment of canine CTLA-4 (Accession XP_038303218) and amino acids 146-384 comprise a fragment of equine IgG1 heavy chain containing mutated the constant region.

[00190] The inventors of the instant application have surprisingly found that by substituting one or more amino acid residues in the Fc region of equine IgG4, advantageous characteristics may be introduced into equine IgGs, for example reducing or completely knocking out CDC, ADCC and ADCP activities or increasing or decreasing binding affinity to canine Fcγ receptor and C1q.

[00191] Accordingly, in various embodiments, an Fc region of equine IgG4 may comprise one or more mutations or substitutions, including at locations 229, 234, 235, 237, 329, 330, and/or 331. In various embodiments, an Fc region of equine IgG4 may comprise one or more mutations and/or substitutions selected from among L234A, Q235A, G237A, P239S, A330Q, A330S, P331S, using EU Index numbering as in Kabat. For example, various combinations of substitutions in a Fc region of equine IgG4 can include one or more of the following, referred to herein by short designations: PSS (A330S, P331S), SQP (P239S, A330Q), Win (L234A,

Q235A, G237A), WinPSS (L234A, Q235A, G237A; A330S, P331S), SAP (P329S), and WinSAP (L234A, Q235A, G237A; P329S).

[00192] As further examples, mutated equine IgG4 Fc can be comprised within a recombinantly modified protein, *e.g.*, an equine IgG heavy chain comprising a rituximab variable region and a mutated constant region, or a fusion protein comprising an effector region (such as a CTLA-4 sequence) fused to an equine IgG heavy chain comprising a mutated constant region. Examples of such proteins include the following sequences:

Canine CTLA-4 Equine IgG4 fusion PSS (A330S, P331S) SEQ ID NO: 53

```

METDTLLLVV LLLWVPGSTG DMHVAQPAVV LASSRGVASF VCEYGSSGNA AEVRVTVLRQ 60
AGSQMTEVCA ATYTVEDELA FLDDSTCTGT SSGNKVNLTQ QGLRAMDTGL YICKVELMYP 120
PPYYVGMGNG TQIYVIDPEP CPDSDVIKEC NGGCPAECLQ VGPSVFIFPP KPKDVLMISS 180
TPTVTCVVVD VGHDFPDVQF NWYVDGVETH TATTEPKQEQ FNSTYRVVSV LPIQHKDWLS 240
GKEFKCKVNN KALPSSVERT ISKPTGQPRE PQVYVLAPHR DELSKNKVSV TCLVKDFYPT 300
DIDIEWKSNG QPEPETKYST TPAQLSDSGS YFLYSKLTVE TNRWQQGTTF TCAVMHEALH 360
NHYTEKSVSK SPGK 374

```

where amino acids 22-145 comprise a fragment of canine CTLA-4 (Accession XP_038303218) and amino acids 146-374 comprise a fragment of equine IgG1 heavy chain containing mutated the constant region.

Canine CTLA-4 Equine IgG4 fusion SQP (P239S, A330Q) SEQ ID NO:54

METDTLLLWV LLLWVPGSTG DMHVAQPAVV LASSRGVASF VCEYGSSGNA AEVRVTVLRQ 60
 AGSQMTEVCA ATYTVEDELA FLDDSTCTGT SSGNKVNLT I QGLRAMDTGL YICKVELMYP 120
 PPIYVGMGNG TQIYVIDPEP CPDSDVIKEC NGGCPAECLQ VGPSVFIFPP KPKDVLMI SR 180
 TPTVTCVVVD VGHDFPDVQF NWYVDGVETH TATTEPKQEQ FNSTYRVVSV LPIQHKDWLS 240
 GKEFKCKVNN KAL**SQ**PVERT ISKPTGQPRE PQVYVLAPHR DELSKNKVSV TCLVKDFYPT 300
 DIDIEWKSNG QPEPETKYST TPAQLSDSGS YFLYSKLTVE TNRWQQGTTF TCAVMHEALH 360
 NHYTEKSVSK SP GK 374

where amino acids 22-145 comprise a fragment of canine CTLA-4 (Accession XP_038303218) and amino acids 146-374 comprise a fragment of equine IgG1 heavy chain containing mutated the constant region.

Canine CTLA-4 Equine IgG4 fusion WIN_PSS (L234A, Q235A, G237A; A330S, P331S) SEQ ID NO: 55

METDTLLLWV LLLWVPGSTG DMHVAQPAVV LASSRGVASF VCEYGSSGNA AEVRVTVLRQ 60
 AGSQMTEVCA ATYTVEDELA FLDDSTCTGT SSGNKVNLT I QGLRAMDTGL YICKVELMYP 120
 PPIYVGMGNG TQIYVIDPEP CPDSDVIKEC NGGCPAE**CAA** **G**APSVFIFPP KPKDVLMI SR 180
 TPTVTCVVVD VGHDFPDVQF NWYVDGVETH TATTEPKQEQ FNSTYRVVSV LPIQHKDWLS 240
 GKEFKCKVNN KAL**PSS**VERT ISKPTGQPRE PQVYVLAPHR DELSKNKVSV TCLVKDFYPT 300
 DIDIEWKSNG QPEPETKYST TPAQLSDSGS YFLYSKLTVE TNRWQQGTTF TCAVMHEALH 360
 NHYTEKSVSK SP GK 374

where amino acids 22-145 comprise a fragment of canine CTLA-4 (Accession XP_038303218) and amino acids 146-374 comprise a fragment of equine IgG1 heavy chain containing mutated the constant region.

Canine CTLA-4 Equine IgG4 fusion WIN_SAP (L234A, Q235A, G237A; P329S)**SEQ ID NO: 56**

METDTLLLV LLLWVPGSTG DMHVAQPAVV LASSRGVASF VCEYGSSGNA AEVRVTVLRQ 60
 AGSQMTEVCA ATYTVEDELA FLDDSTCTGT SSGNKVNLT I QGLRAMDTGL YICKVELMYP 120
 PPYYVGMGNG TQIYVIDPEP CPDSDVIKEC NGGCPAEC**AA** **G**APSVFIFPP FIFPP KPKDVLMI SR 180
 TPTVTCVVVD VGHDFPDVQF NWYVDGVETH TATTEPKQEQ FNSTYRVVSV LPIQHKDWLS 240
 GKEFKCKVNN KAL**S**APVERT ISKPTGQPRE PQVYVLAPHR DELSKNKVSV TCLVKDFYPT 300
 DIDIEWKSNG QPEPETKYST TPAQLSDSGS YFLYSKLTVE TNRWQQGTTF TCAVMHEALH 360
 NHYTEKSVSK SPGK 374

where amino acids 22-145 comprise a fragment of canine CTLA-4 (Accession
 XP_038303218) and amino acids 146-374 comprise a fragment of equine IgG1 heavy chain
 containing mutated the constant region.

Canine CTLA-4 Equine IgG4 fusion SAP (P329S) SEQ ID NO: 57

METDTLLLV LLLWVPGSTG DMHVAQPAVV LASSRGVASF VCEYGSSGNA AEVRVTVLRQ 60
 AGSQMTEVCA ATYTVEDELA FLDDSTCTGT SSGNKVNLT I QGLRAMDTGL YICKVELMYP 120
 PPYYVGMGNG TQIYVIDPEP CPDSDVIKEC NGGCPAEC**LQ** VGPSVFIFPP KPKDVLMI SR 180
 TPTVTCVVVD VGHDFPDVQF NWYVDGVETH TATTEPKQEQ FNSTYRVVSV LPIQHKDWLS 240
 GKEFKCKVNN KAL**S**APVERT ISKPTGQPRE PQVYVLAPHR DELSKNKVSV TCLVKDFYPT 300
 DIDIEWKSNG QPEPETKYST TPAQLSDSGS YFLYSKLTVE TNRWQQGTTF TCAVMHEALH 360
 NHYTEKSVSK SPGK 374

where amino acids 22-145 comprise a fragment of canine CTLA-4 (Accession
 XP_038303218) and amino acids 146-374 comprise a fragment of equine IgG1 heavy chain
 containing mutated the constant region.

Canine CTLA-4 Equine IgG4 fusion WIN (L234A, Q235A, G237A) SEQ ID NO:

58

```
METDTLLLWV LLLWVPGSTG DMHVAQPAVV LASSRGVASF VCEYGSSGNA AEVRVTVLRQ 60
AGSQMTEVCA ATYTVEDELA FLDDSTCTGT SSGNKVNLTI QGLRAMDTGL YICKVELMYP 120
PPYYVGMGNG TQIYVIDPEP CPDSDVIKEC NGGCPAECAA GAPSVFIFPP KPKDVLMISR 180
TPTVTCTVVVD VGHDFPDVQF NWYVDGVETH TATTEPKQEQ FNSTYRVVSV LPIQHKDWLS 240
GKEFKCKVNN KALPAPVERT ISKPTGQPRE PQVYVLAPHR DELSKNKVSV TCLVKDFYPT 300
DIDIEWKSNG QPEPETKYST TPAQLSDSGS YFLYSKLTVE TNRWQQGTFE TCAVMHEALH 360
NHYTEKSVSK SPGK 374
```

where amino acids 22-145 comprise a fragment of canine CTLA-4 (Accession XP_038303218) and amino acids 146-374 comprise a fragment of equine IgG1 heavy chain containing mutated the constant region.

[00193] The inventors of the instant application have surprisingly found that by substituting one or more amino acid residues in the Fc region of equine IgG7, advantageous characteristics may be introduced into equine IgGs, for example reducing or completely knocking out CDC, ADCC and ADCP activities or increasing or decreasing binding affinity to canine Fcγ receptor and C1q.

[00194] Accordingly, in various embodiments, an Fc region of equine IgG7 may comprise one or more mutations or substitutions, including at locations 229, 234, 235, 237, 329, 330, and/or 331. In various embodiments, an Fc region of equine IgG7 may comprise one or more mutations and/or substitutions selected from among L234A, S235A, V236G, G237A, P239S, P329S, A330S, A330Q, P331S, using EU Index numbering as in Kabat. For example, various combinations of substitutions in a Fc region of equine IgG7 can include one or more of the following, referred to herein by short designations: PSS (A330S, P331S), SAP (P329S), SQP

(P239S, A330Q), WIN (L234A, S235A, V236G, G237A), WIN_PSS (L234A, S235A, V236G, G237A; A330S, P331S), and WIN_SAP (L234A, S235A, V236G, G237A; P329S).

[00195] As further examples, mutated equine IgG7 Fc can be comprised within a recombinantly modified protein, *e.g.*, an equine IgG heavy chain comprising a rituximab variable region and a mutated constant region, or a fusion protein comprising an effector region (such as a CTLA-4 sequence) fused to an equine IgG heavy chain comprising a mutated constant region. Examples of such proteins include the following sequences:

Canine CTLA-4 Equine IgG7 fusion PSS (A330S, P331S) SEQ ID NO: 59

```

METDTLLLV LLLWVPGSTG DMHVAQPAVV LASSRGVASF VCEYGSSGNA AEVRVTVLRQ 60
AGSQMTEVCA ATYTVEDELA FLDDSTCTGT SSGNKVNLTQ QGLRAMDTGL YICKVELMYP 120
PPYYVGMGNG TQIYVIDPEP CPDSDVIKEC GGCPTCPECL SVGPSVFIFP PKPKDVLMS 180
RTPTVTCVVV DVGHDFFDVQ FNWYVDGVET HTATTEPKQE QNNSTYRVVS ILAIQHKDWL 240
SGKEFKCKVN NQALPSSVQK TISKPTGQPR EPQVYVLAPH RDELSKNKVS VTCLVKDFYP 300
TDIDIEWKSN GQPEPETKYS TTPAQLDSDG SYFLYSKLTV ETNRWQQGTT FTCAMHEAL 360
HNHYTEKSVS KSPGK 375

```

where amino acids 22-145 comprise a fragment of canine CTLA-4 (Accession XP_038303218) and amino acids 146-375 comprise a fragment of equine IgG1 heavy chain containing mutated the constant region.

Canine CTLA-4 Equine IgG7 fusion SAP (P329S) SEQ ID NO: 60

METDTLLLWV LLLWVPGSTG DMHVAQPAVV LASSRGVASF VCEYGSSGNA AEVRVTVLRQ 60
 AGSQMTEVCA ATYTVEDELA FLDDSTCTGT SSGNKVNLT I QGLRAMDTGL YICKVELMYP 120
 PPYYVGMGNG TQIYVIDPEP CPDSDVIKEC GGCPTCPECL SVGPSVFIFP PKPKDVLMI 180
 RTPTVTCVVV DVGHDFFPDVQ FNWYVDGVET HTATTEPKQE QNNSTYRVVS ILAIQHKDWL 240
 SGKEFKCKVN NQALSAPVQK TISKPTGQPR EPQVYVLAPH RDELSKNKVS VTCLVKDFYP 300
 TDIDIEWKSN GQPEPETKYS TTPAQLSDG SYFLYSKLT V ETNRWQQGTT FTCAVMHEAL 360
 HNHYTEKSVS KSPGK 375

where amino acids 22-145 comprise a fragment of canine CTLA-4 (Accession XP_038303218) and amino acids 146-375 comprise a fragment of equine IgG1 heavy chain containing mutated the constant region.

Canine CTLA-4 Equine IgG7 fusion SQP (P239S, A330Q) SEQ ID NO: 61

METDTLLLWV LLLWVPGSTG DMHVAQPAVV LASSRGVASF VCEYGSSGNA AEVRVTVLRQ 60
 AGSQMTEVCA ATYTVEDELA FLDDSTCTGT SSGNKVNLT I QGLRAMDTGL YICKVELMYP 120
 PPYYVGMGNG TQIYVIDPEP CPDSDVIKEC GGCPTCPECL SVGPSVFIFP PKPKDVLMI 180
 RTPTVTCVVV DVGHDFFPDVQ FNWYVDGVET HTATTEPKQE QNNSTYRVVS ILAIQHKDWL 240
 SGKEFKCKVN NQALSQPVQK TISKPTGQPR EPQVYVLAPH RDELSKNKVS VTCLVKDFYP 300
 TDIDIEWKSN GQPEPETKYS TTPAQLSDG SYFLYSKLT V ETNRWQQGTT FTCAVMHEAL 360
 HNHYTEKSVS KSPGK 375

where amino acids 22-145 comprise a fragment of canine CTLA-4 (Accession XP_038303218) and amino acids 146-375 comprise a fragment of equine IgG1 heavy chain containing mutated the constant region.

Canine CTLA-4 Equine IgG7 fusion WIN (L234A, S235A, V236G, G237A) SEQ**ID NO: 62**

METDTLLLLWV LLLWVPGSTG DMHVAQPAVV LASSRGVASF VCEYGSSGNA AEVRVTVLRQ 60
 AGSQMTEVCA ATYTVEDELA FLDDSTCTGT SSGNKVNLT I QGLRAMDTGL YICKVELMYP 120
 PPYYVGMGNG TQIYVIDPEP CPDSDVIKEC GGCPTCPECA **AG**APSVFIFP PKPKDVLMI 180
 RTPTVTCVVV DVGHDFFPDVQ FNWYVDGVET HTATTEPKQE QNNSTYRVVS ILAIQHKDWL 240
 SGKEFKCKVN NQALPAPVQK TISKPTGQPR EPQVYVLAPH RDELSKNKVS VTCLVKDFYP 300
 TDIDIEWKSN GQPEPETKYS TTPAQLDSDG SYFLYSKLTV ETNRWQQGTT FTCAMHEAL 360
 HNHYTEKSVS KSPGK 375

where amino acids 22-145 comprise a fragment of canine CTLA-4 (Accession XP_038303218) and amino acids 146-375 comprise a fragment of equine IgG1 heavy chain containing mutated the constant region.

Canine CTLA-4 Equine IgG7 fusion WIN_PSS (L234A, S235A, V236G, G237A;**A330S, P331S) SEQ ID NO: 63**

METDTLLLLWV LLLWVPGSTG DMHVAQPAVV LASSRGVASF VCEYGSSGNA AEVRVTVLRQ 60
 AGSQMTEVCA ATYTVEDELA FLDDSTCTGT SSGNKVNLT I QGLRAMDTGL YICKVELMYP 120
 PPYYVGMGNG TQIYVIDPEP CPDSDVIKEC GGCPTCPECA **AG**APSVFIFP PKPKDVLMI 180
 RTPTVTCVVV DVGHDFFPDVQ FNWYVDGVET HTATTEPKQE QNNSTYRVVS ILAIQHKDWL 240
 SGKEFKCKVN NQALP**SS**VQK TISKPTGQPR EPQVYVLAPH RDELSKNKVS VTCLVKDFYP 300
 TDIDIEWKSN GQPEPETKYS TTPAQLDSDG SYFLYSKLTV ETNRWQQGTT FTCAMHEAL 360
 HNHYTEKSVS KSPGK 375

where amino acids 22-145 comprise a fragment of canine CTLA-4 (Accession XP_038303218) and amino acids 146-375 comprise a fragment of equine IgG1 heavy chain containing mutated the constant region.

Canine CTLA-4 Equine IgG7 fusion WIN_SAP (L234A, S235A, V236G, G237A; P329S) SEQ ID NO: 64

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METDTLLLLWV LLLWVPGSTG DMHVAQPAVV LASSRGVASF VCEYGSSGNA AEVRVTVLRQ 60
AGSQMTEVCA ATYTVEDELA FLDDSTCTGT SSGNKVNLTI QGLRAMDTGL YICKVELMYP 120
PPYYVGMNG TQIYVIDPEP CPDSDVIKEC GGCPTCPECA AGAPSVFIFP PKPKDVLMS 180
RTPTVTCVVV DVGHDFFDVQ FNWYVDGVET HTATTEPKQE QNNSTYRVVS ILAIQHKDWL 240
SGKEFKCKVN NQALSAPVQK TISKPTGQPR EPQVYVLAPH RDELSKNKVS VTCLVKDFYP 300
TDIDIEWKSN GQPEPETKYS TTPAQLDSDG SYFLYSKLTV ETNRWQQGTT FTCAMHEAL 360
HNHYTEKSVS KSPGK 375

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where amino acids 22-145 comprise a fragment of canine CTLA-4 (Accession XP_038303218) and amino acids 146-375 comprise a fragment of equine IgG1 heavy chain containing mutated the constant region.

Methods for Making Antibody Molecules of the Invention

[00196] Methods for making antibody molecules are well known in the art and fully described in U.S. Patents 8,394,925; 8,088,376; 8,546,543; 10,336,818; and 9,803,023 and U.S. Patent Application Publication 20060067930, which are incorporated by reference herein in their entirety. Any suitable method, process, or technique, known to one of skilled in the art, can be used. An antibody molecule having a variant Fc region of the invention may be generated according to the methods well known in the art. In some embodiments, the variant Fc region can be fused to a heterologous polypeptide of choice, such as an antibody variable domain or binding domain of a receptor or ligand.

[00197] With the advent of methods of molecular biology and recombinant technology, a person of skilled in the art can produce antibody and antibody-like molecules by recombinant means and thereby generate gene sequences that code for

specific amino acid sequences found in the polypeptide structure of the antibodies. Such antibodies can be produced by either cloning the gene sequences encoding the polypeptide chains of said antibodies or by direct synthesis of said polypeptide chains, with assembly of the synthesized chains to form active tetrameric (H₂L₂) structures with affinity for specific epitopes and antigenic determinants. This has permitted the ready production of antibodies having sequences characteristic of neutralizing antibodies from different species and sources.

[00198] Regardless of the source of the antibodies, or how they are recombinantly constructed, or how they are synthesized, in vitro or in vivo, using transgenic animals, large cell cultures of laboratory or commercial size, using transgenic plants, or by direct chemical synthesis employing no living organisms at any stage of the process, all antibodies have a similar overall 3 dimensional structure. This structure is often given as H₂L₂ and refers to the fact that antibodies commonly comprise two light (L) amino acid chains and 2 heavy (H) amino acid chains. Both chains have regions capable of interacting with a structurally complementary antigenic target. The regions interacting with the target are referred to as “variable” or “V” regions and are characterized by differences in amino acid sequence from antibodies of different antigenic specificity. The variable regions of either H or L chains contain the amino acid sequences capable of specifically binding to antigenic targets.

[00199] As used herein, the term “antigen binding region” refers to that portion of an antibody molecule which contains the amino acid residues that interact with an antigen and confer on the antibody its specificity and affinity for the antigen. The antibody binding region includes the “framework” amino acid residues necessary to maintain the

proper conformation of the antigen-binding residues. Within the variable regions of the H or L chains that provide for the antigen binding regions are smaller sequences dubbed “hypervariable” because of their extreme variability between antibodies of differing specificity. Such hypervariable regions are also referred to as “complementarity determining regions” or “CDR” regions. These CDR regions account for the basic specificity of the antibody for a particular antigenic determinant structure.

[00200] The CDRs represent non-contiguous stretches of amino acids within the variable regions but, regardless of species, the positional locations of these critical amino acid sequences within the variable heavy and light chain regions have been found to have similar locations within the amino acid sequences of the variable chains. The variable heavy and light chains of all antibodies each have three CDR regions, each non-contiguous with the others. In all mammalian species, antibody peptides contain constant (*i.e.*, highly conserved) and variable regions, and, within the latter, there are the CDRs and the so-called “framework regions” made up of amino acid sequences within the variable region of the heavy or light chain but outside the CDRs.

