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Fortsættes ...

Description

The present invention relates to a polypeptide comprising a mutated Fc region and having functional activity mediated by the modified Fc region as compared to a parent polypeptide.

An antibody consists of a tetramer of heavy and light chains. The two light chains are identical to each other, and the two heavy chains are identical and connected by disulfide bridges. There are five types of heavy chains (alpha, gamma, delta, epsilon, mu), which determine the immunoglobulin classes (IgA, IgG, IgD, IgE, IgM). The light chain group comprises two subtypes, lambda and kappa.

The IgGs are soluble antibodies that can be found in blood and other body fluids. IgG is a Y-shaped glycoprotein with an approximate molecular weight of 150 kDa, composed of two heavy chains and two light chains. Each chain is made up of a constant region and a variable region. The two carboxy-terminal domains of the heavy chains form the Fc fragment, while the amino-terminal domains of the heavy and light chains recognize the antigen and are called the Fab fragment.

Fc fusion proteins are created by combining an antibody Fc fragment with a protein domain that provides specificity for a given therapeutic target. Examples are combinations of the Fc fragment with any type of therapeutic proteins or fragments thereof.

Therapeutic antibodies and Fc fusion proteins are used today to treat various diseases, such as rheumatoid arthritis, psoriasis, multiple sclerosis and many forms of cancer. Therapeutic antibodies may be monoclonal or polyclonal antibodies. Monoclonal antibodies are obtained from a single antibody producing cell line, which shows identical specificity for a single antigen. Therapeutic Fc fusion proteins are used or developed as medicinal products against autoimmune diseases, such as etanercept (Enbrel from Amgen, which is a TNF receptor

bonded to an Fc fragment) or Alefacept (Amevive from Biogen Idec, which is LFA-3 bonded to the Fc portion of human IgG1).

For the Fc antibodies and fusion proteins to be able to exert their effects, it is necessary for the Fc fragment to have the best possible activity (for example antibody-dependent cell cytotoxicity, complement-dependent cytotoxicity or antibody-dependent cellular phagocytosis), i.e. for it to be optimized. Such optimization would make it possible to obtain a protein having improved activity and/or efficacy and/or decreased side effects.

WO2010/106180 describes Fc variants comprising mutations.

The applicant has now developed specific Fc fragments exhibiting improved activity. These fragments can be used in therapy, with a view to giving the product containing them better efficacy.

The present invention thus provides a variant of a parent polypeptide having optimized properties relating to the functional activity mediated by the Fc region.

The present invention relates to a method according to claim 1, and to a polypeptide comprising a mutated Fc region according to claim 6. Such a polypeptide is called "polypeptide according to the invention" in the present application.

The description discloses a polypeptide comprising a mutated Fc region and having functional activity mediated by the Fc region that is modified as compared to that of a parent polypeptide, said Fc region comprising at least one mutation selected from among: G316D, K326E, N315D, N361H, P396L, T350A, V284L, V323I, P352S, A378V, Y436H, V266M, N421T, G385R, K326T, H435R, K447N, N434K, K334N, V397M, E283G, A378T, F423L, A431V, F423S, N325S, P343S, K290E, S375R, F405V, K322E, K340E, N389S, F243I, T307P, N389T, S442F, K248E, Y349H, N286I, T359A, S383R, K334R, T394P, V259A, T393A, P352L, Q418P, V302A, L398P, F423P, S442P, V363I, S383N, S254F, K320E, G402D, I253F, V284A, A431T, N315H, Y319H,

C226Y, F405L, T393I, N434S, R255W, A287T, N286Y, A231V, K274R, V308G, K414R, M428T, E345G, F243L, P247T, Q362R, S440N, Y278H, D312G, V262A, V305A, K246R, V308I, E380G, N276S, K439Q, S267G, F423Y, A231T, K320R, L410R, K320M, V412M, T307N, T366A, P230S, Y349S, A339T, K246E, K274E, A231P, I336T, S298N, L234P, S267N, V263A, E333G, V308A, K439R, K392R, S440G, V397I, I336V, Y373D, K288E, L309P, P227S, V379A, K288R, K320T, V282A, I377T, N421S and C261R,
the numbering being that of the EU index or equivalent in Kabat.

Also disclosed is a polypeptide comprising a mutated Fc region and having functional activity mediated by the Fc region that is modified as compared to that of a parent polypeptide, said Fc region comprising at least one combination of 2 mutations, said combination being selected from among:
(i) a mutation selected from among 307N, 326E, 326T, 334N, 334R, 352L, 378V, 378T, 394P, 396L, 397M and 421T;
(ii) at least one mutation selected from among 226Y, 227S, 230S, 231V, 234P, 243I, 243L, 246R, 246E, 247T, 248E, 253F, 254F, 255W, 259A, 261R, 262A, 263A, 266M, 267N, 267G, 274E, 274R, 276S, 278H, 282A, 283G, 284L, 286I, 286Y, 287T, 288E, 288R, 290E, 298N, 302A, 305A, 307P, 308A, 308I, 308G, 309P, 312G, 315D, 316D, 319H, 320T, 320R, 320M, 322E, 323I, 325S, 333G, 334N, 334R, 336T, 339T, 340E, 343S, 345G, 349S, 349H, 350A 352S, 359A, 361H, 362R, 363I, 366A, 373D, 375R, 377T, 378V, 378T, 379A, 380G, 383R, 385R, 389S, 389T, 392R, 393A, 393I, 394P, 396L, 397I, 397M, 398P, 405V, 405L, 410R, 412M, 414R, 421T, 421S, 423L, 423Y, 423S, 423P, 428T, 431V, 431T, 434K, 434S, 435R, 436H, 439R, 440G, 440N, 442F, 442P, and 447N,
the numbering being that of the EU index or equivalent in Kabat and with the condition that the mutation (i) does not take place on the same amino acid as the mutation (ii).

Preferably, the mutation (i) is selected from among 378V, 396L and 397M. Preferably, the polypeptide further comprises a mutation selected from among 248E, 326T, 333G and 423Y.

