VARIANT FC-POLYPEPTIDES WITH ENHANCED BINDING TO THE NEONATAL FC RECEPTOR

PRIORITY

This application claims the benefit of US Provisional Application Nos. 61/578,780, 61/585,993, and 61/729,050 filed December 21, 2011, January 12, 2012, and November 21, 2012, respectively, each of which are incorporated herein in their entirety.

FIELD

The invention relates to polypeptides comprising variant Fc-fragments that bind to the neonatal Fc receptor with higher affinity and/or greater binding activity compared to a control Fc-fragment. The invention further relates to methods of isolating, making, and using such polypeptides.

BACKGROUND

Therapeutic monoclonal antibodies have been used successfully as treatments for a variety of diseases. The relatively long *in vivo* half life of antibodies is mediated at least in part by the interaction of the Fc region of the antibody with the neonatal Fc receptor (FcRn). *See, e.g.,* Ghetie et al. (1996), Eur. J. Immunol. 26: 690-96. FcRn binds to the Fc region of IgG antibodies with nanomolar affinity (K_D ≈ 100 nM) at a pH of less than or equal to 6.0, but does not bind at the pH of blood, *i.e.,* about pH 7.4. Tesar and Björkman (2001), Curr. Opin. Struct. Biol. 20(2): 226-233. Upon internalization of an antibody by a cell, for example by pinocytosis, an IgG can be bound by FcRn in the acidic environment of an endosome. *Id.* When bound by FcRn within the endosome, the IgG will be directed back to the cell surface, as opposed to entering a default catabolic pathway within the endosome. *Id.* In the generally physiologic pH environment at the cell surface, the IgG can dissociate from FcRn and re-enter the circulation. This process allows the antibody to return to the circulation following internalization within a cell, as opposed to being degraded in the cell within an endosome.

There is a need in the art for therapeutic antibodies with increased *in vivo* half lives so as to decrease dosing amounts and/or frequencies. Such antibodies are advantageous because of increased patient convenience, and therefore also possibly increased patient compliance, and/or decreased cost. In the current cost-conscious health care environment, cost can be a determining factor in the practical utility of a therapeutic product.

SUMMARY

Provided are variant Fc-fragments that bind to FcRn with higher affinity and/or higher binding activity than does a control Fc-fragment at a slightly acidic pH and that bind to FcRn with about the same affinity as or lower than a control Fc-fragment, that is, little or no binding activity, at a physiologic pH. Also provided are variant Fc-polypeptides, which contain a variant Fc-fragment as well as a binding region that binds to a target molecule. It is demonstrated herein that variant Fc-polypeptides

containing variant Fc-fragments with the binding properties mentioned above also have longer *in vivo* half lives than control Fc-polypeptides. Further provided are nucleic acids that encode these Fc-fragments and Fc-polypeptides and methods of making these proteins using these nucleic acids. Also included are methods for extending the *in vivo* half life of an Fc-polypeptide and methods for identifying variant Fc-fragments that bind to FcRn with higher affinity at pH 5-6 and bind FcRn with comparable or lower affinity at physiologic pH as compared to a control Fc-fragment.

Described here is a variant Fc-polypeptide comprising a human IgG1, IgG2, IgG3, or IgG4 variant Fc-fragment, wherein the variant Fc-fragment comprises an insertion of 3 to 20, 10 to 20, 20 to 40, 40 to 60, or 60 to 80 amino acids within or adjacent to Loop 5, 8, and/or 10 of the variant Fc-fragment, wherein the variant Fc-polypeptide binds to a human neonatal Fc receptor (hFcRn) with higher affinity and/or higher binding activity at a pH from about 5.0 to about 6.0 and/or at a pH of about 5.0, 5.2, 5.5, 5.7, or 6.0 than a control Fc-polypeptide that has the same amino acid sequence as the variant Fc-polypeptide except that it does not contain the insertion within or adjacent to Loop 5, 8, and/or 10 and wherein the variant Fc-polypeptide binds to the human neonatal Fc receptor (hFcRn) with approximately the same or lower affinity or binding activity as compared to the control Fc-polypeptide, that is, little or no binding activity, at a physiologic pH and/or at a pH of about 7.4, 7.5, or 7.6. The insertion within or adjacent to the variant Fc-fragment can be at least six amino acids long, not more than 25 amino acids long, from 6 to 16 amino acids long, and/or at least 12 amino acids long. In some embodiments, the insertion does not contain methionine and/or tryptophan residues. The insertion within or adjacent to the variant Fc-fragment can comprise at least one cysteine among the first four inserted amino acids and at least one cysteine among the last four inserted amino acids. Optionally, the insertion can lack cysteine residues other than any cysteine residues occurring in the first or last four amino acids of the insertion. In some embodiments, the insertion is within or adjacent to Loop 10 of the variant Fc-fragment, optionally between amino acids 384 and 385 of the variant Fc-fragment using the EU numbering system shown in Table 1. The insertion can be within amino acids 383 to 387 using the EU numbering system. In some embodiments, the insertion in the variant human Fc-fragment can be between amino acids 382 and 383, 383 and 384, 385 and 386, 386 and 387, 387 and 388, 388 and 389, 389 and 390, or 390 and 391 using the EU numbering system shown in Table 1. Alternatively, amino acids 384-386 can be deleted, and the insertion can be between amino acids 383 and 387 using the EU numbering system shown in Table 1. The insertion in the variant Fc-fragment can comprise the amino acid sequence of any one of SEQ ID NO:13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 90-356, and 359-379. Further, the insertion in the variant Fc-fragment can comprise the amino acid sequence of any one of the following: SEQ ID NOs:41-67; SEQ ID NOs:90-246; SEQ ID NOs:247-

356; SEQ ID NOs:367, 369, 372, 373, and 375-379; amino acids 4-9 of any one of SEQ ID NOs:41-53, 67, 90-162, 90-163, 165-215, 164-214, 217-247, 216-246, 359-379 and 392-399, 401-409, 411-426, 428-439, 441-447, 449-453, 456, 459, 461, 464, and 470, 475, 477-489, 491-496; amino acids 4-10 of SEQ ID NO:164, 252, and 490, and amino acids 4-8 of SEQ ID NO:215 or 216; amino acids 4-11 of any one of SEQ ID NOs: 248-288, 290-306, 342, 400, 410, 427, 440, 448, 454, 455, 457, 458, 460, 465-469, 471-474, and 476; amino acids 4-12 of SEQ ID NO:289 or 307; and amino acids 4-13 of any one of SEQ ID NOs:308-341 and 343-356.

Further described herein is a variant Fc-polypeptide comprising a human IgG1, IgG2, IgG3 or IgG4 variant Fc-fragment, wherein the variant Fc-fragment comprises an insertion of 3 to 20, 10 to 20, 20 to 40, 40 to 60, or 60 to 80 amino acids within or adjacent to Loop 10, wherein the variant Fc-polypeptide binds to a hFcRn with higher affinity and/or higher binding activity at a pH in a range from about 5.0 to about 6.0 and/or at a pH of about 5.0, 5.2, 5.5, 5.7, or 6.0 than a control Fc-polypeptide that is the same as the variant Fc-polypeptide except that it does not contain the insertion within or adjacent to Loop 10 and wherein the variant Fc-polypeptide binds to the hFcRn with approximately the same or lower affinity as compared to the control Fc-polypeptide, that is, with little or no binding activity, at a physiologic pH and/or at a pH of about 7.4, 7.5, or 7.6. The insertion can be at least six amino acids long, can be not more than 25 amino acids long, can be from 6 to 16 amino acids long, can be at least 12 amino acids long, and/or can contain at least one cysteine among the first four amino acids of the insertion and at least one cysteine among the last four amino acids of the insertion. The insertion can lack cysteine residues at other positions within the insertion. The insertion can lack methionine and/or tryptophan residues. The insertion in the variant Fc-fragment can be within amino acids 383-387 according to the EU numbering system. The insertion in the variant Fc-fragment can be between amino acids 382 and 383, 383 and 384, 384 and 385, 385 and 386, 386 and 387, 387 and 388, 388 and 389, 389 and 390, or 390 and 391 using the EU numbering system shown in Table 1. Alternatively amino acids 384-386 can be deleted, and the insertion can occur between amino acids 383 and 387, using the EU numbering system. The insertion in the variant Fc-fragment can comprise the amino acid sequence of any one of SEQ ID NO:13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 90-356, 359-379, and 392-496. Further, the insertion in the variant Fc-fragment can comprise the amino acid sequence of any one of the following: SEQ ID NOs:41-67; SEQ ID NOs:90-246; SEQ ID NOs:247-356; SEQ ID NOs:367, 369, 372, 373, and 375-379; amino acids 4-9 of any one of SEQ ID NOs:41-53, 67, 90-162, 90-163, 165-215, 164-214, 217-247, 216-246, 359-379 and 392-399, 401-409, 411-426, 428-439, 441-447, 449-453, 456, 459, 461, 464, and 470, 475, 477-489, 491-496; amino acids 4-10 of SEQ ID NO:164, 252, and 490, and amino acids 4-8 of SEQ ID NO:215 or 216; amino acids 4-11 of any one of SEQ ID NOs:

248-288, 290-306, 342, 400, 410, 427, 440, 448, 454, 455, 457, 458, 460, 465-469, 471-474, and 476; amino acids 4-12 of SEQ ID NO:289 or 307; and amino acids 4-13 of any one of SEQ ID NOs:308-341 and 343-356. The variant Fc-polypeptide can be a variant Fc fusion protein comprising a non-antibody polypeptide. In particular embodiments, a control Fc fusion protein for the variant Fc fusion protein can be alefacept, rilonacept, aflibercept, etanercept, romiplostim, or abatacept. In other embodiments, the variant Fc-polypeptide can comprise a heavy chain variable region (V_H) and/or a light chain variable region (V_L) of any antibody and may also comprise a first heavy chain constant region (C_H1) and a light chain constant region (C_L). In some embodiments, the variant Fc-polypeptide can comprise (a) a heavy chain comprising a V_H region, a first heavy chain constant region (C_H1), a hinge region, a C_H2 region, and a C_H3 region, and (b) a light chain comprising a V_L region and a light chain constant region (C_L). The variant Fc-polypeptide can be monovalent. The variant Fc-polypeptide can be a dimer or can be a tetramer.

In further embodiments, described herein are nucleic acids encoding the variant Fc-polypeptides or the variant Fc-fragments described herein, as well as the insertions within or adjacent to the loops of the variant Fc-fragments. Also described are host cells containing such nucleic acids. Also contemplated are methods of making a variant Fc-polypeptide or variant Fc-fragment comprising (a) introducing a nucleic acid encoding the variant Fc-polypeptide or Fc-fragment into a host cell, (b) culturing the host cell comprising the nucleic acid under conditions such that the nucleic acid is expressed, and (c) recovering the expressed variant Fc-polypeptide or Fc-fragment from the culture medium or the cell mass, wherein the variant Fc-fragment, or the variant Fc-polypeptide that contains a variant Fc-fragment, comprises an insertion of 3 to 20, 10 to 20, 20 to 40, 40 to 60, or 60 to 80 amino acids within or adjacent to Loop 5, 8, and/or 10 of the variant Fc-fragment, and wherein the variant Fc-polypeptide or Fc-fragment binds to a hFcRn with higher affinity and or higher binding activity at a pH in a range from about 5.0 to about 6.0 and/or at a pH of about 5.0, 5.2, 5.5, 5.7, or 6.0 than a control Fc-polypeptide or Fc-fragment and wherein the variant Fc-polypeptide or Fc-fragment binds to the human neonatal Fc receptor (hFcRn) with approximately the same or lower affinity or binding activity as compared to the control Fc-polypeptide or control Fc-fragment, that is, with little or no binding activity, at a physiologic pH and/or at a pH of about 7.4, 7.5, or 7.6. .

Also described are methods for making any of the variant Fc-fragments or Fc-polypeptides described herein comprising (a) introducing a nucleic acid encoding the variant Fc-polypeptide or Fc-fragment into a host cell, (b) culturing the host cell comprising the nucleic acid under conditions such that the nucleic acid is expressed, and (c) recovering the expressed variant Fc-polypeptide or Fc-fragment from the culture medium or the cell mass.

In a further embodiment, described herein is a method for extending the half life of an Fc-polypeptide comprising a human IgG Fc-fragment comprising the following steps: selecting a site within or adjacent to Loop 5, 8, and/or 10 for insertion; and inserting a peptide into the selected site, wherein the peptide comprises an amino acid sequence selected from the group consisting of: SEQ ID NOs:41-67; SEQ ID NOs:90-246; SEQ ID NOs:247-356; SEQ ID NOs:367, 369, 372, 373, and 375-379; amino acids 4-9 of any one of SEQ ID NOs:41-53, 67, 90-162, 90-163, 165-215, 164-214, 217-247, 216-246, 359-379 and 392-399, 401-409, 411-426, 428-439, 441-447, 449-453, 456, 459, 461, 464, and 470, 475, 477-489, 491-496; amino acids 4-10 of SEQ ID NO:164, 252, and 490, and amino acids 4-8 of SEQ ID NO:215 or 216; amino acids 4-11 of any one of SEQ ID NOs: 248-288, 290-306, 342, 400, 410, 427, 440, 448, 454, 455, 457, 458, 460, 465-469, 471-474, and 476; amino acids 4-12 of SEQ ID NO:289 or 307; and amino acids 4-13 of any one of SEQ ID NOs:308-341 and 343-356. The selected site can be within or adjacent to Loop 10, and the insertion site can be between amino acids 384 and 385, numbered according to the EU numbering system. The insertion site can be within amino acids 383 to 387 using the EU numbering system. The insertion in the variant Fc-fragment can be between amino acids 382 and 383, 383 and 384, 384 and 385, 385 and 386, 386 and 387, 387 and 388, 388 and 389, 389 and 390, or 390 and 391 using the EU numbering system shown in Table 1. Alternatively amino acids 384-386 can be deleted, and the insertion can occur between amino acids 383 and 387, using the EU numbering system.

Further provided herein is a method for identifying a human IgG variant Fc-fragment that confers a longer *in vivo* half life on a variant Fc-polypeptide that comprises the variant Fc-fragment, as compared to a control Fc-polypeptide, comprising the following steps: (a) creating a library of nucleic acids encoding Fc-fragments containing an insertion comprising 4-20, 10 to 20, 20-40, 40-60, or 60-80 randomized amino acids within or adjacent to Loop 10; (b) screening Fc-fragments encoded by the library to identify the variant Fc-fragments that (i) bind to human FcRn with higher affinity and/or higher binding activity at pH 5.5, and/or at a pH from about 5-6, than a control Fc-fragment and (ii) bind to human FcRn at the same or lower affinity or binding activity compared to the control Fc-fragment, that is, with little or no binding activity, at physiologic pH and/or at a pH of 7.4, 7.5, or 7.6; (c) constructing a nucleic acid encoding a variant Fc-polypeptide comprising a variant Fc-fragment identified in (b), wherein the concentration of a control Fc-polypeptide, which comprises a control Fc-fragment rather than the variant Fc-fragment, is known to decrease linearly over time when administered to an animal *in vivo*; (d) introducing the nucleic acid of (c) into a host cell and culturing the host cell under conditions such that the variant Fc-polypeptide encoded by the nucleic acid can be expressed; (e) recovering the variant Fc-polypeptide from the cell mass or cell culture medium; (f) administering the variant Fc-polypeptide to an animal and administering the control Fc-polypeptide to another animal; and (g)

monitoring the concentrations of the variant and control Fc-polypeptides in peripheral blood over time subsequent to administration, thereby identifying a variant Fc-fragment that confers a longer *in vivo* half life on a variant Fc-polypeptide. The insertion of step (a) can be between positions 384 and 385 using the EU numbering system as illustrated in Table 1. The insertion site can be within amino acids 383 to 387 using the EU numbering system. The insertion in the variant Fc-fragment can be between amino acids 382 and 383, 383 and 384, 384 and 385, 385 and 386, 386 and 387, 387 and 388, 388 and 389, 389 and 390, or 390 and 391 using the EU numbering system shown in Table 1. Alternatively amino acids 384-386 can be deleted, and the insertion can occur between amino acids 383 and 387, using the EU numbering system.

In another aspect, a method is provided for treating a chronic disease comprising administering to a patient in need thereof a variant Fc-polypeptide as described herein.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Ribbon diagram of the predicted three dimensional structure of portions of human FcRn (hFcRn) and a human IgG1 Fc-fragment that come in closest contact upon binding of FcRn to the Fc-fragment. At top is a ribbon diagram of a portion of the tertiary structure of hFcRn. Below is a ribbon diagram of a portion of a human IgG1 Fc-fragment that comes in closest contact with FcRn when FcRn is bound to it. Loops are shown as strings, whereas alpha helices and beta sheets are shown as ribbons. The six sites at which insertions were made are indicated by the names of the eight libraries that were constructed as described below, *i.e.,* L1, L2A, L2B, L3, L4, L5, L6A, and L6B.

Figure 2: Format of the insertion libraries. Above is shown the sequence of a human IgG1 Fc-fragment (SEQ ID NO:1). Amino acids within loops in which, or adjacent to which, insertions were made are indicated by underlining and boldface type. Library names, *i.e.,* L1, L2A, etc., appear above the region into which insertions were made for that particular library. The format of the insertions is indicated below. At far left are the library names, *i.e.,* L1, L2A, etc. Just to the right of the library names is the original sequence around the insertion site prior to the insertion of any amino acids. Italic letters indicate amino acids that are deleted prior to insertion. A vertical dashed line indicates the insertion site in those libraries in which no amino acids were deleted. At far right is the sequence around the insertion site with the insertion in place. The designations "$(19R)_5$" or "$(19R)_6$" means five or six, respectively, randomized amino acids, which can be any of the 19 amino acids other than cysteine. The designation "$(20R)_8$" means eight randomized amino acids, which can be any of the twenty amino acids.

Figure 3: Association and dissociation curves at pH 6 and pH 7.4, respectively, of variant Fc-fragments from library L5. The curves at left show the response detected using the ForteBio Octet®

system as explained in Example 4 where the portions of the curves to the left of the central vertical line show the relative amounts of association of the various Fc-fragments to FcRn at pH 6 and the portions of the curves to the right of the central vertical line show the dissociation of the Fc-fragments from FcRn at pH 7.4. The table at right provides the maximal binding response detected at pH 6 for each variant and for a wild type Fc-fragment (FcWT).

Figure 4: Average concentrations of antibodies containing variant and control Fc-fragments as a function of time post-injection in cynomolgus monkeys. The x axis indicates time in hours post-injection, and the y axis indicates the concentration of the antibody in nanograms per milliliter (ng/mL) in peripheral blood of cynomolgus monkeys that have been injected with an antibody.

Figure 5: Sequences of variants of Loop 10. The amino acid sequences of wild type or variant versions of a loop in a human IgG1 Fc-fragment, plus three adjacent amino acids on either side, are shown. The amino acid sequence shown is from amino acid residues 166-178, in the numbering scheme of Figure 2, or amino acids 381-393 according to EU numbering (SEQ ID NO:563) as shown in this figure and in Table 1. The unaltered, wild type loop region sequence is designated "wild type sequence." The sequence of this loop region in Fc variant 5-1 (encoded by an isolate from library L5) is designated "5-1" (SEQ ID NO:564). Inserted amino acid sequences are shown in **boldface with underlining**. The sequence of this same loop region from various variants of 5-1, which have the same peptide inserted in different locations within or adjacent to this loop, in some cases with some loop amino acids deleted, are shown below.

Figure 6: Association and dissociation curves at pH 6 and pH 7.4, respectively, of variant Fc-fragments having the same insertion at different positions of Loop 10. At left are shown the association curves at pH 6 (to the left of the central vertical line) and dissociation curves at pH 7.4 (to the right of the central vertical line) of the various variant Fc-fragments and a wild type Fc-fragment (FcWT). At right the maximal binding response detected for each variant at pH 6 is shown in tabular form.

Figure 7: Association and dissociation curves at pH 6 and pH 7.4 of variant Fc-fragments from library L5 screened in yeast. At left are shown the association curves at pH 6 (to the left of the central vertical line) and dissociation curves at pH 7.4 (to the right of the central vertical line) of the various variant Fc-fragments and a wild type Fc-fragment (FcWT). At right the maximal binding response detected for each variant at pH 6 is shown in tabular form.

Figure 8: Association and dissociation curves at pH 6 and pH 7.4 of variant Fc-fragments from library L-8 and L-10 screened in yeast. At left are shown the association curves at pH 6 (to the left of the central vertical line) and dissociation curves at pH 7.4 (to the right of the central vertical line) of the various variant Fc-fragments and a wild type Fc-fragment (FcWT). At right the maximal binding response detected for each variant at pH 6 is shown in tabular form.

Figure 9: Average concentrations of antibodies containing variant and control Fc-fragments as a function of time post-injection in cynomolgus monkeys. The x axis indicates time in hours post-injection, and the y axis indicates the concentration of the antibody in nanograms per milliliter (ng/mL) in peripheral blood of cynomolgus monkeys that have been injected with an antibody.

Figure 10: Average concentrations of antibodies containing variant and control Fc-fragments as a function of time post-injection in cynomolgus monkeys. The x axis indicates time in hours post-injection, and the y axis indicates the concentration of the antibody in nanograms per milliliter (ng/mL) in peripheral blood of cynomolgus monkeys that have been injected with an antibody. As indicated, filled circles represent individual data points for cynomolgus monkeys injected with Y-5-112, and the solid line represents the mean of these data. Similarly, triangles represent individual data points for cynomolgus monkeys injected with Antibody Y, and the dashed line represents the mean of these data.

BRIEF DESCRIPTION OF THE SEQUENCES

SEQUENCE LISTING NUMBER	DESCRIPTION
SEQ ID NO:1	Amino acid sequence of a human IgG1 Fc region
SEQ ID NO:2	Nucleotide sequence encoding a human IgG1 Fc region
SEQ ID NO:3	Amino acid sequence of a human IgG2 Fc region
SEQ ID NO:4	Nucleotide sequence encoding a human IgG2 Fc region
SEQ ID NO:5	Amino acid sequence of a human IgG3 Fc region
SEQ ID NO:6	Nucleotide sequence encoding a human IgG3 Fc region
SEQ ID NO:7	Amino acid sequence of a human IgG4 Fc region
SEQ ID NO:8	Nucleotide sequence encoding a human IgG4 Fc region
SEQ ID NO:9	Amino acid sequence of human FcRn alpha chain
SEQ ID NO:10	Amino acid sequence of human FcRn β-2-microglobulin chain
SEQ ID NO:11	Amino acid sequence of the extracellular region of mature human p75 tumor necrosis factor receptor
SEQ ID NO:12	Amino acid sequence of the extracellular region of mature human (CTLA4)
SEQ ID NO:13	Amino acid sequence of randomized insertion sequence (CXXXXXXC)
SEQ ID NO:14	Amino acid sequence of randomized insertion sequence (CXXXXXXXC)
SEQ ID NO:15	Amino acid sequence of randomized insertion sequence (CXXXXXXXXC)
SEQ ID NO:16	Amino acid sequence of randomized insertion sequence (GCXXXXXXCG)
SEQ ID NO:17	Amino acid sequence of randomized insertion sequence (GCXXXXXXXCG)
SEQ ID NO:18	Amino acid sequence of randomized insertion sequence (GCXXXXXXXXCG)
SEQ ID NO:19	Amino acid sequence of randomized insertion sequence (GGCXXXXXXCGG)
SEQ ID NO:20	Amino acid sequence of randomized insertion sequence (GGCXXXXXXXCGG)
SEQ ID NO:21	Amino acid sequence of randomized insertion sequence (GGCXXXXXXXXCGG)
SEQ ID NO:22	Amino acid sequence of randomized insertion sequence (GGGCXXXXXXCGGG)
SEQ ID NO:23	Amino acid sequence of randomized insertion sequence (GGGCXXXXXXXCGGG)
SEQ ID NO:24	Amino acid sequence of randomized insertion sequence (GGGCXXXXXXXXCGGG)
SEQ ID NO:25	Nucleotide sequence of the Fc-fragment-encoding portion of library L1
SEQ ID NO:26	Amino acid sequence encoded by SEQ ID NO:25
SEQ ID NO:27	Nucleotide sequence of the Fc-fragment-encoding portion of library L2A
SEQ ID NO:28	Amino acid sequence encoded by SEQ ID NO:27
SEQ ID NO:29	Nucleotide sequence of the Fc-fragment-encoding portion of library L2B

SEQUENCE LISTING NUMBER	DESCRIPTION
SEQ ID NO:30	Amino acid sequence encoded by SEQ ID NO:29
SEQ ID NO:31	Nucleotide sequence of the Fc-fragment-encoding portion of library L3
SEQ ID NO:32	Amino acid sequence encoded by SEQ ID NO:31
SEQ ID NO:33	Nucleotide sequence of the Fc-fragment-encoding portion of library L4
SEQ ID NO:34	Amino acid sequence encoded by SEQ ID NO:33
SEQ ID NO:35	Nucleotide sequence of the Fc-fragment-encoding portion of library L5
SEQ ID NO:36	Amino acid sequence encoded by SEQ ID NO:35
SEQ ID NO:37	Nucleotide sequence of the Fc-fragment-encoding portion of library L6A
SEQ ID NO:38	Amino acid sequence encoded by SEQ ID NO:37
SEQ ID NO:39	Nucleotide sequence of the Fc-fragment-encoding portion of library L6B
SEQ ID NO:40	Amino acid sequence encoded by SEQ ID NO:39
SEQ ID NO:41	Amino acid sequence of the insertion in variant Fc-fragment Fc-5-51
SEQ ID NO:42	Amino acid sequence of the insertion in variant Fc-fragment Fc-5-69
SEQ ID NO:43	Amino acid sequence of the insertion in variant Fc-fragment Fc-5-104
SEQ ID NO:44	Amino acid sequence of the insertion in variant Fc-fragment Fc-5-106
SEQ ID NO:45	Amino acid sequence of the insertion in variant Fc-fragment Fc-5-112
SEQ ID NO:46	Amino acid sequence of the insertion in variant Fc-fragment Fc-5-55
SEQ ID NO:47	Amino acid sequence of the insertion in variant Fc-fragment Fc-5-60
SEQ ID NO:48	Amino acid sequence of the insertion in variant Fc-fragment Fc-5-70
SEQ ID NO:49	Amino acid sequence of the insertion in variant Fc-fragment Fc-5-79
SEQ ID NO:50	Amino acid sequence of the insertion in variant Fc-fragment Fc-5-85
SEQ ID NO:51	Amino acid sequence of the insertion in variant Fc-fragment Fc-5-95
SEQ ID NO:52	Amino acid sequence of the insertion in variant Fc-fragment Fc-5-97
SEQ ID NO:53	Amino acid sequence of the insertion in variant Fc-fragment Fc-5-99
SEQ ID NO:54	Amino acid sequence of the middle six amino acids of the insertion in Fc-5-51
SEQ ID NO:55	Amino acid sequence of the middle six amino acids of the insertion in Fc-5-69
SEQ ID NO:56	Amino acid sequence of the middle six amino acids of the insertion in Fc-5-104
SEQ ID NO:57	Amino acid sequence of the middle six amino acids of the insertion in Fc-5-106
SEQ ID NO:58	Amino acid sequence of the middle six amino acids of the insertion in Fc-5-112
SEQ ID NO:59	Amino acid sequence of the middle six amino acids of the insertion in Fc-5-55
SEQ ID NO:60	Amino acid sequence of the middle six amino acids of the insertion in Fc-5-60
SEQ ID NO:61	Amino acid sequence of the middle six amino acids of the insertion in Fc-5-70
SEQ ID NO:62	Amino acid sequence of the middle six amino acids of the insertion in Fc-5-79
SEQ ID NO:63	Amino acid sequence of the middle six amino acids of the insertion in Fc-5-85
SEQ ID NO:64	Amino acid sequence of the middle six amino acids of the insertion in Fc-5-95
SEQ ID NO:65	Amino acid sequence of the middle six amino acids of the insertion in Fc-5-97
SEQ ID NO:66	Amino acid sequence of the middle six amino acids of the insertion in Fc-5-99
SEQ ID NO:67	Amino acid sequence of the insertion in variant Fc-fragment 5-1
SEQ ID NO:68	Nucleotide sequence of the forward primer used to construct a vector encoding variant Fc-polypeptide 5-1-1
SEQ ID NO:69	Nucleotide sequence of the reverse primer used to construct a vector encoding variant Fc-polypeptide 5-1-1
SEQ ID NO:70	Nucleotide sequence of the forward primer used to construct a vector encoding variant Fc-polypeptide 5-1-2
SEQ ID NO:71	Nucleotide sequence of the reverse primer used to construct a vector encoding variant Fc-polypeptide 5-1-2
SEQ ID NO:72	Nucleotide sequence of the forward primer used to construct a vector encoding variant Fc-polypeptide 5-1-3
SEQ ID NO:73	Nucleotide sequence of the reverse primer used to construct a vector encoding variant Fc-polypeptide 5-1-3
SEQ ID NO:74	Nucleotide sequence of the forward primer used to construct a vector encoding variant Fc-polypeptide 5-1-4
SEQ ID NO:75	Nucleotide sequence of the reverse primer used to construct a vector encoding variant Fc-polypeptide 5-1-4
SEQ ID NO:76	Nucleotide sequence of the forward primer used to construct a vector encoding variant Fc-polypeptide 5-1-5
SEQ ID NO:77	Nucleotide sequence of the reverse primer used to construct a vector encoding variant Fc-polypeptide 5-1-5
SEQ ID NO:78	Nucleotide sequence of the forward primer used to construct a vector encoding variant Fc-polypeptide 5-1-6
SEQ ID NO:79	Nucleotide sequence of the reverse primer used to construct a vector encoding variant Fc-polypeptide 5-1-6
SEQ ID NO:80	Nucleotide sequence of the forward primer used to construct a vector encoding variant Fc-polypeptide 5-1-7
SEQ ID NO:81	Nucleotide sequence of the reverse primer used to construct a vector encoding variant Fc-polypeptide 5-1-7
SEQ ID NO:82	Nucleotide sequence of the forward primer used to construct a vector encoding variant Fc-polypeptide 5-1-8
SEQ ID NO:83	Nucleotide sequence of the reverse primer used to construct a vector encoding variant Fc-polypeptide 5-1-8
SEQ ID NO:84	Nucleotide sequence of the forward primer used to construct a vector encoding variant Fc-polypeptide 5-1-9
SEQ ID NO:85	Nucleotide sequence of the reverse primer used to construct a vector encoding variant Fc-polypeptide 5-1-9