[00201] The present invention further provides a vector including at least one of the nucleic acids described above. Because the genetic code is degenerate, more than one codon can be used to encode a particular amino acid. Using the genetic code, one or more different nucleotide sequences can be identified, each of which would be capable of encoding the amino acid. The probability that a particular oligonucleotide will, in fact, constitute the actual encoding sequence can be estimated by considering abnormal base pairing relationships and the frequency with which a particular codon is actually used (to encode a particular amino acid) in eukaryotic or prokaryotic cells expressing an

antibody or portion. Such “codon usage rules” are disclosed by Lathe, et al., 183 J. Molec. Biol. 1-12 (1985). Using the “codon usage rules” of Lathe, a single nucleotide sequence, or a set of nucleotide sequences that contains a theoretical “most probable” nucleotide sequence capable of encoding feline, equine, or canine IgG sequences can be identified. It is also intended that the antibody coding regions for use in the present invention could also be provided by altering existing antibody genes using standard molecular biological techniques that result in variants of the antibodies and peptides described herein. Such variants include, but are not limited to deletions, additions and substitutions in the amino acid sequence of the antibodies or peptides.

[00202] For example, one class of substitutions is conservative amino acid substitutions. Such substitutions are those that substitute a given amino acid in a feline antibody peptide by another amino acid of like characteristics. Typically seen as conservative substitutions are the replacements, one for another, among the aliphatic amino acids Ala, Val, Leu, and Ile; interchange of the hydroxyl residues Ser and Thr, exchange of the acidic residues Asp and Glu, substitution between the amide residues Asn and Gln, exchange of the basic residues Lys and Arg, replacements among the aromatic residues Phe, Tyr, and the like. Guidance concerning which amino acid changes are likely to be phenotypically silent is found in Bowie *et al.*, 247 *Science* 1306-10 (1990).

[00203] Variant feline antibodies or peptides may be fully functional or may lack function in one or more activities. Fully functional variants typically contain only conservative variations or variations in non-critical residues or in non-critical regions. Functional variants can also contain substitution of similar amino acids that result in no

change or an insignificant change in function. Alternatively, such substitutions may positively or negatively affect function to some degree. Non-functional variants typically contain one or more non-conservative amino acid substitutions, deletions, insertions, inversions, or truncation or a substitution, insertion, inversion, or deletion in a critical residue or critical region.

[00204] Amino acids that are essential for function can be identified by methods known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis.

Cunningham *et al.*, 244 *Science* 1081-85 (1989). The latter procedure introduces single alanine mutations at every residue in the molecule. The resulting mutant molecules are then tested for biological activity such as epitope binding or in vitro ADCC activity. Sites that are critical for ligand-receptor binding can also be determined by structural analysis such as crystallography, nuclear magnetic resonance, or photoaffinity labeling. Smith *et al.*, 224 *J. Mol. Biol.* 899-904 (1992); de Vos *et al.*, 255 *Science* 306-12 (1992).

[00205] Moreover, polypeptides often contain amino acids other than the twenty “naturally occurring” amino acids. Further, many amino acids, including the terminal amino acids, may be modified by natural processes, such as processing and other post-translational modifications, or by chemical modification techniques well known in the art. Known modifications include, but are not limited to, acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent crosslinks, formation of cystine, formation of pyroglutamate, formylation,

gamma carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination. Such modifications are well known to those of skill in the art and have been described in great detail in the scientific literature. Several particularly common modifications, glycosylation, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation and ADP ribosylation, for instance, are described in most basic texts, such as *Proteins-Structure and Molecular Properties* (2nd ed., T. E. Creighton, W. H. Freeman & Co., N.Y., 1993). Many detailed reviews are available on this subject, such as by Wold, *Posttranslational Covalent Modification of proteins*, 1-12 (Johnson, ed., Academic Press, N.Y., 1983); Seifter *et al.* 182 *Meth. Enzymol.* 626-46 (1990); and Rattan *et al.* 663 *Ann. NY Acad. Sci.* 48-62 (1992).

[00206] In another aspect, the invention provides antibody derivatives. A “derivative” of an antibody contains additional chemical moieties not normally a part of the protein. Covalent modifications of the protein are included within the scope of this invention. Such modifications may be introduced into the molecule by reacting targeted amino acid residues of the antibody with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues. For example, derivatization with bifunctional agents, well-known in the art, is useful for cross-linking the antibody or fragment to a water-insoluble support matrix or to other macromolecular carriers.

[00207] Derivatives also include radioactively labeled monoclonal antibodies that are labeled. For example, with radioactive iodine (²⁵¹I, ¹³¹I), carbon (¹⁴C), sulfur (³⁵S),

indium, tritium (H^3) or the like; conjugates of monoclonal antibodies with biotin or avidin, with enzymes, such as horseradish peroxidase, alkaline phosphatase, beta-D-galactosidase, glucose oxidase, glucoamylase, carboxylic acid anhydrase, acetylcholine esterase, lysozyme, malate dehydrogenase or glucose 6-phosphate dehydrogenase; and also conjugates of monoclonal antibodies with bioluminescent agents (such as luciferase), chemoluminescent agents (such as acridine esters) or fluorescent agents (such as phycobiliproteins).

[00208] Another derivative bifunctional antibody of the invention is a bispecific antibody, generated by combining parts of two separate antibodies that recognize two different antigenic groups. This may be achieved by crosslinking or recombinant techniques. Additionally, moieties may be added to the antibody or a portion thereof to increase half-life *in vivo* (e.g., by lengthening the time to clearance from the blood stream. Such techniques include, for example, adding PEG moieties (also termed pegylation), and are well-known in the art. See U.S. Patent. Appl. Pub. No. 20030031671.

[00209] In some embodiments, the nucleic acids encoding a subject antibody are introduced directly into a host cell, and the cell is incubated under conditions sufficient to induce expression of the encoded antibody. After the subject nucleic acids have been introduced into a cell, the cell is typically incubated, normally at 37° C., sometimes under selection, for a period of about 1-24 hours in order to allow for the expression of the antibody. In one embodiment, the antibody is secreted into the supernatant of the media in which the cell is growing. Traditionally, monoclonal antibodies have been produced as native molecules in murine hybridoma lines. In addition to that technology, the

present invention provides for recombinant DNA expression of the antibodies. This allows the production of antibodies, as well as a spectrum of antibody derivatives and fusion proteins in a host species of choice.

[00210] A nucleic acid sequence encoding at least one antibody, portion or polypeptide of the invention may be recombined with vector DNA in accordance with conventional techniques, including blunt-ended or staggered-ended termini for ligation, restriction enzyme digestion to provide appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and ligation with appropriate ligases. Techniques for such manipulations are disclosed, *e.g.*, by Maniatis et al., MOLECULAR CLONING, LAB. MANUAL, (Cold Spring Harbor Lab. Press, NY, 1982 and 1989), and Ausubel et al. 1993 *supra*, may be used to construct nucleic acid sequences which encode an antibody molecule or antigen binding region thereof.

[00211] A nucleic acid molecule, such as DNA, is said to be “capable of expressing” a polypeptide if it contains nucleotide sequences which contain transcriptional and translational regulatory information and such sequences are “operably linked” to nucleotide sequences which encode the polypeptide. An operable linkage is a linkage in which the regulatory DNA sequences and the DNA sequence sought to be expressed are connected in such a way as to permit gene expression as peptides or antibody portions in recoverable amounts. The precise nature of the regulatory regions needed for gene expression may vary from organism to organism, as is well known in the analogous art. *See, e.g.*, Sambrook *et al.*, 2001 *supra*; Ausubel *et al.*, 1993 *supra*.

[00212] The present invention accordingly encompasses the expression of an antibody or peptide, in either prokaryotic or eukaryotic cells. Suitable hosts include bacterial or eukaryotic hosts including bacteria, yeast, insects, fungi, bird and mammalian cells either in vivo, or in situ, or host cells of mammalian, insect, bird or yeast origin. The mammalian cell or tissue may be of human, primate, hamster, rabbit, rodent, cow, pig, sheep, horse, goat, dog or cat origin. Any other suitable mammalian cell, known in the art, may also be used.

[00213] In one embodiment, the nucleotide sequence of the invention will be incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host. Any of a wide variety of vectors may be employed for this purpose. *See, e.g., Ausubel et al., 1993 supra.* Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to “shuttle” the vector between host cells of different species.

[00214] Example prokaryotic vectors known in the art include plasmids such as those capable of replication in *E.coli* (such as, for example, pBR322, ColEI, pSCIOI, pACYC 184, .pi.vX). Such plasmids are, for example, disclosed by Maniatis et al., 1989 *supra*; Ausubel et al, 1993 *supra*. Bacillus plasmids include pC194, pC221, pT127, etc. Such plasmids are disclosed by Gryczan, in THE MOLEC. BIO. OF THE BACILLI 307-329 (Academic Press, NY, 1982).

[00215] Suitable Streptomyces plasmids include pIJOI (Kendall et al., 169 J. Bacterial. 4177-83 (1987), and Streptomyces bacteriophages such as phLC31 (Chater et

al., in SIXTH INT'L SYMPOSIUM ON ACTINOMYCETALES BIO. 45-54

(Akademiai Kiado, Budapest,

[00216] 30 Hungary 1986). Pseudomonas plasmids are reviewed in John et al., 8 Rev. Infect. Dis. 693-704 (1986); Izaki, 33 Jpn. J. Bacteriol. 729-42 (1978); and Ausubel et al., 1993 *supra*.

[00217] Alternatively, gene expression elements useful for the expression of cDNA encoding antibodies or peptides include, but are not limited to, (a) viral transcription promoters and their enhancer elements, such as the SV40 early promoter (Okayama et al., 3 Mol. Cell. Biol. 280 (1983), Rous sarcoma virus LTR (Gorman et al., 79 Proc. Natl. Acad. Sci., USA 6777 (1982), and Moloney murine leukemia virus LTR (Grosschedl et al., 41 Cell 885 (1985); (b) splice regions and polyadenylation sites such as those derived from the SV40 late region (Okayama et al., 1983), and (c) polyadenylation sites such as in SV40 (Okayama et al., 1983).

[00218] Immunoglobulin cDNA genes can be expressed as described by Weidle *et al.*, 51 Gene 21 (1987), using as expression elements the SV40 early promoter and its enhancer, the mouse immunoglobulin H chain promoter enhancers, SV40 late region mRNA splicing, rabbit S-globin intervening sequence, immunoglobulin and rabbit S-globin polyadenylation sites, and SV40 polyadenylation elements. For immunoglobulin genes comprised of part cDNA, part genomic DNA (Whittle et al., 1 Protein Engin. 499 (1987)), the transcriptional promoter can be human cytomegalovirus, the promoter enhancers can be cytomegalovirus and mouse/human immunoglobulin, and mRNA splicing and polyadenylation regions can be the native chromosomal immunoglobulin sequences.

[00219] In an embodiment, for expression of cDNA genes in rodent cells, the transcriptional promoter is a viral LTR sequence, the transcriptional promoter enhancers are either or both the mouse immunoglobulin heavy chain enhancer and the viral LTR enhancer, the splice region contains an intron of greater than 31 bp, and the polyadenylation and transcription termination regions are derived from the native chromosomal sequence corresponding to the immunoglobulin chain being synthesized. In other embodiments, cDNA sequences encoding other proteins are combined with the above-recited expression elements to achieve expression of the proteins in mammalian cells.

[00220] Each fused gene can be assembled in, or inserted into, an expression vector. Recipient cells capable of expressing the immunoglobulin chain gene product are then transfected singly with a peptide or H or L chain-encoding gene or are co-transfected with H and L chain gene. The transfected recipient cells are cultured under conditions that permit expression of the incorporated genes and the expressed immunoglobulin chains or intact antibodies or fragments are recovered from the culture.

[00221] In one embodiment, the fused genes encoding the peptide or H and L chains, or portions thereof are assembled in separate expression vectors that are then used to cotransfect a recipient cell. Alternatively the fused genes encoding the H and L chains can be assembled on the same expression vector. For transfection of the expression vectors and production of the antibody, the recipient cell line may be a myeloma cell. Myeloma cells can synthesize, assemble and secrete immunoglobulins encoded by transfected immunoglobulin genes and possess the mechanism for glycosylation of the immunoglobulin. Myeloma cells can be grown in culture or in the peritoneal cavity of a

mouse, where secreted immunoglobulin can be obtained from ascites fluid. Other suitable recipient cells include lymphoid cells such as B lymphocytes of feline or non-feline origin, hybridoma cells of feline or non-feline origin, or interspecies heterohybridoma cells.

[00222] The expression vector carrying an antibody construct or polypeptide of the invention can be introduced into an appropriate host cell by any of a variety of suitable means, including such biochemical means as transformation, transfection, conjugation, protoplast fusion, calcium phosphate-precipitation, and application with polycations such as diethylaminoethyl (DEAE) dextran, and such mechanical means as electroporation, direct microinjection, and microprojectile bombardment. Johnston *et al.*, 240 *Science* 1538 (1988).

[00223] Yeast may provide substantial advantages over bacteria for the production of immunoglobulin H and L chains. Yeasts carry out post-translational peptide modifications including glycosylation. A number of recombinant DNA strategies now exist which utilize strong promoter sequences and high copy number plasmids which can be used for production of the desired proteins in yeast. Yeast recognizes leader sequences of cloned mammalian gene products and secretes peptides bearing leader sequences (*i.e.*, pre-peptides). Hitzman *et al.*, 11th Int'l Conference on Yeast, Genetics & Molec. Biol. (Montpellier, France, 1982).

[00224] Yeast gene expression systems can be routinely evaluated for the levels of production, secretion and the stability of peptides, antibodies, fragments and regions thereof. Any of a series of yeast gene expression systems incorporating promoter and termination elements from the actively expressed genes coding for glycolytic enzymes

produced in large quantities when yeasts are grown in media rich in glucose can be utilized. Known glycolytic genes can also provide very efficient transcription control signals. For example, the promoter and terminator signals of the phosphoglycerate kinase (PGK) gene can be utilized. A number of approaches can be taken for evaluating optimal expression plasmids for the expression of cloned immunoglobulin cDNAs in yeast. See Vol. II DNA Cloning, 45-66, (Glover, ed.,) IRL Press, Oxford, UK 1985).

[00225] Bacterial strains can also be utilized as hosts for the production of antibody molecules or peptides described by this invention. Plasmid vectors containing replicon and control sequences which are derived from species compatible with a host cell are used in connection with these bacterial hosts. The vector carries a replication site, as well as specific genes which are capable of providing phenotypic selection in transformed cells. A number of approaches can be taken for evaluating the expression plasmids for the production of antibodies, fragments and regions or antibody chains encoded by the cloned immunoglobulin cDNAs in bacteria (see Glover, 1985 supra; Ausubel, 1993 supra; Sambrook, 2001 supra; Colligan et al., eds. Current Protocols in Immunology, John Wiley & Sons, NY, N.Y. (1994-2001); Colligan et al., eds. Current Protocols in Protein Science, John Wiley & Sons, NY, N.Y. (1997-2001).

[00226] Host mammalian cells may be grown *in vitro* or *in vivo*. Mammalian cells provide posttranslational modifications to immunoglobulin protein molecules including leader peptide removal, folding and assembly of Heavy and Light chains, glycosylation of the antibody molecules, and secretion of functional antibody protein. Mammalian cells which can be useful as hosts for the production of antibody proteins, in addition to the cells of lymphoid origin described above, include cells of fibroblast origin, such as Vero

(ATCC CRL 81) or CHO-K1 (ATCC CRL 61) cells. Many vector systems are available for the expression of cloned peptide H and L chain genes in mammalian cells (see Glover, 1985 supra). Different approaches can be followed to obtain complete H2L2 antibodies. It is possible to co-express H and L chains in the same cells to achieve intracellular association and linkage of H and L chains into complete tetrameric H2L2 antibodies and/or peptides. The co-expression can occur by using either the same or different plasmids in the same host. Genes for both H and L chains and/or peptides can be placed into the same plasmid, which is then transfected into cells, thereby selecting directly for cells that express both chains. Alternatively, cells can be transfected first with a plasmid encoding one chain, for example the L chain, followed by transfection of the resulting cell line with an H chain plasmid containing a second selectable marker. Cell lines producing peptides and/or H2L2 molecules via either route could be transfected with plasmids encoding additional copies of peptides, H, L, or H plus L chains in conjunction with additional selectable markers to generate cell lines with enhanced properties, such as higher production of assembled H2L2 antibody molecules or enhanced stability of the transfected cell lines.

[00227] For long-term, high-yield production of recombinant antibodies, stable expression may be used. For example, cell lines, which stably express the antibody molecule may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with immunoglobulin expression cassettes and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in enriched media, and then are switched to a selective media. The selectable marker in the

recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into a chromosome and grow to form foci which in turn can be cloned and expanded into cell lines. Such engineered cell lines may be particularly useful in screening and evaluation of compounds/components that interact directly or indirectly with the antibody molecule.

[00228] Once an antibody of the invention has been produced, it may be purified by any method known in the art for purification of an immunoglobulin molecule, for example, by chromatography (*e.g.*, ion exchange, affinity, particularly affinity for the specific antigen after Protein A, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. In many embodiments, antibodies are secreted from the cell into culture medium and harvested from the culture medium.

[00229] An antibody having one or more substitutions as described herein can be any suitable antibody known to one of skilled in the art. In one example, the antibody is an anti-IL31 antibody. In another example, the antibody is an anti-NGF antibody.

[00230] Anti-IL31 antibodies, without the substitution described herein, are well known in the art and fully described in, for example, U.S. Patents 10,526,405; 10,421,807; 9,206,253; and 8,790,651. Also, anti-NGF antibodies, without the substitution described herein, are also well known in the art and fully described in, for example, U.S. Patents 10,125,192; 10,093,725; 9,951,128; 9,617,334; and 9,505,829.

[00231] In one embodiment, an anti-IL31 antibody of the invention (*i.e.*, antibody having the substitution) reduces, inhibits, or neutralizes an IL-31-mediated pruritic or allergic condition. In another embodiment, the anti-IL31 antibody of the invention reduces, inhibits, or neutralizes IL-31 activity. VL, VH, and CDR sequences of the anti-IL31 antibodies are well known in the art and fully described in, for example, U.S. Patents 11,530,262; 10,526,405; 10,421,807; 9,206,253; and 8,790,651. In one embodiment, an anti-IL31 antibody of the invention may include a variable light chain comprising an amino acid sequence set forth therein. In another embodiment, the anti-IL31 antibody of the invention may include a variable heavy chain comprising the amino acid sequence set forth therein.

[00232] In one embodiment, the mutant anti-NGF antibody of the invention (*i.e.*, antibody having the substitution) reduces, inhibits, or neutralizes NGF activity in an animal, and/or enhanced ability to inhibit NGF binding to Trk A and p75, in order to treat an NGF-mediated pain or condition. VL, VH, and CDR sequences of anti-NGF antibodies are also well known in the art and fully described in, for example, U.S. Patents 10,125,192; 10,093,725; 9,951,128; 9,617,334; and 9,505,829. In one example, the anti-NGF antibody of the invention may include at least one of the complementary determining region (CDR) sequences set forth therein.

Pharmaceutical and Veterinary Applications

[00233] The invention also provides a pharmaceutical composition comprising molecules of the invention and one or more pharmaceutically acceptable carriers. More specifically, the invention provides for a pharmaceutical composition comprising a

pharmaceutically acceptable carrier or diluent and, as active ingredient, an antibody or peptide according to the invention.

[00234] “Pharmaceutically acceptable carriers” include any excipient which is nontoxic to the cell or animal being exposed thereto at the dosages and concentrations employed. The pharmaceutical composition may include one or additional therapeutic agents.

[00235] “Pharmaceutically acceptable” refers to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for contact with the tissues of animals without excessive toxicity, irritation, allergic response, or other problem complications commensurate with a reasonable benefit/risk ratio.

[00236] Pharmaceutically acceptable carriers include solvents, dispersion media, buffers, coatings, antibacterial and antifungal agents, wetting agents, preservatives, buffers, chelating agents, antioxidants, isotonic agents and absorption delaying agents.

[00237] Pharmaceutically acceptable carriers include water; saline; phosphate buffered saline; dextrose; glycerol; alcohols such as ethanol and isopropanol; phosphate, citrate and other organic acids; ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; EDTA; salt forming counterions such as sodium; and/or nonionic surfactants such as TWEEN, polyethylene glycol (PEG), and PLURONICS; isotonic agents such as sugars, polyalcohols such as mannitol and sorbitol, and sodium chloride; as well as combinations thereof.

[00238] The pharmaceutical compositions of the invention may be formulated in a variety of ways, including for example, liquid, semi-solid, or solid dosage forms, such as liquid solutions (*e.g.*, injectable and infusible solutions), dispersions or suspensions, liposomes, suppositories, tablets, pills, or powders. In some embodiments, the compositions are in the form of injectable or infusible solutions. The composition can be in a form suitable for intravenous, intraarterial, intramuscular, subcutaneous, parenteral, transmucosal, oral, topical, or transdermal administration. The composition may be formulated as an immediate, controlled, extended or delayed release composition.

[00239] The compositions of the invention can be administered either as individual therapeutic agents or in combination with other therapeutic agents. They can be administered alone, but are generally administered with a pharmaceutical carrier selected on the basis of the chosen route of administration and standard pharmaceutical practice. Administration of the antibodies disclosed herein may be carried out by any suitable means, including parenteral injection (such as intraperitoneal, subcutaneous, or intramuscular injection), orally, or by topical administration of the antibodies (typically carried in a pharmaceutical formulation) to an airway surface. Topical administration to an airway surface can be carried out by intranasal administration (*e.g.*, by use of dropper, swab, or inhaler). Topical administration of the antibodies to an airway surface can also be carried out by inhalation administration, such as by creating respirable particles of a pharmaceutical formulation (including both solid and liquid particles) containing the antibodies as an aerosol suspension, and then causing the subject to inhale the respirable particles. Methods and apparatus for administering respirable

particles of pharmaceutical formulations are well known, and any conventional technique can be employed.