Preferably, the mutation (ii) is selected from among 226Y, 227S, 230S, 231V, 234P, 243I, 243L, 246R, 246E, 247T, 248E, 253F, 254F, 255W, 259A, 261R, 262A, 263A, 266M, 267G, 274E, 274R, 276S, 278H, 282A, 283G, 284L, 286I, 286Y, 287T, 288E, 288R, 290E, 298N, 302A, 305A, 307P, 308A, 308I, 308G, 309P, 312G, 316D, 319H, 320T, 320R, 320M, 322E, 323I, 325S, 333G, 334N, 334R, 336T, 339T, 340E, 343S, 345G, 349S, 349H, 350A 352S, 359A, 361H, 362R, 363I, 366A, 373D, 375R, 377T, 378T, 379A, 380G, 383R, 385R, 389S, 389T, 392R, 393A, 393I, 394P, 396L, 397I, 398P, 405V, 405L, 410R, 412M, 414R, 421T, 421S, 423L, 423Y, 423S, 423P, 428T, 431V, 431T, 434K, 434S, 435R, 436H, 439R, 440G, 440N, 442F, 442P and 447N.

Throughout the present application, the numbering of the residues in the Fc region is that of the immunoglobulin heavy chain according to the EU index or equivalent in Kabat et al. (Sequences of proteins of immunological interest, 5th ed. Public Health Service, National Institutes of Health, Bethesda, Maryland, 1991). The expression "EU index or equivalent in Kabat" refers to the EU numbering of the residues of the human IgG1, IgG2, IgG3 or IgG4 antibody. This is illustrated on the IMGT website (http://www.imgt.org/IMGTScientificChart/Numbering/Hu IGHGnber.html).

The term "polypeptide" or "protein" is understood to mean a sequence comprising at least 100 covalently attached amino acids.

The term "amino acid" is understood to mean one of the 20 natural amino acids or unnatural analogues.

The term "position" means a position in the sequence of a polypeptide. For the Fc region, the positions are numbered according to the EU index or equivalent in Kabat.

The term "antibody" is used in the common sense. It corresponds to a tetramer which comprises at least one Fc region and two

variable regions. The term "antibodies" covers in particular full-length immunoglobulins, monoclonal antibodies, multi-specific antibodies, chimaeric antibodies, humanized antibodies and entirely human antibodies. The amino-terminal portion of each heavy chain comprises a variable region of about 100 to 110 amino acids that is responsible for antigen recognition. In each variable region, three loops are grouped together to form an antigen binding site. Each one of the loops is called a complementarity-determining region (hereinafter referred to as a "CDR"). The carboxy-terminal portion of each heavy chain defines a constant region primarily responsible for effector function.

IgGs have several subclasses, in particular IgG1, IgG2, IgG3 and IgG4. The IgM subclasses are in particular IgM1 and IgM2. Thus, the term "isotype" is understood to mean one of the subclasses of immunoglobulins defined by the chemical and antigenic characteristics of their constant regions. The known isotypes of human immunoglobulins are IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgM1, IgM2, IgD and IgE.

Full-length IgGs are tetramers and are made up of two identical pairs of two immunoglobulin chains, each pair having a light chain and a heavy chain, each light chain comprising the VL and CL domains, and each heavy chain comprising the VH, Cγ1 (also called CH1), Cγ2 (also called CH2), and Cγ3 (also called CH3) domains. In the context of a human IgG1, "CH1" refers to positions 118 to 215, "CH2" refers to positions 231 to 340 and "CH3" refers to positions 341 to 447 according to the EU index or equivalent in Kabat. The IgG heavy chain also comprises an N-terminal flexible hinge domain which refers to positions 216 to 230 in the case of IgG1. The lower hinge domain refers to positions 226 to 230 according to the EU index or equivalent in Kabat. The term "variable region" is understood to mean the region of an immunoglobulin which comprises one or more Ig domains substantially encoded by any one of the VK, Vλ and/or VH genes which make up the kappa, lambda and heavy immunoglobulin chains, respectively. The variable regions comprise the

complementarity-determining regions (CDRs) and framework regions (FRs).

The term "Fc" or "Fc region" denotes the constant region of an antibody excluding the first immunoglobulin constant region domain (CH1). Thus, Fc refers to the last two constant region domains (CH2 and CH3) of IgG1, and to the N-terminal flexible hinge of these domains. For a human IgG1, the Fc region corresponds to the residue C226 up to its carboxy-terminal end, i.e. the residues from position 226 to 447, where the numbering is according to the EU index or equivalent in Kabat. The Fc region used may further comprise a portion of the upper hinge region, located between positions 216 to 226 according to the EU index or equivalent in Kabat; in this case, the Fc region used corresponds to the residues of position 216 to 447, 217 to 447, 218 to 447, 219 to 447, 220 to 447, 221 to 447, 222 to 447, 223 to 447, 224 to 447 or 225 to 447, where the numbering is according to the EU index or equivalent in Kabat. Preferably in this case, the Fc region used corresponds to the residues from position 216 to 447, where the numbering is according to the EU index or equivalent in Kabat.

Preferably, the Fc region used is selected from among the sequences SEQ ID NO: 1 to 10.

The term "parent polypeptide" is understood to mean a reference polypeptide. Said parent polypeptide may be of natural or synthetic origin. In the context of the present invention, the parent polypeptide comprises an Fc region, called "parent Fc region". This Fc region may be selected from among the group of wild-type Fc regions, fragments thereof and mutants thereof. Preferably, the parent polypeptide comprises a human Fc region, preferably an Fc region of a human IgG1. The parent polypeptide may comprise pre-existing amino acid modifications in the Fc region (e.g., an Fc mutant) as compared to wild-type Fc regions. Advantageously, the parent polypeptide is an isolated Fc region (i.e. an Fc fragment as such), a sequence derived from an isolated Fc region, an antibody, a fusion protein comprising an

Fc region, or an Fc conjugate, this list not being limiting. The term "sequence derived from an isolated Fc region" is understood to mean a sequence comprising at least two isolated Fc regions linked together, such as an scFc (single chain Fc) or an Fc multimer. The term "fusion protein comprising an Fc region" is understood to mean a polypeptide sequence fused to an Fc region, said polypeptide sequence preferably being selected from among variable regions of any antibody, sequences for binding a receptor to its ligand, adhesion molecules, ligands, enzymes, cytokines and chemokines. The term "Fc conjugate" is understood to mean a compound which is the result of the chemical coupling of an Fc region with a conjugation partner. The conjugation partner may be protein or non-protein. The coupling reaction generally uses functional groups on the Fc region and the conjugation partner. Various bonding groups are known in the prior art as being suitable for the synthesis of a conjugate; for example, homo- or heterobifunctional bonding groups are well known (see Pierce Chemical Company Catalogue, 2005-2006, Technical section on crosslinking agents, pages 321-350). Suitable conjugation partners include therapeutic proteins, tags, cytotoxic agents such as chemotherapeutic agents, toxins, and active fragments thereof. Suitable toxins and fragments thereof include in particular diphtheria toxin, exotoxin A, ricin, abrin, saporin, gelonin, calicheamicin, auristatins E and F and mertansine.