SEQUENCE LISTING NUMBER	DESCRIPTION
SEQ ID NO:86	Nucleotide sequence of the forward primer used to construct a vector encoding variant Fc-polypeptide 5-1-10
SEQ ID NO:87	Nucleotide sequence of the reverse primer used to construct a vector encoding variant Fc-polypeptide 5-1-10
SEQ ID NO:88	Amino acid sequence of the insertion in library L-8
SEQ ID NO:89	Amino acid sequence of the insertion in library L-10
SEQ ID NO:90	Amino acid sequence of insertion in variant Fc-fragment 5y-1
SEQ ID NO:91	Amino acid sequence of insertion in variant Fc-fragment 5y-2
SEQ ID NO:92	Amino acid sequence of insertion in variant Fc-fragment 5y-3
SEQ ID NO:93	Amino acid sequence of insertion in variant Fc-fragment 5y-4
SEQ ID NO:94	Amino acid sequence of insertion in variant Fc-fragment 5y-5
SEQ ID NO:95	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-6
SEQ ID NO:96	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-7
SEQ ID NO:97	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-8
SEQ ID NO:98	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-9
SEQ ID NO:99	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-10
SEQ ID NO:100	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-11
SEQ ID NO:101	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-12
SEQ ID NO:102	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-13
SEQ ID NO:103	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-14
SEQ ID NO:104	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-15
SEQ ID NO:105	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-16
SEQ ID NO:106	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-17
SEQ ID NO:107	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-18
SEQ ID NO:108	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-19
SEQ ID NO:109	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-20
SEQ ID NO:110	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-21
SEQ ID NO:111	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-22
SEQ ID NO:112	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-23
SEQ ID NO:113	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-24
SEQ ID NO:114	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-25
SEQ ID NO:115	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-26
SEQ ID NO:116	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-27
SEQ ID NO:117	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-28
SEQ ID NO:118	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-29
SEQ ID NO:119	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-30
SEQ ID NO:120	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-31
SEQ ID NO:121	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-32
SEQ ID NO:122	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-33
SEQ ID NO:123	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-34
SEQ ID NO:124	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-35
SEQ ID NO:125	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-36
SEQ ID NO:126	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-37
SEQ ID NO:127	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-38
SEQ ID NO:128	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-39
SEQ ID NO:129	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-40
SEQ ID NO:130	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-41
SEQ ID NO:131	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-42
SEQ ID NO:132	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-43
SEQ ID NO:133	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-44
SEQ ID NO:134	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-45
SEQ ID NO:135	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-46
SEQ ID NO:136	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-47
SEQ ID NO:137	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-48
SEQ ID NO:138	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-49
SEQ ID NO:139	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-50
SEQ ID NO:140	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-51
SEQ ID NO:141	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-52

SEQUENCE LISTING NUMBER	DESCRIPTION
SEQ ID NO:142	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-53
SEQ ID NO:143	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-54
SEQ ID NO:144	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-55
SEQ ID NO:145	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-56
SEQ ID NO:146	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-57
SEQ ID NO:147	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-58
SEQ ID NO:148	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-59
SEQ ID NO:149	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-60
SEQ ID NO:150	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-61
SEQ ID NO:151	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-62
SEQ ID NO:152	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-63
SEQ ID NO:153	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-64
SEQ ID NO:154	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-65
SEQ ID NO:155	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-66
SEQ ID NO:156	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-67
SEQ ID NO:157	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-68
SEQ ID NO:158	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-69
SEQ ID NO:159	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-70
SEQ ID NO:160	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-71
SEQ ID NO:161	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-72
SEQ ID NO:162	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-73
SEQ ID NO:163	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-74
SEQ ID NO:164	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-75
SEQ ID NO:165	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-76
SEQ ID NO:166	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-77
SEQ ID NO:167	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-78
SEQ ID NO:168	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-79
SEQ ID NO:169	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-80
SEQ ID NO:170	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-81
SEQ ID NO:171	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-82
SEQ ID NO:172	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-83
SEQ ID NO:173	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-84
SEQ ID NO:174	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-85
SEQ ID NO:175	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-86
SEQ ID NO:176	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-87
SEQ ID NO:177	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-88
SEQ ID NO:178	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-89
SEQ ID NO:179	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-90
SEQ ID NO:180	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-91
SEQ ID NO:181	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-92
SEQ ID NO:182	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-93
SEQ ID NO:183	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-94
SEQ ID NO:184	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-95
SEQ ID NO:185	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-96
SEQ ID NO:186	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-97
SEQ ID NO:187	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-98
SEQ ID NO:188	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-99
SEQ ID NO:189	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-100
SEQ ID NO:190	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-101
SEQ ID NO:191	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-102
SEQ ID NO:192	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-103
SEQ ID NO:193	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-104
SEQ ID NO:194	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-105
SEQ ID NO:195	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-106
SEQ ID NO:196	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-107
SEQ ID NO:197	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-108

SEQUENCE LISTING NUMBER	DESCRIPTION
SEQ ID NO:198	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-109
SEQ ID NO:199	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-110
SEQ ID NO:200	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-111
SEQ ID NO:201	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-112
SEQ ID NO:202	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-113
SEQ ID NO:203	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-114
SEQ ID NO:204	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-115
SEQ ID NO:205	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-116
SEQ ID NO:206	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-117
SEQ ID NO:207	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-118
SEQ ID NO:208	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-119
SEQ ID NO:209	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-120
SEQ ID NO:210	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-121
SEQ ID NO:211	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-122
SEQ ID NO:212	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-123
SEQ ID NO:213	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-124
SEQ ID NO:214	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-125
SEQ ID NO:215	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-126
SEQ ID NO:216	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-127
SEQ ID NO:217	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-128
SEQ ID NO:218	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-129
SEQ ID NO:219	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-130
SEQ ID NO:220	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-131
SEQ ID NO:221	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-132
SEQ ID NO:222	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-133
SEQ ID NO:223	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-134
SEQ ID NO:224	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-135
SEQ ID NO:225	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-136
SEQ ID NO:226	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-137
SEQ ID NO:227	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-138
SEQ ID NO:228	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-139
SEQ ID NO:229	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-140
SEQ ID NO:230	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-141
SEQ ID NO:231	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-142
SEQ ID NO:232	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-143
SEQ ID NO:233	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-144
SEQ ID NO:234	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-145
SEQ ID NO:235	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-146
SEQ ID NO:236	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-147
SEQ ID NO:237	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-148
SEQ ID NO:238	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-149
SEQ ID NO:239	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-150
SEQ ID NO:240	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-151
SEQ ID NO:241	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-152
SEQ ID NO:242	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-153
SEQ ID NO:243	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-154
SEQ ID NO:244	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-155
SEQ ID NO:245	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-156
SEQ ID NO:246	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-157
SEQ ID NO:247	Amino acid sequence of sequence of insertion in variant Fc-fragment 5y-158
SEQ ID NO:248	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-1
SEQ ID NO:249	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-2
SEQ ID NO:250	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-3
SEQ ID NO:251	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-4
SEQ ID NO:252	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-5
SEQ ID NO:253	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-6

SEQUENCE LISTING NUMBER	DESCRIPTION
SEQ ID NO:254	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-7
SEQ ID NO:255	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-8
SEQ ID NO:256	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-9
SEQ ID NO:257	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-10
SEQ ID NO:258	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-11
SEQ ID NO:259	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-12
SEQ ID NO:260	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-13
SEQ ID NO:261	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-14
SEQ ID NO:262	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-15
SEQ ID NO:263	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-16
SEQ ID NO:264	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-17
SEQ ID NO:265	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-18
SEQ ID NO:266	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-19
SEQ ID NO:267	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-20
SEQ ID NO:268	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-21
SEQ ID NO:269	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-22
SEQ ID NO:270	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-23
SEQ ID NO:271	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-24
SEQ ID NO:272	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-25
SEQ ID NO:273	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-26
SEQ ID NO:274	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-27
SEQ ID NO:275	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-28
SEQ ID NO:276	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-29
SEQ ID NO:277	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-30
SEQ ID NO:278	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-31
SEQ ID NO:279	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-32
SEQ ID NO:280	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-33
SEQ ID NO:281	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-34
SEQ ID NO:282	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-35
SEQ ID NO:283	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-36
SEQ ID NO:284	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-37
SEQ ID NO:285	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-38
SEQ ID NO:286	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-39
SEQ ID NO:287	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-40
SEQ ID NO:288	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-41
SEQ ID NO:289	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-42
SEQ ID NO:290	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-43
SEQ ID NO:291	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-44
SEQ ID NO:292	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-45
SEQ ID NO:293	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-46
SEQ ID NO:294	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-47
SEQ ID NO:295	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-48
SEQ ID NO:296	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-49
SEQ ID NO:297	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-50
SEQ ID NO:298	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-51
SEQ ID NO:299	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-52
SEQ ID NO:300	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-53
SEQ ID NO:301	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-54
SEQ ID NO:302	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-55
SEQ ID NO:303	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-56
SEQ ID NO:304	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-57
SEQ ID NO:305	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-58
SEQ ID NO:306	Amino acid sequence of sequence of insertion in variant Fc-fragment 8y-59
SEQ ID NO:307	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-1
SEQ ID NO:308	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-2
SEQ ID NO:309	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-3

SEQUENCE LISTING NUMBER	DESCRIPTION
SEQ ID NO:310	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-4
SEQ ID NO:311	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-5
SEQ ID NO:312	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-6
SEQ ID NO:313	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-7
SEQ ID NO:314	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-8
SEQ ID NO:315	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-9
SEQ ID NO:316	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-10
SEQ ID NO:317	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-11
SEQ ID NO:318	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-12
SEQ ID NO:319	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-13
SEQ ID NO:320	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-14
SEQ ID NO:321	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-15
SEQ ID NO:322	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-16
SEQ ID NO:323	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-17
SEQ ID NO:324	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-18
SEQ ID NO:325	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-19
SEQ ID NO:326	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-20
SEQ ID NO:327	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-21
SEQ ID NO:328	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-22
SEQ ID NO:329	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-23
SEQ ID NO:330	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-24
SEQ ID NO:331	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-25
SEQ ID NO:332	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-26
SEQ ID NO:333	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-27
SEQ ID NO:334	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-28
SEQ ID NO:335	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-29
SEQ ID NO:336	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-30
SEQ ID NO:337	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-31
SEQ ID NO:338	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-32
SEQ ID NO:339	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-33
SEQ ID NO:340	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-34
SEQ ID NO:341	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-35
SEQ ID NO:342	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-36
SEQ ID NO:343	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-37
SEQ ID NO:344	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-38
SEQ ID NO:345	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-39
SEQ ID NO:346	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-40
SEQ ID NO:347	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-41
SEQ ID NO:348	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-42
SEQ ID NO:349	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-43
SEQ ID NO:350	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-44
SEQ ID NO:351	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-45
SEQ ID NO:352	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-46
SEQ ID NO:353	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-47
SEQ ID NO:354	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-48
SEQ ID NO:355	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-49
SEQ ID NO:356	Amino acid sequence of sequence of insertion in variant Fc-fragment 10y-50
SEQ ID NO:357	Nucleotide sequence of a primer
SEQ ID NO:358	Nucleotide sequence of a primer
SEQ ID NO:359	Amino acid sequence of the peptide insertion in variant Fc-fragment 5-57
SEQ ID NO:360	Amino acid sequence of the peptide insertion in variant Fc-fragment 5-64
SEQ ID NO:361	Amino acid sequence of the peptide insertion in variant Fc-fragment 5-66
SEQ ID NO:362	Amino acid sequence of the peptide insertion in variant Fc-fragment 5-73
SEQ ID NO:363	Amino acid sequence of the peptide insertion in variant Fc-fragment 5-92
SEQ ID NO:364	Amino acid sequence of the peptide insertion in variant Fc-fragment 5-96
SEQ ID NO:365	Amino acid sequence of the peptide insertion in variant Fc-fragment 5-110

SEQUENCE LISTING NUMBER	DESCRIPTION
SEQ ID NO:366	Amino acid sequence of the peptide insertion in variant Fc-fragment 5-113
SEQ ID NO:367	Amino acid sequence of the peptide insertion in variant Fc-fragment Fc-5-69- WIF
SEQ ID NO:368	Amino acid sequence of the peptide insertion in variant Fc-fragment Fc-5-69- WIY
SEQ ID NO:369	Amino acid sequence of the peptide insertion in variant Fc-fragment Fc-5-106- M4A
SEQ ID NO:370	Amino acid sequence of the peptide insertion in variant Fc-fragment Fc-5-106- M4G
SEQ ID NO:371	Amino acid sequence of the peptide insertion in variant Fc-fragment Fc-5-106- M4H
SEQ ID NO:372	Amino acid sequence of the peptide insertion in variant Fc-fragment Fc-5-106-M4I
SEQ ID NO:373	Amino acid sequence of the peptide insertion in variant Fc-fragment Fc-5-106-M4L
SEQ ID NO:374	Amino acid sequence of the peptide insertion in variant Fc-fragment Fc-5-106- M4N
SEQ ID NO:375	Amino acid sequence of the peptide insertion in variant Fc-fragment Fc-5-106- M4Q
SEQ ID NO:376	Amino acid sequence of the peptide insertion in variant Fc-fragment Fc-5-106- M4S
SEQ ID NO:377	Amino acid sequence of the peptide insertion in variant Fc-fragment Fc-5-106- M4T
SEQ ID NO:378	Amino acid sequence of the peptide insertion in variant Fc-fragment Fc-5-106- M4V
SEQ ID NO:379	Amino acid sequence of the peptide insertion in variant Fc-fragment Fc-5-110
SEQ ID NO:380	Nucleotide sequence of forward PCR primer used to make Antibody X-5y-8
SEQ ID NO:381	Nucleotide sequence of reverse PCR primer used to make Antibody X-5y-8
SEQ ID NO:382	Nucleotide sequence of forward PCR primer used to make Antibody X-5y-132
SEQ ID NO:383	Nucleotide sequence of reverse PCR primer used to make Antibody X-5y-132
SEQ ID NO:384	Nucleotide sequence of forward PCR primer used to make Antibody X-5y-38
SEQ ID NO:385	Nucleotide sequence of reverse PCR primer used to make Antibody X-5y-38
SEQ ID NO:386	Nucleotide sequence of forward PCR primer used to make Antibody X-5y-91
SEQ ID NO:387	Nucleotide sequence of reverse PCR primer used to make Antibody X-5y-91
SEQ ID NO:388	Nucleotide sequence of forward PCR primer used to make Antibody X-5y-119
SEQ ID NO:389	Nucleotide sequence of reverse PCR primer used to make Antibody X-5y-119
SEQ ID NO:390	Nucleotide sequence of forward PCR primer used to make Antibody X-5y-127
SEQ ID NO:391	Nucleotide sequence of reverse PCR primer used to make Antibody X-5y-127
SEQ ID NO:392	Amino acid sequence of a peptide insertion in a variant Fc polypeptide with a positive ELISA score
SEQ ID NO:393	Amino acid sequence of a peptide insertion in a variant Fc polypeptide with a positive ELISA score
SEQ ID NO:394	Amino acid sequence of a peptide insertion in a variant Fc polypeptide with a positive ELISA score
SEQ ID NO:395	Amino acid sequence of a peptide insertion in a variant Fc polypeptide with a positive ELISA score
SEQ ID NO:396	Amino acid sequence of a peptide insertion in a variant Fc polypeptide with a positive ELISA score
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SEQ ID NO:421	Amino acid sequence of a peptide insertion in a variant Fc polypeptide with a positive ELISA score

SEQUENCE LISTING NUMBER	DESCRIPTION
SEQ ID NO:422	Amino acid sequence of a peptide insertion in a variant Fc polypeptide with a positive ELISA score
SEQ ID NO:423	Amino acid sequence of a peptide insertion in a variant Fc polypeptide with a positive ELISA score
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SEQ ID NO:471	Amino acid sequence of a peptide insertion in a variant Fc polypeptide with a positive ELISA score
SEQ ID NO:472	Amino acid sequence of a peptide insertion in a variant Fc polypeptide with a positive ELISA score
SEQ ID NO:473	Amino acid sequence of a peptide insertion in a variant Fc polypeptide with a positive ELISA score
SEQ ID NO:474	Amino acid sequence of a peptide insertion in a variant Fc polypeptide with a positive ELISA score
SEQ ID NO:475	Amino acid sequence of a peptide insertion in a variant Fc polypeptide with a positive ELISA score
SEQ ID NO:476	Amino acid sequence of a peptide insertion in a variant Fc polypeptide with a positive ELISA score
SEQ ID NO:477	Amino acid sequence of a peptide insertion in a variant Fc polypeptide with a positive ELISA score

SEQUENCE LISTING NUMBER	DESCRIPTION
SEQ ID NO:478	Amino acid sequence of a peptide insertion in a variant Fc polypeptide with a positive ELISA score
SEQ ID NO:479	Amino acid sequence of a peptide insertion in a variant Fc polypeptide with a positive ELISA score
SEQ ID NO:480	Amino acid sequence of a peptide insertion in a variant Fc polypeptide with a positive ELISA score
SEQ ID NO:481	Amino acid sequence of a peptide insertion in a variant Fc polypeptide with a positive ELISA score
SEQ ID NO:482	Amino acid sequence of a peptide insertion in a variant Fc polypeptide with a positive ELISA score
SEQ ID NO:483	Amino acid sequence of a peptide insertion in a variant Fc polypeptide with a positive ELISA score
SEQ ID NO:484	Amino acid sequence of a peptide insertion in a variant Fc polypeptide with a positive ELISA score
SEQ ID NO:485	Amino acid sequence of a peptide insertion in a variant Fc polypeptide with a positive ELISA score
SEQ ID NO:486	Amino acid sequence of a peptide insertion in a variant Fc polypeptide with a positive ELISA score
SEQ ID NO:487	Amino acid sequence of a peptide insertion in a variant Fc polypeptide with a positive ELISA score
SEQ ID NO:488	Amino acid sequence of a peptide insertion in a variant Fc polypeptide with a positive ELISA score
SEQ ID NO:489	Amino acid sequence of a peptide insertion in a variant Fc polypeptide with a positive ELISA score
SEQ ID NO:490	Amino acid sequence of a peptide insertion in a variant Fc polypeptide with a positive ELISA score
SEQ ID NO:491	Amino acid sequence of a peptide insertion in a variant Fc polypeptide with a positive ELISA score
SEQ ID NO:492	Amino acid sequence of a peptide insertion in a variant Fc polypeptide with a positive ELISA score
SEQ ID NO:493	Amino acid sequence of a peptide insertion in a variant Fc polypeptide with a positive ELISA score
SEQ ID NO:494	Amino acid sequence of a peptide insertion in a variant Fc polypeptide with a positive ELISA score
SEQ ID NO:495	Amino acid sequence of a peptide insertion in a variant Fc polypeptide with a positive ELISA score
SEQ ID NO:496	Amino acid sequence of a peptide insertion in a variant Fc polypeptide with a positive ELISA score
SEQ ID NO:497	Nucleotide sequence of a forward PCR primer used to make Fc variant Fc-5-69-W1F
SEQ ID NO:498	Nucleotide sequence of a reverse PCR primer used to make Fc variant Fc-5-69-W1F
SEQ ID NO:499	Nucleotide sequence of a forward PCR primer used to make Fc variant Fc-5-69-W1Y
SEQ ID NO:500	Nucleotide sequence of a reverse PCR primer used to make Fc variant Fc-5-69-W1Y
SEQ ID NO:501	Nucleotide sequence of a forward PCR primer used to make Fc variant Fc-5-106-M4A
SEQ ID NO:502	Nucleotide sequence of a reverse PCR primer used to make Fc variant Fc-5-106-M4A
SEQ ID NO:503	Nucleotide sequence of a forward PCR primer used to make Fc variant Fc-5-106-M4G
SEQ ID NO:504	Nucleotide sequence of a reverse PCR primer used to make Fc variant Fc-5-106-M4G
SEQ ID NO:505	Nucleotide sequence of a forward PCR primer used to make Fc variant Fc-5-106-M4H
SEQ ID NO:506	Nucleotide sequence of a reverse PCR primer used to make Fc variant Fc-5-106-M4H
SEQ ID NO:507	Nucleotide sequence of a forward PCR primer used to make Fc variant Fc-5-106-M4I
SEQ ID NO:508	Nucleotide sequence of a reverse PCR primer used to make Fc variant Fc-5-106-M4I
SEQ ID NO:509	Nucleotide sequence of a forward PCR primer used to make Fc variant Fc-5-106-M4L
SEQ ID NO:510	Nucleotide sequence of a reverse PCR primer used to make Fc variant Fc-5-106-M4L
SEQ ID NO:511	Nucleotide sequence of a forward PCR primer used to make Fc variant Fc-5-106-M4N
SEQ ID NO:512	Nucleotide sequence of a reverse PCR primer used to make Fc variant Fc-5-106-M4N
SEQ ID NO:513	Nucleotide sequence of a forward PCR primer used to make Fc variant Fc-5-106-M4Q
SEQ ID NO:514	Nucleotide sequence of a reverse PCR primer used to make Fc variant Fc-5-106-M4Q
SEQ ID NO:515	Nucleotide sequence of a forward PCR primer used to make Fc variant Fc-5-106-M4S
SEQ ID NO:516	Nucleotide sequence of a reverse PCR primer used to make Fc variant Fc-5-106-M4S
SEQ ID NO:517	Nucleotide sequence of a forward PCR primer used to make Fc variant Fc-5-106-M4T
SEQ ID NO:518	Nucleotide sequence of a reverse PCR primer used to make Fc variant Fc-5-106-M4T
SEQ ID NO:519	Nucleotide sequence of a forward PCR primer used to make Fc variant Fc-5-106-M4V
SEQ ID NO:520	Nucleotide sequence of a reverse PCR primer used to make Fc variant Fc-5-106-M4V
SEQ ID NO:521	Nucleotide sequence of a forward PCR primer used to construct libraries L1, L2A, L2B, L3, L4, and L5
SEQ ID NO:522	Nucleotide sequence of a reverse PCR primer used to construct library L1
SEQ ID NO:523	Nucleotide sequence of a reverse PCR primer used to construct libraries L2A and L2B
SEQ ID NO:524	Nucleotide sequence of a reverse PCR primer used to construct library L3
SEQ ID NO:525	Nucleotide sequence of a reverse PCR primer used to construct library L4
SEQ ID NO:526	Nucleotide sequence of a reverse PCR primer used to construct library L5
SEQ ID NO:527	Nucleotide sequence of a reverse PCR primer used to construct libraries L1, L2A, L2B, L3, L4, and L5
SEQ ID NO:528*	Nucleotide sequence of a forward PCR primer used to construct library L1
SEQ ID NO:529*	Nucleotide sequence of a forward PCR primer used to construct library L2A
SEQ ID NO:530*	Nucleotide sequence of a forward PCR primer used to construct library L2B
SEQ ID NO:531*	Nucleotide sequence of a forward PCR primer used to construct library L3
SEQ ID NO:532*	Nucleotide sequence of a forward PCR primer used to construct library L4
SEQ ID NO:533*	Nucleotide sequence of a forward PCR primer used to construct library L5

SEQUENCE LISTING NUMBER	DESCRIPTION
SEQ ID NO:534	Nucleotide sequence of a forward PCR primer used to construct libraries L1, L2A, L2B, L3, L4, and L5
SEQ ID NO:535	Nucleotide sequence of a reverse PCR primer used to construct libraries L1, L2A, L2B, L3, L4, and L5
SEQ ID NO:536	Nucleotide sequence of a forward PCR primer used to construct library L6A
SEQ ID NO:537#	Nucleotide sequence of a forward PCR primer used to construct library L6B
SEQ ID NO:538	Nucleotide sequence of a reverse PCR primer used to construct libraries L6A and L6B
SEQ ID NO:539	Nucleotide sequence of forward PCR primer used to construct Antibody X-5-51
SEQ ID NO:540	Nucleotide sequence of reverse PCR primer used to construct Antibody X-5-51
SEQ ID NO:541	Nucleotide sequence of forward PCR primer used to construct Antibody X-5-69
SEQ ID NO:542	Nucleotide sequence of reverse PCR primer used to construct Antibody X-5-69
SEQ ID NO:543	Nucleotide sequence of forward PCR primer used to construct Antibody X-5-104
SEQ ID NO:544	Nucleotide sequence of reverse PCR primer used to construct Antibody X-5-104
SEQ ID NO:545	Nucleotide sequence of forward PCR primer used to construct Antibody X-5-106
SEQ ID NO:546	Nucleotide sequence of reverse PCR primer used to construct Antibody X-5-106
SEQ ID NO:547	Nucleotide sequence of forward PCR primer used to construct Antibody X-5-112
SEQ ID NO:548	Nucleotide sequence of reverse PCR primer used to construct Antibody X-5-112
SEQ ID NO:549	Amino acid sequence of amino acids 36-41 of SEQ ID NO:1, which correspond to amino acids 251-256 in the EU numbering scheme
SEQ ID NO:550	Amino acid sequence from amino acid 251-256 in the EU numbering scheme in the Fc fragments encoded by Library L1
SEQ ID NO:551	Amino acid sequence of amino acids 92-99 of SEQ ID NO:1, which correspond to amino acids 307-314 in the EU numbering system
SEQ ID NO:552	Amino acid sequence from amino acid 307-314 in the EU numbering scheme in the Fc fragments encoded by Library L2A
SEQ ID NO:553	Amino acid sequence from amino acid 307-314 in the EU numbering scheme in the Fc fragments encoded by Library L2B
SEQ ID NO:554	Amino acid sequence of amino acids 213-221 of SEQ ID NO:1, which correspond to 428-436 in the EU numbering scheme
SEQ ID NO:555	Amino acid sequence from amino acid 428-436 in the EU numbering scheme in the Fc fragments encoded by Library L3
SEQ ID NO:556	Amino acid sequence of amino acids 68-72 of SEQ ID NO:1, which correspond to amino acids 283-287 in the EU numbering scheme
SEQ ID NO:557	Amino acid sequence from amino acid 283-287 in the EU numbering scheme in the Fc fragments encoded by Library L4
SEQ ID NO:558	Amino acid sequence of amino acids 169-174 of SEQ ID NO:1, which correspond to amino acids 384-389 in the EU numbering system
SEQ ID NO:559	Amino acid sequence from amino acid 384-389 in the EU numbering scheme in the Fc fragments encoded by Library L5
SEQ ID NO:560	Amino acid sequence of amino acids 141-147 of SEQ ID NO:1, which correspond to amino acids 356-362 in the EU numbering system
SEQ ID NO:561	Amino acid sequence from amino acid 356-362 in the EU numbering scheme in the Fc fragments encoded by Library L6A
SEQ ID NO:562	Amino acid sequence from amino acid 356-362 in the EU numbering scheme in the Fc fragments encoded by Library L6B
SEQ ID NO:563*	Amino acid sequence of amino acids 166 to 178 of SEQ ID NO:1, corresponding to amino acids 381 to 393 in the EU numbering system
SEQ ID NO:564*	Amino acid sequence corresponding to amino acids 381 to 393 is the EU numbering system of variant Fc fragment 5-1
SEQ ID NO:565*	Amino acid sequence corresponding to amino acids 381 to 393 is the EU numbering system of variant Fc fragment 5-1-1
SEQ ID NO:566*	Amino acid sequence corresponding to amino acids 381 to 393 is the EU numbering system of variant Fc fragment 5-1-2
SEQ ID NO:567*	Amino acid sequence corresponding to amino acids 381 to 393 is the EU numbering system of variant Fc fragment 5-1-3
SEQ ID NO:568*	Amino acid sequence corresponding to amino acids 381 to 393 is the EU numbering system of variant Fc fragment 5-1-4
SEQ ID NO:569*	Amino acid sequence corresponding to amino acids 381 to 393 is the EU numbering system of variant Fc fragment 5-1-5

SEQUENCE LISTING NUMBER	DESCRIPTION
SEQ ID NO:570[$]	Amino acid sequence corresponding to amino acids 381 to 393 is the EU numbering system of variant Fc fragment 5-1-6
SEQ ID NO:571[$]	Amino acid sequence corresponding to amino acids 381 to 393 is the EU numbering system of variant Fc fragment 5-1-7
SEQ ID NO:572[$]	Amino acid sequence corresponding to amino acids 381 to 393 is the EU numbering system of variant Fc fragment 5-1-8
SEQ ID NO:573[$]	Amino acid sequence corresponding to amino acids 381 to 393 is the EU numbering system of variant Fc fragment 5-1-9
SEQ ID NO:574[$]	Amino acid sequence corresponding to amino acids 381 to 393 is the EU numbering system of variant Fc fragment 5-1-10

DETAILED DESCRIPTION

Changes in a therapeutic protein that increase *in vivo* half-life can be useful because such proteins can be dosed in lower amounts and/or less frequently. The instant invention provides variant Fc-polypeptides comprising variant Fc-fragments that bind to FcRn with increased affinity and/or binding activity at slightly acidic pHs, such as, for example, pH 5.0-6.0, and do not bind well to FcRn at physiologic pHs of about 7.2 to 7.6. As demonstrated herein, such variant Fc-polypeptides containing the variant Fc-fragments can have longer *in vivo* half lives as compared to control Fc-polypeptides. Provided are variant Fc-polypeptides comprising variant human Fc-fragments, which contain at least one insertion within or adjacent to a loop, which bind to FcRn with enhanced affinity and/or binding activity at a pH of from about 5 to 6 as compared to control Fc-polypeptides that differ only in that they do not contain the insertion(s). Also provided are nucleic acids encoding proteins containing such variant Fc-fragments and variant Fc-polypeptides, host cells containing such nucleic acids, and methods of making variant Fc-polypeptides and variant Fc-fragments. Further, methods of extending the half life of an Fc-polypeptide are provided, and methods of identifying variant Fc-fragments that bind to FcRn with higher affinity and/or binding activity at pH 5-6 with lower or comparable affinity and/or binding activity at a physiologic pH as compared to control Fc-fragments are also provided.

A variant Fc-fragment as described herein contains short insertions at selected sites in loops. FcRn interacts with an Fc-fragment. An immunoglobulin domain, such as the C_H2 or C_H3 domain, has a barrel-shaped structure comprising beta sheet portions alternating with loops. Hunkapiller and Hood (1989), Adv. Immunol. 44: 1-63; Williams and Barclay (1988), Ann. Rev. Immunol. 6: 381-405. Insertion sites in an Fc-fragment were chosen in loops that are part of the area that comes into relatively close contact with FcRn when it is bound to the Fc-fragment or in loops that are adjacent to these areas. Figure 1 shows ribbon diagrams of portions of FcRn (top) and an Fc-fragment (bottom) and indicates the positions in the Fc-fragment of the sites selected for insertions.

As will be shown below, many insertions in library L5 have the desired properties of enhanced binding to FcRn at pH 5 to 6 and little or no binding to FcRn at physiologic pH. Other libraries, including

libraries L1, L2B, L3, and L4 (*see* Figures 1 and 2), yielded no insertions that had the desired properties even though approximately the same number of variants were screened. Libraries L2A, L6A, and L6B yielded some insertions having the desired properties, although the numbers were much smaller than the numbers obtained from library L5. Surprisingly, the library that produced the most numerous insertions with the desired properties, *i.e.,* library L5, lay in a loop region that is not part of the area that comes in to closest contact with FcRn. Figure 1.