[00240] In some desired embodiments, the antibodies are administered by parenteral injection. For parenteral administration, antibodies or molecules can be formulated as a solution, suspension, emulsion or lyophilized powder in association with a pharmaceutically acceptable parenteral vehicle. For example, the vehicle may be a solution of the antibody or a cocktail thereof dissolved in an acceptable carrier, such as an aqueous carrier such vehicles are water, saline, Ringer's solution, dextrose solution, trehalose or sucrose solution, or 5% serum albumin, 0.4% saline, 0.3% glycine and the like. Liposomes and nonaqueous vehicles such as fixed oils can also be used. These solutions are sterile and generally free of particulate matter. These compositions may be sterilized by conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjustment agents and the like, for example sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate, etc. The concentration of antibody in these formulations can vary widely, for example from less than about 0.5%, usually at or at least about 1% to as much as 15% or 20% by weight and will be selected primarily based on fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected. The vehicle or lyophilized powder can contain additives that maintain isotonicity (*e.g.*, sodium chloride, mannitol) and chemical stability (*e.g.*, buffers and preservatives). The formulation is sterilized by commonly used techniques. Actual methods for preparing parenterally administrable

compositions will be known or apparent to those skilled in the art and are described in more detail in, for example, REMINGTON'S PHARMA. SCI. (15th ed., Mack Pub. Co., Easton, Pa., 1980).

[00241] The antibodies or molecules of the invention can be lyophilized for storage and reconstituted in a suitable carrier prior to use. This technique has been shown to be effective with conventional immune globulins. Any suitable lyophilization and reconstitution techniques can be employed. It will be appreciated by those skilled in the art that lyophilization and reconstitution can lead to varying degrees of antibody activity loss and that use levels may have to be adjusted to compensate. The compositions containing the present antibodies or a cocktail thereof can be administered for prevention of recurrence and/or therapeutic treatments for existing disease. Suitable pharmaceutical carriers are described in the most recent edition of REMINGTON'S PHARMACEUTICAL SCIENCES, a standard reference text in this field of art. In therapeutic application, compositions are administered to a subject already suffering from a disease, in an amount sufficient to cure or at least partially arrest or alleviate the disease and its complications.

[00242] Effective doses of the compositions of the present invention, for treatment of conditions or diseases as described herein vary depending upon many different factors, including, for example, but not limited to, the pharmacodynamic characteristics of the particular agent, and its mode and route of administration; target site; physiological state of the animal; other medications administered; whether treatment is prophylactic or therapeutic; age, health, and weight of the recipient; nature

and extent of symptoms kind of concurrent treatment, frequency of treatment, and the effect desired.

[00243] Single or multiple administrations of the compositions can be carried out with dose levels and pattern being selected by the treating veterinarian. In any event, the pharmaceutical formulations should provide a quantity of the antibody(ies) of this invention sufficient to effectively treat the subject. In some embodiments, the composition is administered bimonthly, once-in-three months, once-in-four months, once-in-five months, once-in-six months, or once- in-seven months.

[00244] Treatment dosages may be titrated using routine methods known to those of skill in the art to optimize safety and efficacy. The pharmaceutical compositions of the invention may include a “therapeutically effective amount.” A “therapeutically effective amount” refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result. A therapeutically effective amount of a molecule may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the molecule to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the molecule are outweighed by the therapeutically beneficial effects.

[00245] In another aspect, the compositions of the invention can be used, for example, in the treatment of various diseases and disorders. As used herein, the terms “treat” and “treatment” refer to therapeutic treatment, including prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) an undesired physiological change associated with a disease or condition. Beneficial or

desired clinical results include, but are not limited to, alleviation of symptoms, diminishment of the extent of a disease or condition, stabilization of a disease or condition (*i.e.*, where the disease or condition does not worsen), delay or slowing of the progression of a disease or condition, amelioration or palliation of the disease or condition, and remission (whether partial or total) of the disease or condition, whether detectable or undetectable. Those in need of treatment include those already with the disease or condition as well as those prone to having the disease or condition or those in which the disease or condition is to be prevented.

[00246] The composition having mutant molecule of the invention can be used to treat any suitable disease or disorder. For example, a mutant anti-IL31 antibody of the invention can be used to treat an IL-31-mediated pruritic or allergic condition. The examples of IL-31-mediated pruritic condition include, for example, but not limited to, atopic dermatitis, eczema, psoriasis, scleroderma, and pruritis. The examples of IL-31-mediated allergic condition include, for example, but not limited to, allergic dermatitis, summer eczema, urticaria, heaves, inflammatory airway disease, recurrent airway obstruction, airway hyper-responsiveness, chronic obstruction pulmonary disease, and inflammatory processes resulting from autoimmunity.

[00247] A mutant anti-NGF antibody of the invention can be used to treat an NGF-mediated pain or a condition. The examples of a pain include, for example, but not limited to, a chronic pain, an inflammatory pain, a post-operative incision pain, a neuropathic pain, a fracture pain, an osteoporotic fracture pain, a post-herpetic neuralgia, a cancer pain, a pain resulting from burns, a pain associated with wounds, a

pain associated with trauma, a neuropathic pain, a pain associated with a musculoskeletal disorder, a rheumatoid arthritis, an osteoarthritis, an ankylosing spondylitis, a seronegative (non-rheumatoid) an arthropathies, a non-articular rheumatism, a periarticular disorder, or a peripheral neuropathy. In a particular embodiment, the pain is an osteoarthritis pain.

[00248] All patents and literature references cited in the present specification are hereby incorporated by reference in their entirety.

[00249] The following examples are offered to illustrate, but not to limit, the claimed embodiments. It is to be understood that the examples and embodiments described herein are for illustrative purposes only, and persons skilled in the art will recognize various parameters that can be altered without departing from the spirit of the disclosure or the scope of the appended claims.

EXAMPLES

[00250] **Example 1—CDC Cell-Based Assay of Feline IgG Fc Effector Function**

[00251] The cell-based complement-dependent cytotoxicity (CDC) assay was developed and employed to characterize the effectiveness of two CTLA4 feline IgG subclass Fc fusion proteins in mediating CDC and to investigate Fc region mutations in the subclasses. The assay can be used in identifying key residues in the Fc region that determine CDC activity of the feline IgG subclasses. The assay utilizes CHO target cells engineered to express canine CD80 which binds to CTLA4 on the Fc fusion proteins. These target cells have been used in past canine ADCC assays and were utilized in the CDC assay due to their dependability (Bergeron, McCandless et al. 2014).

[00252] Incubation of the fusion protein-bound target cells with feline complement-preserved serum can result in Fc binding on the fusion proteins to complement component C1q initiating

the complement cascade, ultimately forming membrane attack complexes. The pore-forming complexes mediate cell lysis of the target cells measured by loss of cell viability. If there is no Fc binding to C1q there is no resultant cell lysis/death.

[00253] Methods. CD80-expressing CHO cells (target cells) were plated at 40,000 cells/well in CD CHO media in round-bottomed 96-well plates. Titrated fusion proteins in CD CHO media were added to the target cells and allowed to bind for 60 minutes at 37°C. Feline complement preserved serum (25% in CD CHO media) was added to the plates for 60 minutes at 37°C. Cell viability was then measured using CellTiter-Glo and data were expressed as “cell viability % of control” calculated using no fusion protein + complement preserved serum controls.

[00254] Results. As shown in Figure 1 and Table 1, Feline IgG1a and IgG1b Fc subclasses showed robust and potent CDC activity with EC50 values 66.0 and 68.9 ng/mL respectively. Feline IgG2 Fc subclass showed no CDC effector function activity. Feline IgG1a and IgG1b Fc subclasses showed CDC activity. Feline IgG2 Fc subclass showed no CDC activity.

[00255] Table 1. CDC effects induced by feline IgG Fc wildtype subclasses.

CTLA4 Feline IgG Fusion Protein	CDC EC50 Value
CTLA4 Fc flgG1a	66.0 ng/mL
CTLA4 Fc flgG1b	68.9 ng/mL
CTLA4 Fc flgG2	No CDC

[00256] As shown in Figures 2A and 2B, wild type feline IgG1a Fc subclass showed robust and potent CDC activity, but several Fc mutations investigated all showed diminished CDC activity to varying degrees. In Figure 2A, feline IgG1a WT Fc CTLA4 fusion protein both showed robust concentration-dependent CDC activity. The Win mutation showed a slight right

shift of the concentration response curve. In Figure 2B, all seven of the listed Win plus mutations showed significant (60-75%) knock down of CDC effector function.

[00257] As shown in Figure 3, wild type feline IgG1a Fc subclass showed robust and potent CDC activity, while several Fc mutations investigated all showed diminished CDC activity to varying degrees. Again, the Win mutation showed a slight right shift of the concentration response curve. CDC effector function was successively knocked down as the mutations went from DANG < DANG PSS < WinDANG < WinDANGPSS.

[00258] As shown in Figure 4, wild type IgG1a Fc subclasses showed robust and potent CDC activity. However, several Fc mutations investigated all showed diminished CDC activity to varying degrees. Again, the Win mutation showed a slight right shift of the concentration response curve. The mutations GSP, GAP, DANG_GAP and WinDANG_GAP all knocked down CDC effector function 60-70%.

[00259] As shown in Figure 5, wild type IgG1a Fc subclass showed robust and potent CDC activity. However, the Fc mutations investigated showed varying effects on CDC effector function ranging from no effect to completely knocking out CDC effector function. The mutations KAPA and WinKAPA both showed dramatic knock down of CDC effector function with WinKAPA showing complete knockout of effector function. However, the mutations D270G and D270S showed little effect on CDC effector function.

[00260] Example 2—ADCP Assay of Feline IgG Effector Function

[00261] The antibody dependent cellular phagocytosis (ADCP) assay utilizes CHO target cells engineered to express canine CD80, which binds to canine CTLA4 on the Fc fusion proteins. The Fc region of the fusion protein may then bridge this complex to Fc gamma receptors on activated macrophage effector cells, which have the capacity to phagocytose the

target cell. ADCP is measured by fluorescent intensity and/or fluorescent area of a pH-sensitive fluorescent dye-stained target cells within the population of effector macrophages in the co-culture, wherein fluorescent cells are indicative of an effector cell that has successfully internalized a target cell into the acidic lysosome.

[00262] Methods. Canine CD80-expressing CHO cells (CD80 target cells) or wild-type CHO cells which do not express CD80 (parental target cells) were stained with pHrodo red dye for 30 minutes at 37 degrees C. Stained cells were subsequently incubated with feline CTLA4-Fc fusion proteins for 60 minutes to mediate CTLA4:CD80 binding. 30,000 target cells were then added to 7,000 pre-plated Fcwf-4 effector cells (feline macrophage cell line). Co-cultures were maintained for 6-8 hours at 37 C as they were monitored and analyzed by a SX5 Incucyte live cell imaging system. Fluorescent area and/or intensity was quantified using a predefined processing definition in the Incucyte software and expressed as such.

[00263] Results. As shown in Figure 5, wild type IgG1a showed concentration dependent ADCP activity. The Fc mutations investigated either showed little effect or a slight knock down of ADCP activity compared to the wild type. The GSP mutation in the Fc showed significant knockdown of ADCP activity.

[00264] As shown in Figure 6, wild type IgG1a showed concentration dependent ADCP activity. The Fc mutations investigated showed varying effects on ADCP activity compared to the wild type. The WinDANG_PSS mutation in the Fc showed knockdown of ADCP activity. However, the WinPSS mutation in the Fc showed an enhancement of ADCP activity.

[00265] As shown in Figure 7, wild type IgG1a showed concentration-dependent ADCP activity. Several Fc mutations investigated either showed little effect or a slight knock down of ADCP activity compared to the wild type. The Win and WinDANG mutations have little or no

effect on ADCP activity compared to wild type IgG1a, but the WinDANG_GAP mutation in the Fc showed knockdown of ADCP activity.

[00266] As shown in Figure 8, wild type IgG1a showed concentration-dependent ADCP activity. The Fc mutations investigated either showed little effect or a dramatic knock down of ADCP activity compared to the wild type. The KAPA mutation in the Fc showed a dramatic knockout of ADCP activity. The D270G and D270S mutations showed no effect on ADCP activity.

[00267] As shown in Figure 9, wild type IgG1a showed concentration-dependent ADCP activity. The Fc mutations investigated showed moderate knock down of ADCP activity compared to the wild type. The DANG_GAP and WinKAPA mutation in the Fc showed a very strong knock down of ADCP activity.

[00268] Example 3—ADCC Assay of Feline IgG Effector Function

[00269] The ADCC cell-based assay was developed and employed to characterize the effectiveness of feline IgG1a subclass Fc rituximab chimera IgGs in mediating ADCC and to investigate Fc region mutations in the subclass IgG1a. This will help define key residues in the Fc region that determine ADCC activity of the feline IgG subclasses. The assay utilizes specially engineered HiBiT Ramos target cells (Promega) and express human CD20 which binds to our rituximab chimera IgGs. These target cells have been used in past human ADCC assays (Promega).

[00270] Incubation of rituximab chimera-bound target cells with cultured activated PBMCs can result in Fc binding on the IgGs to FcγRIII mediating granzyme and perforin release from the NK cells within the PBMC population. The actions of these proteins will result in cytotoxicity and lysis of the target cells bound by the IgGs. Upon killing of the target cell, the

HiBiT fusion protein is released and binds extracellular LgBiT to create a functional NanoBiT® Luciferase enzyme (Promega). Luminescence is measured using a luciferase substrate and the GloMax® Discover System (Promega). If there is no Fc binding to FcγRIII there is no resultant target cell death measured.

[00271] Methods. HiBiT Ramos cells (target cells) were plated at 1,000 cells/well in EMEM media in 384-well plates. Titrated fusion proteins in EMEM media were added to the target cells and allowed to bind for 60 minutes at 37°C. Single donor canine PBMCs (effector cells), cultured overnight in RPMI 1640 medium + IL-2 and IL-15, were added to the plates for 5 hours at 37°C using an effector:target cell ratio (E:F Ratio) of 25:1. Cells death was then quantified using the Promega Nano-Glo HiBiT Extracellular Detection System. Data were expressed as "Fold Induction" normalized to controls (minus effector PBMCs). Figure 13 summarizes the mutations investigated using the ADCC assay.

[00272] Results. As shown in Figure 10, wild type feline IgG1a showed concentration-dependent ADCC activity. The Fc mutations investigated either showed a slight knock down or an enhancement of ADCP activity compared to the wild type. The Win mutation in the Fc showed knockdown of ADCC activity. Both the KAPA and DE mutations showed enhancement of ADCC activity, especially the DE mutation.

[00273] As shown in Figure 11, wild type IgG1a showed concentration-dependent ADCP activity. The Fc mutations investigated showed varying knockdown to knockout effects on ADCC activity compared to the wild type. The DANG and DANG_GAP mutations in the Fc showed knockdown of ADCC activity. The WinDANG_GAP mutation in the Fc showed a knockout of ADCC activity.

[00274] As shown in Figure 12, wild type IgG1a showed concentration-dependent ADCC activity. The Fc mutations investigated showed knock down of ADCC activity compared to the wild type. The WinKAPA, WinKA and WinPA mutations in the Fc showed knockdown of ADCC activity.

[00275] **Example 4—In Silico Modeling of Feline IgG Fc Regions**

[00276] The Fc regions of the feline backbone subclasses, IgG1a and IgG1b were first designed using their respective CH2 and CH3 regions. The protein modeling feature of Alphafold 2.2 developed by Deepmind was implemented to model the 3D structure of each of wild-type (WT) and mutant constructs of each feline backbone subclass and allotypes.

[00277] **Methods.** Molecular Operating Environment (MOE) developed by Chemical Computing Group (MOE2019.0102) provides a flexible and automated graphical user interface for protein modeling. To analyze the difference in structures, the sequence-to-profile alignment algorithm uses a scoring algorithm to rank the sequence templates and scores higher than 85% ensure the selection of protein templates with physically realistic structures. The model was then optimized using the same pipeline and the structural stability of the models was verified using Ramachandran Plots, which checks the stereochemical quality of a protein structure.

[00278] This method was performed on wild-type (WT) constructs and mutations in the following positions: M234, L235, G237, S239, D265, D270, N297, K322, P329, S330, P331, and I332. The models were overlayed, and the mutated residues were displayed in ball and stick form (Fig. 14A). An RMSD plot was generated to calculate the root mean square deviation of the two WT structures, Feline-IgG1a_WT and Feline-IgG1b_WT relative to each other (Fig. 14B). An RMSD value of 2.0Å or lower is considered the standard for considering two structures to be alike. Results indicated that Feline IgG1a construct was identical in its fold to the feline IgG1b

subclass with average RMSD value for the structure being 1.05 Å. RMSDs at the twelve positions where mutational scanning was performed was also noted in Table 2A with values ranging from 0.2-0.9 Å.

[00279] Table 2A. Root Mean Square Deviation (RMSD) comparisons of positions where mutations were performed in the protein models of WT Feline IgG1a and IgG1b.

Residue in IgG1a WT	Residue in IgG1b WT	RMSD (Å)
M234	P234	0.5
L235	L235	0.9
G237	G237	0.6
S239	S239	0.4
D265	D265	0.4
D270	D270	0.2
N297	N297	0.4
K322	R322	0.3
P329	P329	0.4
S330	S330	0.4
P331	P331	0.3
I332	I332	0.4

[00280] The method described above was performed on the wild-type (WT) constructs and mutations in the following positions: G236, G267, P268, S298, N324, E333, R334 and E345. The models were overlayed, and the mutated residues were displayed in ball and stick form (Fig 14C). The root mean square deviation of the two WT structures, Feline-IgG1a_WT and Feline-IgG1b_WT relative to each other was calculated. RMSDs at the twelve positions where mutational scanning was performed is shown in Table 2B with values ranging from 0.1-0.7 Å.

An RMSD value of 2.0Å or lower is considered the standard for considering two structures to be alike. Results indicated that Feline IgG1a construct was identical in its fold to the feline IgG1b subclass with average RMSD value for the structure being 1.05Å.

[00281] Table 2B. Root Mean Square Deviation (RMSD) comparisons of positions where mutations were performed in the protein models of WT Feline IgG1a and IgG1b.

Residue in IgG1a WT	Residue in IgG1b WT	RMSD (Å)
G236	G236	0.7
G267	G267	0.4
P268	P268	0.4
S298	S298	0.4
N324	N324	0.3
E333	E333	0.4
R334	R334	0.3
E345	E345	0.1

[00282] Results. Molecular modeling of all mutations across the two IgG1 allotypes, IgG1a and IgG1b of feline backbones were performed and validated using MOE2019.0102. The Fc fold and residue conformation of between subclasses were shown to have RMSDs less than 2Å, indicating that the protein structures have very high identity and therefore mutations in the positions in IgG1a would function in a similar manner when extrapolated to IgG1b.

[00283] Example 5—CDC Cell-Based Assay of Canine IgG Fc Effector Function

[00284] The cell-based complement-dependent cytotoxicity (CDC) assay was developed and employed to characterize the effectiveness of two CTLA4 canine IgG subclass Fc fusion proteins in mediating CDC and to investigate Fc region mutations in the subclasses. The assay

can be used in identifying key residues in the Fc region that determine CDC activity of the feline IgG subclasses. The assay utilizes CHO target cells engineered to express canine CD80 which binds to CTLA4 on the Fc fusion proteins. These target cells have been used in past canine ADCC assays and were utilized in the CDC assay due to their dependability (Bergeron, McCandless et al. 2014).

[00285] Methods. Incubation of the fusion protein-bound target cells with feline complement-preserved serum can result in Fc binding on the fusion proteins to complement component C1q initiating the complement cascade, ultimately forming membrane attack complexes. The pore-forming complexes mediate cell lysis of the target cells measured by loss of cell viability. If there is no Fc binding to C1q there is no resultant cell lysis/death.

[00286] CD80-expressing CHO cells (target cells) were plated at 40,000 cells/well in CD CHO media in round-bottomed 96-well plates. Titrated fusion proteins in CD CHO media were added to the target cells and allowed to bind for 60 minutes at 37°C. Complement preserved serum (25% final concentration in CD CHO media) was added to the plates for 60 minutes at 37°C. Cell viability was then measured using CellTiter-Glo and data were expressed as “cell viability % of control” calculated using no fusion protein + complement preserved serum controls.

[00287] Results. As shown in Figure 15 and Table 3, Canine IgG2 Fc subclass showed robust and potent CDC activity with an EC50 value of 298.7 ng/mL. Canine IgG1 Fc subclass showed no CDC effector function activity.

[00288] Table 3. CDC effects induced by canine IgG1 and IgG2 Fc wildtype subclasses.

CTLA4 Canine IgG Fusion Protein	CDC EC50 Value
CTLA4 Fc flgG1	No CDC
CTLA4 Fc flgG2	298.7 ng/mL

[00289] As shown in Figure 16, wild type canine IgG2 Fc subclass showed robust and potent CDC activity. The Fc mutations investigated showed no effect, diminished, or completely knocked out CDC activity. The D270S mutation of the Fc showed no effect on CDC activity. The Win mutation showed a slight right shift of the concentration response curve. The D70G mutation showed a knockdown of CDC activity. The six mutations GSP, GAP, DANG_GAP, WIN_DANG_GAP, KAPA and WIN_KAPA showed complete knockout of CDC effector function.

[00290] Example 6—ADCP Assay of Canine IgG Effector Function

[00291] The antibody dependent cellular phagocytosis (ADCP) assay utilizes CHO target cells engineered to express canine CD80, which binds to canine CTLA4 on the Fc fusion proteins. The Fc region of the fusion protein may then bridge this complex to Fc gamma receptors on activated macrophage effector cells, which have the capacity to phagocytose the target cell. ADCP is measured by fluorescent intensity and/or fluorescent area of a pH-sensitive fluorescent dye-stained target cells within the population of effector macrophages in the co-culture, wherein fluorescent cells are indicative of an effector cell that has successfully internalized a target cell into the acidic lysosome.