Advantageously, the parent polypeptide - and therefore the polypeptide according to the invention - consists of an Fc region.

Advantageously, the parent polypeptide - and therefore the polypeptide according to the invention - is an antibody.

Finally, preferably, the parent polypeptide - and therefore the polypeptide according to the invention - is a polypeptide produced in the milk of transgenic animals.

The term "mutation" is understood to mean a change of at least one amino acid of the sequence of a polypeptide, in particular a change of at least one amino acid of the Fc region of the parent polypeptide. The mutated polypeptide thus obtained is a variant polypeptide; it is a polypeptide according to the invention. Such a polypeptide comprises a mutated Fc region, this compared to the parent polypeptide. Preferably, the mutation is a substitution, an insertion or a deletion of at least one amino acid. The term "substitution" is understood to mean the replacement of an amino acid at a particular position in a parent polypeptide sequence by another amino acid. For example, the substitution N434S refers to a variant polypeptide, in this case a variant in which asparagine at position 434 is replaced by serine. The term "amino acid insertion" or "insertion" is understood to mean the addition of an amino acid at a particular position in a parent polypeptide sequence. For example, the insertion G>235-236 denotes an insertion of glycine between positions 235 and 236. The term "amino acid deletion" or "deletion" is understood to mean the removal of an amino acid at a particular position in a parent polypeptide sequence. For example, E294del denotes the removal of glutamic acid at position 294. Preferably, the following mutation wording is used: "434S" or "N434S", and means that the parent polypeptide comprises asparagine at position 434, which is replaced by serine in the variant. In the case of a combination of substitutions, the preferred format is: "2591/315D/434Y" or "V259I/N315D/N434Y". This means that there are three substitutions in the variant, at positions 259, 315 and 434, and that the amino acid at position 259 of the parent polypeptide, namely valine, is replaced by isoleucine, that the amino acid at position 315 of the parent polypeptide, namely asparagine, is replaced by aspartic acid and that the amino acid at position 434 of the parent polypeptide, namely asparagine, is replaced by tyrosine.

The polypeptide according to the invention has functional activity mediated by the Fc region that is modified as compared to that of the parent polypeptide.

The expression "functional activity mediated by the Fc region" is understood to mean in particular the effector functions. The functional activity mediated by the Fc region thus comprises in particular antibody-dependent cell cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), antibody-dependent cellular phagocytosis (ADCP), endocytosis activity, cytokine secretion, or a combination of at least two of these activities. Preferably, the functional activity mediated by the Fc region considered in the invention is selected from among ADCC, CDC and a combination thereof. This functional activity can be evaluated by methods well known in the prior art, such as those described in the examples (see in particular points 4.4 and 4.5 of the examples).

The functional activity mediated by the Fc region of the polypeptide according to the invention is increased as compared to that of the parent polypeptide.

The polypeptide according to the invention has functional activity mediated by the Fc region that is increased as compared to that of the parent polypeptide. Preferably, the polypeptide according to the invention has functional activity mediated by the Fc region that is increased, as compared to that of the parent polypeptide, by a ratio of at least 2, preferably greater than 5, preferably greater than 10, preferably greater than 15, preferably greater than 20, preferably greater than 25, and preferably greater than 30.

Preferably, the mutated Fc region of the polypeptide according to the invention has a modified affinity for at least one of the Fc region (FcR) receptors selected from among complement C1q, FcgRIIIa (CD16a), FcgRIIa (CD32a) and FcgRIIb (CD32b). Complement C1q is indeed involved in CDC activity. The FcgRIIIa receptor (CD16a) is for its part involved in ADCC; it has a V/F polymorphism at position 158. The FcgRIIa receptor (CD32a) is for its part involved in platelet activation and phagocytosis; it has an H/R polymorphism at position 131. Finally, the FcgRIIb

receptor (CD32b) is involved in the inhibition of cellular activity.

Preferentially, said mutated Fc region has an increased affinity for at least one of the FcRs. Preferentially, the affinity is increased, as compared to that of the parent Fc, by a ratio of at least 2, preferably greater than 5, preferably greater than 10, preferably greater than 15, preferably greater than 20, preferably greater than 25, and preferably greater than 30. In other words, the affinity of the mutated Fc region for an FcR is greater than that of the parent polypeptide. Alternatively, said mutated Fc region has a decreased affinity for at least one of the FcRs. Preferentially, the affinity is decreased, as compared to that of the parent Fc, by a ratio of at least 2, preferably greater than 5, preferably greater than 10, preferably greater than 15, preferably greater than 20, preferably greater than 25, and preferably greater than 30. In other words, the affinity of the mutated Fc region for an FcR is less than that of the parent polypeptide.

The affinity of a polypeptide comprising an Fc region, for an FcR, can be evaluated by methods well known in the prior art. For example, a person skilled in the art can determine the affinity (Kd) using surface plasmon resonance (SPR). Alternatively, a person skilled in the art may perform an appropriate ELISA assay. An appropriate ELISA assay makes it possible to compare the binding strengths of the parent Fc and of the mutated Fc. The detected signals specific to the mutated Fc and to the parent Fc are compared. The binding affinity can be determined either by evaluating the whole polypeptides or by evaluating the Fc regions isolated therefrom.

Also disclosed is a mutated Fc region of the polypeptide comprising from 1 to 20 mutations as compared to the parent polypeptide, preferably from 2 to 20 mutations. The expression "from 1 to 20 amino acid modifications" encompasses 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 and 20 amino acid mutations. Preferably, it comprises from 1 to 15

mutations, preferably from 2 to 15 mutations, preferably from 1 to 10 mutations, as compared to the parent polypeptide, preferably from 2 to 10 mutations.