Definitions

The term **"affinity,"** as meant herein, refers to binding affinity measured as an EC_{50} measured using a BIAcore® T100 (or a similar instrument) as explained in Example 3. "Higher affinity" of a variant Fc-polypeptide (containing a variant Fc-fragment), as compared to an control Fc-polypeptide (containing a control Fc-fragment), for FcRn means that the variant Fc-polypeptide has an EC_{50} that is no more than 50% of the EC_{50} of the control Fc-polypeptide. Similarly, "lower affinity" of a variant Fc-polypeptide, as compared to a control Fc-polypeptide, means that the variant Fc-polypeptide has an EC_{50} that is more than 150% of that of a control Fc-polypeptide. Affinity of a variant Fc-polypeptide is considered "substantially the same" as that of a control Fc-polypeptide if its EC_{50} is 50-150% of the EC_{50} of the control Fc-polypeptide.

The term **"binding activity,"** as meant herein, refers to binding activity of an Fc-polypeptide to an FcRn measured using streptavidin biosensors coated with biotinylated human FcRn (hFcRn) in the Octet Red® system as described in Example 4 where association occurs at pH 6 and dissociation occurs at pH 7.4. Binding activity of a variant Fc-polypeptide at pH 6 is considered to be "higher than" that of a control Fc-polypeptide if the maximal response observed is at least 1.5, 1.75, or 2.0 times the maximal response observed for the control Fc-polypeptide. The very small amount of residual binding response, *i.e.,* the binding response remaining after most of the protein has dissociated from hFcRn at pH 7.4, observed using a control Fc-polypeptide comprising a wild type Fc-fragment is referred to as "little or no binding activity." A variant Fc-polypeptide is also considered to have "little or no binding activity" at pH 7.4 if the residual binding response detected after some or all of the protein has dissociated at pH 7.4 is no more than 0.2 or 0.1 nanometers more than that detected using a control Fc-polypeptide using the ForteBio system as described in Example 4.

An **"amino acid,"** as meant herein, refers to any one of the twenty L-amino acids commonly found in human proteins, which are the following: alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine.

An **"antibody,"** as meant herein, is a protein containing at least one heavy or light chain immunoglobulin variable region. For example, an scFv, a molecule comprising two or more scFv's, and the domain antibodies consisting essentially of a single immunoglobulin variable region (as described in,*e.g.,* US Patent 7,563,443) are "antibodies" as meant herein. In many cases, an antibody includes a heavy and a light chain variable region plus a human IgG Fc region. Thus, the term "antibody" encompasses full length antibodies containing two full length heavy and two full length light chains, such as naturally-occurring IgG, IgA, IgD, IgD, or IgM antibodies found in mammals. Carayannopoulos and Capra, Ch. 9 *in* FUNDAMENTAL IMMUNOLOGY, 3rd ed., Paul, ed., Raven Press, New York, 1993, pp. 284-286); the portions of this reference that describe antibodies are incorporated herein by reference. Exemplary antibodies include adalimumab, (HUMIRA®, Abbott Laboratories), infliximab (REMICADE®, Centocor Ortho Biotech Inc.), ustekinumab (STELLARA®, Centocor), golimumab (SIMPONI®, Centocor Ortho Biotech), canakinumab (ILARIS®, Novartis Pharmceuticals Corporation), ofatumumab (ARZERRA®, Glaxo Group Ltd.), tocilizumab (ACTEMRA®, Chugai Seiyaku Kabushiki Corp., Japan), belimumab (BENLYSTA®, LYMPHOSTAT-B®, Human Genome Sciences, Inc.), bevacizumab (AVASTIN®, Genentech), cetuximab (ERBITUX®, ImClone Systems Inc.), efungumab (MYCOGRAB®, Novartis AB Corp.), efalizumab, (RAPTIVA®, Genentech Inc.), etaracizumab (ABEGRIN®, Medimmune LLC), gemtuzumab ozogamicin (MYLOTARG®, Wyeth), girentuximab (RENCAREX®, Wilex AG Corp., Germany), natalizumab (TYSABRI®, Elan Pharmceuticals, Inc.), omalizumab (XOLAIR®, Novartis AG Corp., Switzerland), oregovomab (OVAREX®,AltaRex Corp., Canada), palivizumab (SYNAGIS®, ABBOSYNAGIS®, Medimmune Inc.). panitumumab (VECTIBIX®, Amgen Inc.), ranibizumab (LUCENTIS®, Genentech Inc.), rituximab (MABTHERA®, RITUXAN®, Idec Pharmaceuticals Corp.) tefibazumab (AUREXIS®, Inhibitex Corp.), tositumomab, (BEXXAR®, GlaxoSmithKline Beecham Corp.), trastuzumab (HERCEPTIN®, Genentech Inc.) and denosumab (PROLIA® or XGEVA®, Amgen Inc.), among many others. Such IgG antibodies can be of the IgG1, IgG2, IgG3, or IgG4 isotype and can be chimeric, human or humanized antibodies. Further, the term "antibody" includes dimeric antibodies containing two heavy chains and no light chains, such as the naturally-occurring antibodies found in camels, other dromedary species, and sharks. *See, e.g.,* Muyldermans *et al.*, 2001, J. Biotechnol. 74:277-302; Desmyter *et al.,* 2001, J. Biol. Chem. 276:26285-90; Streltsov *et al.* (2005), Protein Science 14: 2901-2909. An antibody can be monospecific (that is, binding to only one kind of antigen) or multispecific (that is, binding to more than one kind of antigen). In some embodiments, an antibody can be bispecific (that is, binding to two different kinds of antigen). Further, an antibody can be monovalent, bivalent, or multivalent, meaning that it can bind to one or two or more antigen molecules at once. Some of the possible formats for such antibodies include monospecific or bispecific

full length antibodies, monospecific monovalent antibodies (as described in International Application W0 2009/089004 and US Application Publication 2007/0105199, the relevant portions of which are incorporated herein by reference, and in US Application Publication 2005/0227324, the relevant portions of which are incorporated herein by reference) that may inhibit or activate the molecule to which they bind, bivalent monospecific or bispecific dimeric Fv-Fc, scFv-Fc, or diabody Fc, monospecific monovalent scFv-Fc/Fc's, and the multispecific binding proteins and dual variable domain immunoglobulins described in US Publication 2009/0311253 (the relevant portions of which are incorporated herein by reference), among many other possible antibody formats.

A **"binding region,"** as meant herein, is a region included in an "Fc-polypeptide," a "control Fc-polypeptide," or "variant Fc-polypeptide," as described herein, that binds to a target molecule, such as, for example, a protein that is expressed at high levels on a cancer cell, a cell mediating an autoimmune or inflammatory condition, an infected cell, an infectious agent, or a cell mediating an immune effector function, for example, an NK cell. A binding region can contain a heavy and/or light chain immunoglobulin variable (V_H and/or V_L) region or a non-immunoglobulin polypeptide. Exemplary binding regions include, for example, an Fv (which comprises a V_H and a V_L region joined by a linker) or a soluble form of a human receptor that binds to a target molecule.

An **"Fc-fragment,"** as meant herein, is a polypeptide that consists of part or all of a hinge region plus the C_H2 and C_H3 regions of an antibody plus, optionally, regions found downstream from the C_H3 region in some naturally occurring isotypes such as IgA or IgM antibodies. The antibody can be of the IgG isotype, including IgG1, IgG2, IgG3, or IgG4 isotypes, or of the IgM, IgE, IgD or IgA isotype. The antibody can be of human or animal origin. For example, the antibody can be from a mammal, such as a mouse, rat, hamster, rabbit, goat, or sheep, or from a camelid species or a shark. Sequences of human IgG1, IgG2, IgG3, and IgG4 Fc-fragments are disclosed in SEQ ID NOs: 1, 3, 5, and 7, respectively. These sequences are also shown in Table 1 below. In general, Fc-fragments form dimers via interactions between C_H3 domains within two Fc-fragments, which are stabilized by disulfide bonds occurring between cysteine residues in the hinge regions. An Fc-fragment referred to herein as an **"Fc region"** is specifically defined as a dimeric Fc-fragment, although it is recognized that Fc-fragments will generally dimerize under physiologic conditions. Dimers can be dissociated in strongly reducing conditions.

An **"Fc-polypeptide,"** as meant herein, is a protein that comprises an Fc-fragment and a binding region. Fc-polypeptides include antibodies, Fc fusion proteins, and antibodies or fusion proteins that contain an additional "pharmacologically active moiety," which is a non-peptide organic moiety (*i.e.,* "small molecule") or a peptide, which can act as a toxin and/or can mimic, antagonize, or agonize the activity of a biological pathway, covalently conjugated or fused to the Fc-polypeptide. Fc-

polypeptides, like Fc-fragments, ordinarily form dimers that are stabilized by disulfide bonds between cysteine residues in the hinge regions of two Fc-polypeptides.

A **"control Fc-fragment,"** as meant herein, is an Fc-fragment that is the same as the "variant Fc-polypeptide" to which it is being compared except that it does not have an insertion in a loop as the variant Fc-fragment does. Thus, the "control Fc-fragment" has unmodified sequence found in a naturally-occurring Fc-fragment in the loop where the variant Fc-fragment has an insertion that increases the affinity of the variant Fc-fragment for FcRn at pH 5 to 6. A control Fc-fragment may contain modifications relative to a naturally-occurring Fc-fragment other than the insertion(s). For example, the heterodimerizing alterations or other minor modifications described below can be present in both the variant Fc-fragment and the control Fc-fragment to which it is being compared. Hence, the only difference between a "control Fc-fragment" and a "variant Fc-fragment" is the insertion within or adjacent to a loop in the variant Fc-fragment. A **"control Fc region"** is defined a dimer comprising two control Fc-fragments, although a control Fc-fragment would generally be expected to exist in a dimeric form.

A **"variant Fc-fragment,"** as meant herein is an Fc-fragment that includes an insertion of no more than 80, no more than 60, no more than 40, or no more than 20 amino acids within or adjacent to a loop of the Fc-fragment. The loops in a human IgG Fc-fragment are shown in Table 1, and the loops into which insertions were made are shown in Figure 2. Loops in other Fc-fragments are known in the art. Numerous sequences are known in the art. *See, e.g.,* Kabat et al., Sequences of Proteins of Immunological Interest, Public Health Service N.I.H., Bethesda, MD, 1991, the portions of which relate to the sequences and tertiary structure of hinge, C_H2, and C_H3 regions are incorporated herein reference. Kabat et al. provides alignments of numerous sequences. Given the alignments provided in Kabat et al., the highly conserved structure of immunoglobulin domains, and the locations of loops provided herein, as well as abundant further information available in the art, loops could be located in an Fc-fragment of any isotype from any species. For example, the Protein Data Bank website contains abundant information on tertiary structures of many proteins. In some embodiments, the inserted amino acids can replace from one to ten amino acids that naturally occur in the loop. The inserted amino acids can be of a fewer, greater, or the same in number as the amino acids that are removed. In other embodiments, all amino acids originally in the loop remain, and the inserted amino acids are simply added. In some embodiments, a variant human IgG Fc-fragment can comprise other "additional minor modifications," that is, the insertion, deletion, or substitution of no more than sixteen amino acids at locations other than the loop in which the insertion occurs. In some embodiments, there can be no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 insertions, deletions, or substitutions of a single amino acid at locations other than the loop in which the insertion occurs. In other embodiments,

a variant Fc-fragment does not comprise any additional minor modifications. In some embodiments, the additional minor modifications can be, for example the "heterodimerizing alterations" described below, which facilitate the formation of heterodimeric Fc regions. In some embodiments, these additional minor modifications can include conservative amino acid substitutions.

Conservative amino acid substitutions include the following: (1) valine, leucine or isoleucine for alanine; (2) lysine, glutamine, or asparagine for arginine; (3) glutamine, glutamate, or aspartate for asparagine; (4) glutamate, asparagine, or glutamine for aspartate; (5) serine or alanine for cysteine; (6) asparagine, glutamate, or aspartate for glutamine; (7) aspartate, gluatamine, or asparagine for glutamate; (8) proline or alanine for glycine; (9) asparagine, glutamine, lysine, or arginine for histidine; (10) leucine, valine, methionine, alanine, or phenylalanine for isoleucine; (11) isoleucine, valine, methionine, alanine, or phenylalanine for leucine; (12) arginine, asparagine, or glutamine for lysine; (13) leucine, phenylalanine, or isoleucine for methionine; (14) leucine, valine, isoleucine, alanine, or tyrosine for phenylalanine; (15) alanine for proline; (16) threonine, alanine, or cysteine for serine; (17) serine for threonine; (18) tyrosine or phenylalanine for tryptophan; (19) tryptophan, phenylalanine, threonine, or serine for tyrosine; and (20) isoleucine, methionine, leucine, phenylalanine, or alanine for valine.

In addition, in some embodiments, a variant Fc-fragment can contain an additional "pharmacologically active moiety," which is a non-peptide organic moiety (*i.e.,* "small molecule") or a peptide, which can act as a toxin and/or can mimic, antagonize, or agonize the activity of a biological pathway, covalently conjugated or fused to the Fc-fragment. If a variant Fc-fragment contains such a pharmacologically active moiety, then its "control Fc-fragment" also contains such a moiety.

In other embodiments, the variant human IgG Fc-fragment comprises no alteration outside of those occurring in the loop at which the insertion occurs. Like an "Fc-fragment," a "variant Fc-fragment" can be of the IgG isotype, including IgG1, IgG2, IgG3, or IgG4 isotypes, or of the IgM, IgE, IgD or IgA isotype. It can be of human or animal origin. For example, an Fc-fragment can be from a mammal, such as a mouse, rat, hamster, rabbit, goat, or sheep, or from a camelid species or a shark can be modified to produce a variant Fc-fragment. As with other Fc-fragments, a variant Fc-fragment is normally dimeric, and a **"variant Fc region"** is specifically defined as a dimeric variant Fc-fragment.

A **"variant Fc-polypeptide,"** as meant herein, comprises a variant Fc-fragment and a binding region. Optionally, a variant Fc-polypeptide can contain multiple binding regions. A variant Fc-polypeptide can be a multimer, such as a dimer, trimer, tetramer, or higher order multimer. In many cases, the variant Fc-fragment portion of the Fc-polypeptide can form disulfide bonds so as to dimerize. Variant Fc-polypeptides include, for example, dimeric Fc fusion proteins, tetrameric full length antibodies (containing two heavy and two light chains), dimeric scFv-Fc's, etc. They can be

heteromultimers or homomultimers, such as heterodimers or homodimers. A variant Fc-polypeptide can be an antibody, an Fc-fusion protein, or in some embodiments an antibody or an Fc-fusion protein comprising an additional, non-antibody pharmacologically active moiety. Such a "pharmacologically active moiety" can be covalently conjugated or fused to the Fc-polypeptide and can be a non-peptide organic moiety (*i.e.,* "small molecule") or a peptide, which can act as a toxin and/or can mimic, antagonize, or agonize the activity of a biological pathway.

An **"Fc fusion protein,"** as meant herein, is a protein containing an Fc-fragment fused to another, non-antibody polypeptide. The non-antibody polypeptide comprises a binding region that binds to a target molecule and does not comprise a heavy or light chain variable region of an antibody. The binding region of an Fc fusion protein can comprise a non-antibody polypeptide such as a soluble portion of a receptor or one or more peptides that bind to a target molecule (such as, for example, a "monomer domain" as defined in US Patent 7,820,790 that binds to a target protein, which can be selected as discussed in US Patent 7,820,790), or other polypeptides. The portions of US Patent 7,820,790 describing monomer domains and how they are selected are incorporated herein by reference. In specific embodiments, an Fc fusion protein can be etanercept (ENBREL® sold by Amgen Inc.), romiplostim (NPLATE® sold by Amgen Inc.), alefacept (AMEVIVE®, Biogen Corp.), abatacept (ORENCIA® sold by Bristol –Myers Squibb), rilonacept (ARCALYST®, sold by Regeneron), or aflibercept (EYLEA™, sold by Regeneron). Other polypeptides that can be part of a binding region of an Fc fusion protein include polypeptides comprising scaffold domains that have been randomized in certain positions and subjected to selection for binding to a certain target molecule. Such scaffold domains include, for example, T-lymphocyte associated protein-4 (CTLA-4; Nuttall *et al.* (1999), Proteins 36: 217-227), the Z domain of Staphylococcal protein 1 (Nord *et al.* (1995), Protein Eng. 8: 601-608), green fluorescent protein, and the tenth type III domain of human fibronectin (FN3; Koide *et al.* (1998), J. Mol. Biol. 284: 1141-1151; Karatan *et al.* (2004), Chem. & Biol. 11: 835-844). The portions of these references describing the scaffold domains and their use to generate binding domains are incorporated herein by reference. Fc fusion proteins, like other proteins containing Fc-fragments can form multimers, which can be dimers. These multimers can be heteromultimers or homomultimers. An Fc fusion protein can be heterodimeric or homodimeric and bispecific or monospecific.

A **"variant Fc fusion protein,"** as meant herein, is an Fc fusion protein that includes a variant Fc-fragment.

A **"control Fc fusion protein,"** as meant herein, is Fc fusion protein that has the same amino acid sequence as the variant Fc fusion protein to which it is being compared except for the insertion in the loop of the Fc-fragment portion of the variant Fc fusion protein.

A **"full length IgG antibody,"** as meant herein, is an IgG antibody comprising two complete heavy chains and two complete light chains.

"Heterodimerizing alterations" generally refer to alterations in the two chains, that is, the A and B chains, of a dimeric Fc region that facilitate the formation of heterodimeric Fc regions, that is, Fc regions in which the A chain and the B chain of the Fc region do not have identical amino acid sequences. Heterodimerizing alterations can be asymmetric, that is, an A chain having a certain alteration can pair with a B chain having a different alteration. These alterations facilitate heterodimerization and disfavor homodimerization. Whether hetero- or homo-dimers have formed can be assessed by size differences as determined by polyacrylamide gel electrophoresis in situations where one polypeptide chain is an Fc-fragment (that is, lacking sequences other than the hinge, C_H2 and C_H3 regions) and the other is an scFv-Fc. One example of such paired heterodimerizing alterations are the so-called "knobs and holes" substitutions. *See, e.g.,* US Patent 7,695,936 and US Patent Application Publication 2003/0078385, the portions of which describe such mutations are incorporated herein by reference. As meant herein, an Fc region that contains one pair of knobs and holes substitutions, contains one substitution in one Fc-fragment (designated the A chain) and another in the other Fc-fragment (designated the B chain). For example, the following knobs and holes substitutions in the A and B chains of an IgG1 Fc region have been found to increase heterodimer formation as compared with that found with unmodified A and B chains: 1) Y407T in one chain and T366Y in the other; 2) Y407A in one chain and T366W in the other; 3) F405A in one chain and T394W in the other; 4) F405W in one chain and T394S in the other; 5) Y407T in one chain and T366Y in the other; 6) T366Y and F405A in one chain and T394W and Y407T in the other; 7) T366W and F405W in one chain and T394S and Y407A in the other; 8) F405W and Y407A in one chain and T366W and T394S in the other; and 9) T366W in one polypeptide of the Fc and T366S, L368A, and Y407V in the other. As meant herein, mutations are denoted in the following way. The amino acid (using the one letter code) normally present at a given position in the Fc-fragment using the EU numbering system (see Table 1 below) is followed by the EU position, which is followed by the alternate amino acid that is present at that position. For example, Y407T means that the tyrosine normally present at EU position 407 is replaced by a threonine. Alternatively or in addition to such alterations, substitutions creating new disulfide bridges can facilitate heterodimer formation. *See, e.g.,* US Patent Application Publication 2003/0078385, the portions of which describe such mutations are incorporated herein by reference. Such alterations in an IgG1 Fc region include, for example, the following substitutions: Y349C in one Fc-fragment and S354C in the other; Y349C in one Fc-fragment and E356C in the other; Y349C in one Fc-fragment and E357C in the other; L351C in one Fc-fragment and S354C in the other; T394C in one Fc-fragment and E397C in the other; or D399C in one Fc-fragment and K392C in the other. Similarly,

substitutions changing the charge of a one or more residue, for example, in the C_H3-C_H3 interface, can enhance heterodimer formation as explained in WO 2009/089004, the portions of which describe such substitutions are incorporated herein by reference. Such substitutions are referred to herein as "charge pair substitutions," and an Fc region containing one pair of charge pair substitutions contains one substitution in the A chain and a different substitution in the B chain. General examples of charge pair substitutions include the following: 1) K409D or K409E in one chain plus D399K or D399R in the other; 2) K392D or K392E in one chain plus D399K or D399R in the other; 3) K439D or K439E in one chain plus D356K or D356R in the other; and 4) K370D or K370E in one chain plus E357K or E357R in the other. In addition, the substitutions R355D, R355E, K360D, or K360R in both chains can stabilize heterodimers when used with other heterodimerizing alterations. Specific charge pair substitutions can be used either alone or with other charge pair substitutions. Specific examples of single pairs of charge pair substitutions and combinations thereof include the following: 1) K409E in one chain plus D399K in the other; 2) K409E in one chain plus D399R in the other; 3) K409D in one chain plus D399K in the other; 4) K409D in one chain plus D399R in the other; 5) K392E in one chain plus D399R in the other; 6) K392E in one chain plus D399K in the other; 7) K392D in one chain plus D399R in the other; 8) K392D in one chain plus D399K in the other; 9) K409D and K360D in one chain plus D399K and E356K in the other; 10) K409D and K370D in one chain plus D399K and E357K in the other; 11) K409D and K392D in one chain plus D399K, E356K, and E357K in the other; 12) K409D and K392D on one chain and D399K on the other; 13) K409D and K392D on one chain plus D399K and E356K on the other; 14) K409D and K392D on one chain plus D399K and D357K on the other; 15) K409D and K370D on one chain plus D399K and D357K on the other; 16) D399K on one chain plus K409D and K360D on the other; and 17) K409D and K439D on one chain plus D399K and E356K on the other. Any of the these heterodimerizing alterations can be part of a variant Fc region as described herein, which can bind to FcRn with a higher affinity than does a control Fc region.

A **"loop,"** as meant herein, is a portion of a tertiary structure in an Fc-fragment that links beta strands that make up the beta sheets of the core immunoglobulin fold. A loop may contain no more than two consecutive residues designated as being involved in a beta sheet (as in Loops 10 and 11; *see* Table 1 below) and can also contain residues designated as a helix, a turn, an isolated β bridge, or a bend. The loop regions of human IgG Fc regions, as meant herein, are shown in Table 1.

An **"insertion within a loop"** or **"within"** any stretch of amino acids, for example, an insertion within Loop 10, is an insertion of 3-35, 3-20, 6-20, 6-15, 6-12, 3-15, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 amino acids between two adjacent amino acids within a loop or between an amino acid within a loop and an adjacent amino acid that is not part of the loop. An "insertion within a loop" also encompasses situations where amino acids are removed from a loop, and other amino acids are

inserted into the loop. The number of amino acids inserted into the loop can be 3-35, 3-22, 4-20, 6-18, 6-15, 6-12, 3-15, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 amino acids, and the number of amino acids removed from the loop can be less than, the same as, or more than the number of amino acids inserted into the loop. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids can be removed from the loop. In addition, an "insertion within a loop" encompasses embodiments where more than one insertion occurs within the same loop, such as insertions between two different pairs of amino acids that are within or adjacent to a loop, as described above, or where one or more non-adjacent amino acids within the loop are deleted and replaced with fewer, the same number, or more amino acids than were deleted. Library L3 (as shown in Figure 2) is one example of such an insertion scheme.

An **"insertion within or adjacent to a loop,"** is like an "insertion within a loop," as described above, except that it also includes situations where the insertion is between a first amino acid that is adjacent to the loop and another amino acid that is adjacent to this first amino acid and is outside the loop.

A **"physiologic pH,"** as meant herein, is a pH from about 7.2-7.6.

An **"scFv –Fc/Fc"** is a dimeric protein consisting essentially of an scFv-Fc plus an Fc-fragment that are linked by one or more disulfide bonds. Further, the Fc region formed by the Fc-fragment and the scFv-Fc can contain "heterodimerizing alterations" in the C_H3 domains, such as one, two, three, or more pairs of charge pair substitutions, as described.

A **"peptide,"** as meant herein, is a short polypeptide. Generally, a peptide is from 2 to 80, 2 to 60, 2 to 50, 2 to 40, 2 to 30, or 2-20 amino acids in length.

A **"target molecule,"** as meant herein, is a molecule to which the binding region of an Fc-polypeptide, as described herein, binds. In some embodiments, a target molecule is, for example, a protein that is expressed at high levels on a cancer cell or is expressed on a cell mediating an autoimmune or inflammatory condition or on an infected cell, on an infectious agent, or on a cell mediating an immune effector function, for example, an NK cell.

A **"therapeutically effective amount"** of a variant Fc-polypeptide that has higher affinity for FcRn than a control Fc-polypeptide, as described herein, is an amount that has the effect of, for example, reducing or eliminating the tumor burden of a cancer patient or reducing or eliminating the symptoms of any disease condition that the protein is used to treat. A therapeutically effective amount need not completely eliminate all symptoms of the condition, but may reduce severity of one or more symptoms or delay the onset of more serious symptoms or a more serious disease that can occur with some frequency following the treated condition. Alternatively, a therapeutically effective amount of a protein containing a modified Fc-polypeptide is sufficient to detectably affect the expression of a relevant biomarker when it is administered to a patient in need of treatment *in vivo.*

"Treatment" of any disease mentioned herein encompasses an alleviation of at least one symptom of the disease, a reduction in the severity of the disease, or the delay or prevention of disease progression to more serious symptoms that may, in some cases, accompany the disease or lead to at least one other disease. Treatment need not mean that the disease is totally cured. A useful therapeutic agent needs only to reduce the severity of a disease, reduce the severity of one or more symptoms associated with the disease or its treatment, or delay the onset of more serious symptoms or a more serious disease that can occur with some frequency following the treated condition.

Variant Fc-polypeptides

Provided are variant Fc-polypeptides that bind to FcRn with greater affinity and/or binding activity at pHs from 5.0 to 6.0 than do control Fc-polypeptides. Such variant Fc-polypeptides, like control Fc-polypeptides, bind to FcRn with low affinity and/or binding activity, if at all, at pHs from about 7.2 to about 7.6. These variant Fc-polypeptides can (before modification) be of human or animal origin and can be of an IgG isotype, including IgG1, IgG2, IgG3, or IgG4. The FcRn can also be of human or animal origin. These variant Fc-polypeptides further comprise a binding region that can bind to a target molecule, as described herein, and can be used to treat a condition that is mediated, at least in part, by the target molecule. In some embodiments, the target molecule may not directly mediate the condition treated, but binding to the target molecule can serve to localize the Fc-polypeptide or target molecule(s) it binds to. For example, a bispecific Fc-polypeptide could bridge two different cell types, *e.g.,* a cancer cell and an immune system cell, each of which expresses a target molecule on its surface that the Fc-polypeptide can bind to. The variant Fc-polypeptides can be antibodies or Fc fusion proteins comprising a variant Fc-fragment. A variant Fc-polypeptide could, in addition, be an antibody that contains an additional, non-antibody binding region. Optionally, a variant Fc-polypeptide can contain a human IgG variant Fc-fragment.

In Table 1 below, the amino acid sequences of human IgG1, IgG2, IgG3, and IgG4 Fc-polypeptides (SEQ ID NOs: 1, 3, 5, and 7, respectively) are aligned. Sequences are numbered according to the EU system of Edelman *et al.* (1969), Proc. Natl. Acad. Sci. 63: 78-85, the relevant portions of which are incorporated herein by reference. As shown in Table 1, a single number can be used to refer to a position that is analogous in all four human IgG isotypes, even though a linear numbering scheme would force one to name a different number for this position in each isotype. For example, the asparagine at position 297 is a well-known and highly conserved glycosylation site present in all human IgG antibodies that is referred to as asparagine 297, regardless of the human IgG isotype under discussion.

Human IgG3 antibodies have a much longer hinge region than other human IgG isotypes, and only the most carboxy-terminal portion of this hinge region is shown in Table 1. The human IgG1, IgG2, and IgG4 sequences shown in Table 1 include the entire hinge region. As is apparent in Table 1, the hinge region of a human IgG4 antibody is three amino acids shorter than that of an IgG1 antibody. Human IgG2 antibodies have a slightly shorter hinge region and a gap of one amino acid near the amino terminal end of the C_H2 region as compared to human IgG1. Amino acids 216-447 shown in the IgG1 line and amino acids 224-447 in the IgG4 line of Table 1 correspond to amino acids 1-232 of SEQ ID NO:1 and 6-229 of SEQ ID NO:7, respectively. Amino acids 216 to 447 in the IgG3 line of Table 1 correspond to amino acids 48-279 of SEQ ID NO:5. Amino acids 237 to 447 in the IgG2 line of Table 1 correspond to amino acids 18 to 228 of SEQ ID NO:3.

In Table 1, amino acids that are located in loops, as meant herein, are shown in boldface and are underlined. Each loop is labeled with a number, *i.e.,* "Loop 1," "Loop 2," etc., beneath it. In more detail, loops occur the following locations: Loop 1, amino acids 29-43 of SEQ ID NO:1, amino acids 25-39 of SEQ ID NO:3, amino acid 76-90 of SEQ ID NO:5, and amino acids 26-40 of SEQ ID NO:7; Loop 2, amino acids 50-58 of SEQ ID NO:1, amino acids 46-54 of SEQ ID NO:3, amino acids 97-105 of SEQ ID NO:5, and amino acids 47-55 of SEQ ID NO:7; Loop 3, amino acids 70-72 of SEQ ID NO:1, amino acids 66-68 of SEQ ID NO:3, amino acids 117-119 of SEQ ID NO:5, and amino acids 67-69 of SEQ ID NO:7; Loop 4, amino acids 80-84 of SEQ ID NO:1, amino acids 76-80 of SEQ ID NO:3, amino acids 127-131 of SEQ ID NO:5, and amino acids 77-81 of SEQ ID NO:7; Loop 5, amino acids 92-103 of SEQ ID NO:1, amino acids 88-99 of SEQ ID NO:3, amino acids 139-150 of SEQ ID NO:5, and amino acids 89-100 of SEQ ID NO:7; Loop 6, amino acids 110-116 of SEQ ID NO:1, amino acids 106-112 of SEQ ID NO:3, amino acids 157-163 of SEQ ID NO:5, and amino acids 107-113 of SEQ ID NO:7; Loop 7, amino acids 122-131 of SEQ ID NO:1, amino acids 118-127 of SEQ ID NO:3, amino acids 169-178 of SEQ ID NO:5, and amino acids 119-128 of SEQ ID NO:7; Loop 8, amino acids 137-146 of SEQ ID NO:1, amino acids 133-142 of SEQ ID NO:3, amino acids 184-193 of SEQ ID NO:5, and amino acids 134-143 of SEQ ID NO:7; Loop 9, amino acids 158-162 of SEQ ID NO:1, amino acids 154-158 of SEQ ID NO:3, amino acids 205-209 of SEQ ID NO:5, and amino acids 155-159 of SEQ ID NO:7; Loop 10, amino acids 169-175 of SEQ ID NO:1, amino acids 165-171 of SEQ ID NO:3, amino acids 216-222 of SEQ ID NO:5, and amino acids 166-172 of SEQ ID NO:7; Loop 11, amino acids 179-188 of SEQ ID NO:1, amino acids 175-184 of SEQ ID NO:3, amino acids 226-235 of SEQ ID NO:5, and amino acids 176-185 of SEQ ID NO:7; Loop 12, amino acids 199-207 of SEQ ID NO:1, amino acids 195-203 of SEQ ID NO:3, amino acids 246-254 of SEQ ID NO:5, and amino acids 196-204 of SEQ ID NO:7; Loop 13, amino acids 214-221 of SEQ ID NO:1, amino acids 210-217 of SEQ ID NO:3, amino acids 261-268 of SEQ ID NO:5, and amino acids 211-218 of SEQ ID NO:7.