[00292] **Methods.** Canine CD80-expressing CHO cells (CD80 target cells) or wild-type CHO cells which do not express CD80 (parental target cells) were stained with pHrodo red dye for 30 minutes at 37 degrees C. Stained cells were subsequently incubated with canine CTLA4-Fc fusion proteins for 60 minutes to mediate CTLA4:CD80 binding. 30,000 target cells were then

added to 7,000 pre-plated DH-82 cells (canine macrophage cell line). Co-cultures were maintained for 6-8 hours at 37 °C and they were monitored and analyzed by a SX5 Incucyte live cell imaging system. Fluorescent area and/or intensity was quantified using a predefined processing definition in the Incucyte software and expressed as such.

[00293] Results. As shown in Figure 17, wild type IgG2 showed concentration-dependent ADCP activity. The Fc mutations investigated either showed knockout of ADCP activity compared to the wild type. The Win and GAP mutations in the Fc showed knockout of ADCP activity.

[00294] As shown in Figure 18, wild type IgG2 showed concentration-dependent ADCP activity. The Fc mutations investigated showed knockdown effects on ADCP activity compared to the wild type. The DANG_GAP and WIN_DANG_GAP mutations in the Fc showed knockdown of ADCP activity.

[00295] As shown in Figure 19, wild type IgG2 showed concentration-dependent ADCP activity. The Fc mutations investigated either showed knockdown or knockout of ADCP activity compared to the wild type. The KAPA mutation in the Fc showed knockdown of ADCP activity. The GSP mutation showed knockout of ADCP activity.

[00296] As shown in Figure 20, wild type IgG2 showed concentration-dependent ADCP activity. The Fc mutations investigated showed moderate knock down of ADCP activity compared to the wild type. The DANG_GAP and WinKAPA mutation in the Fc showed a moderate knockdown to almost complete knockout of ADCP activity, respectively.

[00297] As shown in Figure 21, wild type IgG2 showed concentration dependent ADCP activity. The Fc mutations investigated either showed knockdown of ADCP activity or little

effect compared to the wild type. The D270G mutation in the Fc showed knockdown of ADCP activity. The D270S mutation showed no effect on ADCP activity.

[00298] Example 7—ADCC Assay of Canine IgG Effector Function

[00299] The ADCC cell-based assay was developed and employed to characterize the effectiveness of canine subclass IgG2a Fc rituximab chimera IgGs in mediating ADCC and to investigate Fc region mutations in the subclasses. This will help define key residues in the Fc region that determine ADCC activity of the feline IgG subclasses. The assay utilizes specially engineered HiBiT Ramos target cells (Promega) and express human CD20 which binds to our rituximab chimera IgGs. These target cells have been used in past human ADCC assays (Promega).

[00300] Incubation of the rituximab chimera-bound target cells with cultured activated PBMCs can result in Fc binding on the IgGs to FcγRIII mediating granzyme and perforin release from the NK cells within the PBMC population. The actions of these proteins will result in cytotoxicity and lysis of the target cells bound by the IgGs. Upon killing of the target cell, the HiBiT fusion protein is released and binds extracellular LgBiT to create a functional NanoBiT® Luciferase enzyme. Luminescence is measured using a luciferase substrate and the GloMax® Discover System. If there is no Fc binding to FcγRIII there is no resultant target cell death measured.

[00301] Methods. HiBiT Ramos cells (target cells) were plated at 1,000 cells/well in EMEM media in 384-well plates. Titrated fusion proteins in EMEM media were added to the target cells and allowed to bind for 60 minutes at 37°C. Single donor canine PBMCs (effector cells), cultured overnight in RPMI 1640 medium + IL-2 and IL-15, were added to the plates for 5 hours at 37°C using an effector:target cell ratio (E:F Ratio) of 25:1. Cells death was then quantified

using the Promega Nano-Glo HiBiT Extracellular Detection System. Data were expressed as "Fold Induction" normalized to controls (minus effector PBMCs).

[00302] Results. As shown in Figure 22, wild type canine IgG2 showed concentration-dependent ADCC activity. The Fc mutations investigated either showed a slight knock down or knockout of ADCC activity compared to the wild type. The GAP mutation showed a knockdown of ADCC activity. The Win, DANG, DANG_GAP and WinDANG_GAP mutations showed a knockout of ADCC activity.

[00303] As shown in Figure 23, wild type IgG2 showed concentration-dependent ADCC activity. The Fc mutations investigated showed varying knockdown to knockout effects on ADCC activity compared to the wild type. The Win mutation in the Fc showed knockout of ADCC activity. The KAPA, DE, DAE and DLE mutations showed slight enhancement of ADCC activity.

[00304] As shown in Figure 24, wild type IgG2 showed concentration-dependent ADCC activity. The Fc mutations investigated either showed knock down of ADCC activity compared to the wild type. The WinKAPA, WinKA and WinPA mutations in the Fc showed knockdown of ADCC activity with WinKAPA showing the most effective knockout of ADCC activity.

[00305] Example 8—Canine Fcγ receptor and C1q binding assay.

[00306] Binding of canine WT IgG2 (IgG 65) and mutant Fc were measured by surface plasmon resonance. IgGs were used as the ligand and captured to a sensor chip for analysis of canine Fcγ receptor and C1q binding. 10 mM HEPES, 150mM NaCl, 3mM EDTA and 0.05% v/v Surfactant P20 pH 7.4 was used as titration and method running buffer. Canine Fcγ receptors and Human C1q were used as analytes and flowed over captured Fc mutant IgGs. Mutations were made in several locations listed in Table 4.

[00307] **Table 4**—Listing of mutation locations in canine IgG2 assayed for effect on canine Fcγ receptor and C1q binding, using EU Index numbering as in Kabat.

231	232	233	234	235	236	237	238	239	240	262	263
264	265	266	267	268	269	270	271	294	295	296	297
298	299	300	301	322	323	324	325	326	327	328	329
330	331	332	333	334	335	336	337	338	339	340	422
423	441										

[00308] **Table 5**—Listing of mutations in canine IgG2 assayed for effect on canine Fcγ receptor and C1q binding, using EU Index numbering as in Kabat.

A231C	A231D	A231E	A231G	A231H	A231K
A231N	A231Q	A231R	A231S	A231T	A231V
A231Y	P232A	P232C	P232D	P232E	P232F
P232G	P232H	P232I	P232K	P232L	P232M
P232N	P232Q	P232R	P232S	P232T	P232V
P232W	P232Y	E233A	E233C	E233D	E233F
E233G	E233H	E233I	E233K	E233L	E233M
E233N	E233P	E233Q	E233R	E233S	E233T
E233V	E233W	E233Y	M234A	M234C	M234D
M234E	M234F	M234G	M234H	M234I	M234K
M234L	M234N	M234P	M234Q	M234R	M234S
M234T	M234W	M234Y	L235A	L235C	L235D
L235E	L235F	L235G	L235H	L235I	L235K
L235M	L235N	L235P	L235Q	L235R	L235S

L235T	L235V	L235W	L235Y	G236A	G236C
G236D	G236E	G236F	G236H	G236I	G236K
G236L	G236M	G236N	G236P	G236Q	G236R
G236S	G236T	G236V	G236W	G236Y	G237A
G237C	G237D	G237E	G237F	G237H	G237I
G237K	G237L	G237M	G237N	G237P	G237Q
G237R	G237S	G237T	G237V	G237W	G237Y
P238A	P238C	P238D	P238E	P238F	P238G
P238H	P238I	P238K	P238L	P238M	P238N
P238Q	P238R	P238S	P238T	P238V	P238W
P238Y	S239A	S239C	S239D	S239E	S239F
S239G	S239H	S239I	S239K	S239L	S239M
S239N	S239P	S239Q	S239R	S239T	S239V
S239W	S239Y	V240A	V240C	V240D	V240E
V240F	V240G	V240H	V240I	V240K	V240L
V240M	V240N	V240P	V240Q	V240R	V240S
V240T	V240W	V240Y	V262A	V262C	V262D
V262E	V262F	V262G	V262H	V262K	V262L
V262M	V262Q	V262R	V262S	V262T	V262W
V262Y	V263A	V263C	V263E	V263F	V263G
V263H	V263I	V263K	V263L	V263M	V263N
V263P	V263Q	V263S	V263T	V263W	V263Y
V264A	V264C	V264D	V264E	V264F	V264G
V264H	V264I	V264K	V264L	V264M	V264N

V264P	V264Q	V264R	V264S	V264T	V264W
V264Y	D265A	D265C	D265E	D265F	D265G
D265H	D265I	D265K	D265L	D265M	D265N
D265P	D265Q	D265R	D265S	D265T	D265V
D265W	D265Y	L266A	L266C	L266D	L266E
L266F	L266G	L266H	L266I	L266K	L266M
L266N	L266P	L266Q	L266R	L266S	L266T
L266V	L266W	L266Y	D267A	D267C	D267E
D267F	D267G	D267H	D267I	D267K	D267L
D267M	D267N	D267P	D267Q	D267R	D267S
D267T	D267V	D267W	D267Y	P268A	P268C
P268D	P268E	P268F	P268G	P268H	P268I
P268K	P268L	P268M	P268N	P268Q	P268R
P268S	P268T	P268V	P268W	P268Y	E269A
E269C	E269D	E269F	E269G	E269H	E269I
E269K	E269L	E269M	E269N	E269P	E269Q
E269R	E269S	E269T	E269V	E269W	E269Y
D270A	D270C	D270F	D270G	D270H	D270I
D270K	D270L	D270M	D270N	D270P	D270Q
D270R	D270S	D270V	D270W	D270Y	P271C
P271F	P271G	P271H	P271I	P271K	P271L
P271M	P271N	P271Q	P271R	P271S	P271T
P271Y	E294A	E294C	E294D	E294F	E294G
E294H	E294I	E294K	E294L	E294M	E294N

E294P	E294Q	E294R	E294S	E294T	E294V
E294W	E294Y	Q295A	Q295D	Q295E	Q295F
Q295G	Q295K	Q295L	Q295N	Q295P	Q295R
Q295S	Q295T	Q295V	Q295W	Q295Y	F296A
F296C	F296D	F296E	F296G	F296H	F296I
F296K	F296L	F296M	F296N	F296P	F296Q
F296R	F296S	F296T	F296V	N297A	N297C
N297D	N297E	N297F	N297G	N297H	N297I
N297K	N297L	N297M	N297P	N297Q	N297R
N297S	N297T	N297V	N297W	N297Y	G298A
G298C	G298D	G298E	G298F	G298H	G298I
G298K	G298L	G298M	G298N	G298P	G298Q
G298R	G298S	G298T	G298V	G298W	G298Y
T299A	T299C	T299D	T299E	T299F	T299G
T299H	T299I	T299K	T299L	T299M	T299N
T299P	T299Q	T299R	T299S	T299V	T299W
T299Y	Y300A	Y300C	Y300D	Y300E	Y300H
Y300I	Y300K	Y300L	Y300M	Y300N	Y300P
Y300Q	Y300R	Y300S	Y300T	Y300V	Y300W
R301A	R301C	R301D	R301E	R301F	R301G
R301H	R301I	R301L	R301P	R301Q	R301V
R301W	R301Y	V323A	V323C	V323D	V323E
V323F	V323G	V323H	V323K	V323L	V323M
V323N	V323P	V323Q	V323R	V323S	V323T

V323W	V323Y	N324A	N324C	N324D	N324E
N324F	N324G	N324H	N324I	N324K	N324L
N324M	N324P	N324Q	N324R	N324S	N324T
N324V	N324W	N324Y	N325A	N325C	N325D
N325E	N325F	N325G	N325H	N325I	N325K
N325L	N325M	N325P	N325Q	N325R	N325S
N325T	N325V	N325W	N325Y	K326A	K326D
K326E	K326F	K326I	K326L	K326N	K326R
K326S	K326T	K326V	K326W	A327C	A327D
A327E	A327F	A327G	A327H	A327I	A327K
A327L	A327M	A327N	A327P	A327Q	A327R
A327S	A327T	A327V	A327W	A327Y	L328A
L328C	L328D	L328E	L328F	L328G	L328H
L328I	L328K	L328M	L328N	L328P	L328Q
L328R	L328S	L328T	L328V	L328W	L328Y
K322A	K322C	K322D	K322E	K322F	K322G
K322H	K322I	K322L	K322M	K322N	K322P
K322Q	K322R	K322S	K322T	K322V	K322W
K322Y	P329A	P329C	P329D	P329E	P329F
P329G	P329H	P329I	P329K	P329L	P329M
P329N	P329Q	P329R	P329S	P329T	P329V
P329W	P329Y	S330A	S330C	S330D	S330E
S330F	S330G	S330H	S330I	S330K	S330L
S330M	S330N	S330P	S330Q	S330R	S330T

S330V	S330W	S330Y	P331A	I332A	I332D
I332E	I332F	I332G	I332H	I332K	I332L
I332M	I332N	I332P	I332R	I332T	I332V
I332W	I332Y	E333A	E333D	E333H	E333I
E333K	E333N	E333R	E333S	E333V	E333Y
R334A	R334C	R334D	R334E	R334F	R334G
R334L	R334M	R334N	R334P	R334Q	R334S
R334T	R334V	R334W	R334Y	T335C	T335H
T335I	T335K	T335L	T335P	I336D	I336E
I336G	I336K	I336N	I336Y	S337D	S337P
K338A	K338C	K338D	K338E	K338F	K338G
K338H	K338I	K338L	K338M	K338N	K338P
K338Q	K338R	K338S	K338V	K338W	K338Y
A339D	A339F	A339G	A339N	A339P	A339R
A339S	A339T	A339V	A339W	A339Y	R340G
T422K	T422R	F423P	L441P		

[00309] Kinetics and affinities were determined using Cytiva 8K evaluation software. Blank runs containing buffer only were subtracted out from all runs. Flow cells were regenerated using 10mM Glycine pH 1.5. Runs were performed at room temperature. Mutations (Table 5) made at respective canine mutant Fc IgG positions have a marked effect on the kinetics and/or affinity of the IgG to canine Fcγ receptors and/or human C1q when compared against the respective WT Fc kinetic and/or affinity values (Table 6).

[00310] Table 6—Binding kinetics and affinities of canine Fcγ receptors and/or human C1q to WT IgG2 and mutant canine IgG2 with indicated substitutions in Fc. (LS=Low Signal. NBO=No binding observed.)

ID No.	EU numbering system		KD (M)			
	Codon usage mutation	AA mutation	1:1 binding	Steady State	1:1 binding	1:1 binding
			canFcγR1	canFcγR2b	canFcγR3	hC1q
1	WT 1	WT 1	4.64E-09	2.89E-06	4.81E-07	2.18E-09
2	TGC	A231C	-	6.51E-07	-	7.09E-09
3	GAC	A231D	-	-	-	6.97E-09
4	GAG	A231E	-	-	-	1.02E-08
5	GGC	A231G	-	-	1.11E-06	7.61E-09
6	CAC	A231H	-	-	-	5.43E-09
7	AAG	A231K	-	1.24E-05	8.98E-07	5.26E-09
8	AAC	A231N	-	-	-	2.03E-08
9	CAG	A231Q	-	-	-	8.55E-09
10	AGG	A231R	-	8.12E-05	1.45E-06	1.15E-08
11	TCC	A231S	-	-	-	7.76E-09
12	ACC	A231T	-	7.59E-07	-	-
13	GTG	A231V	-	-	-	1.47E-09
14	TAC	A231Y	-	-	8.90E-07	-
15	GCC	P232A	-	-	8.00E-07	9.63E-09
16	TGC	P232C	-	-	-	1.45E-08
17	GAC	P232D	2.32E-09	7.96E-07	7.50E-07	6.90E-09
18	GAG	P232E	-	9.75E-07	-	3.17E-08
19	TTC	P232F	-	-	2.37E-07	4.08E-09
20	GGC	P232G	-	-	1.01E-06	8.20E-07
21	CAC	P232H	-	-	1.23E-06	3.91E-08
22	ATC	P232I	-	1.00E-06	-	4.25E-09
23	AAG	P232K	9.36E-09	LS	LS	3.29E-07
24	CTG	P232L	-	7.74E-07	-	-
25	ATG	P232M	-	-	-	4.50E-09
26	AAC	P232N	-	-	9.34E-07	5.99E-08
27	CAG	P232Q	-	-	7.97E-07	7.98E-09
28	AGG	P232R	9.84E-09	LS	LS	3.10E-07
29	TCC	P232S	-	-	1.26E-06	8.08E-09
30	ACC	P232T	-	-	7.57E-07	8.90E-09
31	GTG	P232V	-	-	-	5.42E-09
32	TGG	P232W	-	7.91E-07	2.45E-07	-
33	TAC	P232Y	-	-	-	7.15E-09
34	GCC	E233A	8.57E-09	LS	1.65E-06	3.81E-08

35	TGC	E233C	LS	LS	-	4.94E-09
36	GAC	E233D	-	-	-	5.05E-09
37	TTC	E233F	9.87E-09	LS	1.54E-06	1.81E-08
38	GGC	E233G	9.48E-09	LS	1.03E-06	LS
39	CAC	E233H	1.00E-08	LS	2.86E-06	1.58E-08
40	ATC	E233I	8.74E-09	LS	LS	LS
41	AAG	E233K	1.26E-08	LS	LS	LS
42	CTG	E233L	9.20E-09	LS	9.14E-07	1.74E-08
43	ATG	E233M	9.07E-09	LS	1.41E-06	3.11E-08
44	AAC	E233N	1.03E-08	LS	LS	LS
45	CCC	E233P	9.05E-09	LS	1.09E-06	3.99E-09
46	CAG	E233Q	-	LS	1.68E-06	1.26E-08
47	AGG	E233R	1.18E-08	LS	LS	LS
48	TCC	E233S	9.29E-09	LS	LS	LS
49	ACC	E233T	9.60E-09	LS	LS	LS
50	GTG	E233V	9.78E-09	LS	LS	2.82E-08
51	TGG	E233W	9.19E-09	LS	6.07E-07	3.99E-09
52	TAC	E233Y	1.08E-08	LS	LS	1.03E-08
53	GCC	M234A	-	-	1.49E-06	1.34E-08
54	TGC	M234C	-	LS	LS	LS
55	GAC	M234D	LS	3.23E-07	LS	3.23E-08
56	GAG	M234E	6.26E-09	2.51E-07	LS	1.02E-08
57	TTC	M234F	-	1.01E-06	8.21E-07	1.03E-08
58	GGC	M234G	LS	LS	LS	LS
59	CAC	M234H	8.01E-09	-	9.05E-07	1.94E-08
60	ATC	M234I	-	-	1.95E-07	-
61	AAG	M234K	1.08E-08	LS	LS	LS
62	CTG	M234L	-	-	-	9.20E-09
63	AAC	M234N	9.39E-09	-	9.43E-07	-
64	CCC	M234P	-	-	-	LS
65	CAG	M234Q	-	-	-	1.07E-08
66	AGG	M234R	9.82E-09	LS	LS	LS
67	TCC	M234S	7.95E-09	-	1.27E-06	3.62E-08
68	ACC	M234T	-	-	-	7.14E-09
69	TGG	M234W	6.90E-09	8.38E-07	-	-
70	TAC	M234Y	-	-	LS	2.92E-08
71	GCC	L235A	NBO	-	8.60E-07	LS
72	TGC	L235C	NBO	LS	LS	LS
73	GAC	L235D	NBO	3.57E-07	7.79E-07	LS
74	GAG	L235E	NBO	5.94E-07	-	LS
75	TTC	L235F	LS	LS	-	-
76	GGC	L235G	NBO	LS	LS	LS
77	CAC	L235H	LS	LS	-	3.52E-08
78	ATC	L235I	8.78E-09	-	-	6.30E-09

79	AAG	L235K	NBO	LS	LS	LS
80	ATG	L235M	7.71E-09	-	8.00E-07	6.44E-09
81	AAC	L235N	NBO	-	LS	LS
82	CCC	L235P	NBO	-	7.66E-07	-
83	CAG	L235Q	NBO	-	6.05E-07	LS
84	AGG	L235R	NBO	LS	LS	NBO
85	TCC	L235S	NBO	8.20E-06	LS	LS
86	ACC	L235T	NBO	LS	-	LS
87	GTG	L235V	8.04E-09	-	-	4.14E-08
88	WT 2	WT 2	6.14E-09	1.65E-04	4.22E-06	1.74E-08
89	TGG	L235W	LS	LS	-	-
90	TAC	L235Y	LS	-	-	-
91	GCC	G236A	1.23E-08	1.16E-05	LS	LS
92	TGC	G236C	NBO	NBO	LS	NBO
93	GAC	G236D	1.42E-08	-	LS	-
94	GAG	G236E	1.15E-08	4.47E-06	LS	-
95	TTC	G236F	1.42E-08	-	LS	-
96	CAC	G236H	LS	-	LS	LS
97	ATC	G236I	1.40E-08	LS	LS	LS
98	AAG	G236K	NBO	NBO	LS	NBO
99	CTG	G236L	1.99E-08	LS	LS	-
100	ATG	G236M	1.83E-08	LS	LS	1.05E-06
101	AAC	G236N	LS	LS	LS	-
102	CCC	G236P	NBO	LS	LS	LS
103	CAG	G236Q	2.12E-08	LS	LS	LS
104	AGG	G236R	NBO	NBO	LS	NBO
105	TCC	G236S	1.55E-08	-	LS	LS
106	ACC	G236T	1.27E-08	LS	LS	-
107	GTG	G236V	1.50E-08	-	LS	LS
108	TGG	G236W	9.13E-09	1.68E-07	LS	1.18E-09
109	TAC	G236Y	1.55E-08	-	LS	2.49E-09
110	GCC	G237A	1.49E-08	-	LS	9.67E-07
111	TGC	G237C	NBO	NBO	LS	NBO
112	GAC	G237D	1.19E-08	LS	LS	NBO
113	GAG	G237E	1.08E-08	LS	LS	NBO
114	TTC	G237F	LS	LS	LS	-
115	CAC	G237H	2.33E-08	NBO	LS	NBO
116	ATC	G237I	NBO	LS	LS	LS
117	AAG	G237K	NBO	NBO	LS	NBO
118	CTG	G237L	NBO	LS	LS	NBO
119	ATG	G237M	3.39E-08	LS	LS	NBO
120	AAC	G237N	2.67E-08	LS	LS	NBO
121	CCC	G237P	NBO	LS	LS	NBO
122	CAG	G237Q	LS	LS	LS	NBO