Also disclosed is a mutated Fc region of the polypeptide comprising at least one combination of 2 mutations, said combination being selected from among:
(i) a mutation selected from among 378V, 396L and 397M;
(ii) at least one mutation selected from among 231V, 248E, 286I, 286Y, 290E, 298N, 308A, 315D, 316D, 326E, 333G, 334N, 334R, 336T, 352S, 361H, 366A, 378T, 396L, 397M, 412M, 421T, 423Y and 447N,
the numbering being that of the EU index or equivalent in Kabat and with the condition that the mutation (i) does not take place on the same amino acid as the mutation (ii).

Also disclosed is a mutated Fc region of the polypeptide comprising at least one combination of 2 mutations, said combination being selected from among:
(i) a mutation selected from among 378V, 396L and 397M;
(ii) at least one mutation selected from among 248E, 316D, 326E, 333G, 378T, 396L and 421T,
the numbering being that of the EU index or equivalent in Kabat and with the condition that the mutation (i) does not take place on the same amino acid as the mutation (ii).

Also disclosed is a polypeptide comprising a mutated Fc region having ADCC and CDC functional activities that are increased as compared to that of a parent polypeptide, characterized in that said Fc region comprises at least one combination of 2 mutations, said combination being selected from among:
(i) a mutation selected from among 378V, 396L and 397M;
(ii) at least one mutation selected from among 248E, 316D, 326E, 333G, 378T, 396L and 421T,
the numbering being that of the EU index or equivalent in Kabat and with the condition that the mutation (i) does not take place on the same amino acid as the mutation (ii).

Also disclosed is a mutated Fc region of the polypeptide comprising at least one combination of 2 mutations, said combination being selected from among:
(i) the 378V mutation;
(ii) at least one mutation selected from among 298N and 336T,
the numbering being that of the EU index or equivalent in Kabat and with the condition that the mutation (i) does not take place on the same amino acid as the mutation (ii).

Also disclosed is a mutated Fc region of the polypeptide comprising at least one combination of 2 mutations, said combination being selected from among:
(i) a mutation selected from among 378V, 396L and 397M;
(ii) at least one mutation selected from among 231V, 286I, 286Y, 290E, 315D, 334N, 352S, 361H, 366A, 378T, 397M, 412M, 421T and 423Y,
the numbering being that of the EU index or equivalent in Kabat and with the condition that the mutation (i) does not take place on the same amino acid as the mutation (ii).

Also disclosed is a polypeptide comprising a mutated Fc region having an ADCC functional activity that is increased as compared to that of a parent polypeptide, characterized in that said Fc region comprises at least one combination of 2 mutations, said combination being selected from among:
(i) a mutation selected from among 378V, 396L and 397M;
(ii) at least one mutation selected from among 231V, 286I, 286Y, 298N, 290E, 315D, 334N, 336T, 352S, 361H, 366A, 378T, 397M, 412M, 421T and 423Y,
the numbering being that of the EU index or equivalent in Kabat and with the condition that the mutation (i) does not take place on the same amino acid as the mutation (ii).

Preferably, the mutated Fc region of the polypeptide according to the invention comprises at least one combination of 2 mutations, said combination being selected from among:
(i) the 378V mutation;

(ii) at least one mutation selected from among 248E, 308A, 334R and 447N,
the numbering being that of the EU index or equivalent in Kabat and with the condition that the mutation (i) does not take place on the same amino acid as the mutation (ii).

Preferably, the polypeptide according to the invention comprises a mutated Fc region having a CDC functional activity that is increased as compared to that of a parent polypeptide, characterized in that said Fc region comprises at least one combination of 2 mutations, said combination being selected from among:
(i) the 378V mutation;
(ii) at least one mutation selected from among 248E, 308A, 334R and 447N,
the numbering being that of the EU index or equivalent in Kabat and with the condition that the mutation (i) does not take place on the same amino acid as the mutation (ii).

Also disclosed is a mutated Fc region of the polypeptide comprising a combination of mutations selected from among the combinations:
K320E/T394P/G402D
K290E/K320E/T350A/P396L
T359A/S383R/V397M

Also disclosed is a mutated Fc region of the polypeptide having improved affinity for complement C1q, and comprising at least one combination of 2 mutations, said combination comprising:
i) a mutation selected from among 378V, 378T, 396L, 421T, 334R and 326E; and
ii) at least one mutation selected from among 361H, 290E, 316D, 248E, 410R, 421T, 334R, 394P, 307P, 447N, 378V, 284L, 421T, 396L, 286I, 315D and 397M,
the numbering being that of the EU index or equivalent in Kabat and with the condition that the mutation (i) does not take place on the same amino acid as the mutation (ii).

Also disclosed is a mutated Fc region of the polypeptide having improved affinity for the FcgRIIIa receptor (CD16a), and comprising at least one combination of 2 mutations, said combination comprising:
i) a mutation selected from among 378V, 326E, 397M, 334N and 396L; and
ii) at least one mutation selected from among 316D, 397M, 334N, 248E, 231V, 246R, 336T, 421T, 361H, 366A, 439R, 290E, 394P, 307P, 378V, 378T, 286I, 286Y and 298N,
the numbering being that of the EU index or equivalent in Kabat and with the condition that the mutation (i) does not take place on the same amino acid as the mutation (ii).

Also disclosed is a mutated Fc region of the polypeptide having increased affinity for the FcgRIIa receptor (CD32a), and comprising at least one combination of 2 mutations, said combination comprising:
i) a mutation selected from among 378V, 326E, 397M, 307N, 394P, 326T, 396L and 334N; and
ii) at least one mutation selected from among: 316D, 334R, 334N, 323I, 231V, 246R, 336T, 378T, 286Y, 286I, 352S, 383R, 359A, 421T, 361H, 315D, 366A, 290E, 307P and 439R,
the numbering being that of the EU index or equivalent in Kabat and with the condition that the mutation (i) does not take place on the same amino acid as the mutation (ii).

Preferably, the mutation (ii) is selected from among: 316D, 334R, 334N, 323I, 231V, 246R, 336T, 378T, 286Y, 286I, 352S, 383R, 359A, 421T, 361H, 366A, 290E, 307P and 439R.

Also disclosed is a mutated Fc region of the polypeptide having increased affinity for the FcgRIIb receptor (CD32b), and comprising at least one combination of 2 mutations, said combination comprising:
i) a mutation selected from among 326E, 326T, 378V, 397M, 352L, 394P, 396L and 421T; and
ii) at least one mutation selected from among 316D, 334R, 248E, 334N, 418P, 231V, 320E, 402D, 359A, 383R, 421T and 361H,

the numbering being that of the EU index or equivalent in Kabat and with the condition that the mutation (i) does not take place on the same amino acid as the mutation (ii).