Table 1: Alignment of human IgG Fc regions (alignment discloses SEQ ID NOS 1 and 3, residues 48-279 of SEQ ID NO: 5, and SEQ ID NO: 7, respectively, in order of appearance)

```
                225       235       245       255       265       275
                 *         *         *         *         *         *
IgG1  EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF
IgG2  ERKCCVE---CPPCPAPPVA-GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQF
IgG3  EPKSCDTPPPCPRCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQF
IgG4  ESKYG---PPCPSCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQF
DSSP      S SS S  S      SSS EEEEE   HHHHH TTS   EEEEEE   TT    EE
                                        Loop 1              Loop2

                285       295       305       315       325       335
                 *         *         *         *         *         *
IgG1  NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKT
IgG2  NWYVDGMEVHNAKTKPREEQFNSTFRVVSVLTVVHQDWLNGKEYKCKVSNKGLPAPIEKT
IgG3  KWYVDGVEVHNAKTKPREEQYNSTFRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKT
IgG4  NWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKT
DSSP  EEEETTEEE   EEEEEEE TTS EEEEEEE   HHHHHHT  EEEEEE TTSSS EEEE
                    Loop 3     Loop 4         Loop 5          Loop 6

                345       355       365       375       385       395
                 *         *         *         *         *         *
IgG1  ISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
IgG2  ISKTKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
IgG3  ISKTKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESSGQPENNYNTTP
IgG4  ISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
DSSP  E       B   EEEEE   GGGGGSSEEEEEEEEEEEBSS  EEEEEETTEE   EEE
       Loop 7           Loop 8            Loop 9      Loop 10

                405       415       425       435       445
                 *         *         *         *         *
IgG1  PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
IgG2  PMLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
IgG3  PMLDSDGSFFLYSKLTVDKSRWQQGNIFSCSVMHEALHNRFTQKSLSLSPGK
IgG4  PVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGK
DSSP   EE TTS EEEEEEEEEEHHHHHTT  EEEEEE TTSGGG EEEEE   S
       Loop 11           Loop 12       Loop 13
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The markings in the fifth line of each aligned group, designated "DSSP" (which stands for Dictionary of Protein Secondary Structure) were taken from the 1HZH (chain H) structure available from the Protein Data Bank, have the following meanings: "G" designates a 3-turn helix (3_{10} helix), which has a minimum length 3 residues; "H" designates a 4-turn helix (α helix), which has a minimum length of 4 residues; "I" designates a 5-turn helix (π helix), which has a minimum length of 5 residues; "T" designates a hydrogen bonded turn (3, 4 or 5 turn); "E" designates an extended strand in parallel and/or anti-parallel β-sheet conformation, which has a minimum length of 2 residues; "B" designates a residue in an isolated β-bridge (single pair β-sheet hydrogen bond formation); "S" designates a bend (the only non-hydrogen-bond based assignment). Amino acid residues that are not in any of the above

conformations are assigned a blank space in the "DSSP" line. These designations are standard in the art and available in the website for the Protein Data Bank (PDB).

The hinge region of the human IgG Fc-fragments runs from amino acid 216 to 230 as numbered in Table 1. The C_H2 region extends from amino acid 231 to 340, and the C_H3 region extends from amino acid 341 to 447. It is apparent from the alignment of Table 1 that the sequences of the human IgG Fc-fragments are highly conserved. Most of the sequence differences occur in the hinge region, and very few sequence differences occur in the C_H2 and C_H3 regions. Thus, results obtained using variant Fc-polypeptides comprising a human IgG variant Fc-fragment comprising insertions in the C_H2 or C_H3 region of one human IgG subtype will likely be applicable to other human IgG subtypes.

The insertions in the variant human IgG Fc-polypeptides described herein can occur at locations known to be in loops. The insertions can occur within or adjacent to any of Loops 1-13 (as shown in Table 1 above) of an Fc-fragment portion of the Fc-polypeptide. In some embodiments, insertions of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 or 1-18, 6-16, 10-16, 12-14, 3-20, 20-30, 30-50, or 50-80 amino acids can be made between two adjacent amino acids, at least one of which is included in a loop of an Fc-fragment. In some embodiments, two cysteines are non-randomly included in the insertion, one each among the first and last four amino acids of the insertion, so as to constrain the amino acids between the cysteines into a tight loop bounded by a disulfide bond. In some embodiments the first three amino acids of the insertion are Gly-Gly-Cys, and the last three amino acids are Cys-Gly-Gly. An insertion of an additional 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, or 10-30 amino acids can be inserted between these two cysteines. In still other embodiments, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids from the loop are deleted and replaced by an insertion of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 or 1-18, 6-16, 10-16, 12-14, 15-30, 30-50, or 50-80 amino acids and/or by an insertion that contains two cysteines among the first and last four amino acids of the insertion. The number of amino acids deleted can be the same, less, or greater in number than the amino acids inserted. In other embodiments, more than one insertion can occur in a single loop. The variant Fc-polypeptides, which contain the insertions, can bind to human FcRn with a higher affinity at pH 5 to 6 than does a control Fc-polypeptide. The inserted amino acids can include any of all 20 amino acids or any of all amino acids other than cysteine. Further, a variant Fc-fragment can comprise an insertion in only one loop or in more than one loop, such as in 2, 3, or 4 loops.

Insertions within or adjacent to Loops 5, 8, or 10 of an Fc-fragment can increase the binding affinity of such a variant Fc-fragment for human FcRn at pH 5 to 6. In specific embodiments, an insertion can be made in a human IgG Fc-fragment within or adjacent to Loop 8 between amino acids 358 and 359 or within or adjacent to Loop 10 between amino acids 384 and 385 (using the EU numbering scheme as illustrated in Table 1). The insertion can contain from 4-20, 1-18, 6-16, 10-16,

12-14, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 or amino acids. Optionally, the amino acids may be limited to all amino acids other than cysteine. In some embodiments, the insertion can contain a cysteine among the first and the last four amino acids of the insertion, and the inserted amino acids can otherwise be limited to amino acids other than cysteine. In some embodiments, the insertion can contain no cysteines. In some embodiments, the insertion can have a formula selected from the group consisting of CXXXXXXC (SEQ ID NO:13), CXXXXXXXC (SEQ ID NO:14), CXXXXXXXXC (SEQ ID NO:15), GCXXXXXXCG (SEQ ID NO:16), GCXXXXXXXCG (SEQ ID NO:17), GCXXXXXXXXCG (SEQ ID NO:18), GGCXXXXXXCGG (SEQ ID NO:19), GGCXXXXXXXCGG (SEQ ID NO:20), and GGCXXXXXXXXCGG (SEQ ID NO:21), GGGCXXXXXXCGGG (SEQE ID NO:22), GGGCXXXXXXXCGGG (SEQ ID NO:23), and GGGCXXXXXXXXCGGG (SEQ ID NO:24), where X represents any amino acid except cysteine. Since the actual insertion results from a screening process as described in the examples below, it is the randomized amino acids that likely play a dominant role in the observed changes in the properties of the Fc-fragment. As such, it is contemplated that a variant Fc-fragment having an insertion containing only the randomized portions of insertions which, like those above, contain some non-random amino acids can also have the desired properties. Examples of such sequence include the middle six amino acids from SEQ ID NOs: 41-53, which are shown as SEQ ID NOs: 54-66.

In another embodiment, amino acids 308 and 309 (using the EU numbering as shown in Table 1) within or adjacent to Loop 5 are deleted and replaced from 4-20, 1-18, 6-16, 10-16, 12-14, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 15-20, 20-40, 40-60, or 60-80 amino acids, which may be limited to all amino acids other than cysteine. In some embodiments, the insertion can contain a cysteine among the first and the last four amino acids of the insertion, and the inserted amino acids can otherwise be limited to amino acids other than cysteine. The insertion can have a formula selected from the group consisting of SEQ ID NO:13-24.

These variant Fc-fragments, as well as Fc-polypeptides that contain them, comprising the amino acid insertions described above and below, can bind to human FcRn at pH 5 to 6 with higher affinity and/or binding activity as compared to a control Fc-fragment and can bind to human FcRn at physiologic pH with an affinity and/or binding activity comparable to or lower than that of control Fc-fragments. Further, these variant Fc-fragments can be further altered and screened for alterations that confer even higher affinity and/or binding activity for human FcRn at pH 5 to 6, lower affinity and/or binding activity for human FcRn at physiologic pH, or other desirable properties, such as stability upon storage. In the Examples below, insertions of particular amino acid sequences (*e.g,* SEQ ID NOs:41-66 and 90-246) at defined sites within an Fc-fragment are shown to be effective at increasing affinity and/or binding activity of the Fc-fragment for human FcRn at pH 5 to 6.

Variant Fc-polypeptides comprising human IgG variant Fc-fragments also comprise a binding region that binds to a target molecule. Binding of such variant Fc-polypeptides to the target molecule can modulate, optionally antagonize or agonize, the biological activity of the target molecule and/or can serve to localize the Fc-polypeptide to a location where the target molecule is expressed. An Fc-polypeptide can include more than one binding region, such as two, three, or four binding regions in an Fc-polypeptide. Since an Fc-polypeptide can multimerize forming, in some cases, dimers, trimers, or tetramers, multimerization of different Fc-polypeptides can form multimeric Fc-polypeptides that can bind to more than one target molecule. If there are plural binding regions in an Fc-polypeptide, they can bind to the same or different target molecules or portions of a target molecule. Modulation of the activity of the target molecule can affect the course of a disease or condition that is mediated at least in part by the target molecule. Human IgG variant Fc-polypeptides can comprise a binding region that includes one or more antibody variable regions that bind to one or more target molecules. Such human IgG variant Fc-polypeptides can, for example, be full length antibodies, such as human, humanized, or chimeric IgG antibodies, scFc-Fv's, monovalent forms of antibodies such as those described in International Application Publication WO 2005/063816 and US Application Publication 2007/0105199 (the relevant portions of which are incorporated herein by reference), which comprise a dimeric Fc region, as well as V_H, C_H1, V_L, C_L and hinge regions. Further forms are described, for example, in Figure 2 of US Application Publication 2010/0286374, which is incorporated herein by reference along with the text describing and/or referring to Figure 2, and in US Patent 5,837,821, which describes "minibodies" comprising an scFv linked to an Fc region. The portions of US Patent 5,837,821 describing minibodies are incorporated herein by reference. Alternatively, the binding region can comprise all or part of a non-antibody protein, optionally a human protein, that binds to the target molecule. For example, the binding region could comprise an extracellular portion a receptor such as, for example, the extracellular region of the human p75 tumor necrosis factor receptor (SEQ ID NO:13) or the human T-lymphocyte associated protein-4 (CTLA4) protein (SEQ ID NO:14).

The binding region can comprise one or more peptides that bind to a target molecule (such as, for example, a "monomer domain" as defined in US Patent 7,820,790 that binds to a target protein, which can be selected as discussed in US Patent 7,820,790), or other peptides. The portions of US Patent 7,820,790 describing monomer domains and how they are selected are incorporated herein by reference. One example of such a peptide is the binding portion of the Fc fusion protein romiplostim (NPLATE®, Amgen Inc., Thousand Oaks, California). Prescribing Information, NPLATE®, Amgen Inc., 2008-2011. Other polypeptides that can be part of a binding region of an Fc fusion protein include polypeptides comprising scaffold domains that have been randomized in certain positions and subjected to selection for binding to a certain target molecule. Such scaffold domains include, for

example, CTLA-4 (Nuttall *et al.* (1999), Proteins 36: 217-227), the Z domain of Staphylococcal protein 1 (Nord *et al.* (1995), Protein Eng. 8: 601-608), green fluorescent protein, and the tenth type III domain of human fibronectin (FN3; Koide *et al.* (1998), J. Mol. Biol. 284: 1141-1151; Karatan *et al.* (2004), Chem. & Biol. 11: 835-844). The portions of these references that describe the scaffold domains and their use to generate binding domains are incorporated herein by reference.

A target molecule, to which a binding region binds, can be a molecule where the modulation of the biological activity of the molecule can affect the course of a disease or condition or can be a molecule localized in a diseased area, for example a protein expressed on the surface of cancer cells. In an autoimmune or inflammatory condition, the target molecule can be a molecule acting in a pathway that plays a role in mediating the condition. A target molecule can be a molecule that is localized such that binding to the target molecule will place an Fc-polypeptide appropriately such that it can affect the course of a disease. For example, if an Fc-polypeptide includes a toxin, placing it near a cancer cell by means of a binding region that binds to a protein highly expressed on the cancer cells can be advantageous. Target molecules include, for example, general classes of proteins such as soluble ligands, receptor bound ligands, soluble receptors, membrane bound receptors, membrane channel proteins, soluble and membrane bound proteins, and non-protein antigens, including extracellular and intracellular target molecules. Exemplary target molecules include, without limitation, the following human proteins: tumor necrosis factor, tumor necrosis factor receptor, interleukin-1, interleukin-6 (IL-6), IL-6 receptor, CD80/86, CD20, CD33, CD52, interleukin-12, interleukin-23, interleukin-17, HER2, HER2 neu receptor, epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), B cell activating factor (BAFF), RANK ligand, OR51E2, claudins, CDH3, CD22, complement factors, and sclerostin, among many others.

Depending in part on the host cell in which a variant Fc-polypeptide is produced and/or the purification methods used, the variant Fc-polypeptide can further comprise additional amino acid sequences such as, for example, a signal sequence facilitating secretion in eukaryotic cells (which is removed in mature forms of the protein), an N-terminal methionine to facilitate translation in bacteria, and/or a tag sequence (for example, a polyhistidine tag or a FLAG® tag) to facilitate protein purification or identification.

Nucleic Acids Encoding Libraries of Variant Fc-fragments

Also described herein are libraries of nucleic acids encoding Fc-fragments having insertions, which are at least partially randomized, that occur within or adjacent to loops of the Fc-fragments. Figure 2 illustrates the format for each of the eight libraries that were made, as described below in the Examples. The sequences of these libraries and the amino acid sequences encoded by them are

disclosed in SEQ ID NOs:25-40. Such libraries are useful for panning in order to select individual nucleic acids that encode variant human IgG Fc-fragments that bind with enhanced affinity to FcRn at pH 5.0 to 6.0 as compared to a control Fc-fragment. Such variant human IgG Fc-polypeptides may also bind to FcRn poorly if at all at pH 7.2 to 7.6, as does a control Fc-fragment.

In some embodiments, these libraries of nucleic acids encoding Fc-fragments with insertions in regions encoding loop regions (as set forth in Table 1) can be of human or animal origin, including nucleic acids encoding mouse, rat, rabbit, or monkey Fc-fragments or Fc-fragments from a camelid species. In some embodiments the encoded Fc-fragment can be an IgG Fc-fragment, such as an IgG1, IgG2, IgG3, or IgG4 Fc-fragment, or an Fc-fragment of an IgA, IgE, IgD, or IgM isotype. The insertions in the portions of the nucleic acids encoding loops can be within or adjacent to the nucleotide sequences encoding Loop 1, Loop 2, Loop 3, Loop 4, Loop 5, Loop 6, Loop 7, Loop 8, Loop 9, Loop 10, Loop 11, Loop 12, or Loop 13, as shown in Table 1.

The size of the library as compared to the number of different variants in the library is an important consideration. In a best case, the panning procedure to which the library is subjected will have a high probability of including all different variants that are included in the library. The number of different variants in the library depends on the number of randomized positions in the library and the number of different amino acids that can be used at each randomized position. For example, a library that has six randomized positions that can contain any of 19 different amino acids can theoretically have $19^6 \approx 4.7 \times 10^7$ different variants. If, for example, 10^9 independent phage or bacteria expressing these variants are panned, there is a very high probability that all isolates with the properties selected for in the screen will be recovered. *See, e.g.,* Grossman and Turner, Mathematics for the Biological Sciences, Chapter 2, Section 2.9, pp. 97-104, Macmillan Publishing Co., Inc., New York and Collier Macmillan Publishers, London, 1974, which section is incorporated herein by reference. Libraries of a size that make it possible to screen enough isolates to detect rare isolates with the desired properties are contemplated. Thus, libraries encoding from 10^5 to 10^{13}, 10^6 to 10^{12}, or 10^7 to 10^{10} different variants are contemplated.

Libraries with randomized insertions within or adjacent to nucleotide sequences encoding Loops 5, 8, and 10 of the Fc-fragment-encoding portion can be advantageous. Such libraries can encode Fc-fragments with insertions within or adjacent to Loops 5, 8, or 10 that contain 20-40, 4-20, 1-18, 6-16, 10-16, 12-14, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 randomized amino acids. These randomized amino acids can be flanked by cysteine residues. In some embodiments, additional non-randomized amino acids can also be inserted before or after the randomized amino acids. In some embodiments, the encoded randomized amino acids can be preceded by a Gly-Gly-Cys and followed by a Cys-Gly-Gly.

Nucleic Acids Encoding Variant Fc-polypeptides, Fc-fragments, and Insertions

Also described are nucleic acids encoding specific variant Fc-fragments, which can be part of a variant Fc-polypeptide, and nucleic acids encoding insertions within or adjacent to loops of variant Fc-fragments. Nucleotide sequences encoding Fc-fragments are known in the art (*see, e.g.,* Kabat et al., Sequences of Proteins of Immunological Interest, Public Health Service N.I.H., Bethesda, MD, 1991), and sequences encoding human IgG1, IgG2, IgG3, and IgG4 Fc-fragments (SEQ ID NOs: 2, 4, 6, and 8, respectively) are reported herein. The locations of loops within Fc-fragments are delineated (Table 1), and various kind of insertions into these regions are described herein. Sequences encoding amino acid insertions that increase binding to FcRn at pHs in the range of 5 to 6 are also reported (*see, e.g.,* SEQ ID NOs:41-53).

More generally, described herein are nucleic acids encoding a variant Fc-fragment having an insertion within or adjacent to a loop, wherein the Fc-fragment can bind to human FcRn with higher affinity at pH 5-6 than a control Fc-fragment. The positions at which such insertions occur in include Loops 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or 13 withn the Fc-polypepetide.

Nucleic acids encoding variant Fc-polypeptides can be used to produce variant Fc-polypeptides by introducing the nucleic acids into host cells, including prokaryotic or eukaryotic host cells, and culturing the cells under conditions such that the protein(s) encoded by the nucleic acids will be expressed. The variant Fc-polypeptide can be recovered from the cell mass or the culture supernatant. Such polypeptides can be purified by methods well known in the art such as, for example, Protein A or Protein G affinity chromatography, size exclusion chromatography, ion exchange chromatography, and hydrophobic interaction chromatography, among other available methods.

Methods of Isolating and Making Variant Fc-fragments and Fc-polypeptides

Variant Fc-fragments can be isolated using screening procedures described in detail in the Examples below. In broad terms, a library of nucleic acids encoding Fc-fragments with insertions including randomized amino acids within or adjacent to a loop is prepared. This library is then introduced into a virus or a cell, which is cultured under conditions such that the nucleic acids from the library can be expressed, so that the virus (which can, for example, be a baculovirus or a filamentous phage) or cell (which can, for example, be a bacterial, yeast, or mammalian cell), can display the Fc-fragments containing the insertions. In an alternate embodiment, the library can be translated *in vitro* in a ribosome display system such that Fc-fragments with the desired properties, and the nucleic acids encoding them, can be selected out from this mixture. Alternatively, a CIS display system can be used.

See, e.g., Odegrip *et al.* (2004), Proc. Natl. Acad. Sci. 101(9): 2806-2810, the relevant portions of which are incorporated herein by reference. In the methods of the invention, variant Fc-fragments that can bind to human FcRn with higher affinity as compared to a control Fc-fragment within the pH range from 5 to 6 can be enriched for by panning with FcRn as described in the examples below and screened for using, for example, enzyme-linked immunosorbent assay (ELISA), which may be performed initially on groups of isolates or on single isolates. If ELISA is initially performed on groups of isolates, these isolates can be amplified and rescreened individually to obtain individual isolates expressing a variant Fc-fragment with enhanced affinity to human FcRn at pH 5-6 and with lower or about the same affinity for human FcRn at physiologic pH as compared to a control Fc-fragment.

In further steps, nucleic acids encoding the variant Fc-fragments can be isolated from phage or cells, such as bacteria, yeast or mammalian cells, or amplified from RNA in the case of ribosome display. These nucleic acids can be sequenced to determine the exact position and nature of the insertion. Using this information, nucleic acids encoding a desired variant Fc-polypeptide or variant Fc-fragment can be constructed using methods well-known in the art. Further modification of the nucleic acid encoding the variant Fc-fragment or Fc-polypeptide can be undertaken if desired.

Such nucleic acids can be inserted into vectors appropriate for the host cell in which the Fc-polypeptide or Fc-fragment will be expressed. These nucleic acids can be introduced into the host cells by any of the methods well-known in the art. Host cells that could be used include bacteria, including *Escherichia coli,* yeast, including *Saccharomyces cerevisiae or Pichia pastoris,* insect cells, plant cells, and mammalian cells, including Chinese hamster ovary (CHO) cells, 293 cells, and baby hamster kidney (BHK) cells, among many others. These host cells can be cultured under conditions such that the introduced nucleic acids will be expressed, and the Fc-polypeptide or Fc-fragment can be recovered from the culture supernatant or the cell mass.

Generally, the procedure used to introduce the nucleic acids into the host cells may depend upon the host cell into which the nucleic acids are to be introduced. Methods of introducing nucleic acids into bacteria are well-known in the art. For example, electroporation or calcium choride transformation are commonly used. Methods for introduction of nucleic acids into yeast are well-known in the art and include, for example, transformation methods using lithium acetate and polyethylene glycol. Methods for introducing heterologous polynucleotides into mammalian cells are well known in the art and include, but are not limited to, dextran-mediated transfection, calcium phosphate precipitation, polybrene mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the DNA into nuclei.

Expression vectors used in any of the host cells will contain sequences for plasmid maintenance and for cloning and expression of exogenous nucleotide sequences. Such sequences will

typically include one or more of the following nucleotide sequences: a promoter, one or more enhancer sequences, an origin of replication, a transcriptional termination sequence, a complete intron sequence containing a donor and acceptor splice site, a sequence encoding a leader sequence for polypeptide secretion, a ribosome binding site, a polyadenylation sequence, a polylinker region for inserting the nucleic acid encoding the polypeptide to be expressed, and a selectable marker element.

Therapeutic Uses of Variant Fc-polypeptides

Variant Fc-polypeptides described herein can be used as human therapeutics for a variety of conditions. Which variant Fc-polypeptide is appropriate for which condition can be determined by the binding region(s) of the Fc-polypeptide, as well as possibly other aspects of its structure such as, for example, an attached toxic moiety. Variant Fc-polypeptides can have increased *in vivo* half lives as compared to control Fc-polypeptides and may therefore require lower and/or less frequent dosing than control Fc-polypeptides. Thus, variant Fc-polypeptides might be well suited to treat chronic conditions, where less frequent dosing is particularly desirable. However, a variant Fc-polypeptide can be used as a treatment of most or all conditions that a control Fc-polypeptide (which has the same amino acid sequence as the variant Fc-polypeptide except that it does not have the insertion in a loop) can be used to treat. The variant Fc-polypeptides described herein can also be used concurrently with other medications used for treating the condition being treated.