123	AGG	G237R	NBO	NBO	LS	NBO
124	TCC	G237S	1.37E-08	LS	LS	LS
125	ACC	G237T	1.30E-08	LS	LS	NBO
126	GTG	G237V	LS	LS	LS	LS
127	TGG	G237W	1.65E-07	LS	LS	LS
128	TAC	G237Y	LS	LS	LS	LS
129	GCC	P238A	-	LS	LS	NBO
130	TGC	P238C	NBO	LS	LS	NBO
131	GAC	P238D	1.49E-08	LS	LS	NBO
132	GAG	P238E	1.14E-08	LS	LS	NBO
133	TTC	P238F	-	LS	LS	NBO
134	GGC	P238G	1.99E-08	NBO	LS	NBO
135	CAC	P238H	1.40E-08	NBO	LS	NBO
136	ATC	P238I	-	LS	LS	NBO
137	AAG	P238K	NBO	NBO	LS	NBO
138	CTG	P238L	-	NBO	LS	NBO
139	ATG	P238M	-	LS	LS	NBO
140	AAC	P238N	1.40E-08	LS	LS	NBO
141	CAG	P238Q	1.42E-08	LS	LS	NBO
142	AGG	P238R	NBO	LS	LS	NBO
143	TCC	P238S	1.62E-08	LS	LS	NBO
144	ACC	P238T	1.34E-08	LS	LS	NBO
145	GTG	P238V	1.06E-08	LS	LS	NBO
146	TGG	P238W	LS	LS	LS	NBO
147	TAC	P238Y	1.20E-08	LS	LS	NBO
148	GCC	S239A	-	-	3.32E-05	-
149	TGC	S239C	-	-	LS	LS
150	GAC	S239D	1.29E-09	5.76E-07	-	-
151	GAG	S239E	1.80E-09	4.64E-07	-	-
152	TTC	S239F	1.15E-08	LS	LS	LS
153	GGC	S239G	-	-	LS	NBO
154	CAC	S239H	1.35E-08	LS	LS	LS
155	ATC	S239I	-	6.66E-07	4.21E-05	LS
156	AAG	S239K	LS	LS	LS	-
157	CTG	S239L	-	4.78E-07	4.04E-05	LS
158	ATG	S239M	-	3.27E-06	-	-
159	AAC	S239N	8.84E-09	-	3.26E-05	-
160	CCC	S239P	-	LS	LS	NBO
161	CAG	S239Q	-	-	5.88E-05	-
162	AGG	S239R	2.09E-08	LS	LS	-
163	ACC	S239T	-	-	6.33E-05	-
164	GTG	S239V	-	3.10E-06	LS	-
165	TGG	S239W	1.00E-08	-	LS	LS
166	TAC	S239Y	1.33E-08	-	LS	LS

167	GCC	V240A	-	-	6.23E-07	-
168	TGC	V240C	-	-	-	1.14E-08
169	GAC	V240D	LS	LS	LS	LS
170	GAG	V240E	-	-	1.35E-05	1.19E-09
171	TTC	V240F	-	-	LS	7.48E-08
172	GGC	V240G	-	-	-	LS
173	CAC	V240H	-	-	-	2.03E-09
174	ATC	V240I	-	-	-	1.34E-09
175	AAG	V240K	NBO	LS	NBO	NBO
176	CTG	V240L	-	-	-	7.01E-09
177	ATG	V240M	-	-	-	7.63E-09
178	AAC	V240N	-	-	-	3.99E-09
179	CCC	V240P	-	LS	2.43E-05	-
180	CAG	V240Q	-	-	-	8.62E-10
181	AGG	V240R	NBO	LS	NBO	NBO
182	WT 3	WT 3	5.25E-09	1.17E-06	4.13E-07	8.36E-09
183	TCC	V240S	-	-	1.98E-07	-
184	ACC	V240T	-	-	-	-
185	TGG	V240W	9.66E-09	NBO	NBO	LS
186	TAC	V240Y	NBO	NBO	NBO	NBO
187	GCC	V262A	-	LS	LS	1.75E-08
188	TGC	V262C	-	4.26E-05	LS	2.77E-08
189	GAC	V262D	-	9.25E-05	LS	1.40E-08
190	GAG	V262E	-	NBO	NBO	1.13E-07
191	TTC	V262F	-	1.46E-05	LS	1.71E-07
192	GGC	V262G	-	7.52E-06	NBO	3.54E-08
193	CAC	V262H	-	2.48E-05	LS	2.12E-08
194	AAG	V262K	-	9.55E-05	LS	1.66E-08
195	CTG	V262L	-	-	2.61E-07	-
196	ATG	V262M	-	2.99E-04	-	-
197	CAG	V262Q	4.17E-09	-	LS	2.54E-08
198	AGG	V262R	-	1.30E-04	NBO	LS
199	TCC	V262S	-	1.24E-04	LS	1.45E-08
200	ACC	V262T	-	-	LS	LS
201	TGG	V262W	-	1.80E-04	LS	LS
202	TAC	V262Y	-	1.82E-04	NBO	NBO
203	GCC	V263A	-	-	NBO	LS
204	TGC	V263C	-	-	LS	1.62E-08
205	GAG	V263E	NBO	NBO	NBO	NBO
206	TTC	V263F	-	1.78E-04	NBO	LS
207	GGC	V263G	-	9.29E-05	NBO	LS
208	CAC	V263H	NBO	NBO	NBO	NBO
209	ATC	V263I	-	-	2.09E-07	5.58E-09
210	AAG	V263K	NBO	NBO	NBO	NBO

211	CTG	V263L	-	1.79E-04	LS	LS
212	ATG	V263M	-	3.78E-04	LS	-
213	AAC	V263N	LS	NBO	NBO	NBO
214	CCC	V263P	NBO	NBO	NBO	NBO
215	CAG	V263Q	NBO	NBO	NBO	NBO
216	TCC	V263S	-	2.22E-05	LS	LS
217	ACC	V263T	-	8.81E-05	-	6.36E-09
218	TGG	V263W	NBO	NBO	NBO	NBO
219	TAC	V263Y	NBO	NBO	NBO	NBO
220	GCC	V264A	-	NBO	NBO	LS
221	TGC	V264C	-	NBO	NBO	LS
222	GAC	V264D	LS	NBO	NBO	LS
223	GAG	V264E	LS	NBO	NBO	LS
224	TTC	V264F	-	NBO	NBO	-
225	GGC	V264G	-	NBO	NBO	LS
226	CAC	V264H	-	NBO	NBO	LS
227	ATC	V264I	-	-	1.78E-07	5.74E-09
228	AAG	V264K	4.15E-08	NBO	NBO	LS
229	CTG	V264L	-	1.79E-04	NBO	-
230	ATG	V264M	-	1.15E-04	NBO	-
231	AAC	V264N	LS	2.05E-04	NBO	-
232	CCC	V264P	1.35E-08	NBO	NBO	LS
233	CAG	V264Q	-	NBO	NBO	3.79E-08
234	AGG	V264R	NBO	NBO	NBO	NBO
235	TCC	V264S	-	NBO	NBO	1.54E-08
236	ACC	V264T	-	2.25E-04	NBO	-
237	TGG	V264W	-	NBO	NBO	1.85E-08
238	WT 4	WT 4	3.48E-09	8.34E-06	2.58E-07	7.66E-09
239	TAC	V264Y	-	LS	NBO	-
240	GCC	D265A	LS	NBO	NBO	3.82E-07
241	TGC	D265C	7.29E-09	NBO	NBO	2.08E-08
242	GAG	D265E	5.07E-09	NBO	NBO	3.68E-07
243	TTC	D265F	NBO	NBO	NBO	NBO
244	GGC	D265G	NBO	NBO	NBO	LS
245	CAC	D265H	LS	NBO	NBO	LS
246	ATC	D265I	NBO	NBO	NBO	NBO
247	AAG	D265K	NBO	NBO	NBO	LS
248	CTG	D265L	NBO	NBO	NBO	NBO
249	ATG	D265M	NBO	NBO	NBO	LS
250	AAC	D265N	LS	NBO	NBO	-
251	CCC	D265P	LS	NBO	NBO	NBO
252	CAG	D265Q	LS	NBO	NBO	LS
253	AGG	D265R	NBO	NBO	NBO	LS
254	TCC	D265S	LS	NBO	NBO	1.45E-07

255	ACC	D265T	7.75E-09	NBO	NBO	NBO
256	GTG	D265V	NBO	NBO	NBO	NBO
257	TGG	D265W	NBO	NBO	NBO	NBO
258	TAC	D265Y	NBO	NBO	NBO	NBO
259	GCC	L266A	-	NBO	NBO	NBO
260	TGC	L266C	-	NBO	NBO	NBO
261	GAC	L266D	6.90E-09	NBO	NBO	NBO
262	GAG	L266E	6.00E-09	NBO	NBO	NBO
263	TTC	L266F	7.11E-09	NBO	NBO	NBO
264	GGC	L266G	1.56E-09	LS	NBO	NBO
265	CAC	L266H	LS	NBO	NBO	NBO
266	ATC	L266I	1.69E-09	-	-	-
267	AAG	L266K	NBO	NBO	NBO	NBO
268	ATG	L266M	-	-	LS	NBO
269	AAC	L266N	-	NBO	NBO	NBO
270	CCC	L266P	LS	NBO	NBO	NBO
271	CAG	L266Q	8.27E-09	NBO	NBO	NBO
272	AGG	L266R	NBO	NBO	NBO	NBO
273	TCC	L266S	7.84E-09	NBO	NBO	NBO
274	ACC	L266T	7.79E-09	NBO	NBO	NBO
275	GTG	L266V	2.45E-09	-	LS	-
276	TGG	L266W	6.37E-09	NBO	NBO	NBO
277	TAC	L266Y	7.00E-09	NBO	NBO	NBO
278	GCC	D267A	-	6.31E-05	-	NBO
279	TGC	D267C	9.58E-09	NBO	NBO	NBO
280	GAG	D267E	-	5.54E-05	-	2.23E-08
281	TTC	D267F	6.75E-09	NBO	NBO	NBO
282	GGC	D267G	6.80E-09	LS	NBO	NBO
283	CAC	D267H	7.19E-09	NBO	NBO	NBO
284	ATC	D267I	LS	NBO	LS	NBO
285	AAG	D267K	NBO	NBO	NBO	NBO
286	CTG	D267L	-	NBO	NBO	NBO
287	ATG	D267M	NBO	NBO	NBO	NBO
288	AAC	D267N	6.21E-09	NBO	LS	NBO
289	CCC	D267P	6.59E-09	NBO	NBO	NBO
290	CAG	D267Q	-	NBO	NBO	NBO
291	AGG	D267R	NBO	NBO	NBO	NBO
292	TCC	D267S	-	6.45E-05	-	NBO
293	ACC	D267T	6.66E-09	NBO	LS	NBO
294	GTG	D267V	-	NBO	LS	NBO
295	TGG	D267W	-	NBO	LS	NBO
296	TAC	D267Y	-	NBO	LS	NBO
297	GCC	P268A	6.53E-09	1.40E-04	LS	-
298	TGC	P268C	-	1.61E-04	LS	-

299	GAC	P268D	-	-	1.36E-07	3.33E-09
300	GAG	P268E	-	6.98E-05	LS	3.37E-09
301	TTC	P268F	-	5.01E-04	LS	9.93E-10
302	GGC	P268G	-	1.03E-04	-	-
303	CAC	P268H	-	5.71E-04	LS	-
304	ATC	P268I	-	NBO	LS	-
305	AAG	P268K	-	NBO	LS	NBO
306	CTG	P268L	-	NBO	LS	-
307	ATG	P268M	-	NBO	-	5.05E-09
308	AAC	P268N	-	-	-	LS
309	CAG	P268Q	-	2.88E-04	-	-
310	AGG	P268R	-	NBO	LS	NBO
311	TCC	P268S	-	3.09E-05	-	-
312	ACC	P268T	-	2.72E-04	-	-
313	GTG	P268V	-	NBO	LS	-
314	TGG	P268W	-	NBO	LS	6.17E-11
315	TAC	P268Y	-	1.81E-04	LS	5.24E-10
316	GCC	E269A	-	3.22E-05	-	LS
317	TGC	E269C	-	3.51E-04	-	NBO
318	GAC	E269D	-	1.38E-04	-	3.99E-09
319	TTC	E269F	-	4.14E-04	LS	LS
320	GGC	E269G	-	1.90E-04	LS	LS
321	CAC	E269H	-	1.37E-04	LS	NBO
322	ATC	E269I	-	1.21E-04	LS	NBO
323	AAG	E269K	7.73E-09	NBO	LS	NBO
324	CTG	E269L	-	7.75E-04	LS	LS
325	ATG	E269M	-	1.17E-04	LS	LS
326	AAC	E269N	-	3.30E-04	LS	LS
327	CCC	E269P	-	NBO	NBO	NBO
328	CAG	E269Q	-	2.85E-04	LS	LS
329	AGG	E269R	7.17E-09	NBO	NBO	NBO
330	TCC	E269S	-	2.50E-04	LS	NBO
331	ACC	E269T	-	2.30E-04	LS	LS
332	WT 5	WT 5	2.97E-09	1.41E-06	7.17E-07	9.65E-09
333	GTG	E269V	-	-	LS	KO
334	TGG	E269W	-	LS	LS	-
335	TAC	E269Y	-	LS	LS	LS
336	GCC	D270A	-	6.49E-06	LS	NBO
337	TGC	D270C	-	4.55E-07	-	NBO
338	TTC	D270F	-	-	LS	-
339	GGC	D270G	-	LS	-	NBO
340	CAC	D270H	-	-	LS	LS
341	ATC	D270I	-	-	-	LS
342	AAG	D270K	-	LS	LS	NBO

343	CTG	D270L	-	-	-	4.90E-07
344	ATG	D270M	-	-	-	NBO
345	AAC	D270N	-	-	-	2.80E-07
346	CCC	D270P	-	-	LS	NBO
347	CAG	D270Q	-	-	-	NBO
348	AGG	D270R	7.07E-09	NBO	NBO	NBO
349	TCC	D270S	-	8.52E-06	-	4.83E-07
350	GTG	D270V	-	-	-	NBO
351	TGG	D270W	-	-	LS	NBO
352	TAC	D270Y	-	-	LS	-
353	TGC	P271C	-	-	-	NBO
354	TTC	P271F	-	-	-	LS
355	GGC	P271G	-	-	LS	-
356	CAC	P271H	-	-	-	LS
357	ATC	P271I	-	-	-	LS
358	AAG	P271K	-	-	-	NBO
359	CTG	P271L	-	-	-	NBO
360	ATG	P271M	-	-	-	NBO
361	AAC	P271N	-	-	-	NBO
362	CAG	P271Q	-	-	-	NBO
363	AGG	P271R	-	-	LS	NBO
364	TCC	P271S	-	-	-	LS
365	ACC	P271T	-	-	-	NBO
366	TAC	P271Y	-	-	-	LS
367	GCC	E294A	-	3.50E-06	-	NBO
368	TGC	E294C	-	-	-	NBO
369	GAC	E294D	-	NBO	LS	NBO
370	TTC	E294F	-	-	-	NBO
371	GGC	E294G	-	-	LS	NBO
372	CAC	E294H	-	-	-	NBO
373	ATC	E294I	-	-	-	NBO
374	AAG	E294K	-	-	-	NBO
375	CTG	E294L	-	-	2.93E-07	LS
376	ATG	E294M	-	-	2.57E-07	NBO
377	AAC	E294N	-	NBO	-	NBO
378	CCC	E294P	-	-	-	NBO
379	CAG	E294Q	-	-	-	LS
380	AGG	E294R	-	-	-	NBO
381	TCC	E294S	-	LS	LS	NBO
382	ACC	E294T	-	LS	LS	NBO
383	GTG	E294V	-	-	-	LS
384	TGG	E294W	-	LS	-	-
385	TAC	E294Y	-	LS	-	-
386	GCC	Q295A	-	LS	LS	-

387	GAC	Q295D	-	NBO	LS	-
388	GAG	Q295E	-	-	LS	-
389	TTC	Q295F	-	NBO	LS	LS
390	GGC	Q295G	-	NBO	NBO	NBO
391	AAG	Q295K	-	LS	LS	LS
392	CTG	Q295L	-	8.98E-06	-	-
393	AAC	Q295N	-	NBO	NBO	-
394	CCC	Q295P	-	NBO	LS	NBO
395	AGG	Q295R	-	LS	LS	LS
396	TCC	Q295S	-	NBO	LS	-
397	ACC	Q295T	-	NBO	LS	-
398	GTG	Q295V	-	LS	LS	-
399	TGG	Q295W	-	NBO	NBO	NBO
400	TAC	Q295Y	-	NBO	LS	-
401	GCC	F296A	-	-	NBO	-
402	TGC	F296C	-	-	LS	-
403	GAC	F296D	-	-	LS	-
404	GAG	F296E	-	-	LS	-
405	GGC	F296G	-	LS	LS	LS
406	CAC	F296H	-	-	LS	-
407	ATC	F296I	-	-	LS	-
408	AAG	F296K	-	-	LS	-
409	CTG	F296L	-	-	LS	-
410	ATG	F296M	-	-	LS	-
411	AAC	F296N	-	-	LS	-
412	CCC	F296P	LS	NBO	NBO	LS
413	CAG	F296Q	-	-	LS	-
414	AGG	F296R	-	-	LS	-
415	WT 6	WT 6	5.31E-09	1.67E-06	4.10E-07	1.06E-08
416	TCC	F296S	-	-	NBO	NBO
417	ACC	F296T	-	-	LS	LS
418	GTG	F296V	-	-	LS	-
419	GCC	N297A	NBO	NBO	NBO	NBO
420	TGC	N297C	NBO	NBO	NBO	NBO
421	GAC	N297D	NBO	NBO	NBO	NBO
422	GAG	N297E	NBO	NBO	NBO	NBO
423	TTC	N297F	NBO	NBO	NBO	NBO
424	GGC	N297G	NBO	NBO	NBO	NBO
425	CAC	N297H	1.51E-08	NBO	NBO	NBO
426	ATC	N297I	1.39E-08	NBO	NBO	NBO
427	AAG	N297K	NBO	NBO	NBO	NBO
428	CTG	N297L	NBO	NBO	NBO	NBO
429	ATG	N297M	NBO	NBO	NBO	NBO
430	CCC	N297P	NBO	NBO	NBO	NBO

431	CAG	N297Q	NBO	NBO	NBO	NBO
432	AGG	N297R	NBO	NBO	NBO	NBO
433	TCC	N297S	NBO	NBO	NBO	NBO
434	ACC	N297T	NBO	NBO	NBO	NBO
435	GTG	N297V	NBO	NBO	NBO	NBO
436	TGG	N297W	1.28E-08	NBO	NBO	NBO
437	TAC	N297Y	1.14E-08	NBO	NBO	NBO
438	GCC	G298A	2.90E-09	LS	-	-
439	TGC	G298C	1.02E-09	3.95E-07	-	NBO
440	GAC	G298D	-	LS	LS	LS
441	GAG	G298E	-	LS	LS	LS
442	TTC	G298F	-	LS	LS	1.83E-09
443	CAC	G298H	-	LS	LS	LS
444	ATC	G298I	-	LS	LS	7.06E-10
445	AAG	G298K	1.74E-09	-	NBO	NBO
446	CTG	G298L	1.66E-09	3.52E-07	LS	4.49E-09
447	ATG	G298M	1.81E-09	8.65E-07	LS	-
448	AAC	G298N	-	NBO	NBO	NBO
449	CCC	G298P	NBO	NBO	NBO	NBO
450	CAG	G298Q	3.46E-09	LS	LS	LS
451	AGG	G298R	2.41E-09	-	LS	NBO
452	TCC	G298S	-	-	LS	-
453	ACC	G298T	1.79E-09	NBO	NBO	5.72E-09
454	GTG	G298V	-	NBO	LS	3.02E-09
455	TGG	G298W	-	NBO	1.42E-06	-
456	TAC	G298Y	-	4.40E-05	-	1.69E-09
457	GCC	T299A	NBO	-	NBO	NBO
458	TGC	T299C	NBO	NBO	NBO	NBO
459	GAC	T299D	NBO	NBO	NBO	NBO
460	GAG	T299E	NBO	NBO	NBO	NBO
461	TTC	T299F	NBO	NBO	NBO	LS
462	GGC	T299G	NBO	-	NBO	NBO
463	CAC	T299H	NBO	NBO	LS	NBO
464	ATC	T299I	NBO	NBO	NBO	NBO
465	AAG	T299K	NBO	NBO	NBO	NBO
466	CTG	T299L	NBO	NBO	NBO	LS
467	ATG	T299M	NBO	NBO	NBO	LS
468	AAC	T299N	NBO	NBO	NBO	LS
469	CCC	T299P	NBO	NBO	LS	NBO
470	CAG	T299Q	NBO	NBO	NBO	NBO
471	AGG	T299R	NBO	NBO	NBO	NBO
472	TCC	T299S	LS	-	LS	LS
473	GTG	T299V	NBO	NBO	LS	NBO
474	TGG	T299W	NBO	NBO	LS	LS