Also disclosed is a mutated Fc region of the polypeptide having improved CDC activity, and comprising at least one combination of 2 mutations, said combination comprising:
i) a mutation selected from among 378V, 378T, 396L, 421T, 334R and 326E; and
ii) at least one mutation selected from among 361H, 290E, 316D, 248E, 410R, 421T, 334R, 394P, 307P, 447N, 378V, 284L, 421T, 396L, 286I, 315D and 397M,
the numbering being that of the EU index or equivalent in Kabat and with the condition that the mutation (i) does not take place on the same amino acid as the mutation (ii).

Also disclosed is a mutated Fc region of the polypeptide having increased ADCC activity, and comprising at least one combination of 2 mutations, said combination comprising:
i) a mutation selected from among 378V, 326E, 397M, 334N and 396L; and
ii) at least one mutation selected from among 316D, 397M, 334N, 248E, 231V, 246R, 336T, 421T, 361H, 366A, 439R, 290E, 394P, 307P, 378V, 378T, 286I, 286Y and 298N,
the numbering being that of the EU index or equivalent in Kabat and with the condition that the mutation (i) does not take place on the same amino acid as the mutation (ii).

Also disclosed is a mutated Fc region of the polypeptide comprising at least one combination of 3 mutations, said combination comprising:
(i) a mutation selected from among 326E, 326T, 352L, 378V, 378T, 396L, 397M, 421T, 334N, 334R, 307N and 394P ; and
(ii) at least 2 mutations selected from among 226Y, 227S, 230S, 231V, 234P, 243I, 243L, 246R, 246E, 247T, 248E, 253F, 254F, 255W, 259A, 261R, 262A, 263A, 266M, 267N, 267G, 274E, 274R, 276S, 278H, 282A, 283G, 284L, 286I, 286Y, 287T, 288E, 288R, 290E, 298N, 302A, 305A, 307P, 308A, 308I, 308G, 309P, 312G,

315D, 316D, 319H, 320T, 320R, 320M, 322E, 323I, 325S, 333G, 334N, 334R, 336T, 339T, 340E, 343S, 345G, 349S, 349H, 350A 352S, 359A, 361H, 362R, 363I, 366A, 373D, 375R, 377T, 378V, 378T, 379A, 380G, 383R, 385R, 389S, 389T, 392R, 393A, 393I, 394P, 396L, 397I, 397M, 398P, 405V, 405L, 410R, 412M, 414R, 421T, 421S, 423L, 423Y, 423S, 423P, 428T, 431V, 431T, 434K, 434S, 435R, 436H, 439R, 440G, 440N, 442F, 442P and 447N,
the numbering being that of the EU index or equivalent in Kabat and with the condition that the mutation (i) does not take place on the same amino acid as the mutation (ii).

Preferably, the at least 2 mutations (ii) are selected from among 226Y, 227S, 230S, 231V, 234P, 243I, 243L, 246R, 246E, 247T, 248E, 253F, 254F, 255W, 259A, 261R, 262A, 263A, 266M, 267G, 274E, 274R, 276S, 278H, 282A, 283G, 284L, 286I, 286Y, 287T, 288E, 288R, 290E, 298N, 302A, 305A, 307P, 308A, 308I, 308G, 309P, 312G, 316D, 319H, 320T, 320R, 320M, 322E, 323I, 325S, 333G, 334N, 334R, 336T, 339T, 340E, 343S, 345G, 349S, 349H, 350A 352S, 359A, 361H, 362R, 363I, 366A, 373D, 375R, 377T, 378T, 379A, 380G, 383R, 385R, 389S, 389T, 392R, 393A, 393I, 394P, 396L, 397I, 398P, 405V, 405L, 410R, 412M, 414R, 421T, 421S, 423L, 423Y, 423S, 423P, 428T, 431V, 431T, 434K, 434S, 435R, 436H, 439R, 440G, 440N, 442F, 442P, and 447N.

More preferentially, the mutated Fc region of the polypeptide comprises at least one combination of 4 mutations, said combination comprising at least one mutation (i) as described above, and at least 3 mutations (ii) as described above, the numbering being that of the EU index or equivalent in Kabat and with the condition that the mutation (i) does not take place on the same amino acid as the mutation (ii).

More preferentially, the mutated Fc region of the polypeptide comprises at least one combination of 5 mutations, said combination comprising at least one mutation (i) as described above, and at least 4 mutations (ii) as described above, the numbering being that of the EU index or equivalent in Kabat and

with the condition that the mutation (i) does not take place on the same amino acid as the mutation (ii).

A subject of the present invention is also a composition of polypeptides according to the invention, said purified polypeptides possessing, at their Asn297 glycosylation site, N-glycans of which the degree of fucosylation is less than 65%, preferably less than 50%, more preferably less than 40%. Preferably, said purified polypeptides have at their Asn297 glycosylation site a biantennary glycan structure, with short chains, low sialylation, having end mannoses and/or end N-acetylglucosamines that are not intercalated.

More preferentially, said purified polypeptides have a content greater than 60% for the G0+G1+G0F+G1F forms, the G0F+G1F forms being less than 50%. More preferentially, said purified polypeptides have a content greater than 60% for the G0+G1+G0F+G1F forms, the fucose content being less than 65%. More preferentially, said purified polypeptides have a content of less than 40% for the G1F+G0F forms.

A subject of the present invention is also a pharmaceutical composition comprising (i) a polypeptide according to the invention or a composition as described in the preceding paragraph, and (ii) at least one pharmaceutically acceptable excipient.

A subject of the present invention is also the polypeptide according to the invention or the composition as described above, for its use as medicinal product.

As indicated above, advantageously, the parent polypeptide - and therefore the polypeptide according to the invention - is an antibody. In this case, the antibody can be directed against an antigen selected from among a tumour antigen, a viral antigen, a bacterial antigen, a fungal antigen, a toxin, a membrane or circulating cytokine and a membrane receptor.

When the antibody is directed against a tumour antigen, its use is particularly suitable for the treatment of cancers. The term "cancer" is understood to mean any physiological condition characterized by abnormal proliferation of cells. Examples of cancers include in particular carcinomas, lymphomas, blastomas, sarcomas (including liposarcomas), neuroendocrine tumours, mesotheliomas, meningiomas, adenocarcinomas, melanomas, leukaemias and malignant lymphoid pathological conditions, this list not being exhaustive.