For example, an Fc fusion protein that is a variant Fc-polypeptide which contains an insertion in a loop can be used as a treatment for the same conditions that a control Fc-polypeptide is used. However, dosing amount and/or frequency of the variant Fc-polypeptide may be different because of its increased half-life. For example, a variant Fc-polypeptide could be made starting from a therapeutic Fc fusion protein such as etanercept (which is indicated for moderate to severe rheumatoid arthritis, polyarticular juvenile idiopathic arthritis, psoriatic arthritis, ankylosing spondylitis, and moderate to severe plaque psoriasis), abatacept (which is indicated for moderate to severe rheumatoid arthritis, moderate to severe polyarticular juvenile idiopathic arthritis), or romiplostim (which is indicated for chronic immune thrombocytopenic purpura) by inserting one of the insertions that enhance FcRn binding disclosed herein, *e.g.,* SEQ ID NOs: 41-53, in a loop in the Fc-fragment portion of each of these molecules. The insertion could, for example, be made within or adjacent to Loops 5, 8, or 10 of the Fc-fragment portion of the Fc fusion protein. Such variant forms of etanercept , abatacept, or romiplostim could be used to treat the same diseases as unaltered etanercept, abatacept, or romiplostim can be used to treat.

Similarly, if the variant Fc-polypeptide is a therapeutic antibody, such as, for example, adalimumab, ustikinumab, golimumab, natalizumab, infliximab, or denosumab, such a variant form of

the antibody comprising a variant Fc-fragment can be used to treat the same diseases that these antibodies, in an unaltered form, are used to treat. Thus, a variant Fc-fragment, as described herein, can change the dosage amount or frequency of treatment, but not the condition the Fc-polypeptide is used to treat.

Generally, Fc-polypeptides are used to treat a wide variety of diseases including oncologic indications, autoimmune and inflammatory conditions, bone-related conditions, conditions, metabolic conditions, and neurologic conditions such as, for example, chronic pain, among many others.

Pharmaceutical Compositions

The invention includes pharmaceutical compositions comprising the variant Fc-fragments or variant Fc-polypeptides described herein. Such compositions can comprise a therapeutically effective amount of a variant Fc-polypeptide or variant Fc-fragment with one or more additional components such as a physiologically acceptable carrier, excipient, or diluent. Such additional components can include buffers, carbohydrates, polyols, amino acids, chelating agents, stabilizers, and/or preservatives, among many possibilities.

Methods of Administration

The variant Fc-polypeptides or variant Fc-fragments, or pharmaceutical compositions containing these molecules, can administered by any feasible method. Therapeutics that comprise a protein will ordinarily be administered by injection since oral administration, in the absence of some special formulation or circumstance, would lead to hydrolysis of the protein in the acid environment of the stomach. Subcutaneous, intramuscular, intravenous, intraarterial, intralesional, or peritoneal injection are possible routes of administration. Topical administration is also possible, especially for diseases involving the skin. Alternatively, variant Fc-polypeptides or variant Fc-fragments can be administered through contact with a mucus membrane, for example by intra-nasal, sublingual, vaginal, or rectal administration or as an inhalant. Alternatively, certain pharmaceutical compositions comprising a variant Fc-polypeptide or variant Fc-fragment can be administered orally.

Having described the invention in general terms above, the following examples are offered by way of illustration and not limitation.

EXAMPLES

Example 1: Creation of a model of the tertiary structure of human FcRn:human IgG1 Fc

To aid design of the libraries described below, a homology model of the tertiary structure a human FcRn in a complex with a human IgG1 Fc-fragment was created based on the structure of a complex of a rat FcRn and a rat IgG Fc-fragment available in Protein Data Bank (PDB Accession No. 1FRT). The Protein Data Bank (PDB) was searched with amino acid sequences of human IgG1 Fc-fragment (SEQ ID NO:1) and human FcRn α and β-2-microglobulin (β2m) chains, SEQ ID NOs:9 and 10, respectively. Several templates were obtained based on amino acid sequence homology and tertiary structure. FcRn α chain structures (1EXU (chain A), 1FRT (chain A), and 1I1A (chain A)), FcRn β2m chain structures (1CE6 (chain B), 1HSA (chain B), 1YPZ (chain B), 1HHG (chain B) and 1I1A (chain B)), and Fc structures 1HZH (chain H), 1I1A (chain C), 1OQO (chain A), 1T83 (chain A) and 2J6E (chain A)) were selected. The FcRn α structures 1EXU (chain A), 1FRT (chain A) and 1I1A (chain A) were superimposed and the resulting root mean square deviation (RMSD), that is, the average distance between analogous atoms in superimposed protein structures, values are 0.30 Å to 0.76 Å, with the mean value 0.59 Å. The RMSD values between the FcRn β2m domain structures 1CE6 (chain B), 1HSA (chain B), 1YPZ (chain B), 1HHG (chain B), and 1I1A (chain B) range from 0.30 Å to 0.76 Å with the mean value 0.59 Å. The RMSD values between the Fc structures 1HZH (chain H), 1I1A (chain C), 1OQO (chain A), 1T83 (chain A) and 2J6E (chain A) range from 0.30 Å to 0.76 Å with the mean value 0.59 Å. These RMSD values indicate that the template FcRn and Fc structures from different sources share very similar tertiary structures.

Based on these template structures and the amino acid sequences of human FcRn (hFcRn) and human IgG1 Fc-fragment (hIgG1 Fc), a homology model of hFcRn:hIgG1 Fc complex was created using a linux-based molecular modeling module of the computational software Molecular Operating Environment (MOE; Chemical Computing Group, Montreal, Quebec, Canada). Figure 1 shows the portion of this modeled complex where the hIgG1 Fc (below) and the hFcRn (above) come into closest contact, as well as some adjacent areas of these proteins.

Example 2: Construction of the insertion libraries

Eight libraries encoding variant human IgG1 Fc regions, each library having different kinds of insertions, were constructed. Figure 2 shows the sequence of a human IgG1 Fc region and the positions and the format of the six insertion libraries. The positions of the loops into which insertion libraries described below were inserted are indicated in Figures 1 and 2 as L1, L2A, L2B, L3, L4, L5, L6A and L6B. Some insertion sites in the Fc were among the closest points of contact between hIgG1

Fc and hFcRn (such as L1, L2A, L2B, and L3), and others (L4, L5, L6A, and L6B) were at some distance from the closest points of contact between hFc and hFcRn.

As shown in Figure 2, in some of the libraries, such as L1, L2A, L2B, and L3, amino acids within a loop were deleted and replaced with randomized amino acids, in most cases with a slightly greater number of amino acids than had been deleted. The randomized amino acids included all amino acids other than cysteine. In library L2B, six randomized amino acids were preceded by the sequence Gly-Gly-Cys and followed by the sequence Cys-Gly-Gly. Since the two cysteines would be expected to form a disulfide bridge given their proximity, it would be expected that the six randomized amino acids between the cysteines would form a spatially constrained loop. For example, in L2B, six randomized amino acids were preceded by the sequence Gly-Gly-Cys and followed by the sequence Cys-Gly-Gly, thus creating spatially constrained loops containing the randomized amino acids. In other libraries, *i.e.,* L4 and L5, randomized amino acids were inserted without deleting any amino acids normally present.

Two rounds of PCR were used to construct L1, L2A, L2B, L3, L4 and L5 libraries encoding a group of human IgG1 variant Fc-fragments. The template used in the first and second sets of PCR reactions in the first round of reactions was a phagemid vector into which DNA encoding a human IgG1 Fc-fragment (SEQ ID NO:2) had been inserted so as to allow its expression as part of a phage coat protein in appropriate bacterial strains (pIgG1-Fc). The first set of PCR reactions in the first round of reactions used to generate the libraries utilized forward and reverse primers in order to create PCR fragments that contain an ApaLI restriction enzyme site at the upstream end. The following forward primer, which matched vector sequences upstream of the region encoding the Fc-fragment, was used for all of the first set of PCR reactions in the first round of reactions: 5'-GTTCCT TTC TAT TCTCAC-3' (SEQ ID NO:521). The reverse primers, which were within Fc-encoding sequences, were the following: 5'-GAG GGT GTC CTT GGG TTT TGG GGG-3' (SEQ ID NO:522; Library L1); 5'-GGT GAG GAC GCT GAC CAC ACG GTA-3' (SEQ ID NO:523; Libraries L2A and L2B); 5'-ATG CAT CAC GGA GCA TGA GAA GAC-3' (SEQ ID NO:524; Library L3); 5'-ATT ATG CAC CTC CAC GCC GTC CAC-3' (SEQ ID NO:525; Library 4); and 5'-ATT GCT CTC CCA CTC CAC GGC GAT-3' (SEQ ID NO:526; Library L5).

A second set of PCR reactions in the first round of reactions also used pIgG1-Fc as a template and used an oligonucleotide that matched the complement of vector sequences downstream from the Fc-encoding sequences as a reverse primer. This reverse primer had the following sequence: 5'-CCC ATT CAG ATC CTC TTC-3' (SEQ ID NO:527). The forward primers used for these reactions were as follows: Library L1, 5'-AAA CCC AAG GAC ACC CTC $(TRIM)_6$ ACC CCT GAG GTC ACA TGC-3' (SEQ ID NO:528); Library L2A, 5'-GTG GTC AGC GTC CTC ACC $(TRIM)_6$ CAC CAG GAC TGG CTG AAT-3' (SEQ ID NO:529); Library L2B, 5'-GTG GTC AGC GTC CTC ACC GGT GGT TGT $(TRIM)_6$ TGT

GGT GGT CAC CAG GAC TGG CTG AAT-3' (SEQ ID NO:530); Library L3: 5'-TCA TGC TCC GTG ATG CAT $(TRIM)_5$ CAC $(TRIM)_1$ CAC TAC ACG CAG AAG AGC-3' (SEQ ID NO:531); Library L4: 5'-GGC GTG GAG GTG CAT AAT GGT GGT TGT $(TRIM)_6$ TGT GGT GGT GCC AAG ACA AAG CCG CGG-3' (SEQ ID NO:532); and Library L5: 5'-GTG GAG TGG GAG AGC AAT GGT GGT TGT $(TRIM)_6$ TGT GGT GGT GGG CAG CCG GAG AAC AAC-3' (SEQ ID NO:533). In these oligonucleotides, "TRIM" represents a mixture of trinucleotides encoding all amino acids except cysteine (Trimer Phosphoramidite Mix 2, Glen Research Catalog No. 13-1992-95). The mixture is an equimolar mixture of the following trinucleotides: AAA, AAC, ACT, ATC, ATG, CAG, CAT, CCG, CGT, CTG, GAA, GAC, GCT, GGT, GTT, TAC, TCT, TGG, TTC. Hence, codons encoding all amino acids except cysteine were represented approximately equally in the TRIM mixture. Thus, "$(TRIM)_6$" means that six random trinucleotides encoding any amino acid except cysteine are included in the oligo.

In a second round of PCR reactions (one for each library), the products from the first and second sets of PCR reactions from the first round described above served as templates. The primers used were the same for all libraries and were the following: 5'-GTT CCT TTC TAT TCT CAC-3' (forward; SEQ ID NO:534) and 5'-CCC ATT CAG ATC CTC TTC-3' (reverse; SEQ ID NO:535).

Libraries L6A and L6B were constructed in one round of PCR, using pIgG1-Fc as a template. In the PCR reaction described below, an XmaI restriction site (5'-CCCGGG-3') in the coding region for the Fc-fragment was changed to a XhoI restriction site (5'-CTCGAG-3") due to the sequences of the primers. These alterations were silent mutations that did not encode different amino acids from the unmodified pIgG1-Fc and were made because an XhoI plus NotI restriction digestion was more efficient than an XmaI plus NotI digestion. The forward primers used for these reactions were as follows: Library L6A: 5'-CTG CCC CCA TCT CGA GAT GAG CTG GGT TGT $(TRIM)_8$ TGT GGT GGT ACC AAC CAG GTC AGC CTG ACC-3' (SEQ ID NO:536); and library L6B: 5'-CTG CCC CCA TCT CGA GAT GAG CTG GGT TGT $(NNK)_8$ TGT GGT GGT ACC AAC CAG GTC AGC CTG ACC-3' (SEQ ID NO:537). The reverse primer sequence was 5'-GGC CCC GTG ATG GTG ATG ATG-3' (SEQ ID NO:538). In L6B a mixture of trinucleotides called NNK, which contains trinucleotides encoding all twenty amino acids in 32 degenerate codons, was used. In this trinucleotide mixture, "N" represents a randomized position that is either adenosine, guanidine, cytidine, or thymidine and "K" represents a partially constrained position that is either guanidine or thymidine.

For all PCR reactions, PCR core kits were used (Roche, Catalog No. 11 578 553 001) with the following reaction conditions: 95°C for 5 minutes, followed by 30 cycles of 95°C for 45 seconds- 55°C for 45 seconds- 72°C for 90 seconds, and finally 72°C 10 minutes. DNA from the PCR reactions was purified using QIAquick PCR Purification Kits (QIAGEN, 28104). About 200 µg of phagemid vector DNA and 10 µg of purified PCR product (from the third round of PCR reactions) were digested with

ApaLI and NotI (for Libraries L1-L5) and XhoI and NotI (for Libraries L6A and L6B). The digested DNA was gel purified with QIAquick Gel Purification Kits (QIAGEN, 28704).

Digested vector DNA and library PCR products in a molar ratio of 1:2 were ligated using T4 DNA ligase (New England Biolabs). The mixture was incubated at 16°C overnight. DNA was purified by ethanol precipitation. A total of 25 µg DNA was electroporated into 1000 µl of electrocompetent XL1 blue *E. coli* cells (Stratagene) in a 2.5 kV field using 200 Ω resistance and 25 µF capacitance to obtain about 1 x 10^9 *E. coli* transformants. Exact sizes of libraries ranged from 7 x 10^8 (for L5) to 1.8 x 10^9 (for L4). Cells were inoculated in 1000 mL of 2x YT medium (which contains 16 g/L Bacto Tryptone, 10 g/L Bacto Yeast Extract, and 5 g/L NaCl at a pH of 7.0-7.2) containing 100 µg/mL ampicillin and 2% glucose and grown until the OD_{600} was about 0.5. About 3 x 10^9 plaque-forming units per milliliter (PFU/mL) of M13 helper phage were then added, and the culture was incubated at 37°C for 1 hr. Infected cells were then spun down and resuspended with 1000 mL of 2x YT medium containing 100 µg/ml ampicillin and 40µg/mL kanamycin. Cells were grown at 30°C overnight. The phage were then precipitated with PEG 6000.

All libraries used in these transformations, other than L6A and L6B, contained six randomized codons, each of which encoded any of nineteen different amino acids, and therefore would be expected to encode about 4.7 x 10^7 (19^6) different variant Fc-fragments. Similarly, L6A would be expected to encode about 1.76 x 10^{10} (20^8) different variant Fc-fragments, since it contained eight randomized codons that encoded any of 20 different amino acids. For libraries other than Libraries L6A and L6B, given the expected number of different variants in each library, the library sizes of about 10^9 (ranging from 7 x 10^8 to 1.8 x 10^9) were 7-33 times the number of variants.

Example 3: Screening of the Fc loop Libraries

Two rounds of panning for phage expressing variant Fc-fragments that bind to FcRn with increased affinity as compared a wild type Fc-fragment at pH 6.0 (and having a low binding affinity at pH 7.4) were performed as follows. In the first round, phage were resuspended in a liquid containing 0.4 ml 20 mM MES, pH 6.0, 5% skim milk, 0.05% Tween 20. Each of four wells of a MAXISORP™ immunoplate (Nunc, Rochester, NY) was coated with 10 µg of hFcRn and blocked with 5% skim milk, and 100 µL of phage at 5 x 10^{11} PFU/mL were added. After a 1hr incubation at 37°C, wells were washed 3-10 times with 20 mM MES, pH 6.0, 0.2% Tween 20. The phage were eluted by adding 100 µL phosphate buffered saline (PBS), pH 7.4 to each well and incubating the plate for 1 hr at 37°C. These phage were used to reinfect an exponentially growing *E. coli* XL1-blue culture, which was cultured until it reached an OD_{600} of about 0.5. About 3 x 10^9 PFU/mL of M13 helper phage were then added, and the culture was incubated at 37°C for 1 hr. Infected cells were then spun down and

resuspended with 1000 ml of 2x YT medium containing 100 µg/ml ampicillin and 40µg/ml kanamycin. Cells were grown at 30°C overnight. The phage were then precipitated with PEG_{6000}.

Using these phage, a second round of panning was performed using essentially the same methods used in the first round. Stringency of the panning was increased slightly by reducing the concentration of FcRn coated onto the microtiter plates slightly and increasing the number of washes. The eluted phage, after washing several times, were used to reinfect an exponentially growing *E. coli* XL1-blue culture at an OD_{600} of about 0.5, which was then cultured for an hour at 37°C and plated on plates containing about 100 µg/ml ampicillin and kanamycin to obtain colonies.

Single colonies were inoculated into 96-well tissue culture plates containing 120 µL/well 2x YT medium containing 100 µg/mL ampicillin and 2% glucose. The cells were grown at 37°C on a shaker until an OD_{600} of about 0.5 was reached. Then 3 x 10^9/mL M13 helper phage were added to each well and incubated for 1 hr. The cells then were spun down and resuspended with 180 µL/well 2x YT medium containing 100 µg/mL ampicillin and 40 µg/mL kanamycin. The culture was then incubated at 30°C overnight on a shaker.

Approximately 100 µL of biotinylated human FcRn at 2 µg/mL was added to the wells of MAXISORP™ plates coated with 10 µg/mL streptavidin. After washing with PBS plus 0.05% Tween 20 (PBST) five times, phage from the overnight cultures in MES buffer at pH 6.0 or pH 7.4 were added to the plates and incubated at room temperature for 1 hr. A horseradish peroxidase (HRP)-conjugated anti-M13 antibody was used to detect the binding phage, and the plates were scored by a microtiter plate reader using a wavelength in the visible range. Positives were selected based on a higher signal at pH 5.5 (approximately 150% of the signal generated by a control Fc-fragment) and a signal that was comparable to that of a control Fc-fragment or lower at pH 7.4. In a second round of screening in which a single 96 well microtiter plate of isolated colonies was tested for each library, no positives were detected for libraries L2B, L3, or L4. About 20% of the colonies picked for Library L2A were positive, as were about 10% of the colonies picked for libraries L6A and L6B. Library L5 was clearly distinguishable from all other libraries since about 90% of the colonies picked were positive. Hence, library L5 was, by a large margin, the library that produced the most positives. These data indicate that the site at which library 5 is inserted is particularly favorable for isolation of variants that increase binding to FcRn at pH 5-6, while maintaining the low affinity of the Fc-fragment for FcRn at physiologic pHs.

We selected positives from libraries L2A, L5, L6A, and L6B, which were sequenced and further characterized as described below. Binding was assessed by a quantitative enzyme-linked immunoadsorbent assay (ELISA). The ELISA scores reflect binding affinity, with higher scores meaning higher affinity. Typically, scores higher than about 3 ~ 5 indicate binding above background. The sequences of the insertions of these positives and their scores in an ELISA are shown in Table 2

below. For library L5, the insertions are between the N169 and G170 according to the numbering in Figure 2. For libraries 6A and 6B, the insertions are between L143 and T144 according to the numbering in Figure 2.

Table 2: Sequences and ELISA scores of selected positives

Library	Control	ELISA score pH 6	ELISA score pH 7.4	Insertion sequence (SEQ ID NO:)	Isolate identifier
L5		95.4	2.8	GGCVFNMFNCGG (SEQ ID NO:44)	5-106
L5		95.1	15.1	GGCPHMFPWCGG (SEQ ID NO:392)	
L5		92.1	28.4	GGCGHGWIFCGG (SEQ ID NO:393)	
L5		91.0	4.4	GGCVFNMFNCGG (SEQ ID NO:394)	
L5		89.5	23.5	GGCILNFYGCGG (SEQ ID NO:395)	
L5		88.6	8.9	GGCREPHPFCGG (SEQ ID NO:396)	
L5		87.3	1.8	GGCPFEFTQCGG (SEQ ID NO:397)	
L5		86.5	0.4	GGCQLGSMHCGG (SEQ ID NO:398)	
L5		86.3	1.6	GGCYENKTLCGG (SEQ ID NO:399)	
L5		82.5	0.4	GGCHLPFAVCGG (SEQ ID NO:41)	5-51
L5		82.4	2.0	GGCGHEYMWCGG (SEQ ID NO:43)	5-104
L6A		79.5	0.7	GGCRAGYGDASCGG (SEQ ID NO:400)	
L5		79.2	0.6	GGCMVPFSMCGG (SEQ ID NO:401)	
L5		76.7	0.6	GGCWPLQDYCGG (SEQ ID NO:42)	5-69
L5		75.5	0.3	GGCELQERWCGG (SEQ ID NO:402)	
L5		74.8	0.5	GGCPANWGTCGG (SEQ ID NO:403)	
L5		74.2	2.3	GGCMMEFAQCGG (SEQ ID NO:404)	
L5		73.4	0.4	GGCQHHIMQCGG (SEQ ID NO:405)	
L5		73.1	0.4	GGCYQHHMECGG (SEQ ID NO:406)	
L5		72.7	0.5	GGCMQMNKWCGG (SEQ ID NO:364)	5-96
L5		72.3	0.6	GGCMVPFSMCGG (SEQ ID NO:407)	
L5		72	0.4	GGCQKGWVFCGG (SEQ ID NO:408)	
L5		71.2	0.7	GGCVYDVKKCGG (SEQ ID NO:409)	
L6B		68.9	0.8	GGCLKGMHGSACGG (SEQ ID NO:410)	
L5		68.6	0.6	GGCNMLWGSCGG (SEQ ID NO:411)	
L5		67.4	0.4	GGCMQPWAFCGG (SEQ ID NO:412)	
L5		65.6	0.5	GGCMTQYNWCGG (SEQ ID NO:413)	
L5		65.2	0.3	GGCVNTWWSCGG (SEQ ID NO:414)	
L5		64.1	0.5	GGCDGRTKYCGG (SEQ ID NO:363)	5-92
L5		63.6	0.7	GGCYITQKLCGG (SEQ ID NO:415)	
L5		63	0.5	GGCETHYTYCGG (SEQ ID NO:416)	
L5		62.7	0.4	GGCALYPTNCGG (SEQ ID NO:45)	5-112
L5		62.2	0.3	GGCTEQVMWCGG (SEQ ID NO:417)	
L5		61.2	0.4	GGCITEFSHCGG (SEQ ID NO:418)	
L5		61	0.3	GGCQNRSYWCGG (SEQ ID NO:419)	
L5		61	0.5	GGCHGTKQFCGG (SEQ ID NO:420)	

Library	Control	ELISA score pH 6	ELISA score pH 7.4	Insertion sequence (SEQ ID NO:)	Isolate identifier
L5		60.9	0.3	GGCNPHRTPCGG (SEQ ID NO:421)	
L5		60.3	0.3	GGCQHSPPLCGG (SEQ ID NO:422)	
L5		60.2	0.4	GGCNHEETFCGG (SEQ ID NO:423)	
L5		59.6	0.3	GGCQYPRKLCGG (SEQ ID NO:424)	
L5		59.2	0.3	GGCIGPFWWCGG (SEQ ID NO:425)	
L5		59.2	0.3	GGCMQPWINCGG (SEQ ID NO:426)	
L6B		58.9	0.8	GGCVQHKMGVVCGG (SEQ ID NO:427)	
L5		58.5	0.5	GGCEMENAWCGG (SEQ ID NO:428)	
L5		58.3	0.3	GGCPPWPERCGG (SEQ ID NO:429)	
L5		57.8	0.3	GGCGKHWHQCGG (SEQ ID NO:359)	5-57
L5		57.7	0.4	GGCHDPEPFCGG (SEQ ID NO:430)	
L5		57.7	0.5	GGCNEPKYVCGG (SEQ ID NO:431)	
L5		57.7	0.5	GGCDRPVWFCGG (SEQ ID NO:432)	
L5		57.2	0.4	GGCHSFKHFCGG (SEQ ID NO:360)	5-64
L5		57.2	0.7	GGCEIPHSFCGG (SEQ ID NO:433)	
L5		56.7	0.4	GGCMPYEMHCGG (SEQ ID NO:434)	
L5		56.1	0.7	GGCQGMWTWCGG (SEQ ID NO:366)	5-113
L5		55.6	0.7	GGCKRENPYCGG (SEQ ID NO:435)	
L5		55.5	1.4	GGCAERQYYCGG (SEQ ID NO:436)	
L5		55.4	0.3	GGCNVLDLFCGG (SEQ ID NO:437)	
L5		55.2	0.2	GGCKSMISMCGG (SEQ ID NO:438)	
L5		54.9	0.6	GGCHHKQDQCGG (SEQ ID NO:439)	
L6B		54.9	0.6	GGCNATLSGYLCGG (SEQ ID NO:440)	
L5		54.7	0.6	GGCEATMTWCGG (SEQ ID NO:441)	
L5		54.6	0.3	GGCNVLDLFCGG (SEQ ID NO:442)	
L5		54.5	0.3	GGCAQQWHHEYCGG (SEQ ID NO:362)	5-73
L5		54.2	0.7	GGCSRVFKYCGG (SEQ ID NO:443)	
L5		53.2	0.3	GGCHAPQWECGG (SEQ ID NO:444)	
L5		53.2	0.4	GGCPLVRADCGG (SEQ ID NO:445)	
L5		53.2	0.4	GGCMHNEEFCGG (SEQ ID NO:446)	
L5		52.4	1.2	GGCMFETKWCGG (SEQ ID NO:447)	
L6B		52.1	0.4	GGCNMNEWKSGCGG (SEQ ID NO:448)	
L5		51.6	0.4	GGCLQNLYVCGG (SEQ ID NO:449)	
L5		51.3	0.2	GGCQTSMKNCGG (SEQ ID NO:450)	
L5		51.2	0.5	GGCERFHHACGG (SEQ ID NO:361)	5-66
L5		51.1	0.4	GGCNLGHMPCGG (SEQ ID NO:451)	
L5		50.5	1.0	GGCWMWAEECGG (SEQ ID NO:452)	
L5		50.3	0.4	GGCVHNDKLCGG (SEQ ID NO:453)	
L6B		50.1	0.4	GGCYGKAGMRDCGG (SEQ ID NO:454)	
L6B		50	0.3	GGCVSAATSRTCGG (SEQ ID NO:455)	
L5		49.8	0.6	GGCYPQKEICGG (SEQ ID NO:456)	
L6B		49.2	0.4	GGCNQSSSREACGG (SEQ ID NO:457)	
L6A		48.5	0.2	GGCNPVSTGAYCGG (SEQ ID NO:458)	

Library	Control	ELISA score pH 6	ELISA score pH 7.4	Insertion sequence (SEQ ID NO:)	Isolate identifier
L5		48.2	1.8	GGCPGHEFRCGG (SEQ ID NO:459)	
L6B		47.2	0.4	GGCGEYNYVGGCGG (SEQ ID NO:460)	
L5		47.2	0.9	GGCKWSMTKCGG (SEQ ID NO:461)	
L5		47	1.4	GGCDWHRMKCGG (SEQ ID NO:462)	
L5		46.8	3.6	GGCMHSPHACGG (SEQ ID NO:463)	
L5		46.2	0.9	GGCMMWKVNCGG (SEQ ID NO:464)	
L6A		45.2	0.3	GGCFTNYASEKCGG (SEQ ID NO:465)	
L6A		44	0.2	GGCDRFQNVNVCGG (SEQ ID NO:466)	
L6A		43.8	0.6	GGCERHFPALFCGG (SEQ ID NO:467)	
L6B		43.6	0.2	GGCTLGSAPTLCGG (SEQ ID NO:468)	
L6B		43.2	0.3	GGCEMMKNKSGCGG (SEQ ID NO:469)	
L5		42.4	1.6	GGCEASGQICGG (SEQ ID NO:470)	
L6B		42.3	0.2	GGCLRNFMKQSCGG (SEQ ID NO:471)	
L6B		42.2	0.4	GGCPNDTVRDACGG (SEQ ID NO:472)	
L6B		41.8	0.3	GGCSFSRHMGACGG (SEQ ID NO:473)	
L6A		41.2	0.2	GGCAKDQHTGSCGG (SEQ ID NO:474)	
L5		41.1	1.6	GGCLGLRQECGG (SEQ ID NO:475)	
L6B		41	0.2	GGCNMNEWKSGCGG (SEQ ID NO:476)	
L5		38.7	1.6	GGCQQIKEWCGG (SEQ ID NO:477)	
L5		36.1	1	GGCDLPNEMCGG (SEQ ID NO:478)	
L5		35.8	0.7	GGCMFSHPHCGG (SEQ ID NO:479)	
L5		33.7	0.7	GGCAGPYWACGG (SEQ ID NO:480)	
L5		31.1	0.8	GGCEQQFVTCGG (SEQ ID NO:481)	
L5		29.5	1.2	GGCMGWWHLCGG (SEQ ID NO:482)	
L5		28.4	0.5	GGCPQHGEMCGG (SEQ ID NO:483)	
L5		27.7	1.0	GGCYASPHECGG (SEQ ID NO:484)	
L5		27.5	2.1	GGCMPPQWMCGG (SEQ ID NO:485)	
L5		26.9	1.4	GGCDTIGWFCGG (SEQ ID NO:486)	
L5		26.3	0.7	GGCGIFESWCGG (SEQ ID NO:487)	
L5		26.1	0.9	GGCGPYKTECGG (SEQ ID NO:488)	
L5		25.2	0.7	GGCQPQASWCGG (SEQ ID NO:489)	
L5		25	0.4	GGCDRQVTGFCGG (SEQ ID NO:490)	
L5		24.9	1.2	GGSQRAPASCGG (SEQ ID NO:491)	
L5		23.9	1.5	GGCMMREQGCGG (SEQ ID NO:492)	
L5		22	1.0	GGCLLPNMFCGG (SEQ ID NO:493)	
L5		21.7	0.3	GGCCPVYQHCGG (SEQ ID NO:494)	
L5		21.5	1.0	GGCLMSQDLCGG (SEQ ID NO:495)	
L5		21.3	1.4	GGCGGPYVFCGG (SEQ ID NO:496)	
	WT*	13-20	0.5-1.3		
	phg*	0.4-0.7	0.3-1.8		
	media*	0.4–0.7	0.5-0.6		

*Since these control samples were measured multiple times, these results are reported as a range. "WT," "phg," and "media" mean, respectively, phage expressing a control Fc-fragment, phage expressing no Fc-fragment, and no phage.

Example 4: Studies on binding association and dissociation rates

To further characterize some of the variant Fc-fragments identified by the phage ELISA, binding of the variant Fc-fragments to FcRn at pH 6 and 7.4 was characterized for a subset of the identified variant Fc- fragments. DNA encoding selected variant Fc-fragment isolates, all from library L5, was introduced into a mammalian expression vector, which was used to transfect 293 6E cells using deacylated PEI essentially as described by Thomas et al. (Proc. Natl. Acad. Sci. 102(16): 5679-5684, 2005), the relevant portions of which are incorporated herein by reference. Isolates having high ELISA scores for binding to FcRn were selected. The concentrations of variant Fc-fragments in conditioned media (CM) were measured using 1:2 and 1:10 diluted CM samples with Protein A Biosensors (ForteBio, Inc., catalog number 18-5010) on Octet Red® (ForteBio Inc., Menlo Park, California). Concentrations were calculated using a standard curve created with a purified Fc fusion protein.

Biotinylated hFcRn at 100 nM was captured on streptavidin (SA) biosensors (FortéBio Inc., 18-5019) off-line at RT for 2 hours. Using these biotinylated hFcRn-coated SA biosensors in the Octet Red® system, association and dissociation of unlabeled proteins can be detected in real time via diffraction of light. Variant and control Fc-fragment CM samples were diluted to 10 µg/mL at pH 6 or pH 7.4. Three association and dissociation conditions were set up: (1) association at pH 6 and dissociation at pH 6; (2) association at pH 6 and dissociation at pH 7.4; and (3) association at pH 7.4 and dissociation at pH 7.4. SA biosensors coated with biotinylated hFcRN were dipped into buffer at a specific pH for 1 min and then soaked in the samples containing variant Fc-fragments or controls at a specific pH for 5 min to allow binding to FcRn. The Fc biosensors were then soaked in buffer at a specific pH for 5 minutes more to allow the bound Fc-fragments to dissociate from FcRn. Detection of binding and dissociation of the Fc-fragments was possible through the use of bio-layer interferometry as implemented using the Octet Red® system, so that binding and dissociation were detected in real time.