475	TAC	T299Y	NBO	NBO	LS	-
476	GCC	Y300A	-	LS	2.53E-07	LS
477	TGC	Y300C	-	LS	-	LS
478	GAC	Y300D	-	-	2.40E-07	6.51E-10
479	GAG	Y300E	2.75E-09	-	-	-
480	CAC	Y300H	-	-	-	NBO
481	ATC	Y300I	-	-	-	LS
482	AAG	Y300K	-	LS	LS	NBO
483	CTG	Y300L	2.69E-09	-	-	LS
484	ATG	Y300M	-	-	1.69E-07	-
485	AAC	Y300N	-	-	-	LS
486	CCC	Y300P	LS	NBO	NBO	NBO
487	CAG	Y300Q	2.77E-09	-	-	LS
488	AGG	Y300R	3.24E-09	-	LS	NBO
489	TCC	Y300S	-	LS	3.96E-07	LS
490	ACC	Y300T	-	LS	3.58E-07	LS
491	GTG	Y300V	-	LS	-	LS
492	TGG	Y300W	-	-	LS	-
493	GCC	R301A	-	-	1.11E-06	-
494	TGC	R301C	-	LS	LS	-
495	GAC	R301D	-	LS	LS	-
496	GAG	R301E	-	LS	LS	LS
497	TTC	R301F	-	LS	LS	-
498	GGC	R301G	-	LS	LS	-
499	CAC	R301H	-	LS	LS	LS
500	ATC	R301I	-	LS	LS	-
501	CTG	R301L	-	-	LS	-
502	WT 7	WT 7	5.95E-09	1.41E-06	2.09E-07	1.20E-09
503	CCC	R301P	8.46E-09	NBO	LS	NBO
504	CAG	R301Q	-	LS	LS	-
505	GTG	R301V	-	-	LS	-
506	TGG	R301W	-	-	LS	-
507	TAC	R301Y	-	2.62E-05	LS	-
508	GCC	V323A	-	-	-	4.11E-09
509	TGC	V323C	-	-	-	-
510	GAC	V323D	1.13E-08	LS	LS	NBO
511	GAG	V323E	-	LS	LS	NBO
512	TTC	V323F	-	-	-	NBO
513	GGC	V323G	-	-	-	LS
514	CAC	V323H	-	-	6.08E-08	NBO
515	AAG	V323K	1.08E-08	LS	NBO	NBO
516	CTG	V323L	-	-	1.18E-07	NBO
517	ATG	V323M	-	-	-	NBO
518	AAC	V323N	-	LS	LS	NBO

519	CCC	V323P	1.00E-08	5.20E-05	LS	NBO
520	CAG	V323Q	-	7.28E-06	LS	NBO
521	AGG	V323R	LS	NBO	LS	NBO
522	TCC	V323S	-	-	LS	NBO
523	ACC	V323T	-	-	-	LS
524	TGG	V323W	-	LS	LS	NBO
525	TAC	V323Y	-	-	LS	NBO
526	GCC	N324A	-	-	-	NBO
527	TGC	N324C	1.07E-08	-	-	NBO
528	GAC	N324D	-	-	-	NBO
529	GAG	N324E	-	-	LS	NBO
530	TTC	N324F	-	-	-	NBO
531	GGC	N324G	-	-	-	LS
532	CAC	N324H	-	-	-	NBO
533	ATC	N324I	-	-	-	LS
534	AAG	N324K	1.16E-08	LS	LS	NBO
535	CTG	N324L	-	-	-	LS
536	ATG	N324M	-	-	-	NBO
537	CCC	N324P	NBO	NBO	NBO	NBO
538	CAG	N324Q	-	-	-	NBO
539	AGG	N324R	-	LS	LS	NBO
540	TCC	N324S	-	-	-	NBO
541	ACC	N324T	-	-	-	NBO
542	GTG	N324V	-	-	-	NBO
543	TGG	N324W	-	-	-	LS
544	TAC	N324Y	-	-	-	NBO
545	GCC	N325A	-	LS	-	LS
546	TGC	N325C	-	LS	LS	NBO
547	GAC	N325D	-	-	-	LS
548	GAG	N325E	-	-	LS	NBO
549	TTC	N325F	9.14E-09	NBO	LS	NBO
550	GGC	N325G	-	-	LS	NBO
551	CAC	N325H	-	LS	-	NBO
552	ATC	N325I	-	NBO	LS	NBO
553	AAG	N325K	1.34E-08	NBO	LS	NBO
554	CTG	N325L	-	LS	LS	NBO
555	ATG	N325M	-	LS	LS	NBO
556	CCC	N325P	LS	NBO	LS	NBO
557	CAG	N325Q	-	-	LS	NBO
558	AGG	N325R	LS	NBO	LS	NBO
559	TCC	N325S	-	-	-	NBO
560	ACC	N325T	-	-	-	NBO
561	GTG	N325V	1.02E-08	NBO	LS	NBO
562	TGG	N325W	1.05E-08	NBO	LS	NBO

563	TAC	N325Y	1.07E-08	NBO	LS	NBO
564	GCC	K326A	-	-	-	5.69E-10
565	GAC	K326D	-	9.82E-07	5.78E-06	LS
566	GAG	K326E	-	9.12E-07	LS	NBO
567	TTC	K326F	-	-	4.70E-06	5.73E-10
568	ATC	K326I	-	-	-	LS
569	CTG	K326L	-	-	-	LS
570	AAC	K326N	-	9.58E-07	-	5.72E-10
571	AGG	K326R	-	-	-	7.56E-10
572	TCC	K326S	-	-	-	NBO
573	ACC	K326T	-	-	-	NBO
574	GTG	K326V	-	-	-	LS
575	TGG	K326W	-	-	-	9.99E-10
576	TGC	A327C	-	-	-	NBO
577	GAC	A327D	-	-	LS	LS
578	GAG	A327E	-	7.48E-07	-	LS
579	TTC	A327F	-	-	-	5.66E-10
580	GGC	A327G	-	8.20E-07	-	-
581	CAC	A327H	-	-	LS	LS
582	WT 8	WT 8	5.42E-09	5.79E-06	8.54E-07	9.68E-09
583	ATC	A327I	-	-	NBO	NBO
584	AAG	A327K	1.02E-08	NBO	NBO	NBO
585	CTG	A327L	-	-	LS	LS
586	ATG	A327M	-	-	2.95E-05	1.34E-06
587	AAC	A327N	-	-	LS	2.32E-06
588	CCC	A327P	-	LS	NBO	NBO
589	CAG	A327Q	-	-	-	9.53E-07
590	AGG	A327R	1.26E-08	NBO	NBO	NBO
591	TCC	A327S	-	-	7.53E-06	1.14E-06
592	ACC	A327T	9.27E-09	LS	NBO	NBO
593	GTG	A327V	-	LS	NBO	NBO
594	TGG	A327W	-	LS	LS	1.03E-06
595	TAC	A327Y	-	3.22E-05	3.10E-05	-
596	GCC	L328A	-	-	LS	NBO
597	TGC	L328C	-	LS	NBO	NBO
598	GAC	L328D	-	LS	NBO	NBO
599	GAG	L328E	9.25E-09	LS	NBO	NBO
600	TTC	L328F	2.50E-09	1.99E-07	NBO	NBO
601	GGC	L328G	9.21E-09	LS	NBO	NBO
602	CAC	L328H	9.65E-09	NBO	NBO	NBO
603	ATC	L328I	-	6.48E-07	-	NBO
604	AAG	L328K	NBO	NBO	NBO	NBO
605	ATG	L328M	2.79E-09	6.22E-07	LS	NBO
606	AAC	L328N	-	LS	LS	NBO

607	CCC	L328P	NBO	NBO	NBO	NBO
608	CAG	L328Q	-	LS	NBO	NBO
609	AGG	L328R	NBO	NBO	NBO	NBO
610	TCC	L328S	-	-	LS	NBO
611	ACC	L328T	-	-	2.05E-05	NBO
612	GTG	L328V	-	8.81E-07	-	1.71E-06
613	TGG	L328W	-	1.70E-07	NBO	NBO
614	TAC	L328Y	2.10E-09	2.46E-07	NBO	NBO
615	GCC	K322A	-	-	6.26E-06	NBO
616	TGC	K322C	-	-	-	NBO
617	GAC	K322D	-	-	5.16E-06	NBO
618	GAG	K322E	-	-	-	NBO
619	TTC	K322F	-	-	5.48E-06	NBO
620	GGC	K322G	-	-	-	NBO
621	CAC	K322H	-	-	-	NBO
622	ATC	K322I	-	-	-	NBO
623	CTG	K322L	-	-	-	NBO
624	ATG	K322M	-	-	-	NBO
625	AAC	K322N	-	-	-	NBO
626	CCC	K322P	NBO	NBO	NBO	NBO
627	CAG	K322Q	-	-	-	NBO
628	AGG	K322R	-	-	-	NBO
629	TCC	K322S	-	-	LS	NBO
630	ACC	K322T	-	-	-	NBO
631	GTG	K322V	-	-	-	NBO
632	TGG	K322W	-	-	-	NBO
633	TAC	K322Y	-	-	-	NBO
634	GCC	P329A	9.41E-09	NBO	NBO	NBO
635	TGC	P329C	NBO	NBO	NBO	NBO
636	GAC	P329D	NBO	NBO	NBO	NBO
637	GAG	P329E	NBO	NBO	NBO	NBO
638	TTC	P329F	NBO	NBO	NBO	NBO
639	GGC	P329G	1.07E-08	NBO	NBO	NBO
640	CAC	P329H	NBO	NBO	NBO	NBO
641	ATC	P329I	NBO	NBO	NBO	NBO
642	AAG	P329K	NBO	NBO	NBO	NBO
643	CTG	P329L	NBO	NBO	NBO	NBO
644	ATG	P329M	NBO	NBO	NBO	NBO
645	AAC	P329N	NBO	NBO	NBO	NBO
646	CAG	P329Q	NBO	NBO	NBO	NBO
647	AGG	P329R	NBO	NBO	NBO	NBO
648	TCC	P329S	NBO	NBO	NBO	NBO
649	ACC	P329T	NBO	NBO	NBO	NBO
650	GTG	P329V	7.44E-09	NBO	NBO	NBO

651	TGG	P329W	NBO	NBO	NBO	NBO
652	TAC	P329Y	NBO	NBO	NBO	NBO
653	GCC	S330A	-	-	-	1.22E-09
654	TGC	S330C	6.54E-10	3.73E-07	-	NBO
655	GAC	S330D	8.50E-09	LS	-	NBO
656	GAG	S330E	-	-	-	NBO
657	TTC	S330F	-	1.05E-06	-	NBO
658	GGC	S330G	-	-	-	NBO
659	CAC	S330H	-	-	-	1.92E-09
660	ATC	S330I	1.83E-09	9.18E-07	-	NBO
661	AAG	S330K	1.58E-09	-	3.86E-07	NBO
662	CTG	S330L	1.60E-09	9.57E-07	-	NBO
663	ATG	S330M	2.52E-09	-	3.29E-07	LS
664	AAC	S330N	-	9.35E-07	-	NBO
665	CCC	S330P	NBO	NBO	NBO	NBO
666	CAG	S330Q	2.77E-09	-	-	NBO
667	AGG	S330R	1.64E-09	-	-	NBO
668	ACC	S330T	-	-	-	NBO
669	GTG	S330V	1.86E-09	6.28E-07	-	NBO
670	TGG	S330W	-	-	LS	NBO
671	TAC	S330Y	-	1.09E-06	-	NBO
672	GCC	P331A	-	-	-	LS
673	WT 9	WT 9	5.79E-09	2.89E-06	2.08E-07	7.49E-09
674	GCC	I332A	8.63E-09	-	-	-
675	GAC	I332D	-	7.44E-07	-	-
676	GAG	I332E	-	2.06E-07	-	-
677	TTC	I332F	-	LS	LS	-
678	GGC	I332G	LS	LS	LS	LS
679	CAC	I332H	-	LS	LS	LS
680	AAG	I332K	LS	NBO	NBO	LS
681	CTG	I332L	-	-	-	4.27E-09
682	ATG	I332M	-	-	-	3.79E-09
683	AAC	I332N	9.60E-09	LS	LS	2.13E-08
684	CCC	I332P	1.06E-08	LS	NBO	NBO
685	AGG	I332R	LS	NBO	NBO	LS
686	ACC	I332T	-	-	-	1.79E-09
687	GTG	I332V	-	-	-	2.59E-09
688	TGG	I332W	8.51E-09	NBO	NBO	2.31E-08
689	TAC	I332Y	8.27E-09	-	-	3.54E-09
690	GCC	E333A	-	-	-	4.44E-09
691	GAC	E333D	-	-	-	2.70E-09
692	CAC	E333H	-	-	-	2.43E-09
693	ATC	E333I	-	-	-	1.41E-08
694	AAG	E333K	-	-	-	3.83E-09

695	AAC	E333N	-	-	-	2.52E-09
696	AGG	E333R	-	-	-	1.23E-09
697	TCC	E333S	-	-	-	2.73E-09
698	GTG	E333V	-	-	-	1.65E-08
699	TAC	E333Y	-	-	-	7.48E-10
700	GCC	R334A	-	6.97E-07	1.05E-07	-
701	TGC	R334C	-	5.97E-07	9.43E-08	1.07E-08
702	GAC	R334D	-	9.31E-07	1.10E-07	8.98E-09
703	GAG	R334E	-	4.69E-07	7.95E-08	-
704	TTC	R334F	-	-	-	3.70E-09
705	GGC	R334G	-	9.47E-07	9.46E-08	-
706	CTG	R334L	-	6.44E-07	1.13E-07	LS
707	ATG	R334M	-	5.01E-07	1.02E-07	-
708	AAC	R334N	-	9.60E-07	1.34E-07	-
709	CCC	R334P	-	-	-	NBO
710	CAG	R334Q	-	5.95E-07	1.13E-07	-
711	TCC	R334S	-	-	1.17E-07	-
712	ACC	R334T	-	7.47E-07	1.31E-07	3.87E-09
713	GTG	R334V	-	-	1.37E-07	-
714	TGG	R334W	-	-	-	1.33E-08
715	TAC	R334Y	-	-	-	4.04E-09
716	TGC	T335C	-	-	-	1.05E-08
717	CAC	T335H	-	-	-	4.93E-09
718	ATC	T335I	-	-	-	5.45E-09
719	AAG	T335K	-	-	-	6.01E-09
720	CTG	T335L	-	-	-	6.55E-09
721	CCC	T335P	-	-	-	4.29E-09
722	WT 10	WT 10	6.76E-09	9.97E-06	3.51E-07	1.07E-08
723	AAG	T422K	-	-	-	2.39E-09
724	AGG	T422R	-	-	-	1.45E-09
725	CCC	F423P	-	-	-	3.40E-09
726	CCC	L441P	-	-	-	2.22E-09
727	WT 11	WT 11	5.56E-09	5.60E-07	1.52E-07	5.22E-09
728	GAC	I336D	-	-	NBO	-
729	GAG	I336E	-	-	-	1.03E-08
730	GGC	I336G	-	-	LS	-
731	AAG	I336K	-	-	-	9.00E-09
732	AAC	I336N	-	-	LS	-
733	TAC	I336Y	2.36E-09	-	-	1.60E-09
734	GAC	S337D	-	-	-	2.70E-09
735	CCC	S337P	-	-	LS	5.86E-10
736	GCC	K338A	-	-	-	3.06E-09
737	TGC	K338C	-	-	LS	2.14E-09
738	GAC	K338D	-	-	LS	5.24E-10

739	GAG	K338E	-	-	LS	1.05E-09
740	TTC	K338F	-	-	-	1.42E-10
741	GGC	K338G	-	-	-	1.10E-09
742	CAC	K338H	-	-	-	1.54E-09
743	ATC	K338I	-	-	LS	2.07E-09
744	CTG	K338L	-	-	LS	2.27E-09
745	ATG	K338M	-	-	LS	1.25E-09
746	AAC	K338N	-	-	-	4.85E-10
747	CCC	K338P	-	-	-	2.51E-09
748	CAG	K338Q	-	-	LS	-
749	AGG	K338R	1.04E-08	LS	LS	LS
750	TCC	K338S	-	-	-	2.40E-09
751	GTG	K338V	-	LS	LS	-
752	TGG	K338W	-	-	LS	1.23E-10
753	TAC	K338Y	-	-	-	7.85E-10
754	GAC	A339D	-	-	-	2.18E-09
755	TTC	A339F	-	-	-	2.04E-09
756	GGC	A339G	-	-	-	1.13E-09
757	AAC	A339N	-	-	-	1.45E-09
758	CCC	A339P	-	-	-	9.41E-10
759	AGG	A339R	-	-	-	2.63E-09
760	TCC	A339S	-	-	-	2.71E-09
761	ACC	A339T	2.60E-09	-	-	1.09E-09
762	GTG	A339V	3.07E-09	-	-	2.58E-09
763	TGG	A339W	-	-	-	7.13E-10
764	TAC	A339Y	-	-	-	1.06E-09
765	GGC	R340G	-	-	-	2.15E-09

[00311] Example 9—CDC Cell-Based Assay of Equine IgG Fc Effector Function

[00312] The CDC cell-based assay was developed and employed to characterize the effectiveness of the nine CTLA4 equine IgG subclass Fc fusion proteins in mediating CDC and to investigate Fc region mutations in the subclasses. This will help define key residues in the Fc region that determine CDC activity of the equine IgG subclasses. The assay utilizes CHO target cells engineered to express canine CD80 which binds to CTLA4 on the Fc fusion proteins. These target cells have been used in past canine ADCC assays and were utilized in the CDC assay due to their dependability.

[00313] Incubation of the fusion protein-bound target cells with complement-preserved serum can result in Fc binding on the fusion proteins to complement component C1q initiating the complement cascade, ultimately forming membrane attack complexes. The pore-forming complexes mediate cell lysis of the target cells measured by loss of cell viability. If there is no Fc binding to C1q there is no resultant cell lysis/death.

[00314] **Methods.** CD80-expressing CHO cells (target cells) were plated at 40,000 cells/well in CD CHO media in round-bottomed 96-well plates. Titrated fusion proteins in CD CHO media were added to the target cells and allowed to bind for 60 minutes at 37°C.

Equine complement preserved serum (20% final concentration in CD CHO media) was added to the plates for 45 minutes at 37°C. Cell viability was then measured using CellTiter-Glo and data were expressed as “cell viability % of control” calculated using no fusion protein + complement preserved serum controls.

[00315] **Results.** As shown in Figure 26 and Table 7, four of the six equine wild type Fc subclasses showed robust and potent CDC activity. The exceptions were IgG2 and IgG5 both of which showed no CDC activity. EC50 values range from 0.19 to 0.29 µg/mL.

[00316] **Table 7.** CDC effects induced by equine IgG Fc wildtype subclass fusion proteins.

CTLA4 Equine IgG Fusion Protein	CDC EC50 Value
CTLA4 Fc eIgG1	0.29 µg/mL
CTLA4 Fc eIgG2	No CDC
CTLA4 Fc eIgG3	0.21 µg/mL
CTLA4 Fc eIgG4	0.19 µg/mL
CTLA4 Fc eIgG5	No CDC
CTLA4 Fc eIgG7	0.26 µg/mL

[00317] As shown in Figure 27, wild type IgG1 Fc subclass showed robust and potent CDC activity. The Fc mutations investigated showed differing effects on CDC activity. The PAP mutation in the Fc showed did not affect CDC activity compared to WT. The PSS mutation showed slightly enhanced CDC activity. The Win and WinPSS mutations showed knockdown of CDC activity. The SQP, SAP and WinSAP mutations most effectively knocked out CDC activity.

[00318] As shown in Figure 28, wild type IgG4 Fc subclass showed robust and potent CDC activity. The Fc mutations investigated showed differing effects on CDC activity. The Win, PSS and WinPSS mutations in the Fc showed slight knockdown of CDC activity compared to WT. The SQP, SAP and WinSAP mutations knocked out CDC activity.

[00319] As shown in Figure 29, wild type IgG7 Fc subclass showed robust and potent CDC activity. The Fc mutations investigated showed differing effects on CDC activity. The Win mutation in the Fc showed no effect on CDC activity compared to WT. The PSS mutation showed moderate knockdown of CDC activity. The SQP, SAP, WinSAP and WinPSS mutations knocked out CDC activity.

[00320] Example 10—ADCP Assay of Equine IgG Effector Function

[00321] The antibody dependent cellular phagocytosis (ADCP) assay utilizes CHO target cells engineered to express canine CD80, which binds to canine CTLA4 on the Fc fusion proteins. The Fc region of the fusion protein may then bridge this complex to Fc gamma receptors on activated macrophage effector cells, which have the capacity to phagocytose the target cell. ADCP is measured by fluorescent intensity and/or fluorescent area of a pH-sensitive fluorescent dye within the population of effector macrophages in the co-culture, wherein

fluorescent cells are indicative of an effector that has successfully internalized a fluorescent labeled target cell into the acidic lysosome.

[00322] Methods. Canine CD80-expressing CHO cells (CD80 target cells) or wild-type CHO cells which do not express CD80 (parental target cells) were stained with pHrodo red dye for 30 minutes at 37 degrees C. Stained cells were subsequently incubated with equine CTLA4-Fc fusion proteins for 60 minutes to mediate CTLA4:CD80 binding. 40,000 target cells were then added to 8,000 pre-plated equine activated macrophages, isolated and differentiated from PMBCs. Co-cultures were maintained for 6-8 hours at 37 C as they were monitored and analyzed by a SX5 Incucyte live cell imaging system. Fluorescent area and/or intensity was quantified using a predefined processing definition in the Incucyte software and expressed as such.

[00323] Results. As shown in Figure 30, wild type equine IgG1 Fc subclass showed robust and potent ADCP activity. The Fc mutations investigated showed diminished ADCP activity to varying degrees. The Win and SAP mutations in the Fc showed knockdown of ADCP activity.

[00324] As shown in Figure 31, wild type IgG4 Fc subclass showed robust and potent ADCP activity. The Fc mutations investigated showed diminished ADCP activity to varying degrees. . The Win and SAP mutations in the Fc showed knockdown of ADCP activity.