When the antibody is directed against a viral antigen, its use is particularly suitable for the treatment of viral infections. The viral infections are in particular infections caused by HIV, a retrovirus, a Coxsackie virus, smallpox virus, influenza virus, yellow fever virus, West Nile virus, a cytomegalovirus, a rotavirus, or hepatitis B or C virus, this list not being exhaustive. When the antibody is directed against a toxin, its use is particularly suitable for the treatment of bacterial infections, for example infections by tetanus toxin, diphtheria toxin, *Bacillus anthracis* anthrax toxins, or for the treatment of botulinum toxin infections, ricin toxin infections, shigatoxin infections, this list not being exhaustive.

When the antibody is directed against a cytokine, its use is particularly suitable for the treatment of inflammatory and/or autoimmune diseases. Inflammatory and/or autoimmune diseases include in particular thrombotic thrombocytopenic purpura (ITP), graft or organ rejection, graft versus host disease, rheumatoid arthritis, systemic lupus erythematosus, the various types of sclerosis, primary Sjögren syndrome (or Gougerot-Sjögren syndrome), autoimmune polyneuropathies such as multiple sclerosis, type I diabetes, autoimmune hepatitis, ankylosing spondylitis, Reiter's syndrome, gout arthritis, coeliac disease, Crohn's disease, chronic Hashimoto's thyroiditis (hypothyroidism), Addison's disease, autoimmune hepatitis, Graves' disease (hyperthyroidism), ulcerative colitis, vasculitis, such as ANCA (anti-neutrophil cytoplasmic antibody)-associated systemic vasculitis, autoimmune cytopenia and other

haematological complications in adults and children, such as acute or chronic autoimmune thrombocytopenia, autoimmune haemolytic anaemia, haemolytic disease of the newborn (HDN), cold agglutinin disease, autoimmune acquired haemophilia, Goodpasture syndrome, extramembranous nephropathies, autoimmune bullous skin conditions, refractory myasthenia, mixed cryoglobulinemia, psoriasis, juvenile chronic arthritis, inflammatory myositis, dermatomyositis and systemic autoimmune disorders in children, including antiphospholipid syndrome, connective tissue disease, pulmonary autoimmune inflammation, Guillain-Barré syndrome, chronic inflammatory demyelinating polyradiculoneuritis (CIDP), autoimmune thyroiditis, mellitus, myasthenia gravis, autoimmune inflammatory eye disease, neuromyelitis optica (Devic's disease), scleroderma, pemphigus, insulin resistant diabetes, polymyositis, Biermer's anaemia, glomerulonephritis, Wegener's disease, Horton's disease, polyarteritis nodosa and Churg-Strauss syndrome, Still's disease, atrophic polychondritis, Behçet's disease, monoclonal gammopathy, Wegener's granulomatosis, lupus, ulcerative colitis, psoriatic arthritis, sarcoidosis, collagenous colitis, dermatitis herpetiformis, familial Mediterranean fever, IgA glomerulonephritis, Lambert-Eaton myasthenic syndrome, sympathetic ophthalmia, Fiessinger-Leroy-Reiter syndrome and uveo-meningo-encephalitic syndrome.

Other inflammatory diseases are also included, such as acute respiratory distress syndrome (ARDS), acute septic arthritis, adjuvant arthritis, allergic encephalomyelitis, allergic rhinitis, allergic vasculitis, allergy, asthma, atherosclerosis, chronic inflammation due to chronic bacterial or viral infections, chronic obstructive pulmonary disease (COPD), coronary heart disease, encephalitis, inflammatory bowel disease, inflammatory osteolysis, inflammation associated with acute and delayed hypersensitivity reactions, tumour-associated inflammation, peripheral nerve injury or demyelinating diseases, inflammation associated with tissue trauma such as burns and ischaemia, inflammation due to meningitis, multiple organ dysfunction syndrome (MODS), pulmonary fibrosis, septicaemia and

septic shock, Stevens-Johnson syndrome, undifferentiated arthritis, and undifferentiated spondyloarthropathies.

In a particular embodiment of the invention, the autoimmune disease is idiopathic thrombocytopenic purpura (ITP) and chronic inflammatory demyelinating polyradiculoneuritis (CIDP).

Also disclosed is a method to produce a polypeptide comprising an Fc region and having functional activity mediated by the Fc region that is modified as compared to that of a parent polypeptide, said method comprising a step to introduce at least one mutation into said Fc region selected from among:
G316D, K326E, N315D, N361H, P396L, T350A, V284L, V323I, P352S, A378V, Y436H, V266M, N421T, G385R, K326T, H435R, K447N, N434K, K334N, V397M, E283G, A378T, F423L, A431V, F423S, N325S, P343S, K290E, S375R, F405V, K322E, K340E, N389S, F243I, T307P, N389T, S442F, K248E, Y349H, N286I, T359A, S383R, K334R, T394P, V259A, T393A, P352L, Q418P, V302A, L398P, F423P, S442P, V363I, S383N, S254F, K320E, G402D, I253F, V284A, A431T, N315H, Y319H, C226Y, F405L, T393I, N434S, R255W, A287T, N286Y, A231V, K274R, V308G, K414R, M428T, E345G, F243L, P247T, Q362R, S440N, Y278H, D312G, V262A, V305A, K246R, V308I, E380G, N276S, K439Q, S267G, F423Y, A231T, K320R, L410R, K320M, V412M, T307N, T366A, P230S, Y349S, A339T, K246E, K274E, A231P, I336T, S298N, L234P, S267N, V263A, E333G, V308A, K439R, K392R, S440G, V397I, I336V, Y373D, K288E, L309P, P227S, V379A, K288R, K320T, V282A, I377T, N421S and C261R,
the numbering being that of the EU index or equivalent in Kabat.