Association and dissociation rates of the variant Fc-fragments at different pHs were compared to the controls. Higher binding at pH 6, slower off-rate at pH 6, very weak binding at pH 7.4, and faster off-rate at pH 7.4 as compared to the control Fc-fragment were the criteria used for selection of variant Fc-fragments for further characterization. A number of the 61 variant Fc-fragments tested showed more binding and slower dissociation at pH 6 and comparable or faster dissociation at pH 7.4 after binding at pH 6 relative to a control Fc-fragment. Figure 3 shows the binding at pH 6 (to the left of the center vertical line) and dissociation at pH 7.4 (to the right of the center vertical line) curves for 24

variant Fc-fragments tested that had the most favorable properties. To the right of the curves the name of each variant Fc-fragment tested is listed along with the maximal binding response (in nanometers (nm)) observed during the association phase of the experiment. The absolute number for maximal response can vary somewhat from experiment to experiment, and it is not directly proportional to binding constants such as k_{on}, k_{dis}, or K_D (k_{dis}/k_{on}). In Figure 3, the lowest binding response was observed with a wild type Fc-fragment, and all variants shown had higher responses at pH 6. Most of the variant Fc-fragments dissociated rapidly at pH 7.4, as does a wild type Fc-fragment. However, variant 5-1 clearly dissociated much more slowly at pH 7.4 than any other variant, whereas all other variants tested dissociated rapidly at pH 7.4, as did the wild type Fc-fragment. Fc variant 5-85 stood out as the variant with the largest response at pH 6 that also dissociated rapidly at pH 7.4. A number of other variants, such as 5-106, 5-104, 5-112, 5-79, 5-51, and 5-69, among others, also had high responses at pH 6 and rapid dissociation at pH 7.4. Thus, many variant Fc-fragments with improved properties were isolated from library L5.

Example 5: Production of variant Fc-fragments

DNA encoding selected isolates, all from Library L5, was introduced into a mammalian expression vector, which was used to transfect 293 cells using deacylated PEI essentially as described by Thomas et al. (Proc. Natl. Acad. Sci. 102(16): 5679-5684, 2005), the relevant portions of which are incorporated herein by reference. Mammalian cells were chosen due to the ease of expression and post-translational processing in this system. The protein expression and production methods used are described in Durocher et al. (Nucl. Acids Res. 30(2): e9, 2002), the relevant portions of which are incorporated herein by reference. The secreted variant Fc-fragments were purified from the culture media using Protein A affinity chromatograpy and size exclusion chromatography. High performance liquid chromatography (HPLC) was used to check the purity of the Fc-fragments. For general guidance in purification methods, *see* Methods in Molecular Biology: Protein Purification Protocols v.244, Cultler, ed., Humana, New Jersey, 2004. Protein titers of the Fc variants were assessed by Coomassie blue staining of a polyacrlamide gel. All isolates were expressed at adequate levels for testing.

Example 6: Thermal stability of variant Fc-fragments

Differential Scanning Calorimetry (DSC) measures the enthalpy (ΔH) of unfolding due to heat denaturation. Protein (or other macro-) molecules in solution are in equilibrium between the native, folded populations and denatured, unfolded populations. The higher the thermal transition midpoint (T_m), when 50% of the protein molecules are unfolded, the more stable the molecule. DSC is also used to determine the change in heat capacity (ΔCp) of denaturation. DSC experiments were performed with

MicroCal VP-Capillary DSC (GE Helathcare, Piscataway, NJ) in order to measure T_m of the various Fc-fragments. The concentration of purified control (Fc-WT) or variant Fc-fragment in each experiment was 0.5 mg/mL in 10 mM sodium acetate, 9% sucrose at pH 5. The samples were heated from 20°C to 95°C at a heating rate of 60°C/hour. The thermal transition midpoints (Tm) of individual domains were determined when 50% of the individual domain was unfolded. The Tm values of the variant Fc-fragments and the control wild type Fc-fragment are listed in Table 3 below.

Table 3: Tm values of the variant Fc-fragments

Sample Identifier	Tm of C_H2 Domain (°C)	Tm of C_H3 Domain (°C)
Fc-WT	67.7	84.3
Fc-5-55	67.7	77.5
Fc-5-60	67.7	78.4
Fc-5-64	67.4	79.2
Fc-5-69	68.3	76.2
Fc-5-70	67.3	77.7
Fc-5-73	67.7	78.8
Fc-5-79	67.3	76.5
Fc-5-85	66.5	73.4
Fc-5-91	67.4	77.6
Fc-5-92	67.5	79.5
Fc-5-95	67.1	77
Fc-5-96	67.0	79.2
Fc-5-97	67.4	78.2
Fc-5-99	67.0	78.2
Fc-5-101	67.0	78.6
Fc-5-104	67.1	78.0
Fc-5-106	66.6	77.4
Fc-5-112	67.2	77.1
Fc-5-113	68.1	77.9
Fc-5-1	68.4	77.4
Fc-5-51	67.4	77.4
Fc-5-57	67.1	79.1
Fc-5-66	67.5	79

These data indicate that the Tm of the C_H2 domain was not substantially affected by library L5 insertions, which are in the C_H3 domain. Although Tm of the C_H3 domain was slightly decreased in variant Fc-fragments as compared to the control Fc-fragment (Fc-WT), the Tm values of the C_H3 domain in the variant Fc-fragments are still approximately 10 °C higher than the Tm values of the C_H2 domain. Thus, the lower threshold of the thermal stability of the entire Fc domain, that is, the Tm of the C_H2 domain, is not affected.

Example 7: Binding of variant Fc-fragments to human or cynomolgus monkey FcRn

Binding of variant Fc-fragments to human and cynomolgus monkey FcRn was tested using a BIAcore® T100 analysis system (GE Healthcare Bio-Sciences AB Private Limited Liability Company, Uppsala, Sweden) at both pH 5.5 and pH 7.4. The test consisted of incubating various concentrations of the variant Fc-fragments or control Fc-fragments with a fixed amount of human or cynomolgus monkey FcRn under binding conditions and subsequently measuring the amount of free, unbound FcRn in these mixtures by binding to a surface (in a flow cell of a CM5 (Biacore) chip) on which an Fc-fragment was immobilized. The Fc-fragment immobilized on the chip was sufficient in quantity to bind essentially all of the free, unbound FcRn in the mixture. Thus, the amount of unbound FcRn in the mixture was quantitatively ascertained by surface plasmon resonance through use of the BIAcore® T100 analysis system. From this information, an EC_{50}, that is, the concentration of the variant or control Fc-fragment at which 50% of the FcRn in the mixture was bound, was calculated. Initially, a group of 23 variant-Fc-fragments (Fc-5-1 being considered a control) identified in the experiments described above were tested for binding to human FcRn. Based on these data, fourteen variant Fc-fragments were selected and further tested for binding to cynomolgus monkey FcRn.

The experimental protocol is described in more detail below. The Fc-fragments to be tested were produced in 293 cells and purified as described above. A wild-type human control Fc-fragment (Fc-WT) and a variant Fc-fragment (Fc-5-1) were immobilized on the flow cells of a CM5 chip (Biacore) using amine coupling with density around 6000 resonance units (RU). Fc-5-1 was used because it, unlike Fc-WT, binds to FcRn well at pH 7.4. One flow cell with no immobilized protein bound to it was used as a background control. It was possible to use a single flow cell for successive samples by washing between samples with a solution at pH 7.4 to release FcRn bound to the Fc-fragment immobilized on the flow cell.

To obtain a reasonable signal for the different molecules and conditions tested, different assay conditions were used. For assays at pH 5.5, 10 nM of human or cynomolgus monkey FcRn was mixed with serial dilutions of the variant and control Fc-fragments being tested (which ranged from 0.1 ~ 2,000 nM) and incubated for 1 hour at room temperature in 10mM sodium acetate, pH 5.5, 150 mM NaCl, 0.005% P20, 0.1mg/mL BSA. As positive controls, 10 mM human and cynomolgus monkey FcRn were each incubated in the same solution, for the same time, and at the same temperature described immediately above, but without an added Fc-fragment. In these samples, presumably all FcRn was unbound. Binding of the free, unbound FcRn in each of these mixtures to the immobilized Fc-WT and Fc-5-1 was measured by injecting the mixtures over the surfaces on the flow cells and detecting the FcRn bound to the surfaces via surface plasmon resonance.

For assays at pH 7.4, 10 nM human and cynomolgus monkey FcRn was mixed with serial dilutions of the variant and control Fc-fragments being tested (which ranged from 0.1 ~ 2,000 nM) and incubated for 1 hour at room temperature in phosphate buffered saline (PBS) with 0.005% polysorbate 20, 0.1mg/mL BSA. As positive controls, 10 mM human and cynomolgus monkey FcRn were each incubated in the same solution, for the same time, and at the same temperature described immediately above, but without an added Fc-fragment. Amounts of free, unbound FcRn in these mixtures were determined by binding to immobilized Fc-5-1 Fc-fragment, which was measured by injecting these mixtures over a flow cell surface coated with Fc-5-1 and detecting the FcRn bound to the surfaces via surface plasmon resonance. Fc-5-1 was used in these assays because it binds to FcRn with much greater affinity that Fc-WT at pH 7.4.

A decreased FcRn binding response with increasing concentrations of Fc-fragment in the mixtures indicated that FcRn was bound to the Fc-fragment in solution, which blocked FcRn from binding to the immobilized Fc-fragment on the surfaces of the flow cells. Plotting the FcRn binding signal versus Fc-fragment concentrations, EC_{50}'s were calculated using nonlinear regression of one-site competition in GraphPad Prism 5™ software. These results are shown in Table 4.

Table 4. EC_{50} of Fc-fragments for binding to FcRn at pH 5.5 and 7.4

	Human FcRn pH 5.5		Human FcRn pH 7.4		Cynomolgus FcRn pH 5.5		Cynomolgus FcRn pH 7.4	
Sample	EC_{50} (nM)	95% CI (nM)	EC_{50} (nM)	95% CI (nM)	EC_{50} (nM)	95% CI (nM)	EC_{50} (nM)	95% CI (nM)
Fc-WT	290	200~430	>2000		270	200~360	>2000	
Fc-5-1	2.6	1.4~4.7	45	33~61	2.3	1.2~4.5	42	28~63
Fc-5-51	16	13~19	>2000		21	12~36	>2000	
Fc-5-55	49	35~69	>2000		53	46~62	>2000	
Fc-5-57	180	110~310	>2000		ND*		ND	
Fc-5-60	67	48~94	>2000		60	43~85	>2000	
Fc-5-64	210	113~375	>2000		ND		ND	
Fc-5-66	200	170~230	>2000		ND		ND	
Fc-5-69	14	12~17	>2000		18	14~23	>2000	
Fc-5-70	26	20~32	>2000		27	18~39	>2000	
Fc-5-73	190	120~290	>2000		ND		ND	
Fc-5-79	53	45~64	>2000		63	48~81	>2000	
Fc-5-85	18	15~21	>2000		25	14~43	>2000	
Fc-5-91	57	40~110	>2000		ND		ND	
Fc-5-92	75	54~106	>2000		ND		ND	
Fc-5-95	42	31~56	>2000		50	37~67	>2000	
Fc-5-96	76	58~110	>2000		ND		ND	
Fc-5-97	25	19~32	>2000		31	27~36	>2000	
Fc-5-99	15	11~19	>2000		16	10~25	>2000	
Fc-5-101	100	60~170	>2000		ND		ND	

	Human FcRn pH 5.5		Human FcRn pH 7.4		Cynomolgus FcRn pH 5.5		Cynomolgus FcRn pH 7.4	
Fc-5-104	15	3~67	>2000		16	14~19	>2000	
Fc-5-106	5.3	4.5~6.4	>2000		3.6	2.4~5.3	>2000	
Fc-5-110	250	140~460	>2000		ND		ND	
Fc-5-112	46	38~56	>2000		49	34~72	>2000	
Fc-5-113	20	12~33	>2000		27	21~35	>2000	

* ND indicates "not determined"

The data in Table 4 indicate that many of the variant Fc-fragments tested have substantially improved binding to human FcRn at pH 5.5 (*i.e.,* have a substantially lower EC_{50} compared to Fc-WT) and maintain low binding at pH 7.4 (*i.e.,* have a high EC_{50}, like that of Fc-WT). However, five of them (Fc-5-57, Fc-5-64, Fc-5-66, Fc-5-73, and Fc-5-110) did not show much improved binding at pH 5.5. Eight of them (Fc-5-55, Fc-5-60, Fc-5-79, Fc-5-91, Fc-5-92, Fc-5-96, Fc-5-101, and Fc-5-112) showed only around 2~5x improvement in binding at pH 5.5. These data further indicate that EC_{50}'s of the variant Fc-fragments for binding to human and cynomolgus monkey FcRn's are comparable. All variant Fc-fragments tested (other than Fc-5-1, which was considered as a control) maintained low binding to both human and cynomolgus monkey FcRn at pH 7.4.

Example 8: Construction of further modifed variant Fc-fragments

Further modified versions of variant Fc-fragments Fc-5-69 and Fc-5-106 were made. The variants of Fc-5-69 were made as follows. DNA encoding variant Fc-fragment Fc-5-69 was inserted into a mammalian expression vector that could also be propagated in *E. coli*, which was used as a template. For variant Fc-5-69-W1F, the following two primers were used: forward, GAG AGC AAT GGT GGT TGT TTC CCG CTG CAG GAC TAC (SEQ ID NO:497); and reverse, GTA GTC CTG CAG CGG GAA ACA ACC ACC ATT GCT CTC (SEQ ID NO:498). For Fc-5-69-W1Y, the following two primers were used: forward, GAG AGC AAT GGT GGT TGT TAC CCG CTG CAG GAC TAC (SEQ ID NO:499); and reverse, GTA GTC CTG CAG CGG GTA ACA ACC ACC ATT GCT CTC (SEQ ID NO:500). The Quikchange Site-Directed Mutagenesis Kit (Stratagene, 200518) protocol was used. The reaction mixture was 200 nM dNTPs, 100 nM primers, 1ng DNA template, 1μL DNA polymerase and water in a total volume of 50μL. The reaction was run at 95ºC for 30 seconds, then 16 cycles of 95ºC for 30 seconds, 55ºC for 60 seconds, 68ºC for 6 minutes, followed by 68ºC for 10 minutes. Then 1μL of DpnI was added, and the reaction was incubated at 37ºC for 1 hour. Then 2μL of the mixture was used to transform 30μl of XL1-blue supercompetent cells (Stratagene) at 42ºC for 45 seconds. Thereafter, 0.5 mL SOC was added, and the cells were incubated at 37ºC for 1 hour on a shaker at 300 revolutions per minute (rpm). The transformed cells were spread on LB-ampicillin agar plates and incubated at 37ºC overnight.

Individual colonies were picked, and plasmid DNA was prepared and sequenced. The DNA sequences indicated that the variant Fc-fragments had the following inserted amino acid sequences, which differed by one amino acid from the insert in Fc-5-69: Fc-5-69-W1F, GGCFPLQDYCGG (SEQ ID NO:367); and Fc-5-69-W1Y, GGCYPLQDYCGG (SEQ ID NO:368). DNA encoding these variant Fc-fragments was introduced into 293 cells, and purified Fc-fragments were produced as described above for use in Biacore® binding assays performed as described above.

Modified versions of Fc-5-106 were made by similar methods except that the template for the PCR reactions was a DNA encoding Fc-5-106 inserted into a mammalian expression vector. The primers used for the PCR reactions had the following sequences: Fc-5-106-M4A, forward, GGT GGT TGT GTT TTC AAC GCG TTC AAC TGT GGT GGT GGG (SEQ ID NO:501) and reverse, CCC ACC ACC ACA GTT GAA CGC GTT GAA AAC ACA ACC ACC (SEQ ID NO:502); Fc-5-106-M4G, forward, GGT GGT TGT GTT TTC AAC GGG TTC AAC TGT GGT GGT GGG (SEQ ID NO:503), and reverse, CCC ACC ACC ACA GTT GAA CCC GTT GAA AAC ACA ACC ACC (SEQ ID NO:504); Fc-5-106-M4H, forward, GGT GGT TGT GTT TTC AAC CAT TTC AAC TGT GGT GGT GGG (SEQ ID NO:505), and reverse, CCC ACC ACC ACA GTT GAA ATG GTT GAA AAC ACA ACC ACC (SEQ ID NO:506); Fc-5-106-M4I, forward, GGT GGT TGT GTT TTC AAC ATC TTC AAC TGT GGT GGT GGG (SEQ ID NO:507), and reverse, CCC ACC ACC ACA GTT GAA GAT GTT GAA AAC ACA ACC ACC (SEQ ID NO:508); Fc-5-106-M4L, forward, GGT GGT TGT GTT TTC AAC TTG TTC AAC TGT GGT GGT GGG (SEQ ID NO:509), and reverse, CCC ACC ACC ACA GTT GAA CAA GTT GAA AAC ACA ACC ACC (SEQ ID NO:510); Fc-5-106-M4N, forward, GGT GGT TGT GTT TTC AAC AAC TTC AAC TGT GGT GGT GGG (SEQ ID NO:511), and reverse, CCC ACC ACC ACA GTT GAA GTT GTT GAA AAC ACA ACC ACC (SEQ ID NO:512); Fc-5-106-M4Q, forward, GGT GGT TGT GTT TTC AAC CAG TTC AAC TGT GGT GGT GGG (SEQ ID NO:513), and reverse, CCC ACC ACC ACA GTT GAA CTG GTT GAA AAC ACA ACC ACC (SEQ ID NO:514); Fc-5-106-M4S, forward, GGT GGT TGT GTT TTC AAC TCG TTC AAC TGT GGT GGT GGG (SEQ ID NO:515), and reverse, CCC ACC ACC ACA GTT GAA CGA GTT GAA AAC ACA ACC ACC (SEQ ID NO:516); Fc-5-106-M4T, forward, GGT GGT TGT GTT TTC AAC ACG TTC AAC TGT GGT GGT GGG (SEQ ID NO:517), and reverse, CCC ACC ACC ACA GTT GAA CGT GTT GAA AAC ACA ACC ACC (SEQ ID NO:518); Fc-5-106-M4V, forward, GGT GGT TGT GTT TTC AAC GTG TTC AAC TGT GGT GGT GGG (SEQ ID NO:519), and reverse, CCC ACC ACC ACA GTT GAA CAC GTT GAA AAC ACA ACC ACC (SEQ ID NO:520).

PCR reactions were performed as described above and used to transform *E. coli.* Plasmid DNAs from individual colonies were sequenced. Isolates encoding Fc-fragments having the following inserted sequences were selected: Fc-5-106-M4A, GGCVFNAFNCGG (SEQ ID NO:369); Fc-5-106-M4G, GGCVFNGFNCGG (SEQ ID NO:370); Fc-5-106-M4H, GGCVFNHFNCGG (SEQ ID NO:371); Fc-

5-106-M4I, GGCVFNIFNCGG (SEQ ID NO:372); Fc-5-106-M4L, GGCVFNLFNCGG (SEQ ID NO:373); Fc-5-106-M4N, GGCVFNNFNCGG (SEQ ID NO:374); Fc-5-106-M4Q, GGCVFNQFNCGG (SEQ ID NO:375); Fc-5-106-M4S, GGCVFNSFNCGG (SEQ ID NO:376); Fc-5-106-M4T, GGCVFNTFNCGG (SEQ ID NO:377); Fc-5-106-M4V, GGCVFNVFNCGG (SEQ ID NO:378).

These derivatives of Fc-5-69 and Fc-5-106 were made and tested for relative binding affinity to human and cynomolgus monkey FcRn at pH 5.5 and 7.4 using the methods described in Example 7.

Table 5: Binding of variants of 5-69 and Fc 5-106 to human and cynomolgus monkey FcRn

	Human FcRn pH 5.5		Human FcRn pH 7.4		Cynomolgus FcRn pH 5.5		Cynomolgus FcRn pH 7.4	
Sample	EC_{50} (nM)	95% CI (nM)	EC_{50} (nM)	95% CI (nM)	EC_{50} (nM)	95% CI (nM)	EC_{50} (nM)	95% CI (nM)
Fc-WT	270	170~440	>2000		250	130~470	>2000	
Fc-5-69	20	13~30	1100	960~1400				
Fc-5-69- WIF	57	36~89	>2000					
Fc-5-69- WIY	140	75~240	>2000					
Fc-5-106	5.4	3.7~8.0	1400	1100~1700	4.6	3.5~6.1	>2000	
Fc-5-106- M4A	27	16~45	>2000					
Fc-5-106- M4G	100	66~160	>2000					
Fc-5-106- M4H	100	48~230	>2000					
Fc-5-106-M4I	2.6	1.8~3.6	900	760~1100	3.2	2.1~4.8	970	810~1200
Fc-5-106-M4L	2.3	1.5~3.4	650	460~920	2.8	1.6~4.7	710	460~1100
Fc-5-106- M4N	100	52~180	>2000					
Fc-5-106- M4Q	34	17~68	>2000					
Fc-5-106- M4S	58	32~106	>2000					
Fc-5-106- M4T	10	4.4~21	>2000		8.3	5.3~13	>2000	
Fc-5-106- M4V	6.2	3.6~11	>2000		5.0	3.4~7.3	>2000	

Both Fc-5-69 derivatives showed weaker binding affinity than Fc-5-69 itself at pH 5.5. Two Fc-5-106 derivatives (Fc-5-106-M4I and Fc-5-106-M4L) showed higher affinity binding to both human and cynomolgus FcRn at pH 7.4 compared to Fc-5-106. Also, four of the Fc-5-106 derivatives including these two (Fc-5-106-M4I, Fc-5-106-M4L, Fc-5-106-M4T and Fc-5-106-M4V) showed improved or approximately the same binding activity at pH 5.5 compared to Fc-5-106 itself. These four were tested for binding affinity to cynomolgus monkey FcRn. All four had EC_{50}'s for binding to cynomolgus monkey FcRn that were similar to those for human FcRn at both pH 5.5. and 7.4.

Example 9: In vivo Characterization of variant Fc-fragments

To determine whether antibodies containing the variant Fc-fragments identified above have improved pharmacokinetic (PK) properties *in vivo,* an unmodified Antibody X (which is a human IgG2 anti-human IL-23 antibody) and variant versions of Antibody X containing variant Fc-fragments were

tested *in vivo* in cynomolgus monkeys to define pharmacokinetic parameters. Antibody X was selected as an appropriate antibody in which to test the pharmacokinetic parameters of the variant Fc-fragments because it was known to have a linear pK profile and because IL-23 was known to be expressed at low levels *in vivo.* Thus, it was expected that target-related effects on PK parameters would be minimal, making it easier to detect pK effects due to the variant Fc-fragments.

Five variant IgG2 antibodies called (X-5-51, X-5-69, X-5-104, X-5-106, and X-5-112) were made. These IgG2 antibodies had the same insertions at the same positions (according to the alignment in Table1) as the variant IgG1 Fc-fragments Fc-5-51, Fc-5-69, Fc-5-104, Fc-5-106, and Fc-5-112, respectively. More specifically, the insertions were between amino acids 384 and 385 (EU numbering as in Table 1) in the human IgG2 Fc-fragment. A plasmid containing DNA encoding the heavy chain of Antibody X was used as a template for five PCR reactions done using the following primers: for X-5-51, forward, 5'-GAG TGG GAG AGC AAT GGT GGT TGT CAT CTG CCG TTC GCT GTT TGT GGT GGT GGG CAG CCG GAG AAC-3' (SEQ ID NO:539), and reverse, 5'-GTT CTC CGG CTG CCC ACC ACC ACA AAC AGC GAA CGG CAG ATG ACA ACC ACC ATT GCT CTC CCA CTC-3' (SEQ ID NO:540); for X-5-69,forward, 5'-GAG TGG GAG AGC AAT GGT GGT TGT TGG CCG CTG CAG GAC TAC TGT GGT GGT GGG CAG CCG GAG AAC-3' (SEQ ID NO:541), and reverse, 5'-GTT CTC CGG CTG CCC ACC ACC ACA GTA GTC CTG CAG CGG CCA ACA ACC ACC ATT GCT CTC CCA CTC-3' (SEQ ID NO:542); for X-5-104, forward, 5'-GAG TGG GAG AGC AAT GGT GGT TGT GGT CAT GAA TAC ATG TGG TGT GGT GGG CAG CCG GAG AAC-3' (SEQ ID NO:543), and reverse, 5'-GTT CTC CGG CTG CCC ACC ACA CCA CAT GTA TTC ATG ACC ACA ACC ACC ATT GCT CTC CCA CTC-3' (SEQ ID NO:544); for X-5-106, forward, 5'-GAG TGG GAG AGC AAT GGT GGT TGT GTT TTC AAC ATG TTC AAC TGT GGT GGT GGG CAG CCG GAG AAC-3' (SEQ ID NO:545), and reverse, 5'-GTT CTC CGG CTG CCC ACC ACC ACA GTT GAA CAT GTT GAA AAC ACA ACC ACC ATT GCT CTC CCA CTC-3' (SEQ ID NO:546); and for X-5-112, forward,5'-GAG TGG GAG AGC AAT GGT GGT TGT GCT CTG TAC CCG ACT AAC TGT GGT GGT GGG CAG CCG GAG AAC-3' (SEQ ID NO:547), and reverse, 5'-GTT CTC CGG CTG CCC ACC ACC ACA GTT AGT CGG GTA CAG AGC ACA ACC ACC ATT GCT CTC CCA CTC-3' (SEQ ID NO:548). The Quikchange Site-Directed Mutagenesis Kit (Stratagene, 200518) protocol was used. The reaction mixture was 200 nM dNTPs, 100 nM primers, 1 ng DNA template, 1 µL DNA polymerase and water in a total volume of 50 µL. The reaction was run at 95°C for 30 seconds, then 16 cycles of 95°C for 30 seconds, 55°C for 60 seconds, 68°C for 6 minutes, followed by 68°C for 10 minutes. Then 1 µL of DpnI was added, and the reaction was incubated at 37°C for 1 hour. Then 2 µL of the mixture was used to transform 30 µl of XL1-blue supercompetent cells (Stratagene) at 42°C for 45 seconds. Thereafter, 0.5 mL SOC was added, and the cells were incubated at 37°C for 1 hour on a shaker at 300 rpm. The transformed cells

were spread on LB-ampicillin agar plates and incubated at 37°C overnight. Individual colonies were picked, and plasmid DNA was prepared and sequenced to ensure that the isolates chosen had the expected DNA sequence.

Antibodies were prepared in essentially the same way as described above for Fc-fragments, except that the mammalian host cells were transfected with DNAs encoding both the IgG2 heavy chain, including a portion encoding either a variant or a control Fc-fragment, and the light chain of Antibody X. Host cells were incubated under conditions appropriate for expression of the antibodies, and the antibodies were recovered from the culture medium, purified as described above, and used for the following experiments.

Cynomolgus monkeys (n=2/group) received a single intravenous dose of an unmodified or a variant version of Antibody X at a dose of 1 mg/kg and were followed during an 8-week in-life phase. Samples of blood were collected at specified time points over the course of the experiment. Samples were collected at pre-dose, 0.25, 1, 4, 8, 12, 24, 48, 72, 168, 240, 336, 408, 504, 576, 672, 744, 840, 1008, 1176 and 1344 hours post-dose.

An anti-human IgG sandwich ELISA was used to determine systemic concentrations of the injected antibodies by comparison to a standard curve derived from the same molecule. Specifically, a mouse anti-human Fc antibody was diluted in PBS, coated onto plates, and incubated for 2 hours at room temperature. The well contents were discarded, and PBS-Tween 20 (SuperBlock®, Thermo Scientific) was added to the wells as a blocking buffer. After incubation for one hour at room temperature, the plate wells were washed with PBS-Tween-20, and serum samples were added to the wells and incubated for 1 hour with shaking. Wells were again washed, and a horseradish peroxidase labeled mouse anti-human Fc antibody was added to the plates. Following a one hour incubation, wells were washed and developed for 10 minutes using a 3,3',5,5' tetramethylbenzidine (TMB) substrate. The resulting colorimetric reaction was quenched with phosphoric or sulfuric acid added to the plate. Optical densities (ODs) were determined at 450 nm and 650nm, and the OD at 650 nm was subtracted from the OD at 450 nm. The conversion of OD values into concentrations for the study samples was achieved through data regression using a logistic model with weighting set to $1/Y^2$ in Watson LIMS version 7.0.0.04.

Pharmacokinetic analysis was performed using the PK analytical package provided within Watson LIMS, version 7.0.0.04 by. Exposure (area under the curve (AUC)) and clearance (mL/kg/hr) values were derived from this analysis. Concentration vs. time data for the last 5 sampling time points for each cynomolgus monkey was used to calculate half-life (T½) values.

As shown in Figure 4, variant versions of Antibody X containing variant Fc-fragments X-5-51, X-5-69, X-5-104, X-5-106 and X-5-112 all demonstrated higher mAb concentrations in cynomolgus

monkeys at a given sampling time point as compared to unmodified Antibody X. As shown in Table 6, increased exposure values and decreased clearance values were demonstrated for all variant versions of Antibody X tested compared to unmodified Antibody X. In addition, four of the five variant versions of Antibody X had increased half lives. The one variant that did not exhibit an increased half life (X-5-106), may represent a situation where anti-drug antibodies against the injected antibody may have developed in one monkey, since data from one of the two monkeys tested indicated a very short half life (48 hours), whereas data from the other monkey indicated an increased half life (538 hours). Further experimentation to measure the presence of anti-drug antibodies could clarify this issue.

Table 6 : Mean values for half-life, exposure, and clearance

	Unmodified Antibody X	**X- 5-51**	**X-5-69**	**X-5-104**	**X-5-106**	**X-5-112**
T ½ (hours)	335	910	550	477	191	520
AUC - exposure (ug*hr/mL)	3890	6640	4960	5745	6005	7810
Clearance (mL/kg/hr)	0.247	0.109	0.173	0.153	0.154	0.108

Generally, these data indicate that increased binding of a variant Fc-fragment to FcRn at pH 5.5-6.0 (relative to a control Fc-fragment) and rapid dissociation from FcRn at pH 7.4 correlate with a longer *in vivo* half-life of an antibody containing the variant Fc-fragment in cynomolgus monkeys. However, an exact quantitative relationship between the degree of improvement in binding at pH 5.5 or 6 of an IgG1 variant Fc-fragment and the degree of improvement in pharmacokinetic parameters for a full length IgG2 antibody having the same insertion as the variant Fc-fragment is not shown by these data. These data do, however, indicate that X-5-51, X-5-69, X-5-104, and X-5-112 have increased half lives relative to an unaltered Antibody X and suggest that the same is true for X-5-106. Moreover, all variant antibodies tested had lower clearance rates and higher exposure as compared to the control antibody.

Example 10: Construction of variant Fc-fragments with insertions at alternate sites