[00325] As shown in Figure 32, wild type IgG4 Fc subclass showed robust and potent ADCP activity. The Fc mutations investigated showed either no effect or knockout of ADCP activity. The WinSAP mutation in the Fc showed a knockout of ADCP activity.

[00326] As shown in Figure 33, wild type IgG7 Fc subclass showed robust and potent ADCP activity. The Fc mutations investigated showed diminished ADCP activity to varying degrees. The Win and SAP mutations in the Fc showed knockdown of ADCP activity.

[00327] As shown in Figure 34, wild type IgG7 Fc subclass showed robust and potent ADCP activity. The Fc mutations investigated showed diminished ADCP activity. The WinSAP and SQP mutations in the Fc showed knockdown of ADCP activity.

[00328] Example 11—ADCC Assay of Equine IgG Effector Function.

[00329] The ADCC cell-based assay was developed and employed to characterize the effectiveness of the nine CTLA4 equine IgG subclass Fc fusion proteins in mediating ADCC and to investigate Fc region mutations in the subclasses. This will help define key residues in the Fc region that determine ADCC activity of the equine IgG subclasses. The assay utilizes CHO target cells engineered to express canine CD80 which binds to CTLA4 on the Fc fusion proteins. These target cells have been used in past canine ADCC assays and were utilized in the CDC assay due to their dependability.

[00330] **Methods.** CD80-expressing CHO cells (target cells) were plated at 20,000 cells/well in CD CHO media in round-bottomed 96-well plates. Titrated fusion proteins in CD CHO media were added to the target cells and allowed to bind for 60 minutes at 37°C. Single donor equine PBMCs (effector cells), cultured overnight in RPMI 1640 medium + IL-2 and IL-15, were added to the plates for 18-20 hours at 37°C using an effector:target cell ratio (E:F Ratio) of 40-50:1. Cells were then stained fixed and analyzed via flow cytometry quantifying the live/dead stain of the target cells. Data were expressed as “% Dead Target cells” normalized to fusion protein alone (minus effector PBMCs).

[00331] **Results.** As shown in Figure 35 and Table 8, four of the six equine wild type Fc subclasses showed robust and potent ADCC activity. The exceptions were of IgG2 and IgG5 which showed no ADCC activity or greatly diminished ADCC activity, respectively. EC50

values ranged from 16.1 to 108.6 ng/mL. Equine IgG 1, 3, 4, and 7 Fc CTLA4 fusion proteins all showed ADCC activity with varying potency. IgG2 and 5 showed no ADCC activity.

[00332] Table 8. ADCC effects induced by equine IgG Fc wildtype subclass CTLA4 fusion proteins.

CTLA4 Equine IgG Fusion Protein	ADCC EC50 Value
CTLA4 Fc eIgG1	108.6 ng/mL
CTLA4 Fc eIgG2	No ADCC
CTLA4 Fc eIgG3	16.1 ng/mL
CTLA4 Fc eIgG4	53.8 ng/mL
CTLA4 Fc eIgG5	No ADCC
CTLA4 Fc eIgG7	22.5 ng/mL

[00333] As shown in Figures 36 and 37, wild type IgG1 subclass showed robust and potent ADCC activity. The Fc mutations investigated showed differing effects on ADCC activity. The Win, PSS, WinPSS and SQP mutations of the Fc knocked down ADCC effector function activity (Figure 36). The SAP and WinSAP mutations knocked out ADCC effector function activity (Figure 37). The PAP mutation appeared to have little effect on this ADCC effector function activity compared to IgG1 wild type Fc (Figure 37).

[00334] As shown in Figure 38, wild type IgG4 subclass showed robust and potent ADCC activity. The Fc mutations investigated showed differing effects on ADCC activity. The Win, PSS, WinPSS and SQP mutations of the Fc showed no effect on ADCC effector function activity compared to IgG4 wild type Fc. The SAP mutation knocked down ADCC effector function activity and the WinSAP mutation knocked out ADCC effector function activity.

[00335] As shown in Figure 39, wild type IgG7 subclass showed robust and potent ADCC activity. The Fc mutations investigated showed differing effects on ADCC activity. The Win,

SAP and PSS mutations of the Fc knocked down ADCC effector function activity compared to IgG7 wild type Fc. The WinSAP and Win PSS showed greater knockdown of ADCC activity. The SQP mutation knocked out ADCC effector function activity most effectively.

Example 12—In Silico Modeling of Equine IgG Fc Regions

[00336] The Fc regions of the four equine allotypes, IgG1, IgG4a, IgG4b, IgG7a and IgG7b were first designed using their respective CH2 and CH3 regions. The protein modeling feature of Alphafold 2.2 developed by Deepmind was implemented to model the 3D structure of Equine FcRn and each of the wild-type (WT) and mutant constructs of each equine allotype.

[00337] Molecular Operating Environment (MOE) developed by Chemical Computing Group (MOE2019.0102) provides a flexible and automated graphical user interface for protein modeling. To analyze the difference in structures, the sequence-to-profile alignment algorithm uses a scoring algorithm to rank the sequence templates and scores higher than 85% ensure the selection of protein templates with physically realistic structures. The model was then optimized using the same pipeline and the structural stability of the models was verified using Ramachandran Plots, which checks the stereochemical quality of a protein structure.

[00338] The method described above was performed on the Equine wild-type (WT) constructs and the following residues were mutated in IgG1: L234, L235, G237, P329, Q330, P331. The positions for the mutational library are represented in a ball-and-stick form in Fig. 40. The same procedure was also followed to investigate the following residue positions in equine subclasses, IgG4 (IgG4a and IgG4b) and IgG7 (IgG7a and IgG7b): L234, L235, G237, P329, A330, P331. The positions for the mutational library are represented in a ball-and-stick form in Fig. 41 and Fig. 42.

[00339] An RMSD plot was generated to calculate the root mean square deviation of the WT structures, Equine-IgG4a, Equine-IgG4b, Equine-IgG7a and Equine IgG7b relative to each other (Figure 43) . An RMSD value of 2.0Å or lower is considered the standard for considering two structures to be alike. Results indicated that equine IgG4a construct was identical to the equine IgG4b allotype with average RMSD value for the structure being 0.44Å (individual position RMSD in Table 9). Equine IgG7a construct was identical to the equine IgG7b allotype with an average RMSD value of 0.9 Å (individual position RMSD in Table 10).

[00340] **Table 9.** Root Mean Square Deviation (RMSD) comparisons of residues in the protein models of WT Equine IgG4a and IgG4b.

Residue in Equine-IgG4a WT	Residue in Equine-IgG4b WT	RMSD (Å)
L234	L234	1.6
L235	L235	1.5
G237	G237	0.7
P329	P329	0.6
A330	A330	0.5
P331	P331	0.5

[00341] **Table 10.** Root Mean Square Deviation (RMSD) comparisons of residues in the protein models of WT Equine IgG7a and IgG7b.

Residue in Equine-IgG7a WT	Residue in Equine-IgG7b WT	RMSD (Å)
L234	L234	1.6
L235	L235	1.5

G237	G237	0.7
P329	P329	0.9
A330	A330	0.88
P331	P331	0.88

Example 13—CDC Cell-Based Assay of Feline IgG1a, IgG1b, IgG2 and IgG3 Subclasses

[00342] The CDC cell-based assay was developed and employed to characterize the effectiveness of feline IgG subclasses IgG1a, IgG1b, IgG2 and IgG3 Fc mAbs in mediating CDC and to investigate Fc region mutations in the subclasses. This will help identify residues in the Fc region that determine CDC activity of the feline IgG subclasses. The assay utilizes CLBL-1 target cells that express canine CD20 which binds to the anti-canine CD20 test mAbs. These target cells have been used in past canine CDC assays and have been shown to be dependable.

[00343] Incubation of the mAb-bound target cells with complement-preserved serum can result in Fc binding on the mAbs to complement component C1q initiating the complement cascade, ultimately forming membrane attack complexes. The pore-forming complexes mediate cell lysis of the target cells measured by loss of cell viability. If there is no Fc binding to C1q there is no resultant cell lysis/death.

[00344] Briefly, canine CD20-expressing CLBL-1 (target cells) were plated at 40,000 cells/well in complete RPMI 1640 media in round-bottomed 96-well plates. Titrated fusion proteins in complete RPMI 1640 media were added to the target cells and allowed to bind for 60 minutes at 37°C. Feline complement preserved serum (25% in complete RPMI 1640 media) was added to the plates for 60 minutes at 37°C. Cell viability was then measured using CellTiter-Glo and data were expressed as “cell viability % of control” calculated using no fusion protein + complement preserved serum controls.

[00345] Results. As shown in Fig. 44, wild type feline IgG1a Fc subclass showed robust and potent CDC activity. The Fc mutations investigated showed very good to complete knock out CDC activity. All of the mutations of the Fc tested in this figure showed knockout of CDC activity, with the WinKAPA and WinPA mutations showing the greatest knockout.

[00346] As shown in Fig. 45, wild type IgG1b Fc subclass showed robust and potent CDC activity. All of the Fc mutations investigated showed little to no effect on CDC activity. Four of the mutations showed slight knockdown of CDC activity with the M234A, L235A, G237A mutation showing the most knockdown. The S239D, I332E mutation showed slight enhancement of CDC activity. Feline IgG1b WT showed robust concentration-dependent CDC activity. All of the Fc mutations tested showed very little effect on CDC activity. Four of the mutations showed slight knockdown of CDC activity with the M234A, L235A, G237A mutation showing the most knockdown. The S239D, I332E mutation showed slight enhancement of CDC activity.

[00347] As shown in Fig. 46, wild type feline IgG1b Fc subclass showed robust and potent CDC activity. All of the Fc mutations investigated showed knockout of CDC activity to varying degrees. Feline IgG1b WT showed robust concentration-dependent CDC activity. Eight of the Fc mutations tested showed knockout of CDC activity. All five of the mutations tested showed knockdown of CDC activity with the M234A, L235A, G237A, K332A, P331S mutation showing the greatest knockdown of CDC activity.

[00348] As shown in Fig. 47, wild type feline IgG2 Fc subclass Wild Type (WT) and all eight of the Fc mutations investigated showed no CDC activity.

[00349] As shown in Fig. 48, wild type feline IgG3 Fc subclass showed robust and potent CDC activity. All five of the Fc mutations investigated showed little to no effect on CDC activity.

[00350] As shown in Fig. 49, four additional feline IgG3 Fc subclass mutations investigated showed little to no effect on CDC activity and one showed moderate knock down of CDC activity. The D265A_N297G mutation showed moderate knock down of CDC activity.

[00351] **Conclusions.** Among the Fc mutations assayed above, the Fc mutation with the strongest knockout of feline CDC mediated by the feline IgG1a subclass are: WinKAPA and WinKA, which produced greater knockout than WinPA. The Fc mutation that best knocks out feline CDC mediated by the feline IgG1b subclass is M234A_L235A_G237A_K332A_P331S. The Fc mutation that best knocks out feline CDC mediated by the IgG3 subclass is D265A_N297G.

[00352] **Example 14—ADCP Assay of Feline IgG Effector Function**

[00353] The antibody dependent cellular phagocytosis (ADCP) assay was performed as described in Example 2 on feline IgG1a variants.

[00354] **Results.** As shown in Fig. 50, wild type feline IgG1a showed concentration-dependent ADCP activity. The Fc mutations WinKAPA, WinKA, and WinPA showed moderate to strong knock down of ADCP activity compared to the wild type, with the WinPA mutation showing the greatest knockdown.

[00355] **Example 15—CDC Cell-Based Assay of Canine IgG Fc Effector Function**

[00356] The CDC cell-based assay was developed and employed to characterize the effectiveness of canine IgG subclasses IgG2 and IgG3 Fc mAbs in mediating CDC and to investigate Fc region mutations in the subclass. This will help define key residues in the Fc

region that determine CDC activity of the canine IgG subclasses. The assay utilizes CLBL-1 target cells that express canine CD20 which binds to the anti-canine CD20 test mAbs. These target cells have been used in past canine CDC assays and have been shown to be dependable.

[00357] Incubation of the mAb-bound target cells with canine complement-preserved serum can result in Fc binding on the mAbs to complement component C1q initiating the complement cascade, ultimately forming membrane attack complexes. The pore-forming complexes mediate cell lysis of the target cells measured by loss of cell viability. If there is no Fc binding to C1q there is no resultant cell lysis/death.

[00358] Methods. Briefly, canine CD20-expressing CLBL-1 (target cells) were plated at 40,000 cells/well in complete RPMI 1640 media in round-bottomed 96-well plates. Titrated mAbs in complete RPMI 1640 media were added to the target cells and allowed to bind for 60 minutes at 37°C. Canine complement preserved serum (25% in complete RPMI 1640 media) was added to the plates for 60 minutes at 37°C. Cell viability was then measured using CellTiter-Glo and data were expressed as “cell viability % of control” calculated using no fusion protein + complement preserved serum controls.

[00359] Results. As shown in Fig. 51, wild type canine IgG2 Fc subclass showed robust and potent CDC activity. The Win, KAPA, WinKAPA, WinKA, and WinPA mutations of the Fc showed knockout of CDC activity. This differs slightly from the previous canine CDC assay using CD-80 expressing CHO target cells, CTLA4 fusion protein test articles and feline complement preserved serum. In that assay the Win mutation showed only a slight right shift of the response curve and only partial knockdown of CDC activity, whereas here the Win mutation showed an almost complete knockout of CDC activity.

[00360] As shown in Fig. 52, the D270S variation of canine IgG2 did not knockout CDC activity, but D270G did. GSP, GAP, DANG, Win DANG GAP, and DANG GAP mutations all showed knockout of CDC activity. As shown in Fig. 53, of the mutations tested in this set, two showed enhanced CDC activity, four showed little to no effect on CDC activity and one (DLE) showed knockout of CDC activity. The Q345R mutation showed the greatest enhancement of about a half logarithm increase in CDC potency. As shown in Fig. 54, the mutations tested in this set showed varying degrees of knockdown of CDC activity with the triple mutant, D276K_T299E_N324P, showing the greatest knock down in this set.

[00361] As shown in Fig. 55, the mutations of IgG2 tested in this set showed little effect to slight knock down of CDC activity, including the triple mutants. This may be contrasted with Fig. 56, in which the mutations tested in this set all showed knockout of CDC activity, whether a single mutation site, around residues 263, 264, 265 266 or 267, or a combination of mutation sites added to V263W. As shown in Fig. 57, the mutations tested in this set showed knockout of CDC activity whether a single mutation site, around aa 299 or 328, or a combination of mutation sites added to T299E. As shown in Fig. 58, the mutations tested in this set showed knockout of CDC activity whether a single mutation site, or a combination of mutation sites. As shown in Fig. 59, the mutations tested in this set on residue N297 all showed knockdown of CDC activity with the N297A, N297D and N297K mutations showing the greatest knock down. As shown in Fig. 60, of the mutations tested in this set, the Q295G mutation showed little effect on CDC activity. All of the other tested mutations showed enhanced CDC activity with the P268W mutation by far showing the greatest enhancement of about one log unit increase in potency.

[00362] As shown in Fig. 61, of the mutations of IgG2 tested in this set, most of the combination mutations tested that included the V262, V264, P268 sites, showed enhanced CDC

activity. The V262L_Q295N_N324P_Q345R and the V264F_F269A_N324P_Q345R combination mutations showed slight knock down of CDC activity. The P268W_Q345R double mutation by far showed the greatest enhancement of about one log unit.

[00363] As shown in Fig. 62 the mutations of IgG3 tested in this set showed either no effect or great enhancement of CDC activity. As shown in Fig. 63, of the mutations tested in this set, most of the mutations tested showed no effect on CDC activity. The P268W mutation showed great enhancement of CDC activity approaching one log unit increase in potency.

[00364] From these assays, it can be observed that the Fc mutations that best knockout canine CDC mediated by the IgG2 subclass are: Win, WinKAPA, WinKA, Win PA, D270G, GSP, GAP, DLE, and the mutations around positions 238, 263, 265, 266,267, 294, 299 and 328.

[00365] The Fc mutations that best knockout canine CDC mediated by the IgG3 subclass are: N324P, K322A_P331A, S239D_I332E, G236A_S239D_I332E, L234A_L235A_G237A, L234A_L235A_G237A_K322A, and L234A_L235A_G237A_K322A_P331A. The Fc mutations that best enhance canine CDC are: P268W and P268W_Q345R for IgG2 subclass and P268W for IgG3 subclass.

[00366] Example 16—ADCP Assay of Canine IgG Effector Function

[00367] The antibody dependent cellular phagocytosis (ADCP) assay utilizes CHO target cells engineered to express canine CD80 or CLBL-1 target cells that express canine CD20. These cells are stained with a pH-sensitive fluorescent dye which can be imaged as the cells are phagocytosed. Our test articles are either canine CTLA4 IgG2 Fc fusion proteins that bind CD80 or anti-canine CD20 chimeric monoclonal IgG2 antibodies (mAbs). The Fc region of the fusion protein or mAb may then bridge the target cell/test article complex to Fc gamma receptors on DH82 effector cells (a canine macrophage cell line), which have the capacity to phagocytose the

stained target cells. ADCP is measured by fluorescent intensity and/or fluorescent area of the pH-sensitive fluorescent dye within the population of effector DH82 cells in the co-culture, wherein fluorescent cells are indicative of an effector cell that has successfully internalized a fluorescent labeled target cell into the acidic lysosome.

[00368] Methods. Briefly, target cells were stained with pHrodo red dye for 30 minutes at 37°C and were subsequently incubated with our test articles for 60 minutes to mediate test article:target cell binding. The complex was then added, 30,000 cells/well, to pre-plated DH-82 cells, 7,000/well. The plates were maintained for 24 hours at 37°C and were monitored and analyzed by a SX5 Incucyte live cell imaging system. Fluorescent area and/or intensity was quantified using a predefined processing definition in the Incucyte software and expressed as such.

[00369] As shown in Fig. 64, wild type canine IgG2 showed concentration-dependent ADCP activity. The Fc mutations investigated in this set either showed knockdown of ADCP activity or little effect compared to the IgG2 WT. The Win, WinKAPA, WinKA and WinPA mutations in the Fc showed very good knockdown of ADCP activity. The KAPA mutation showed no effect on ADCP activity. As shown in Fig. 65, among the mutations tested in this set, the N297P, N297A, N297D mutations in the Fc showed knockdown of ADCP activity. As shown in Fig. 66, among the mutations tested in this set, the N297F and N297K mutations in the Fc showed knockout of ADCP activity. The N297G mutation showed knockdown of ADCP activity. As shown in Fig. 67, among the mutations tested in this set, the T299I, T299E, T299Q mutations in the Fc showed knockdown of ADCP activity with the T299I showing the least knockdown.

[00370] As shown in Fig. 68, among the mutations of canine IgG2 tested in this set, the T299R mutation in the Fc showed complete knockout of ADCP activity. The N324P mutation robust

enhancement of ADCP activity. The L328K mutation showed no effect on ADCP activity. As shown in Fig. 69, among the mutations tested in this set, the P238L and V264F mutations in the Fc showed knockdown of ADCP activity. The L328P mutation showed no effect on ADCP activity. As shown in Fig. 70, among the mutations tested in this set, the L266P mutation, E269P mutation, and the E294H mutation in the Fc showed knockdown of ADCP activity. As shown in Fig. 71, among the mutations tested in this set, the Q295G and Q295N mutations in the Fc showed knockdown of ADCP activity. The E294N mutation showed no effect on ADCP activity.

[00371] As shown in Fig. 72, among the mutations of canine IgG2 tested in this set, the F296A and F296S mutations in the Fc showed knockout of ADCP activity. The Q295W mutation showed no effect on ADCP activity. As shown in Fig. 73, among the mutations tested in this set, G298M mutation in the Fc showed slight knockdown of ADCP activity. The M234D, G236E, S239L mutation showed knockout of ADCP activity. The L266G, G298L, Y300Q mutation showed no effect on ADCP activity. As shown in Fig. 74, among the mutations tested in this set, the M234E and V262L mutations in the Fc showed knockdown of ADCP activity. The M234E, L325D mutation showed knockout of ADCP activity. As shown in Fig. 75, among the mutations tested in this set, the D267K, T299E mutation in the Fc showed almost complete knockout of ADCP activity. The D267K, N324P mutation and the T299E, N324P mutation showed complete knockout of ADCP activity.

[00372] As shown in Fig. 76, among the mutations of canine IgG2 tested in this set, the V240S, V262L, E294M mutation in the Fc showed knockdown of ADCP activity. The P268W mutation showed no effect on ADCP activity. The V240Q, P268W, G298I mutation showed knockout of ADCP activity. As shown in Fig. 77, among the mutations tested in this set, the D2267K, T299E, N324P and the V263W, T299Q mutations in the Fc showed knockdown of

ADCP activity with the latter showing better knockdown. The V263W, N297A mutation showed no effect on ADCP activity. As shown in Fig. 78, among the mutations tested in this set, the V263H mutation in the Fc showed slight knockdown of ADCP activity. The other mutations showed no effect on ADCP activity. As shown in Fig. 79, among the mutations tested in this set, the D265F mutation in the Fc showed knockdown of ADCP activity. The V263W and V264R mutations showed little to no effect on ADCP activity compared to WT.