Also disclosed is a method to produce a polypeptide comprising an Fc region and having functional activity mediated by the Fc region that is modified as compared to that of a parent polypeptide, said method comprising a step to introduce at least one combination of 2 mutations, said combination being selected from among:
(i) a mutation selected from among 326E, 326T, 352L, 378V, 378T, 396L, 397M, 421T, 334N, 334R, 307N and 394P ; and

(ii) at least one mutation selected from among 226Y, 227S, 230S, 231V, 234P, 243I, 243L, 246R, 246E, 247T, 248E, 253F, 254F, 255W, 259A, 261R, 262A, 263A, 266M, 267N, 267G, 274E, 274R, 276S, 278H, 282A, 283G, 284L, 286I, 286Y, 287T, 288E, 288R, 290E, 298N, 302A, 305A, 307P, 308A, 308I, 308G, 309P, 312G, 315D, 316D, 319H, 320T, 320R, 320M, 322E, 323I, 325S, 333G, 334N, 334R, 336T, 339T, 340E, 343S, 345G, 349S, 349H, 350A 352S, 359A, 361H, 362R, 363I, 366A, 373D, 375R, 377T, 378V, 378T, 379A, 380G, 383R, 385R, 389S, 389T, 392R, 393A, 393I, 394P, 396L, 397I, 397M, 398P, 405V, 405L, 410R, 412M, 414R, 421T, 421S, 423L, 423Y, 423S, 423P, 428T, 431V, 431T, 434K, 434S, 435R, 436H, 439R, 440G, 440N, 442F, 442P, and 447N,
the numbering being that of the EU index or equivalent in Kabat and with the condition that the mutation (i) does not take place on the same amino acid as the mutation (ii).

Preferably, the mutation (ii) is selected from among 226Y, 227S, 230S, 231V, 234P, 243I, 243L, 246R, 246E, 247T, 248E, 253F, 254F, 255W, 259A, 261R, 262A, 263A, 266M, 267G, 274E, 274R, 276S, 278H, 282A, 283G, 284L, 286I, 286Y, 287T, 288E, 288R, 290E, 298N, 302A, 305A, 307P, 308A, 308I, 308G, 309P, 312G, 316D, 319H, 320T, 320R, 320M, 322E, 323I, 325S, 333G, 334N, 334R, 336T, 339T, 340E, 343S, 345G, 349S, 349H, 350A 352S, 359A, 361H, 362R, 363I, 366A, 373D, 375R, 377T, 378T, 379A, 380G, 383R, 385R, 389S, 389T, 392R, 393A, 393I, 394P, 396L, 397I, 398P, 405V, 405L, 410R, 412M, 414R, 421T, 421S, 423L, 423Y, 423S, 423P, 428T, 431V, 431T, 434K, 434S, 435R, 436H, 439R, 440G, 440N, 442F, 442P, and 447N.

Also disclosed is a method to increase the binding of a polypeptide comprising an Fc region to at least one of the Fc region receptors (FcR) selected from among the C1q receptor, FcgRIIIa receptor (CD16a), and FcgRIIa receptor (CD32a), and FcgRIIb receptor (CD32b), said method comprising a step to introduce at least one mutation into said Fc region selected from among:
G316D, K326E, N315D, N361H, P396L, T350A, V284L, V323I, P352S, A378V, Y436H, V266M, N421T, G385R, K326T, H435R, K447N, N434K,

K334N, V397M, E283G, A378T, F423L, A431V, F423S, N325S, P343S, K290E, S375R, F405V, K322E, K340E, N389S, F243I, T307P, N389T, S442F, K248E, Y349H, N286I, T359A, S383R, K334R, T394P, V259A, T393A, P352L, Q418P, V302A, L398P, F423P, S442P, V363I, S383N, S254F, K320E, G402D, I253F, V284A, A431T, N315H, Y319H, C226Y, F405L, T393I, N434S, R255W, A287T, N286Y, A231V, K274R, V308G, K414R, M428T, E345G, F243L, P247T, Q362R, S440N, Y278H, D312G, V262A, V305A, K246R, V308I, E380G, N276S, K439Q, S267G, F423Y, A231T, K320R, L410R, K320M, V412M, T307N, T366A, P230S, Y349S, A339T, K246E, K274E, A231P, I336T, S298N, L234P, S267N, V263A, E333G, V308A, K439R, K392R, S440G, V397I, I336V, Y373D, K288E, L309P, P227S, V379A, K288R, K320T, V282A, I377T, N421S and C261R,

the numbering being that of the EU index or equivalent in Kabat.

Also disclosed is a method to increase the binding of a polypeptide comprising an Fc region to at least one of the Fc region receptors (FcR) selected from among the C1q receptor, FcgRIIIa receptor (CD16a), and FcgRIIa receptor (CD32a), and FcgRIIb receptor (CD32b), said method comprising a step to introduce at least one combination of 2 mutations, said combination being selected from among:

(i) a mutation selected from among 326E, 326T, 352L, 378V, 378T, 396L, 397M, 421T, 334N, 334R, 307N and 394P ; and

(ii) at least one mutation selected from among 226Y, 227S, 230S, 231V, 234P, 243I, 243L, 246R, 246E, 247T, 248E, 253F, 254F, 255W, 259A, 261R, 262A, 263A, 266M, 267N, 267G, 274E, 274R, 276S, 278H, 282A, 283G, 284L, 286I, 286Y, 287T, 288E, 288R, 290E, 298N, 302A, 305A, 307P, 308A, 308I, 308G, 309P, 312G, 315D, 316D, 319H, 320T, 320R, 320M, 322E, 323I, 325S, 333G, 334N, 334R, 336T, 339T, 340E, 343S, 345G, 349S, 349H, 350A 352S, 359A, 361H, 362R, 363I, 366A, 373D, 375R, 377T, 378V, 378T, 379A, 380G, 383R, 385R, 389S, 389T, 392R, 393A, 393I, 394P, 396L, 397I, 397M, 398P, 405V, 405L, 410R, 412M, 414R, 421T, 421S, 423L, 423Y, 423S, 423P, 428T, 431V, 431T, 434K, 434S, 435R, 436H, 439R, 440G, 440N, 442F, 442P, and 447N,

the numbering being that of the EU index or equivalent in Kabat and with the condition that the mutation (i) does not take place on the same amino acid as the mutation (ii).