The fact that many variants from library L5 had desirable properties indicated that the position of these insertions was favorable. The following experiments were done to determine whether other sites within or adjacent to the same loop as the L5 insertion site might have better properties. To test this idea, one of the selected peptides was inserted at different locations in this loop, and the resulting variant Fc-fragments were tested for FcRn binding. The peptide insertion of variant Fc-fragment 5-1 was chosen as the peptide to insert. This peptide has the following amino acid sequence:

GGCGMPIEFCGG (SEQ ID NO:67). As shown in Figure 5 (which uses the EU numbering system as exemplified in Table 1), this peptide was inserted at sites within or adjacent to the library L5 loop other than the library L5 insertion site. Binding of the resulting Fc-fragments to FcRn was assayed using SA biosensors (ForteBio Inc. 18-5019) coated biotinylated huFcRn as described in Example 4 above.

In more detail, the DNA constructs encoding these variant Fc-fragments were made as follows. The Quikchange® Site-Directed Mutagenesis Kit (Stratagene, 200518) protocol was used. The reaction mixture was composed of 200 nM dNTPs, 100 nM primers, 1 ng DNA template, 1 µL DNA polymerase and water to a total volume of 50 µL. The primers used in these reactions for each variant are shown below in Table 7. The DNA template was a cDNA encoding a wild type human IgG1 Fc-polypeptide inserted into a vector. The reaction was run at 95ºC for 30 seconds, then 16 cycles of 95 ºC for 30 seconds, 55ºC for 60 seconds, and 68ºC for 6 minutes, followed by a final cycle of 68ºC for 10 minutes. Then 1 µL of DpnI was then added, and the reaction was incubated at 37ºC for 1 hour. Then 2 µL of the mixture was used to transform 30 µL of XL1-blue supercompetent *E. coli* cells (Stratagene) at 42ºC for 45 seconds. Thereafter, 0.5 mL Super Optimal Broth with Catabolite repression (SOC; containing 2% bacto-tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, and 20 mM glucose) was added, and the cells were incubated at 37ºC for 1 hour on a shaker at 300 revolutions per minute (rpm). The transformed cells were spread on LB-ampicillin agar plates and incubated at 37ºC overnight.

Table 7: Primers used in PCR reactions for construction variant Fc-fragment-encoding DNAs

Variant Fc-fragment designation	Forward Primer	Reverse Primer
5-1-1	5'- GCT GTG GAG TGG GAG GGT GGT TGT GGT ATG CCG ATC GAA TTC TGT GGT GGT AGC AAT GGG CAG CCG-3' (SEQ ID NO:68)	5'- CGG CTG CCC ATT GCT ACC ACC ACA GAA TTC GAT CGG CAT ACC ACA ACC ACC CTC CCA CTC CAC AGC-3' (SEQ ID NO:69)
5-1-2	5'- GTG GAG TGG GAG AGC GGT GGT TGT GGT ATG CCG ATC GAA TTC TGT GGT GGT AAT GGG CAG CCG GAG-3' (SEQ ID NO:70)	5'- CTC CGG CTG CCC ATT ACC ACC ACA GAA TTC GAT CGG CAT ACC ACA ACC ACC GCT CTC CCA CTC CAC-3' (SEQ ID NO:71)
5-1-3	5'- TGG GAG AGC AAT GGG GGT GGT TGT GGT ATG CCG ATC GAA TTC TGT GGT GGT CAG CCG GAG AAC AAC-3' (SEQ ID NO:72)	5'- GTT GTT CTC CGG CTG ACC ACC ACA GAA TTC GAT CGG CAT ACC ACA ACC ACC CCC ATT GCT CTC CCA-3' (SEQ ID NO:73)
5-1-4	5'- GAG AGC AAT GGG CAG GGT GGT TGT GGT ATG CCG ATC GAA TTC TGT GGT GGT CCG GAG AAC AAC TAC-3' (SEQ ID NO:74)	5' GTA GTT GTT CTC CGG ACC ACC ACA GAA TTC GAT CGG CAT ACC ACA ACC ACC CTG CCC ATT GCT CTC-3' (SEQ ID NO:75)
5-1-5	5'- AGC AAT GGG CAG CCG GGT GGT TGT GGT ATG CCG ATC GAA TTC TGT GGT GGT GAG AAC AAC TAC AAG-3' (SEQ ID NO:76)	5'- CTT GTA GTT GTT CTC ACC ACC ACA GAA TTC GAT CGG CAT ACC ACA ACC ACC CGG CTG CCC ATT GCT-3' (SEQ ID NO:77)
5-1-6	5'- AAT GGG CAG CCG GAG GGT GGT TGT GGT ATG CCG ATC GAA	5'-GGT CTT GTA GTT GTT ACC ACC ACA GAA TTC GAT CGG CAT ACC ACA

Variant Fc-fragment designation	Forward Primer	Reverse Primer
	TTC TGT GGT GGT AAC AAC TAC AAG ACC-3' (SEQ ID NO:78)	ACC ACC CTC CGG CTG CCC ATT-3' (SEQ ID NO:79)
5-1-7	5'-GGG CAG CCG GAG AAC GGT GGT TGT GGT ATG CCG ATC GAA TTC TGT GGT GGT AAC TAC AAG ACC ACG-3' (SEQ ID NO:80)	5'-CGT GGT CTT GTA GTT ACC ACC ACA GAA TTC GAT CGG CAT ACC ACA ACC ACC GTT CTC CGG CTG CCC-3' (SEQ ID NO:81)
5-1-8	5'-CAG CCG GAG AAC AAC GGT GGT TGT GGT ATG CCG ATC GAA TTC TGT GGT GGT TAC AAG ACC ACG CCT-3' (SEQ ID NO:82)	5'- AGG CGT GGT CTT GTA ACC ACC ACA GAA TTC GAT CGG CAT ACC ACA ACC ACC GTT GTT CTC CGG CTG-3' (SEQ ID NO:83)
5-1-9	5'- GTG GAG TGG GAG AGC GGT GGT TGT GGT ATG CCG ATC GAA TTC TGT GGT GGT CCG GAG AAC AAC TAC-3' (SEQ ID NO:84)	5'- GTA GTT GTT CTC CGG ACC ACC ACA GAA TTC GAT CGG CAT ACC ACA ACC ACC GCT CTC CCA CTC CAC-3' (SEQ ID NO:85)
5-1-10	5'- GTG GAG TGG GAG AGC GGT GGT GGT TGT GGT ATG CCG ATC GAA TTC TGT GGT GGT GGT CCG GAG AAC AAC TAC-3' (SEQ ID NO:86)	5'- GTA GTT GTT CTC CGG ACC ACC ACC ACA GAA TTC GAT CGG CAT ACC ACA ACC ACC ACC GCT CTC CCA CTC CAC-3' (SEQ ID NO:87)

Individual colonies were picked, and plasmid DNA was prepared and sequenced. DNA encoding these variant Fc-fragments was introduced into 293-6E cells, and Fc-fragments in conditioned media (CM) from these cells were used in ForteBio binding assays performed as described above. *See* Example 4.

The results for binding at pH 6 and dissociation at pH 7 are shown in Figure 6. Two of the variant Fc-fragments, 5-1-10 and 5-1-2, had a greater maximal response than variant 5-1 at pH 6, but also dissociated more slowly at pH 7.4. *See* Figure 6. All other constructs showed a somewhat lesser maximal response, as compared to variant 5-1, at pH 6, but all of these responses were comparable to or higher than that of a wild type Fc-fragment (designated FcWT in Figure 6). Further, dissociation of these constructs at pH 7.4, unlike that of 5-1, was comparable to that of FcWT. Thus, most of the Fc variants, other than 5-1-2 and 5-1-10 (both of which have an insertion between positions 383 and 384), with insertions within or adjacent to Loop 10 dissociated faster at pH 7.4 than variant 5-1. Also, variants 5-1-1, 5-1-2, 5-1-3, 5-1-9, and 5-1-10 clearly had higher maximal responses that did FcWT at pH 6 variant 5-1, whereas variants 5-1-6, 5-1-7, and 5-1-8 had maximal responses comparable to that of FcWT. Variants 5-1-4 and 5-1-5 had only marginally higher responses than did FcWT. These data indicate that insertion of the peptide had more favorable effects at certain sites within Loop 10. Specifically, insertion between positions 382 and 383, 383 and 384, 384 and 385, 385 and 386 (using the EU numbering system as illustrated in Table 1 and Figure 5) produced Fc variants that had a greater maximal response at pH 6 than did FcWT plus rapid dissociation at pH 7.4. In contrast, Fc variants having insertions between positions 388 and 389, 389 and 390, or 390 and 391 had properties

similar to FcWT. Finally, Fc variants having insertions between 386 and 387 or 387 and 388 showed marginally higher responses at pH 6 than Fc WT and rapid dissociation at pH 7.4. In addition, removal of amino acids 384-386 and insertion of the peptide in their place, as was done in variants 5-1-9 and 5-1-10, also improved the properties of the these Fc variants as compared to FcWT. The slightly longer insertion of 5-1-10, which included three rather than the two glycine residues as in 5-1-9 at the beginning and end of the insertion, had a higher response than any other variant, including 5-1-2, which had an insertion (with no accompanying deletion) between positions 383 and 384. These data indicate that insertions of a peptide that can enhance binding to FcRn within the region between positions 382 and 387 can enhance binding of an Fc-fragment to FcRn at pH 6 while preserving its fast dissociation from FcRn at pH 7.4. On the other hand, insertion of such a peptide between positions 388 and 391 did not substantially enhance binding activity and/or affinity of an Fc-fragment to FcRn at pH 6 as compared to FcWT and also had little or no effect on dissociation at pH 7.4. Thus, these data point to a portion of Loop 10, that is, from position 382 to 386, 387, or 388 (EU numbering), as containing particularly favorable sites for making insertions that can enhance binding activity and/or affinity to FcRn at pH 6.

Example 11: Selection of variant Fc-fragment by yeast display

To obtain a different group of variant Fc-polypeptides, a selection was performed using yeast display. To make libraries for screening in yeast, three previously made libraries were used as starting points. One of these was library L5, which is described above. *See* Figure 2 and Example 2. The two other libraries had insertions at exactly the same site as library L5 and were made using the same general methods that were used to make library L5, but the format of the insertions differed in that library L5 encoded insertions with six randomized amino acids while libraries L-8 and L-10 encoded insertions with eight and ten randomized amino acids, respectively. Specifically, these libraries encoded insertions with the following formats: library L-8, GGC(19R)$_8$CGG (SEQ ID NO:88); and library L-10, GGC(19R)$_{10}$CGG (SEQ ID NO:89). These insertions, like those in library L5, are between the "N" at position 169 and the "G" at position 170 according to the numbering scheme in Figure 2. The "(19R)$_8$" and "(19R)$_{10}$" indicate eight and ten, respectively, randomized amino acids, which can be any amino acid other than cysteine.

Libraries L5, L-8 and L-10 were digested with SacII and NotI, and the resulting fragments, which encoded the variant Fc-fragments, were purified and ligated into a vector appropriate for expression in both *Escherichia coli* and *Saccharomyces cerevisiae.* The vector also encoded an HA tag (a short peptide sequence (YPYDVPDYA)(SEQ ID NO:575) from human influenza hemagglutinin) in frame with the insertion, which was used to confirm that the Fc regions were displayed on the surface of

the yeast cells. These three new libraries were introduced into *E. coli*, specifically, into XL1-Blue supercompetent *E. coli* cells. DNA from about 5 x 10^8 *E. coli* transformants was used to transform about 1-2 x 10^9 *S. cerevisiae* cells using the standard lithium acetate method. An overnight culture of *S. cerevisiae* was diluted to an OD_{600} of 0.2-0.3 in 100 milliliters of yeast extract peptone dextrose medium (which contains 20 g/L bacto-peptone, 10 g/L yeast extract, 2% glucose (YPD)) and grown at 30°C shaking at 300 revolutions per minute (rpm) until the OD_{600} reached 1.0-2.0. The cells were then washed with 30 milliliters of water and with 30 milliliters of 100 mM LiOAc. For each library, a transformation mixture containing 1M LiOAc, 50% PEG, single stranded carrier DNA, water, and 25 ug of library DNA was added to cells. Each transformation was heat shocked at 42°C for 45 minutes. The cells were pelleted and then grown in 50 milliliters YPD medium at 30°C for 1 hour. Cells were pelleted again and washed with 30 milliliters SD-leu medium (14.7g/L sodium citrate, 4.29g/L citric acid, 2% dextrose, 6.7g/L yeast nitrogen base (YNB), 1.6 g/L Yeast Synthetic Drop-out Medium Supplements without Leucine (Sigma, Y1376)). The cells were then resuspended in 10 milliliters of SD–leu media. The 10 milliliters of transformed cells were then inoculated into 300 milliliters SD-leu media and grown overnight at 30°C.

Induction of expression of the variant Fc-fragments in the yeast transformants was accomplished by inoculating an aliquot of an overnight culture (about 10^8 cells) into induction medium (5.4g/L Na_2HPO_4, 8.56 g/L NaH_2PO_4, 2% galactose, 6.7 g/L YNB, and 1.6 g/L Yeast Synthetic Drop-out Medium Supplements without Leucine (Sigma, Y1376)) and culturing the cells for about 48 hours at 20 °C.

These induced cells were spun down and resuspended in phosphate buffered saline (PBS) buffer (+0.5% BSA) at pH 7.4. Then 0.5-1 x 10^8 induced cells were incubated for one hour with 1 μM biotinylated FcRn (biotin-FcRn). Cells were then washed and incubated with 2 μg/mL labeled streptavidin (streptavidin (SA)-Alexa Fluor® 647 (Catalog number S32357, Invitrogen)) for 15 minutes. Cells were then washed and incubated with anti-Cy5/Anti-Alexa Fluor® 647 Microbeads (Catalog number 130-091-395, MACS Miltenyi Biotec) for 15 minutes. Cells were washed and separated using LS Columns (Catalog number 130-042-401, MACS Miltenyi Biotec) and a QuadroMACS® Separation Unit (Catalog number 130-091-051, MACS Miltenyi Biotec). Aliquots of the pre-column, flow-thru, washes, and elution fractions were collected for FACS analysis. Flow-thru and washes were collected as our depleted population, which contained cells expressing variant Fc-polypeptides that bound to FcRn with very low affinity at pH 7.4.

This depleted population of cells was then grown overnight at 30 °C in SD-leu medium. Cells were then induced for about 48 hours at 20 °C in induction medium as described above. The

subsequent wash and incubation steps were performed in MES buffer (20 mM MES, 137 mM NaCl, 0.5% BSA) at pH 5.5. Induced cells were incubated on ice with 250 nM biotin-FcRn for one hour. Subsequently, SA-Alexa Fluor® 647 (2 µg/mL) and anti-HA-FITC (1 µg/mL) were added, and, after being washed and put through a cell strainer, the cells were subjected to FACS analysis at pH 5.5. Gates were set to collect cells that exhibited a robust signal for both FITC and Alexa Fluor®. These cells were then cultured overnight at 30 ºC in SD-leu medium and then induced for about 48 hours at 20 ºC in induction medium. Then, in a second round, the induced cells were incubated on ice with 25 nM biotin-FcRn for one hour, and the labeling, washing, straining, FACS, culturing, and inductions steps, as described above, were repeated. Finally, in a third round, the induced cells were incubated on ice with 5 nM biotin-FcRn for one hour, followed by the labeling, washing, straining, FACS and culturing steps described above. Hence, the end result was a population of cells expressing Fc-fragments that could bind FcRn with higher affinity than a wild type Fc-fragment could at pH 5.5.

This population of cells was plated, and 500 colonies from Library L5 and 400 colonies from each of Libraries L-8 and L-10 were selected for further analysis. Each of these individual colonies was cultured at 30 ºC overnight in SD-leu medium as described above. The sequences of the insertions in each of these isolates was determined from yeast plasmid DNA. In all, 317, 265, and 313 unique sequences were found among these isolates in each of libraries L5, L-8, and L-10, respectively. Cells were then induced at 20 ºC for 48 hours in the induction medium described above. The subsequent wash and incubation steps were performed in parallel in MES buffer (20mM MES, 137mM NaCl, 0.5% BSA) at pH 5.5 and in PBS buffer (+0.5% BSA) at pH 7.4. About 5 x 10^5 cells per sample were incubated with 250 nM biotin-FcRn and 1 µg/ml anti-HA-FITC for 1 hour. Then cells were washed and incubated with 2 µg/mL SA-Alexa Fluor® 647 for 15 minutes. Cells were washed and analyzed for binding via FACS to determine whether they bound FcRn strongly at pH 5.5 and weakly at pH 7.4. Individual cell lines showing the highest signals for binding to FcRn at pH 5.5, along with a low signal for binding to FcRn at pH 7.4 and few or no methionine or tryptophan residues, were chosen from each library for further analysis. These selected isolates included 158, 59, and 50 cell lines from L5, L-8, and L-10, respectively, and the sequences of the insertions in these isolates are shown in Table 8 below. (Note that the clone named 5y-37 was not included in the further analysis because it was not re-cloned successfully into the appropriate vector.)

Table 8: Sequences of variant Fc-polypeptides that bind with high affinity at pH 5.5 and low affinity at pH 7.4

LIBRARY L5		LIBRARY L-8		LIBRARY L-10	
Clone	**INSERTION SEQ (SEQ ID NO)**	**Clone**	**INSERTION SEQ (SEQ ID NO)**	**Clone**	**INSERTION SEQ (SEQ ID NO)**

LIBRARY L5		LIBRARY L-8		LIBRARY L-10	
Clone	**INSERTION SEQ (SEQ ID NO)**	**Clone**	**INSERTION SEQ (SEQ ID NO)**	**Clone**	**INSERTION SEQ (SEQ ID NO)**
5y-1	GGCHFDIMNCGG (SEQ ID NO:90)	8y-1	GGCKTWQLLIFCGG (SEQ ID NO:248)	10y-1	GGCAFEFSSAFNCGG (SEQ ID NO:307)
5y-2	GGCVIDFFGCGG (SEQ ID NO:91)	8y-2	GGCEQNWTLYMCGG (SEQ ID NO:249)	10y-2	SGCQTMLTAEGEWCGG (SEQ ID NO:308)
5y-3	GGCDIMIFECGG (SEQ ID NO:92)	8y-3	SGCWPSPYIFPCGG (SEQ ID NO:250)	10y-3	GGCVMDLWPDLEICGG (SEQ ID NO:309)
5y-4	GGCMTEFAICGG (SEQ ID NO:93)	8y-4	GGCWEALQVNNCGG (SEQ ID NO:251)	10y-4	GGCQPLFDDHDTWCGG (SEQ ID NO:310)
5y-5	GGCIQYWQFCGG (SEQ ID NO:94)	8y-5	CGCQAMVVEDLCG (SEQ ID NO:252)	10y-5	GGCPFELVMSDEQCGG (SEQ ID NO:311)
5y-6	GGCPFSWAFCGG (SEQ ID NO:95)	8y-6	GGCPLEWPRISCGG (SEQ ID NO:253)	10y-6	SGCGHGMQMDSVFCGG (SEQ ID NO:312)
5y-7	GGCGFAFMYCGG (SEQ ID NO:96)	8y-7	GGCEPWIMEANCGG (SEQ ID NO:254)	10y-7	SGCDETQSAIWYFCGG (SEQ ID NO:313)
5y-8	GGCPVLLFNCGG (SEQ ID NO:97)	8y-8	GGCPWDQHINFCGG (SEQ ID NO:255)	10y-8	GGCREPEQYWTVWCGG (SEQ ID NO:314)
5y-9	GGCPFTWTKCGG (SEQ ID NO:98)	8y-9	GGCPWYIQMDHCGG (SEQ ID NO:256)	10y-9	SGCQEKKDLYWEYCGG (SEQ ID NO:315)
5y-10	GGCYLYWQFCGG (SEQ ID NO:99)	8y-10	SGCQPWEISYYCGG (SEQ ID NO:257)	10y-10	SGCGQDNDLPWEWCGG (SEQ ID NO:316)
5y-11	GGCVINMFPCGG (SEQ ID NO:100)	8y-11	GGCPVMFLDPRCGG (SEQ ID NO:258)	10y-11	GGCVFQLSFSRSDCGG (SEQ ID NO:317)
5y-12	GGCPFTWNTCGG (SEQ ID NO:101)	8y-12	GGCSSDVLMIFCGG (SEQ ID NO:259)	10y-12	SGCAFDMIWFEGVCGG (SEQ ID NO:318)
5y-13	GGCPFQIGECGG (SEQ ID NO:102)	8y-13	GGCVDEMVIYHCGG (SEQ ID NO:260)	10y-13	SGCAFYWQPWEHSCGG (SEQ ID NO:319)
5y-14	GGCLDIIWMCGG (SEQ ID NO:103)	8y-14	GGCPFMVNLYSCGG (SEQ ID NO: :261)	10y-14	GGCQLSIILTGLPCGG (SEQ ID NO:320)
5y-15	GGCMFVFPACGG (SEQ ID NO:104)	8y-15	GGCESDTMWYFCGG (SEQ ID NO:262)	10y-15	GGCGMLEWSGLQFCGG (SEQ ID NO:321)
5y-16	GGCVMEQLWCGG (SEQ ID NO:105)	8y-16	GGCRSDEIIFFCGG (SEQ ID NO:263)	10y-16	SGCHEKALTYWEFCGG (SEQ ID NO:322)
5y-17	GGCFKEYTWCGG (SEQ ID NO:106)	8y-17	GGCPWDLLLPLCGG (SEQ ID NO:264)	10y-17	GGCFENMQVWYNECGG (SEQ ID NO:323)
5y-18	GGCPKDYHICGG (SEQ ID NO:107)	8y-18	GGCPWAMELVHCGG (SEQ ID NO:265)	10y-18	GGCPEWENQILLFCGG (SEQ ID NO:324)
5y-19	GGCEVMVFPCGG (SEQ ID NO:108)	8y-19	SGCTASMYWEYCGG (SEQ ID NO:266)	10y-19	SGCESWQRDMNYFCGG (SEQ ID NO:325)
5y-20	GGCVFNTVFCGG (SEQ ID NO:109)	8y-20	GGCGLYMDPPYYGG (SEQ ID NO:267)	10y-20	SGCNDQFPMYYLFCGG (SEQ ID NO:326)
5y-21	GGCNLPQEWCGG (SEQ ID NO:110)	8y-21	GGCPVMVMEPYCGG (SEQ ID NO:268)	10y-21	GGCFEDMALQPTQCGG (SEQ ID NO:327)
5y-22	GGCMIAPMYCGG (SEQ ID NO:111)	8y-22	GGCQTEFILEFCGG (SEQ ID NO:269)	10y-22	SGCKGPWQFEFLVCGG (SEQ ID NO:328)

LIBRARY L5		LIBRARY L-8		LIBRARY L-10	
Clone	**INSERTION SEQ (SEQ ID NO)**	**Clone**	**INSERTION SEQ (SEQ ID NO)**	**Clone**	**INSERTION SEQ (SEQ ID NO)**
5y-23	GGCMMLYPMCGG (SEQ ID NO:112)	8y-23	SGCAFQAHGAMCGG (SEQ ID NO:270)	10y-23	GGCEAFSMKFNDFCGG (SEQ ID NO:329)
5y-24	GGCIIGPFLCGG (SEQ ID NO:113)	8y-24	GGCPDFMFRMNCGG (SEQ ID NO:271)	10y-24	GGCVQPAIAMWPFCGG (SEQ ID NO:330)
5y-25	GGCTGMVFFCGG (SEQ ID NO:114)	8y-25	SGCSVWFDTISCGG (SEQ ID NO:272)	10y-25	GGCTDQGRFVLYECGG (SEQ ID NO:331)
5y-26	GGCLMYKNVCGG (SEQ ID NO:115)	8y-26	GGCPWSMEISNCGG (SEQ ID NO:273)	10y-26	GGCPVQEFLWGVYCGG (SEQ ID NO:332)
5y-27	GGCAFGIMWCGG (SEQ ID NO:116)	8y-27	GGCPTWNWEITCGG (SEQ ID NO:274)	10y-27	GGCSNSWEWTLYACGG (SEQ ID NO:333)
5y-28	GGCRHRKKWCGG (SEQ ID NO:117)	8y-28	SGCPWDMHIVDCGG (SEQ ID NO:275)	10y-28	SGCHGLVEWGYMACGG (SEQ ID NO:334)
5y-29	GGCFMGIWQCGG (SEQ ID NO:118)	8y-29	SGCFPWEPAYFCGG (SEQ ID NO:276)	10y-29	SGCEAFGLIFEDFCGG (SEQ ID NO:335)
5y-30	GGCPSLPQFCGG (SEQ ID NO:119)	8y-30	GGCPFGWNVFHCGG (SEQ ID NO:277)	10y-30	GGCANPEFQMWYFCGG (SEQ ID NO:336)
5y-31	GGCPSVFTWCGG (SEQ ID NO:120)	8y-31	GGCPWHMEVNECGG (SEQ ID NO:278)	10y-31	SGCGYEVPIPLFTCGG (SEQ ID NO:337)
5y-32	GGCQEYWEFCGG (SEQ ID NO:121)	8y-32	GGCPFALGMGECGG (SEQ ID NO:279)	10y-32	GGCWFQQFAWRATCGG (SEQ ID NO:338)
5y-33	GGCQWPTEFCGG (SEQ ID NO:122)	8y-33	GGCMFPFMLSNCGG (SEQ ID NO:280)	10y-33	GGCGFELNMISQYCGG (SEQ ID NO:339)
5y-34	GGCIKFFDWCGG (SEQ ID NO:123)	8y-34	GGCAFQFMPAHCGG (SEQ ID NO:281)	10y-34	GGCEPFELRFYHEGCGG (SEQ ID NO:340)
5y-35	GGCEMSFFLCGG SEQ ID NO:124)	8y-35	GGCQIQGFEFTCGG (SEQ ID NO:282)	10y-35	GGCPFQLVWSPAFCGG (SEQ ID NO:341)
5y-36	GGCHSEVEYCGG (SEQ ID NO:125)	8y-36	GGCPMGIILDLCGG (SEQ ID NO:283)	10y-36	SGCAWEIKGIWCGG (SEQ ID NO:342)
5y-37	GGCWEHPHYCGG (SEQ ID NO:126)	8y-37	GGCLMLEPTVTCGG (SEQ ID NO:284)	10y-37	SGCSSIQSWRLWLCGG (SEQ ID NO:343)
5y-38	GGCETYWLFCGG (SEQ ID NO:127)	8y-38	GGCGKNEVAMFCGG (SEQ ID NO:285)	10y-38	GGCGVMQVLNRAHCGG (SEQ ID NO:344)
5y-39	GGCRVPYPSCGG (SEQ ID NO:128)	8y-39	GGCSFLLEIANCGG (SEQ ID NO:286)	10y-39	RGCQVKYYMGEGDCGG (SEQ ID NO:345)
5y-40	GGCGWPFVMCGG (SEQ ID NO:129)	8y-40	GGCDVEKIMIFCGG (SEQ ID NO:287)	10y-40	GGCPVWIPFHWEECGG (SEQ ID NO:346)
5y-41	GGCMLFLESCGG (SEQ ID NO:130)	8y-41	GGCFPMTPWGLCGG (SEQ ID NO:288)	10y-41	SGCLLWQQSMLLFCGG (SEQ ID NO:347)
5y-42	GGCFHVKRWCGG (SEQ ID NO:131)	8y-42	SGCDWYLEWSGNCGG (SEQ ID NO:289)	10y-42	SGCEQQWSWRLYLCGG (SEQ ID NO:348)
5y-43	GGCVWEQEHCGG (SEQ ID NO:132)	8y-43	GGCGVEIMFHGCGG (SEQ ID NO:290)	10y-43	GGCSVQSTWQLWACGG (SEQ ID NO:349)
5y-44	GGCILHFKDCGG (SEQ ID NO:133)	8y-44	GGCMDGLHLYFCGG (SEQ ID NO:291)	10y-44	SGCKYPIFWDTIDCGG (SEQ ID NO:350)

LIBRARY L5		LIBRARY L-8		LIBRARY L-10	
Clone	**INSERTION SEQ (SEQ ID NO)**	**Clone**	**INSERTION SEQ (SEQ ID NO)**	**Clone**	**INSERTION SEQ (SEQ ID NO)**
5y-45	GGCHFEVFQCGG (SEQ ID NO:134)	8y-45	SGCPIFIFDYYCGG (SEQ ID NO:292)	10y-45	SGCVEYQYQMVYFCGG (SEQ ID NO:351)
5y-46	GGCVFEVMQCGG (SEQ ID NO:135)	8y-46	GGCAVWIFSDACGG (SEQ ID NO:293)	10y-46	GGCTDQRWFVLYECGG (SEQ ID NO:352)
5y-47	GGCMTEFSWCGG (SEQ ID NO:136)	8y-47	GGCPWSLHIQQCGG (SEQ ID NO:294)	10y-47	GGCPFWQEWHLSYCGG (SEQ ID NO:353)
5y-48	GGCEGNMRFCGG (SEQ ID NO:137)	8y-48	SGCAFSMLFINCGG (SEQ ID NO:295)	10y-48	SGCYMGYMHLIAECGG (SEQ ID NO:354)
5y-49	GGCKGHMWYCGG (SEQ ID NO:138)	8y-49	SGCLPWELYMFCGG (SEQ ID NO:296)	10y-49	GGCFMGSFSLVYGCGG (SEQ ID NO:355)
5y-50	GGCEAYWQFCGG (SEQ ID NO:139)	8y-50	SGCPFTINFYTCGG (SEQ ID NO:297)	10y-50	SGCPWGFMFPISYCGG (SEQ ID NO:356)
5y-51	GGCVFSRFWCGG (SEQ ID NO:140)	8y-51	GGCPIWFTWSTCGG (SEQ ID NO:298)		
5y-52	GGCMMPFWPCGG (SEQ ID NO:141)	8y-52	GGCQIQVVNPYCGG (SEQ ID NO:299)		
5y-53	GGCIFQFEMCGG (SEQ ID NO:142)	8y-53	GGCAFQIEFLMCGG (SEQ ID NO:300)		
5y-54	GGCKRQMWYCGG (SEQ ID NO:143)	8y-54	GGCAWEIRILGCGG (SEQ ID NO:301)		
5y-55	GGCKTPNPWCGG (SEQ ID NO:144)	8y-55	GGCPYQLVIMWCGG (SEQ ID NO:302)		
5y-56	GGCKAFYPWCGG (SEQ ID NO:145)	8y-56	GGCMFAMHVFGCGG (SEQ ID NO:303)		
5y-57	GGCKMYQYDCGG (SEQ ID NO:146)	8y-57	SGCTVMYTLQIFGG (SEQ ID NO:304)		
5y-58	GGCYPDNMFCGG (SEQ ID NO:147)	8y-58	SGCAHQVYWAFCGG (SEQ ID NO:305)		
5y-59	GGCQVKIFWCGG (SEQ ID NO:148)	8y-59	GGCPNFFNFWFCGG (SEQ ID NO:306)		
5y-60	GGCSIPQEWCGG (SEQ ID NO:149)				
5y-61	GGCKMYQATCGG (SEQ ID NO:150)				
5y-62	GGCQYERWHCGG (SEQ ID NO:151)				
5y-63	GGCRFQHQWCGG (SEQ ID NO:152)				
5y-64	GGCQNMFWQCGG (SEQ ID NO:153)				
5y-65	GGCVMEIVFCGG (SEQ ID NO:154)				
5y-66	GGCILNFNMCGG (SEQ ID NO:155)				

LIBRARY L5		LIBRARY L-8		LIBRARY L-10	
Clone	INSERTION SEQ (SEQ ID NO)	Clone	INSERTION SEQ (SEQ ID NO)	Clone	INSERTION SEQ (SEQ ID NO)
5y-67	GGCMHMDYFCGG (SEQ ID NO:156)				
5y-68	GGCQVMVLPCGG (SEQ ID NO:157)				
5y-69	GGCLFDWPSCGG (SEQ ID NO:158)				
5y-70	GGCKMYHQTCGG (SEQ ID NO:159)				
5y-71	GGCQWLYESCGG (SEQ ID NO:160)				
5y-72	GGCFTNFWLCGG (SEQ ID NO:161)				
5y-73	GGCWEPTHWCGG (SEQ ID NO:162)				
5y-74	GGCAFAMLQCGG (SEQ ID NO:163)				
5y-75	GGCMYQRQAWCGG (SEQ ID NO:164)				
5y-76	GGCPFLWAECGG (SEQ ID NO:165)				
5y-77	GGCMFDHKVCGG (SEQ ID NO:166)				
5y-78	GGCMEIFNFCGG (SEQ ID NO:167)				
5y-79	GGCVMERLWCGG (SEQ ID NO:168)				
5y-80	GGCEYYWQFCGG (SEQ ID NO:169)				
5y-81	GGCPFSWDQCGG (SEQ ID NO:170)				
5y-82	GGCIEYFSWCGG (SEQ ID NO:171)				
5y-83	GGCVFEIMKCGG (SEQ ID NO:172)				
5y-84	GGCESPQYFCGG (SEQ ID NO:173)				
5y-85	GGCHHDFEWCGG (SEQ ID NO:174)				
5y-86	GGCMFPFSWCGG (SEQ ID NO:175)				
5y-87	GGCNTVLQECGG (SEQ ID NO:176)				
5y-88	GGCVFDIMLCGG (SEQ ID NO:177)				

LIBRARY L5		LIBRARY L-8		LIBRARY L-10	
Clone	INSERTION SEQ (SEQ ID NO)	Clone	INSERTION SEQ (SEQ ID NO)	Clone	INSERTION SEQ (SEQ ID NO)
5y-89	GGCMYQQPWCGG (SEQ ID NO:178)				
5y-90	GGCKKLYHYCGG (SEQ ID NO:179)				
5y-91	GGCPHWPFECGG (SEQ ID NO:180)				
5y-92	GGCPIFPMICGG (SEQ ID NO:181)				
5y-93	GGCMSKDLWCGG (SEQ ID NO:182)				
5y-94	GGCMFQMGVCGG (SEQ ID NO:183)				
5y-95	GGCYEWPSYCGG (SEQ ID NO:184)				
5y-96	GGCQMLYMDCGG (SEQ ID NO:185)				
5y-97	GGCTVQVFFCGG (SEQ ID NO:186)				
5y-98	GGCITFPMMCGG (SEQ ID NO:187)				
5y-99	GGCVMYWEYCGG (SEQ ID NO:188)				
5y-100	GGCMWEVLHCGG (SEQ ID NO:189)				
5y-101	GGCMQERSWCGG (SEQ ID NO:190)				
5y-102	GGCVFETIQCGG (SEQ ID NO:191)				
5y-103	GGCQWANSYCGG (SEQ ID NO:192)				
5y-104	GGCKFGQWYCGG (SEQ ID NO:193)				
5y-105	GGCVFDQMWCGG (SEQ ID NO:194)				
5y-106	GGCEVMIFNCGG (SEQ ID NO:195)				
5y-107	GGCESPMFVCGG (SEQ ID NO:196)				
5y-108	GGCITMFQNCGG (SEQ ID NO:197)				
5y-109	GGCVFERMFCGG (SEQ ID NO:198)				
5y-110	GGCGFEIFMCG (SEQ ID NO:199)				

LIBRARY L5		LIBRARY L-8		LIBRARY L-10	
Clone	INSERTION SEQ (SEQ ID NO)	Clone	INSERTION SEQ (SEQ ID NO)	Clone	INSERTION SEQ (SEQ ID NO)
5y-111	GGCLLQFTGCGG (SEQ ID NO:200)				
5y-112	GGCHFQIFQCGG (SEQ ID NO:200)				
5y-113	GGCPFDWDKCGG (SEQ ID NO:202)				
5y-114	GGCVTPLPFCG (SEQ ID NO:203)				
5y-115	GGCYMYMDYCGG (SEQ ID NO:204)				
5y-116	GGCMFEWYVCGG (SEQ ID NO:205)				
5y-117	GGCPFTWRICGG (SEQ ID NO:206)				
5y-118	GGCENDWKMCGG (SEQ ID NO:207)				
5y-119	GGCAFEFIYCGG (SEQ ID NO:208)				
5y-120	GGCPVAVFMCGG (SEQ ID NO:209)				
5y-121	GGCHFDIFDCGG (SEQ ID NO:210)				
5y-122	GGCPPENMFCGG (SEQ ID NO:211)				
5y-123	GGCPFQMGECGG (SEQ ID NO:212)				
5y-124	GGCISGFFWCGG (SEQ ID NO:213)				
5y-125	GGCPFHFQVCGG (SEQ ID NO:214)				
5y-126	GGCMFQIINCGG (SEQ ID NO:215)				
5y-127	GGCQYFLPCGG (SEQ ID NO:216)				
5y-128	GGCHFAVLDCGG (SEQ ID NO:217)				
5y-129	GGCWNVMGLCGG (SEQ ID NO:218)				
5y-130	GGCYTTHELCGG (SEQ ID NO:219)				
5y-131	GGCLYKQVDCGG (SEQ ID NO:220)				
5y-132	GGCVFSALWCGG (SEQ ID NO:221)				

LIBRARY L5		LIBRARY L-8		LIBRARY L-10	
Clone	INSERTION SEQ (SEQ ID NO)	Clone	INSERTION SEQ (SEQ ID NO)	Clone	INSERTION SEQ (SEQ ID NO)
5y-133	GGCPFQFQTCGG (SEQ ID NO:222)				
5y-134	GGCAFLMMDCGG (SEQ ID NO:223)				
5y-135	GGCEVWYEFCGG (SEQ ID NO:224)				
5y-136	GGCAFDIGVCGG (SEQ ID NO:225)				
5y-137	GGCLSPLMWCGG (SEQ ID NO:226)				
5y-138	GGCPFSWVICGG (SEQ ID NO:227)				
5y-139	GGCMLMFQGCGG (SEQ ID NO:228)				
5y-140	GGCQPNHWLCGG (SEQ ID NO:229)				
5y-141	GGCIDTYVWCGG (SEQ ID NO:230)				
5y-142	GGCHFHLMFCGG (SEQ ID NO:231)				
5y-143	GGCQMIFSTCGG (SEQ ID NO:232)				
5y-144	GGCKMYQPDCGG (SEQ ID NO:233)				
5y-145	GGCMWGVFKCGG (SEQ ID NO:234)				
5y-146	GGCGLFGQSCGG (SEQ ID NO:235)				
5y-147	GGCQFNFPWCGG (SEQ ID NO:236)				
5y-148	GGCNIAYPWCGG (SEQ ID NO:237)				
5y-149	GGCKTIPIFCGG (SEQ ID NO:238)				
5y-150	GGCQMELFLCGG (SEQ ID NO:239)				
5y-151	GGCLGAFYWCGG (SEQ ID NO:240)				
5y-152	GGCPFNFASCGG (SEQ ID NO:241)				
5y-153	GGCQFDILWCGG (SEQ ID NO:242)				
5y-154	GGCYYTHELCGG (SEQ ID NO:243)				

LIBRARY L5		LIBRARY L-8		LIBRARY L-10	
Clone	INSERTION SEQ (SEQ ID NO)	Clone	INSERTION SEQ (SEQ ID NO)	Clone	INSERTION SEQ (SEQ ID NO)
5y-155	GGCQQRWRYCGG (SEQ ID NO:244)				
5y-156	GGCLWVDEYCGG (SEQ ID NO:245)				
5y-157	GGCGMLGWFCGG (SEQ ID NO:246)				
5y-158	GGCWEQHYLCGG (SEQ ID NO:247)				