[00373] As shown in Fig. 80, among the mutations of canine IgG2 tested in this set, the D265V, L266K, and D267K mutations in the Fc showed moderate knockdown of ADCP activity. As shown in Fig. 81, among the mutations tested in this set, the Win mutation, and the M234D, I332E mutation in the Fc showed very good knockdown of ADCP activity. The M234D, L328F mutation in the Fc showed moderate knockdown of ADCP activity. As shown in Fig. 82, among the mutations tested in this set, the S239L, I332E mutation, the L328F, I332E mutation, and the M234D, S239L, I332E mutation in the Fc showed moderate to very good knockdown of ADCP activity. As shown in Fig. 83, among the mutations tested in this set, the M234D, L328F, I332E mutation and the M234D, S239L, L328F, I332E mutation in the Fc showed knockdown of ADCP activity. The S239L, L328F, I332E mutation showed no effect on ADCP activity. As shown in Fig. 84, among the mutations tested in this set, the L325D, I332E mutation showed moderate knockdown in ADCP activity. The L325D, S239L mutation, and the L235D, S239L, I332E mutation in the Fc showed very good knockdown to almost knockout of ADCP activity. As shown in Fig. 85, among the mutations tested in this set, the L235D, L328F, I332E mutation, the L235D, S239L, L328F, I332E mutation, and the G236W, S239L mutation in the Fc showed slight to moderate knockdown of ADCP activity.

[00374] As shown in Fig. 86, among the mutations of canine IgG2 tested in this set, the G236W, I332E mutation, the G236W, S239L, I332E mutation and the G236W, L328F, I332E mutation in the Fc showed moderate knockdown of ADCP activity. As shown in Fig. 87, among the mutations tested in this set, the G236W, S239L, L328F, I332E mutation, the M234D, S239L mutation, and the G236E, S239L mutation in the Fc showed slight to moderate knockdown of ADCP activity. As shown in Fig. 88, among the mutations tested in this set, the L235D, G236W mutation, and the G236W, L328F mutation in the Fc showed moderate knockdown of ADCP activity. The L235D, G236W, L328F showed slight knockdown of ADCP activity. As shown in Fig. 89, among the mutations tested in this set, the L266G, G298L mutation and the L266G, S330K mutation in the Fc showed little effect on ADCP activity. The G298L, Y300Q mutation showed moderate knockdown of ADCP activity. As shown in Fig. 90, among the mutations tested in this set, the G298L, S330K mutation and the L266G, G298L, S330K mutation in the Fc showed knockdown of ADCP activity. The Y300Q, S330K mutation showed no effect on ADCP activity.

[00375] As shown in Fig. 91, among the mutations of canine IgG2 tested in this set, the L266G, Y300Q, S330K mutation and the L266G, G298L, Y300Q, S330K mutation in the Fc showed knockdown of ADCP activity. The G298L, Y300Q, S330K mutation showed little to no effect on ADCP activity. As shown in Fig. 92, among the mutations tested in this set, the L266V, G298K mutation, the G298K, S330L mutation, the L266V, S330L mutation and the L266V, G298K, S330L in the Fc showed knockdown of ADCP activity with the L266V, G298K, S330L mutation showing the greatest knockdown.

[00376] As shown in Fig. 91, wild type IgG3 showed concentration-dependent ADCP activity. Among the mutations of canine IgG3 tested in this set, the N324P mutation in the Fc showed no

effect on ADCP activity. The L234A, L235A, G237A, the L234A, L235A, G237A, K322A, P331A, the L234A, L235A, G237A, K322A, P331A mutation, the L234A, L235A, G237A, P331A mutation, and the T299R mutations in the Fc all showed knockout of ADCP activity.

[00377] From these assays, it can be observed that the Fc mutations that best knockout canine ADCP mediated by the IgG2 subclass are: Win, WinKAPA, WinKA, Win PA, N297F, N297K, T299E, T299Q, T299R, FF296A, F296S, M234D_G236E_S239L, M234E_L325D, D267K_N324P, T299E_N324P and V240Q_P268W_G298I. The Fc mutations that best knockout canine ADCP mediated by the IgG3 subclass are: L234A_L235A_G237A, L234A_L235A_G237A_K322A_P331A, L234A_L235A_G237A_K322A_P331A, L234A_L235A_G237A_P331A, and T299R. The Fc mutation that best enhanced canine ADCP is N324P for the IgG2 subclass.

[00378] Example 17—ADCC Assay of Canine IgG Effector Function

[00379] The canine Fc γ RIII binding (ADCC) cell-based assay was developed and employed to characterize the effectiveness of canine subclass IgG2 Fc anti-canine CD20 chimera IgGs in mediating ADCC and to investigate Fc region mutations in the subclass. This will help define key residues in the Fc region that determine ADCC activity of the canine IgG2 subclass. A schema for an ADCC assay is illustrated in Fig. 94. The assay utilizes specially engineered Jurkat “effector cells” (Promega) in which express canine FcR γ RIII extracellularly and express a NFAT-Response Element linked to a luciferase reporter intracellularly. The “target cells” were CLBL-1 cells that express canine CD20. Our test articles were anti-canine CD20 chimera constructs of canine IgG2 wild type (WT) Fc and mutations of the canine IgG2 WT Fc.

[00380] Following engagement with the Fc region of our anti-canine CD20 canine IgG2 test articles that are bound to CLBL-1 “target cells”, the ADCC Bioassay Effector Cells expressing

canine FcγRIII will transduce intracellular signals resulting in NFAT-mediated luciferase activity that can be easily quantified using BioGlo™ Luciferase Assay Reagent (Promega) and a luminometer plate reader. The more binding of the Fc of our test articles to the FcγRIII on the “effector cells, the higher the luminescence. If there is no Fc binding to FcγRIII there is no resultant luminescence measured.

[00381] Methods. Briefly, CLBL-1 cells (target cells) were plated at ~6,000 cells/well in RPMI1640 media in 96-well plates. Titrated test canine mAbs in RPMI 1640 media were added to the target cells and allowed to bind for 60 minutes at 37°C. The engineered “effector cells”, were counted and added to the plates for 6 hours at 37°C using an effector:target cell ratio (E:F Ratio) of 10:1. FcγRIII binding was then quantified using the Promega Bio-Glo Luciferase Assay Reagent Luminescent Detection. Data were expressed as “Fold Increase” normalized to controls (minus effector cells).

[00382] Results. As shown in Fig. 95, wild type canine IgG2 showed concentration-dependent and robust canine FcγRIII binding translating to ADCC activity. The Fc mutations investigated either showed complete knockout of canine FcγRIII binding suggesting translating to ADCC activity. Canine IgG2 Fc showed ADCC activity via strong canine FcγRIII binding. All of the mutations tested in this figure showed complete knockout of ADCC activity.

[00383] As shown in Fig. 96, the Fc mutations investigated in this set either showed complete knockout, knock down or enhancement of canine FcγRIII binding suggesting translating to ADCC activity. The WinKAPA, WinKA and WinPA mutations all showed complete knockout of FcγRIII binding. The KAPA and DAE mutations showed knock down FcγRIII binding. The DLE and DE mutations showed enhancement of FcγRIII binding.

[00384] As shown in Fig. 97, the Fc mutations investigated in this set either showed complete knockout, knock down, no effect or slight enhancement of canine FcγRIII binding suggesting translating to ADCC activity. The D270G, D270S and EFT mutations in the Fc showed knockdown of FcγRIII binding. The YWA mutation showed complete knockout of FcγRIII binding. The Q345R mutation showed no effect on FcγRIII binding compared to WT and the AAA mutation showed enhancement of FcγRIII binding.

[00385] As shown in Fig. 98, the Fc mutations investigated in this set showed complete knockout or enhancement of canine FcγRIII binding suggesting translating to ADCC activity. The Win and L266K mutations in the Fc completely knocked out FcγRIII binding and the V240S_V262L_E294M triple mutation showed enhancement of FcγRIII binding.

[00386] **Conclusions.** From these assays, it can be observed that, the Fc mutations that best knockout FcγRIII binding for ADCC mediated by the canine IgG2 subclass are: Win, WinKAPA, WinKA, Win PA, GSP, GAP, YWA, and L266K. From what is reported here, the Fc mutations that best enhance canine FcγRIII binding for ADCC mediated by the canine IgG2 subclass are: DLE, DE, AAA and V240S_V262L_E294M.

We claim:

1. An Fc region of feline IgG1a comprising one or more substitutions at positions 234, 235, 235, 236, 237, 237, 239, 329, 330, 331, 345, 265, 267, 268, 270, 270, 297, 298, 322, 324, 329, 330, 331, 332, 333, and 334 using EU Index numbering as in Kabat.
2. The Fc region of feline IgG1a of claim 1, comprising one or more substitutions selected from among M234A, L235A, L235Y, G236A, G237A, G237W, S239D, P329G, P329S, S330A, P331A, P331S, E345R, D265A, G267E, P268F, D270G, D270S, N297G, S298A, K322A, N324T, P329G, S330A, S330L, P331A, P331S, I332E, E333A, and R334A using EU Index numbering as in Kabat.
3. The Fc region of feline IgG1a of claim 1, comprising one or more of the following combinations of substitutions: (M234A, L235A, G237A), (P329G), (P331S), (P329G, S330A), (D265A, N297G), (K322A, P331A), (M234A, L235A, G237A, P329G, S330A), (D265A, N297G, P329G, S330A), (M234A, L235A, G237A, D265A, N297G), (M234A, L235A, G237A, D265A, N297G, P329G, S330A), (D265A, N297G, P331S), (M234A, L235A, G237A, P331A), (M234A, L235A, G237A, D265A, N297G, P331S), (M234A, L235A, G237A, P331S), WIN_GSA (M234A, L235A, G237A, P329G, P331A), (M234A, L235A, G237A, P329G), WIN_GSS (M234A, L235A, G237A, P329G, P331S), (M234A, L235A, G237A, P329S, S330A, P331S), (S298A, E333A, R334A), (S239D, S330L, I332E), DE (S239D, I332E), (G236A, S239D, I332E), (L235Y, G237W, S298A), (G267E, P268F, N324T), (D270G), (D270S), and (E345R) using EU Index numbering as in Kabat.

4. An Fc region of feline IgG1b comprising one or more substitutions or combinations of substitutions in positions corresponding to the positions in feline IgG1a in any of claims 1-3.
5. An Fc region of feline IgG3 comprising one or more substitutions or combinations of substitutions in positions corresponding to the positions in feline IgG1a in any of claims 1-3.
6. An Fc region of feline IgG3 comprising substitutions in positions D265A and N297G.
7. An Fc region of canine IgG2 comprising one or more mutations at locations selected from among 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 294, 295, 296, 297, 298, 299, 300, 301, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 422 423, and 441, using EU Index numbering as in Kabat.
8. An Fc region of canine IgG2 of claim 7 comprising one or more substitutions selected from among A231C, A231D, A231E, A231G, A231H, A231K, A231N, A231Q, A231R, A231S, A231T, A231V, A231Y, P232A, P232C, P232D, P232E, P232F, P232G, P232H, P232I, P232K, P232L, P232M, P232N, P232Q, P232R, P232S, P232T, P232V, P232W, P232Y, E233A, E233C, E233D, E233F, E233G, E233H, E233I, E233K, E233L, E233M, E233N, E233P, E233Q, E233R, E233S, E233T, E233V, E233W, E233Y, M234A, M234C, M234D, M234E, M234F, M234G, M234H, M234I, M234K, M234L, M234N, M234P, M234Q, M234R, M234S, M234T, M234W, M234Y, L235A, L235C, L235D, L235E, L235F, L235G, L235H, L235I, L235K, L235M, L235N, L235P, L235Q, L235R, L235S, L235T, L235V, L235W, L235Y, G236A, G236C, G236D, G236E, G236F, G236H, G236I, G236K, G236L, G236M, G236N,

G236P, G236Q, G236R, G236S, G236T, G236V, G236W, G236Y, G237A,
G237C, G237D, G237E, G237F, G237H, G237I, G237K, G237L, G237M,
G237N, G237P, G237Q, G237R, G237S, G237T, G237V, G237W, G237Y,
P238A, P238C, P238D, P238E, P238F, P238G, P238H, P238I, P238K,
P238L, P238M, P238N, P238Q, P238R, P238S, P238T, P238V, P238W,
P238Y, S239A, S239C, S239D, S239E, S239F, S239G, S239H, S239I,
S239K, S239L, S239M, S239N, S239P, S239Q, S239R, S239T, S239V,
S239W, S239Y, V240A, V240C, V240D, V240E, V240F, V240G, V240H,
V240I, V240K, V240L, V240M, V240N, V240P, V240Q, V240R, V240S,
V240T, V240W, V240Y, V262A, V262C, V262D, V262E, V262F, V262G,
V262H, V262K, V262L, V262M, V262Q, V262R, V262S, V262T, V262W,
V262Y, V263A, V263C, V263E, V263F, V263G, V263H, V263I, V263K,
V263L, V263M, V263N, V263P, V263Q, V263S, V263T, V263W, V263Y,
V264A, V264C, V264D, V264E, V264F, V264G, V264H, V264I, V264K,
V264L, V264M, V264N, V264P, V264Q, V264R, V264S, V264T, V264W,
V264Y, D265A, D265C, D265E, D265F, D265G, D265H, D265I, D265K,
D265L, D265M, D265N, D265P, D265Q, D265R, D265S, D265T, D265V,
D265W, D265Y, L266A, L266C, L266D, L266E, L266F, L266G, L266H,
L266I, L266K, L266M, L266N, L266P, L266Q, L266R, L266S, L266T,
L266V, L266W, L266Y, D267A, D267C, D267E, D267F, D267G, D267H,
D267I, D267K, D267L, D267M, D267N, D267P, D267Q, D267R, D267S,
D267T, D267V, D267W, D267Y, P268A, P268C, P268D, P268E, P268F,
P268G, P268H, P268I, P268K, P268L, P268M, P268N, P268Q, P268R,
P268S, P268T, P268V, P268W, P268Y, E269A, E269C, E269D, E269F,

E269G, E269H, E269I, E269K, E269L, E269M, E269N, E269P, E269Q,
E269R, E269S, E269T, E269V, E269W, E269Y, D270A, D270C, D270F,
D270G, D270H, D270I, D270K, D270L, D270M, D270N, D270P, D270Q,
D270R, D270S, D270V, D270W, D270Y, P271C, P271F, P271G, P271H,
P271I, P271K, P271L, P271M, P271N, P271Q, P271R, P271S, P271T,
P271Y, E294A, E294C, E294D, E294F, E294G, E294H, E294I, E294K,
E294L, E294M, E294N, E294P, E294Q, E294R, E294S, E294T, E294V,
E294W, E294Y, Q295A, Q295D, Q295E, Q295F, Q295G, Q295K, Q295L,
Q295N, Q295P, Q295R, Q295S, Q295T, Q295V, Q295W, Q295Y, F296A,
F296C, F296D, F296E, F296G, F296H, F296I, F296K, F296L, F296M,
F296N, F296P, F296Q, F296R, F296S, F296T, F296V, N297A, N297C,
N297D, N297E, N297F, N297G, N297H, N297I, N297K, N297L, N297M,
N297P, N297Q, N297R, N297S, N297T, N297V, N297W, N297Y, G298A,
G298C, G298D, G298E, G298F, G298H, G298I, G298K, G298L, G298M,
G298N, G298P, G298Q, G298R, G298S, G298T, G298V, G298W, G298Y,
T299A, T299C, T299D, T299E, T299F, T299G, T299H, T299I, T299K,
T299L, T299M, T299N, T299P, T299Q, T299R, T299S, T299V, T299W,
T299Y, Y300A, Y300C, Y300D, Y300E, Y300H, Y300I, Y300K, Y300L,
Y300M, Y300N, Y300P, Y300Q, Y300R, Y300S, Y300T, Y300V, Y300W,
R301A, R301C, R301D, R301E, R301F, R301G, R301H, R301I, R301L,
R301P, R301Q, R301V, R301W, R301Y, V323A, V323C, V323D, V323E,
V323F, V323G, V323H, V323K, V323L, V323M, V323N, V323P, V323Q,
V323R, V323S, V323T, V323W, V323Y, N324A, N324C, N324D, N324E,
N324F, N324G, N324H, N324I, N324K, N324L, N324M, N324P, N324Q,

N324R, N324S, N324T, N324V, N324W, N324Y, N325A, N325C, N325D,
N325E, N325F, N325G, N325H, N325I, N325K, N325L, N325M, N325P,
N325Q, N325R, N325S, N325T, N325V, N325W, N325Y, K326A, K326D,
K326E, K326F, K326I, K326L, K326N, K326R, K326S, K326T, K326V,
K326W, A327C, A327D, A327E, A327F, A327G, A327H, A327I, A327K,
A327L, A327M, A327N, A327P, A327Q, A327R, A327S, A327T, A327V,
A327W, A327Y, L328A, L328C, L328D, L328E, L328F, L328G, L328H,
L328I, L328K, L328M, L328N, L328P, L328Q, L328R, L328S, L328T,
L328V, L328W, L328Y, K322A, K322C, K322D, K322E, K322F, K322G,
K322H, K322I, K322L, K322M, K322N, K322P, K322Q, K322R, K322S,
K322T, K322V, K322W, K322Y, P329A, P329C, P329D, P329E, P329F,
P329G, P329H, P329I, P329K, P329L, P329M, P329N, P329Q, P329R,
P329S, P329T, P329V, P329W, P329Y, S330A, S330C, S330D, S330E,
S330F, S330G, S330H, S330I, S330K, S330L, S330M, S330N, S330P,
S330Q, S330R, S330T, S330V, S330W, S330Y, P331A, I332A, I332D,
I332E, I332F, I332G, I332H, I332K, I332L, I332M, I332N, I332P,
I332R, I332T, I332V, I332W, I332Y, E333A, E333D, E333H, E333I,
E333K, E333N, E333R, E333S, E333V, E333Y, R334A, R334C, R334D,
R334E, R334F, R334G, R334L, R334M, R334N, R334P, R334Q, R334S,
R334T, R334V, R334W, R334Y, T335C, T335H, T335I, T335K, T335L,
T335P, I336D, I336E, I336G, I336K, I336N, I336Y, S337D, S337P,
K338A, K338C, K338D, K338E, K338F, K338G, K338H, K338I, K338L,
K338M, K338N, K338P, K338Q, K338R, K338S, K338V, K338W, K338Y,
A339D, A339F, A339G, A339N, A339P, A339R, A339S, A339T, A339V,

A339W, A339Y, R340G, T422K, T422R, F423P, and L441P using EU Index numbering as in Kabat.

9. An Fc region of canine IgG2 of claim 7 comprising one or more substitutions or combinations of substitutions selected from among (P329G), (P329G, S330A), (D265A, N297G), (D265A, N297G; P329G, S330A), (M234A, L235A, G237A), (M234A, L235A, G237A; D265A, N297G; P329G, S330A), (G298A, E333A, R334A), (S239D, I332E), (S239D, S330L, I332E), (G236A, S239D, I332E), (L235Y, G237W, G298A), (D267E, P268F, N324T), (Q345R), (D270G), and (D270S), using EU Index numbering as in Kabat.

10. An Fc region of canine IgG3 comprising one or more substitutions or combinations of substitutions in positions corresponding to the positions in feline IgG2 in any of claims 7-9, using EU Index numbering as in Kabat.

11. An Fc region of canine IgG3 comprising one or more substitutions or combinations of substitutions in positions selected from among N324P, (K322A, P331A), (S239D, I332E), (G236A, S239D, I332E), (L234A, L235A, G237A), (L234A, L235A, G237A, K322A), (L234A, L235A, G237A, K322A, P331A), (L234A, L235A, G237A, P331A), and T299R, using EU Index numbering as in Kabat.

12. An Fc region of equine IgG1 comprising mutations or substitutions at one or more positions selected from among 229, 234, 235, 237, 329, 330, and 331, using EU Index numbering as in Kabat.

13. An Fc region of equine IgG1 of claim 12 comprising one or more substitutions selected from among P229S, L234A, L235A, G237A, P329S, Q330A, Q330S, and P331S, using EU Index numbering as in Kabat.
14. An Fc region of equine IgG1 of claim 12 comprising one or more combinations of substitutions in a Fc region selected from among (P329S, Q330A), (Q330A), (P229S), (L234A, L235A, G237A), (Q330S, P331S), (L234A, L235A, G237A; P329S, Q330A), and (L234A, L235A, G237A, Q330S, P331S), using EU Index numbering as in Kabat.
15. An Fc region of equine IgG4 comprising one or more mutations and/or substitutions at positions selected from among 234, 235, 237, 239, 330, and 331, using EU Index numbering as in Kabat.
16. An Fc region of equine IgG4 of claim 15, comprising one or more substitutions selected from among L234A, Q235A, G237A, P239S, A330Q, A330S, P331S, using EU Index numbering as in Kabat.
17. An Fc region of equine IgG4 of claim 15, comprising one or more substitutions or combinations of substitutions in a Fc region of equine IgG4 selected from among (A330S, P331S), (P239S, A330Q), (L234A, Q235A, G237A), (L234A, Q235A, G237A; A330S, P331S), (P239S), and (L234A, Q235A, G237A; P239S), using EU Index numbering as in Kabat.
18. An Fc region of equine IgG7 comprising one or more mutations and/or substitutions at positions selected from among 234, 235, 236, 237, 239, 330, and 331, using EU Index numbering as in Kabat.

19. An Fc region of equine IgG7 of claim 18, comprising one or more substitutions selected from among L234A, S235A, V236G, G237A, P239S, P329S, A330S, A330Q, P331S, using EU Index numbering as in Kabat.
20. An Fc region of equine IgG7 of claim 18, comprising substitutions or combinations of substitutions in a Fc region of equine IgG7 selected from among (A330S, P331S), (P329S), (P239S, A330Q), (L234A, S235A, V236G, G237A), (L234A, S235A, V236G, G237A; A330S, P331S), and (L234A, S235A, V236G, G237A; P329S).
21. A recombinant polypeptide comprising a modified recombinant feline, canine, or equine IgG Fc region of any of claims 1-20.
22. A recombinant antibody or a molecule comprising a feline, canine, or equine IgG Fc region of any of claims 1-20.
23. A method for producing or manufacturing an antibody or a molecule, the method comprising: providing a vector or a host cell having a nucleic acid sequence that encodes an antibody or molecule of Claim 22.