Selected individual clones from each library were pooled and DNA was isolated using the Zymoprep Yeast Plasmid Miniprep II Kit (D2004, Zymo Research). PCR was performed on the pooled DNA using forward primer (5' GGA AAA GTC GAC TAG ACC ACC ATG GA 3' (SEQ ID NO:357)) and reverse primer (5' CTT TGC GGC CGC TCA TTA TTT 3' (SEQ ID NO:358)). A PCR Core Kit (Roche, Catalog No. 11 578 553 001) was used with the following reaction conditions: 95 °C for 5 minutes, followed by 30 cycles of 95 °C for 45 seconds, 55 °C for 45 seconds, 72 °C for 90 seconds, and finally 72 °C 10 minutes. The PCR reaction and a mammalian expression vector were digested overnight at 37°C with SalI and NotI restriction enzymes (New England Biolabs). The digested DNA was gel purified using the QIAquick Gel Purification Kit (28704, Qiagen). The digested vector DNA and PCR products were ligated using T4 DNA ligase (New England Biolabs) and incubated at 16 °C overnight. The ligated DNA was used to transform XL10-gold Ultracompetent *E. coli* cells (#200315, Stratagene), and individual clones were selected. Plasmid DNA from an *E. coli* clone containing DNA encoding each selected variant Fc-polypeptide were introduced into 293-6E mammalian cells. Specifically, 293-6E cells were seeded at 5 x 10^4 cells/well in poly-D-lysine-coated, 96well flat bottom microtiter plates and incubated overnight at 37°C. Then 200 ng of plasmid DNA was transfected into the cells using the Fugene® HD transfection reagent (#E2312, Promega) and incubated overnight at 37 °C. The following day the growth media was changed to serum free media containing 0.5% Tryptone. Conditioned media was collected after another 6 days of growth at 37°C. The conditioned media, which contained the variant Fc-fragments, was then tested for binding to FcRn using the ForteBio technology described in Example 4. Association and dissociation curves at pH 6 and 7.4, respectively, from variant Fc-fragments having the most favorable binding profiles are shown in Figure 7.

Figure 7 shows association and dissociation profiles for FcRn binding at pH 6 and pH 7.4, respectively, of the 28 best variant Fc-fragments from yeast library L5, as well as a wild type Fc-fragment (FcWT), and Figure 8 shows FcRn binding profiles for the 18 best variant Fc-fragments selected from yeast libraries L-8 and L-10. All of the L5 variant Fc-fragments had a much higher

maximal response at pH 6, that is, higher binding activity, than FcWT. At pH 7.4, all dissociated rapidly, that is, had little or no binding activity, similar to FcWT. All of the L-8 and L-10 variant Fc-fragments had a much higher maximal response at pH 6, that is, higher binding activity, than FcWT. At pH 7.4, all dissociated rapidly, although most showed low levels of residual binding not observed in FcWT. Thus, all of these variant Fc-fragments have favorable properties as compared to FcWT. Therefore, insertions of peptides with different lengths can be successfully used to enhance binding activity to FcRn at pH 6 while preserving rapid dissociation from FcRn at pH 7.4 with little or no residual binding.

Example 12: In vivo Characterization of variant Fc-fragments selected in yeast

To determine whether antibodies containing the variant Fc-fragments identified in the yeast screen described above have improved pharmacokinetic (PK) properties *in vivo,* an unmodified Antibody X (which is a human IgG2 anti-human IL-23 antibody) and variant versions of Antibody X containing variant Fc-fragments were tested *in vivo* in cynomolgus monkeys to define pharmacokinetic parameters. Antibody X was selected as an appropriate antibody in which to test the pharmacokinetic parameters of the variant Fc-fragments because it was known to have a linear pK profile and because IL-23 was known to be expressed at low levels *in vivo.* Thus, it was expected that target-related effects on PK parameters would be minimal, making it easier to detect pK effects due to the variant Fc-fragments.

Six variant IgG2 antibodies called (X-5y-8, X-5y-132, X-5y-38, X-5y-91, X-5y-119, and X-5y-127) were made. These IgG2 antibodies had the same insertions at the same positions as the variant IgG1 Fc-fragments 5y-8, 5y-132, 5y-38, 5y-91, 5y-119, and 5y-127, respectively. A plasmid containing DNA encoding the heavy chain of Antibody X was used as a template for five PCR reactions done using the following primers: for X-5y-8, forward, 5'-GAG TGG GAG AGC AAT GGT GGT TGT CCG GTT CTG CTG TTC AAC TGT GGT GGT GGG CAG CCG GAG AAC-3' (SEQ ID NO:380), and reverse, 5'- GTT CTC CGG CTG CCC ACC ACC ACA GTT GAA CAG CAG AAC CGG ACA ACC ACC ATT GCT CTC CCA CTC -3'(SEQ ID NO:381); for X-5y-132,forward, 5'-GAG TGG GAG AGC AAT GGT GGT TGT GTT TTC TCT GCT CTG TGG TGT GGT GGT GGG CAG CCG GAG AAC-3'(SEQ ID NO:382), and reverse, 5'-GTT CTC CGG CTG CCC ACC ACC ACA CCA CAG AGC AGA GAA AAC ACA ACC ACC ATT GCT CTC CCA CTC-3' (SEQ ID NO:383); for X-5y-38, forward, 5'- GAG TGG GAG AGC AAT GGT GGT TGT GAA ACT TAC TGG TTG TTC TGT GGT GGT GGG CAG CCG GAG AAC-3' (SEQ ID NO:384), and reverse, 5'-GTT CTC CGG CTG CCC ACC ACC ACA GAA CAA CCA GTA AGT TTC ACA ACC ACC ATT GCT CTC CCA CTC-3' (SEQ ID NO:385); for X-5y-91, forward, 5'-GAG TGG GAG AGC AAT GGT GGT TGT CCG CAT TGG CCG TTC GAA TGT GGT GGT GGG CAG CCG GAG AAC-3' (SEQ ID NO:386), and reverse, 5'-GTT CTC CGG CTG CCC ACC ACC ACA TTC

GAA CGG CCA ATG CGG ACA ACC ACC ATT GCT CTC CCA CTC-3' (SEQ ID NO:387); for X-5y-119, forward, 5'-GAG TGG GAG AGC AAT GGT GGT TGT GCT TTC GAA TTC ATC TAC TGT GGT GGT GGG CAG CCG GAG AAC-3' (SEQ ID NO:388), and reverse, 5'- GTT CTC CGG CTG CCC ACC ACC ACA GTA GAT GAA TTC GAA AGC ACA ACC ACC ATT GCT CTC CCA CTC -3' (SEQ ID NO:389); and for X-5y-127, forward, 5'-GAG TGG GAG AGC AAT GGT GGT TGT CAG TAC TTC TTG CCG TGT GGT GGT GGG CAG CCG GAG AAC-3' (SEQ ID NO:390), and reverse 5'-GTT CTC CGG CTG CCC ACC ACC ACA CGG CAA GAA GTA CTG ACA ACC ACC ATT GCT CTC CCA CTC-3' (SEQ ID NO:391). The Quikchange Site-Directed Mutagenesis Kit (Stratagene, 200518) protocol was used. The reaction mixture was 200 nM dNTPs, 100 nM primers, 1ng DNA template, 1 µL DNA polymerase and water in a total volume of 50 µL. The reaction was run at 95°C for 30 seconds, then 16 cycles of 95°C for 30 seconds, 55°C for 60 seconds, 68°C for 6 minutes, followed by 68°C for 10 minutes. Then 1 µL of DpnI was added, and the reaction was incubated at 37°C for 1 hour. Then 2 µL of the mixture was used to transform 30 µl of XL1-blue supercompetent cells (Stratagene) at 42°C for 45 seconds. Thereafter, 0.5 mL SOC was added, and the cells were incubated at 37°C for 1 hour on a shaker at 300 rpm. The transformed cells were spread on LB-ampicillin agar plates and incubated at 37°C overnight. Individual colonies were picked, and plasmid DNA was prepared and sequenced to ensure that the isolates chosen had the expected DNA sequence.

Antibodies were prepared in essentially the same way as described above for Fc-fragments, except that the mammalian host cells were transfected with DNAs encoding both the IgG2 heavy chain, including a portion encoding either a variant or a control Fc-fragment, and the light chain of Antibody X. Host cells were incubated under conditions appropriate for expression of the antibodies, and the antibodies were recovered from the culture medium, purified as described above, and used for the following experiments.

Cynomolgus monkeys (n=2/group) received a single intravenous dose of an unmodified or a variant version of Antibody X at a dose of 1 mg/kg and were followed during an 8-week in-life phase. Antibody X-5-112, which was previously tested, was used as a control, as well as Antibody X itself. Samples of blood were collected at specified time points over the course of the experiment. Samples were collected at pre-dose, 0.25, 1, 4, 8, 12, 24, 48, 72, 168, 240, 336, 408, 504, 576, 672, 744, 840, 1008, 1176 and 1344 hours post-dose. The antibodies were detected in blood samples and the pharmacokinetic analysis was performed essentially as described above in Example 9.

As shown in Figure 9, variant versions of Antibody X containing all variant Fc-fragments tested demonstrated higher mAb concentrations in cynomolgus monkeys at most sampling time points, including all time points beyond 400 hours, compared to unmodified Antibody X. Further, some variants

were comparable to X-5-112, which had previously been tested, including X-5y-8, X-5y-127, and X-5y-91.

Table 11 below shows the half life (T½), exposure (area under the curve (AUC)) and clearance rate(Cl) of Antibody X and variants thereof in each of the cynomolgus monkeys in the study.

Table 11: Individual values for half life (T½), exposure (AUC), and clearance (Cl).

Antibody (monkey identification number)	T½ (hours)	AUC (µg*hr/mL)	Cl (mL/kg/hr)
Antibody X (#201)	269	3356	0.29
Antibody X (#202)	351	3745	0.253
X-5-112 (#203)	469	5843	0.148
X-5-112 (#204)	677	7752	0.099
X-5y-8 (#205)	389	5484	0.168
X-5y-8 (#206)	520	6611	0.13
X-5y-132 (#207)	139	5962	0.167
X-5y-132 (#208)	531	6770	0.127
X-5y-38 (#209)	258	5454	0.178
X-5y-38 (#210)	428	4563	0.199
X-5y-91 (#211)	626	3742	0.213
X-5y-91 (#212)	684	5162	0.151
X-5y-119 (#213)	39	4713	0.212
X-5y-119 (#214)	465	7272	0.12
X-5y-127 (#215)	529	4825	0.176
X-5y-127 (#216)	679	5946	0.132

All variants showed increased half lives and AUC compared to that of Antibody X in at least one of the two monkeys injected with each. In monkeys #207 and #213, X-5y132 and X-5y-119, respectively, had shorter half lives than Antibody X. This could be interpreted to mean that these particular monkeys developed anti-drug antibodies, which led to more rapid clearance of the antibodies. AUC values were also greater than those of Antibody X for most of the variants tested.

Averaged data for these pharmacokinetic parameters, which omits monkeys #207 and #213, is shown in Table 12 below. Values from a previous study reported in Table 6 above are included for Antibodies X and X-5-112, which show that the values are relatively constant from one study to the next.

Table 12: Mean values for half life (T½), exposure (AUC), and clearance (Cl).

Antibody (n)	T½ (hours)	AUC (µg*hr/mL)	Cl (mL/kg/hr)
Antibody X (n=2)*	335	3890	0.247
Antibody X (n=2)	310	3550	0.272
X-5-112 (n=2)*	529	7810	0.108
X-5-112 (n=2)	573	6795	0.123
X-5y-8 (n=2)	455	6044	0.149
X-5y-132 (n=1)#	531	6770	0.127

Antibody (n)	T½ (hours)	AUC (µg*hr/mL)	Cl (mL/kg/hr)
X-5y-38 (n=2)	343	5008	0.185
X-5y-91 (n=2)	655	4452	0.182
X-5y-119 (n=1)#	465	7272	0.12
X=5y-127 (n=2)	604	5386	0.154

*These are values from a previous study reported in Example 9 and Table 6 and included here for comparison.
#Only one of the two monkeys tested in this study was included because it is suspected that anti-drug antibodies are the cause of rapid clearance observed in the other monkey.

These data show that pharmacokinetic parameters for Antibody X and for X-5-112 were relatively constant from one study to the next. They also indicate that half life and exposure are increased in all variants tested. Thus, these data generally indicate that increased binding of a variant Fc-fragment to FcRn at pH 5.5-6.0 (relative to a control Fc-fragment) and rapid dissociation from FcRn at pH 7.4 correlate with a longer *in vivo* half-life of an antibody containing the variant Fc-fragment in cynomolgus monkeys.

Example 13: In vivo characterization of a variant Fc-containing antibody having a non-linear PK profile

To determine whether the PK properties of an antibody having a non-linear PK profile could be improved by the insertion of one of peptides described herein, the insertion in variant Fc fragment Fc-5-112 was transferred into Antibody Y, a human IgG2 antibody having a non-linear PK profile. This variant form of Antibody Y, a variant IgG2 antibody called Y-5-112, was made essentially as described above, except using different PCR primers appropriate for making the required alteration in Antibody Y.

Antibodies were prepared as described above in Example 9. Cynomolgus monkeys (n=2/group) received a single intravenous dose of Antibody Y or Antibody Y-5-112 at a dose of 10 mg/kg and were followed during an 8-week in-life phase. Blood samples were collected at pre-dose, and 0.083 (5 min), 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 36, 48, 72, 96, 120, 144, 168, 192, 216, 240, 264, 288, 312, 336, 384, 432, 480, 528, 576, 624, 672, 720, 768, 816, 864, 912, 960, 1008, 1056, 1104, 1152, 1200, 1248, 1296, and 1344 hours post dose.

Concentrations in cynomolgus monkey serum were determined using a sandwich ELISA. A mouse anti-human antibody was diluted in PBS and added to the wells of a 96-well microtiter plate. After a nominal 5°C incubation lasting overnight or up to three days, the well contents were discarded and a blocking buffer comprised of blocker BLOTTO (Thermo® Scientific) was dispensed into plate wells. After a minimum one-hour incubation at ambient room temperature (ART), plate wells were emptied and washed six times with 1X Wash Solution (2 mM imidazole, 0.02% Tween 20, 0.5 mM EDTA, 160 mM NaCl, which is sold by KPL, Inc. as a 20X solution). Study specimens, assay standards and quality control samples prepared in 100% monkey serum, were diluted 50-fold in blocker BLOTTO

prior to adding to plate wells. The contents of the plate wells were mixed while incubating for 60 minutes on a plate shaker. Next, plate wells were washed and horseradish peroxidase-labeled mouse anti-human antibody was added to the plate wells. After a final one-hour incubation, plate wells were washed and developed using one component 3,3' ,5,5' - tetramethylbenzidine (TMB) substrate solution. The resulting colorimetric reaction was quenched by adding sulfuric acid to plate wells. Optical densities were determined at dual wavelengths of 450 nm and 650 nm, and the value obtained at 650 nm was subtracted from the value obtained at 450 nm. The conversion of OD values into concentrations for the diluted study specimens was achieved through data regression using a logistic model with weighting set to $1/Y^2$ in Watson LIMS version 7.0.0.01.

As shown in Figure10, Antibody Y has a biphasic PK profile with an approximately linear phase extending from 0 hours to about 216 hours. Thereafter, Antibody Y concentration drops off precipitously. Antibody Y-5-112 has a similar profile except that the linear, gradually-descending portion of the profile extends from about 0 hours to about 432 hours. Thus, the insertion in AntibodyY-5-112 apparently decreases the slope of the linear portion of the PK profile, thus increasing overall exposure as compared to Antibody Y.

What is claimed is:

1. A variant Fc-polypeptide comprising a human IgG1, IgG2, IgG3, or IgG4 variant Fc-fragment,

wherein the variant Fc-fragment comprises an insertion of 3 to 20 amino acids within or adjacent to Loop 5, 8, and/or 10 of the variant Fc-fragment,

wherein the variant Fc-polypeptide binds to a human neonatal Fc receptor (hFcRn) with higher binding activity at pH 6.0 than a control Fc-polypeptide that has the same amino acid sequence as the variant Fc-polypeptide except that it does not contain the insertion within or adjacent to Loop 5, 8, and/or 10 and

wherein the variant Fc-polypeptide has little or no binding activity for binding to nFcRn at pH 7.4 and the residual binding response detected at pH 7.4 is no more than 0.1 nanometer more than that detected using the control Fc-polypeptide.

2. The variant Fc-polypeptide of claim 1, wherein the insertion within or adjacent to Loop 5, 8, and/or 10 of the variant Fc-fragment is at least six amino acids long.

3. The variant Fc-polypeptide of claim 2, wherein the insertion within or adjacent to Loop 5, 8, and/or 10 of the variant Fc-fragment is from 6 to 16 amino acids long.

4. The variant Fc-polypeptide of any one of claims 1 to 3, wherein the insertion within or adjacent to Loop 5, 8, and/or 10 of the variant Fc-fragment is at least 12 amino acids long.

5. The variant Fc-polypeptide of any one of claims 1 to 4, wherein the insertion within or adjacent to Loop 5, 8, and/or 10 of the variant Fc-fragment comprises at least one cysteine among the first four inserted amino acids and at least one cysteine among the last four inserted amino acids.

6. The variant Fc-polypeptide of any one of claims 1 to 5, wherein the insertion is within or adjacent to Loop 10 of the variant Fc-fragment.

7. The variant Fc-polypeptide of claim 6, wherein the insertion is within amino acids 383 to 387 using the EU numbering system shown in Table 1.

8. The variant Fc-polypeptide of claim 7,

wherein the insertion in the variant human Fc-fragment is between amino acids 382 and 383, amino acids 383 and 384, amino acids 384 and 385, amino acids 385 and 386, amino acids 386 and 387, or amino acids 387 and 388 of the variant Fc-fragment using the EU numbering system shown in Table 1 or

wherein amino acids 384-386 are deleted and the insertion is between amino acids 383 and 387 using the EU numbering system.

9. The variant Fc-polypeptide of claim 8,

wherein the insertion in the variant human Fc-fragment is between amino acids 382 and 383, amino acids 383 and 384, amino acids 384 and 385, or amino acids 385 and 386 of the variant Fc-fragment using the EU numbering system shown in Table 1 or

wherein amino acids 384-386 are deleted and the insertion is between amino acids 383 and 387 using the EU numbering system.

10. The variant Fc-polypeptide of any one of claims 1 to 9, wherein the variant human Fc-fragment is a variant IgG1 Fc-fragment.

11. The variant Fc-polypeptide of any one of claims 1 to 9, wherein the variant human Fc-fragment is a variant IgG2 Fc-fragment.

12. The variant Fc-polypeptide of any one of claims 1 to 11, wherein the insertion in the variant Fc-fragment has the amino acid sequence of any one of SEQ ID NO:13-24.

13. The variant Fc-polypeptide of any one of claims 1-11, wherein the insertion in the variant Fc-fragment comprises an amino acid sequence of any one of SEQ ID NOs:41-67, 90-356, 359-367, 369, 372, 373, and 375-379.

14. The variant Fc-polypeptide of any one of claims 1-11, wherein the insertion in the variant Fc-fragment comprises an amino acid sequence selected from the group consisting of:

(a) amino acids 4-8 of SEQ ID NO:216;

(b) amino acids 4-9 of and one of SEQ ID NOs: 41-53, 67, 90-163, 165-215, and 217-247, and 359-378;

(c) amino acids 4-10 of SEQ ID NO:164;

(d) amino acids 4-11 of any one of SEQ ID NOs:248-288, 290-306, and 342;

(e) amino acids 4-12 of SEQ ID NO:289 or 307; and

(f) amino acids 4-13 of any one of SEQ ID NOs:308-341 and 343-356.

15. A variant Fc-polypeptide comprising a human IgG1, IgG2, IgG3 or IgG4 variant Fc-fragment,

wherein the variant Fc-fragment comprises an insertion of 3 to 20 amino acids within or adjacent to Loop 10,

wherein the binding activity of the variant Fc-polypeptide to a hFcRn at pH 6.0 is higher than that of a control Fc-polypeptide that is the same as the variant Fc-polypeptide except that it does not contain the insertion within or adjacent to Loop 10 and

wherein the variant Fc-polypeptide has little or no binding activity for binding to hFcRn at pH 7.4 and the residual binding response detected at pH 7.4 is no more than 0.1 nanometer more than that detected using the control Fc-polypeptide.

16. The variant Fc-polypeptide of claim 15, wherein the insertion is at least six amino acids long.

17. The variant Fc-polypeptide of claim 15 or 16, wherein the insertion contains at least one cysteine among the first four amino acids of the insertion and at least one cysteine among the last four amino acids of the insertion.

18. The variant Fc-polypeptide of any one of claims 15 to 17, wherein the insertion is 6 to 16 amino acids long.

19. The variant Fc-polypeptide of any one of claims 15 to 18, wherein the insertion is at least 12 amino acids long.

20. The variant Fc-polypeptide of any one of claims 15 to 19, wherein the insertion is within amino acids 383 to 387 using the EU numbering system shown in Table 1.

21. The variant Fc-polypeptide of claim 20,

wherein the insertion in the variant human Fc-fragment is between amino acids 382 and 383, amino acids 383 and 384, amino acids 384 and 385, amino acids 385 and 386, amino acids 386 and 387, or amino acids 387 and 388 of the variant Fc-fragment using the EU numbering system shown in Table 1 or

wherein amino acids 384-386 are deleted and the insertion is between amino acids 383 and 387 using the EU numbering system.

22. The variant Fc-polypeptide of claim 21, wherein the insertion in the variant Fc-fragment is between amino acids 384 and 385 using the EU numbering system shown in Table 1.

23. The variant Fc-polypeptide of any one of claims 15 to 22, wherein the insertion in the variant Fc-fragment has the amino acid sequence of any one of SEQ ID NOs:13-24.

24. The variant Fc-polypeptide of claim 23, wherein the insertion in the variant Fc-fragment comprises the amino acid sequence of any one of SEQ ID NOs:41, 42, 43, 44, 45, 50, 97, 180, and 216.

25. The variant Fc-polypeptide of any one of claims 15-22, wherein the insertion in the variant Fc-fragment comprises the amino acid sequence of SEQ ID NO:54, 55, 56, 57, or 58.

26. The variant Fc-polypeptide of any one of claims 15 to 25, which comprises a human IgG1 variant Fc-fragment.

27. The variant Fc-polypeptide of any one of claims 15 to 25, which comprises a human IgG2 variant Fc-fragment.

28. The variant Fc-polypeptide of any one of claims 15 to 25, which comprises a human IgG4 variant Fc-fragment.

29. The variant Fc-polypeptide of any one of claims 1 to 28, which is a variant Fc fusion protein comprising a non-antibody polypeptide.

30. The variant Fc-polypeptide of claim 29, wherein a control Fc fusion protein for the variant Fc fusion protein is alefacept, rilonacept, aflibercept, etanercept, romiplostim, or abatacept.

31. The variant Fc-polypeptide of any one of claims 1 to 28, comprising a heavy chain variable region (V_H) and/or a light chain variable region (V_L) of an antibody.

32. The variant Fc-polypeptide of claim 31, which further comprises a first heavy chain constant region (C_H1) and a light chain constant region (C_L).

33. The variant Fc-polypeptide of claim 31 or 32, which comprises a V_H region and a V_L region.

34. The variant Fc-polypeptide of any one of claims 39 to 41, comprising

(a) a heavy chain comprising a V_H region, a first heavy chain constant region (C_H1), a hinge region, a C_H2 region, and a C_H3 region, and

(b) a light chain comprising a V_L region and a light chain constant region (C_L).

35. The variant Fc-polypeptide of any one of claims 31 to 34, which is monovalent.

36. The variant Fc-polypeptide of any one of claims 1 to 35, which is a dimer.

37. The variant Fc-polypeptide of any one of claims 31 to 34, which is a tetramer.

38. A nucleic acid encoding the variant Fc-polypeptide of any one of claims 1 to 37.

39. A host cell containing the nucleic acid of claim 38.

40. A method for making a variant Fc-polypeptide comprising

introducing a nucleic acid encoding the variant Fc-polypeptide into a host cell,

culturing the host cell comprising the nucleic acid under conditions such that the nucleic acid is expressed, and

recovering the expressed variant Fc-polypeptide from the culture medium or the cell mass,

wherein the variant Fc-polypeptide comprises a variant Fc-fragment comprising an insertion of 3 to 20 amino acids within or adjacent to Loop 5, 8, and/or 10 of the variant Fc-fragment, and

wherein the variant Fc-polypeptide has higher binding activity for binding to an hFcRn at pH 6.0 than a control Fc-polypeptide and

wherein the variant Fc-polypeptide has little or no binding activity at pH 7.4 and the residual binding response detected at pH 7.4 is no more than 0.1 nanometer more than that detected using the control Fc-polypeptide.

41. The method of claim 40, wherein the nucleic acid is the nucleic acid of claim 38.

42. A method for extending the half life of an Fc-polypeptide comprising a human IgG Fc-fragment comprising the following steps:

selecting a site within or adjacent to Loop 5, 8, and/or 10 for insertion; and

inserting a peptide into the selected site, wherein the peptide comprises an amino acid sequence selected from the group consisting of:

(a) SEQ ID NOs:41-67, 90-356, 359-367, 369, 372, 373, and 375-379;

(b) amino acids 4-8 of SEQ ID NO:216;

(c) amino acids 4-9 of and one of SEQ ID NOs: 41-53, 67, 90-163, 165-215, and 217-247, and 359-378;

(d) amino acids 4-10 of SEQ ID NO:164;

(e) amino acids 4-11 of any one of SEQ ID NOs:248-288, 290-306, and 342;

(f) amino acids 4-12 of SEQ ID NO:289 or 307; and

(g) amino acids 4-13 of any one of SEQ ID NOs:308-341 and 343-356.

43. The method of claim 42, wherein the site is within or adjacent to Loop 10.

44. The method of claim 43, wherein the insertion site is within amino acids 383 to 387 numbered according to the EU numbering system.

45. The method of claim 44,

wherein the insertion is between amino acids 382 and 383, amino acids 383 and 384, amino acids 384 and 385, or amino acids 385 and 386 of the variant Fc-fragment using the EU numbering system or

wherein amino acids 384-386 are deleted and the insertion is between amino acids 383 and 387 using the EU numbering system.

46. A method for identifying a human IgG variant Fc-fragment that confers a longer *in vivo* half life on a variant Fc-polypeptide that comprises the variant Fc-fragment, as compared to a control Fc-polypeptide, comprising the following steps:

(a) creating a library of nucleic acids encoding Fc-fragments containing an insertion comprising 4-20 randomized amino acids within or adjacent to Loop 10;

(b) screening Fc-fragments encoded by the library to identify the variant Fc-fragments that (i) bind to hFcRn with higher binding activity at pH 6 than a control Fc-fragment and (ii) have little or no binding activity for binding to hFcRn at pH 7.4;

(c) constructing a nucleic acid encoding a variant Fc-polypeptide comprising a variant Fc-fragment identified in (b), wherein the concentration of a control Fc-polypeptide, which comprises a control Fc-fragment rather than the variant Fc-fragment, is known to decrease linearly over time when administered to an animal *in vivo*;

(d) introducing the nucleic acid of (c) into a host cell and culturing the host cell under conditions such that the variant Fc-polypeptide encoded by the nucleic acid can be expressed;

(e) recovering the variant Fc-polypeptide from the cell mass or cell culture medium;

(f) administering the variant Fc-polypeptide to an animal and administering the control Fc-polypeptide to another animal; and

(g) monitoring the concentrations of the variant and control Fc-polypeptides in peripheral blood over time subsequent to administration, thereby identifying a variant Fc-fragment that confers a longer *in vivo* half life and greater exposure on a variant Fc-polypeptide.

47. The method of claim 46, wherein the insertion of step (a) is between positions 384 and 385 using the EU numbering system as illustrated in Table 1.

48. A method for treating a chronic disease comprising administering to a patient in need thereof a variant Fc-polypeptide according to any one of claims 1 to 37.