Preferably, the mutation (ii) is selected from among 226Y, 227S, 230S, 231V, 234P, 243I, 243L, 246R, 246E, 247T, 248E, 253F, 254F, 255W, 259A, 261R, 262A, 263A, 266M, 267G, 274E, 274R, 276S, 278H, 282A, 283G, 284L, 286I, 286Y, 287T, 288E, 288R, 290E, 298N, 302A, 305A, 307P, 308A, 308I, 308G, 309P, 312G, 316D, 319H, 320T, 320R, 320M, 322E, 323I, 325S, 333G, 334N, 334R, 336T, 339T, 340E, 343S, 345G, 349S, 349H, 350A 352S, 359A, 361H, 362R, 363I, 366A, 373D, 375R, 377T, 378T, 379A, 380G, 383R, 385R, 389S, 389T, 392R, 393A, 393I, 394P, 396L, 397I, 398P, 405V, 405L, 410R, 412M, 414R, 421T, 421S, 423L, 423Y, 423S, 423P, 428T, 431V, 431T, 434K, 434S, 435R, 436H, 439R, 440G, 440N, 442F, 442P, and 447N.

The sequences described in this application can be summarized as follows:

SEQ ID NO:	**Protein**
1	Fc region of human IgG1 G1m1,17 (residues 226-447 according to the EU index or equivalent in Kabat) without N-terminal upper hinge region
2	Fc region of human IgG2 without N-terminal upper hinge region
3	Fc region of human IgG3 without N-terminal upper hinge region
4	Fc region of human IgG4 without N-terminal upper hinge region
5	Fc region of human IgG1 G1m3 without N-terminal upper hinge region
6	Fc region of human IgG1 G1m1,17 with N-terminal upper hinge region (residues 216-447 according to the EU index or equivalent in Kabat)

SEQ ID NO:	Protein
7	Fc region of human IgG2 with N-terminal upper hinge region
8	Fc region of human IgG3 with N-terminal upper hinge region
9	Fc region of human IgG4 with N-terminal upper hinge region
10	Fc region of human IgG1 G1m3 with N-terminal upper hinge region
11 and 12	Primers MG_619 and MG_621

The present invention will be better understood on reading the following examples.

EXAMPLES

EXAMPLE 1: Identification of polypeptides with a mutated Fc region according to the invention and characterization of said polypeptides

I. Materials and methods

1. Constructions of human Fc region libraries

The human Fc gene encoding amino acids 226-447 (EU index or equivalent in Kabat), i.e. a polypeptide comprising an Fc region, derived from a human IgG1 heavy chain and having the allotype G1m1,17 (SEQ ID NO: 1), (Poul MA et al., EUR J. Immunol 25 (7): 2005-2009, 1995) was cloned into the phagemid vector pMG58 (pMG58_Fc226) as a BamHI/EcoRI fragment using standard PCR protocols. Several completely randomized libraries were generated using the MUTAGEN™ method (WO02/038756) which uses low fidelity human DNA polymerases (mutases) to introduce random mutations homogeneously over the entire target sequence. Three distinct mutases (pol β, pol η and pol

ι

) were used under different conditions to create complementary mutation profiles. These human polymerases were produced and purified as previously described (Mondon et al. J. Biotechnol 2: 76-82 (2007), Emond et al. Protein Eng des SEI 21:267-274, (2008)).

1.1. Mutagenesis with the MUTAGEN™ process

The MUTAGEN™ process has been described in application WO 02/038756.

Briefly, the human Fc gene (Fc gene) was replicated with mutases using the 5' primer MG_619: 5'-AGTACTGACTCTACCTAGGATCCTGCCCACCGTGC-3' (SEQ ID NO: 11) and 3' primer MG_621: 5'-ACTGCTCGATGTCCGTACTATGCGGCCGCGAATTC-3' (SEQ ID NO: 12). A mixture containing 0.6 µg of plasmid pMG58_Fc226 as a template (wild-type Fc region or selected variants), the MG_619 and MG_621 primers (250 nM each) and the appropriate replication buffer (see details below) was treated for 5 min at 95°C and immediately cooled to 4°C to denature the DNA strands. For pol β, the replication buffer was 50 mM Tris HCl, pH 8.8, 10 mM $MgCl_2$, 10 mM KCl, 1 mM DTT and 1% (v/v) glycerol. The replication buffer for pol η (or pol η and pol ι) was 25 mM Tris HCl, pH 7.2, 5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT and 2.5% (v/v) glycerol. After the denaturation step, the mutagenic replications were carried out by adding 50 µM of dATP/dCTP, 100 µM of dTTP/dGTP and 1 µg of pol β or 100 µM of dNTP and 1 µg of pol η (or pol η and pol

1

, 1 µg of each mutase). The replication reaction was carried out at 37°C for two hours. The replication products were then concentrated and desalted on Microcon columns (Millipore).

1.2. Selective amplification and cloning of mutated fragments

The replication products previously obtained were amplified by selective PCR with tail primers. The primers (MG_619 MG_621) were designed with a tail which is non-complementary to the

template, allowing the specific amplification of the DNA fragments synthesized by the mutases. A fraction of the replication products was added to a mixture containing the PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 10 pmol of the 5' and 3' primers, 200 pM of dNTPs and 1.25 U of Platinum Taq DNA polymerase (Invitrogen). The PCR cycles were as follows, first cycle: 2 min. at 94°C, 10 sec. at 64°C, 30 sec. at 75°C, 1 min. at 94°C, then 30 selective cycles: 20 sec. at 94°C and 30 sec. at 75°C.

The amplified replication products were purified on 1% (w/v) of agarose gels, digested with BamHI and EcoRI, and cloned into the pMG58 vector. The ligation mixtures were transformed into E. coli XL1-Blue electrocompetent cells and then plated out on a 2YT solid medium (16 g/l peptone, 10 g/l yeast extract, 5 g/l NaCl, 15 g/l agar) supplemented with 100 µg/ml ampicillin and 1% (w/v) glucose. After growth, the number of colonies was determined in order to estimate the size of the libraries, and at least 48 clones per library were subjected to a PCR and to high throughput DNA sequencing. The cells were resuspended in 2YT medium with 15% glycerol, frozen and stored at -80°C.

1.3. Construction of the Mut3 library

A first library was obtained using pol β on the wild-type Fc gene and contained 3.2×10^6 clones (called Mut1.1). The DNA of this first library was used to generate the second and third libraries, using pol β (3.8×10^6 clones, Mut1.2) and pol η and i (3.0×10^6 clones, Mut1.3), respectively. This cumulative two-step replication strategy made it possible to increase the mutation rate. The fourth library was produced with pol η alone on the wild-type Fc gene (1.0×10^6 clones, Mut1.4). Finally, these four libraries were proportionally mixed to obtain the final library, called Mut1, representing 1.1×10^7 different clones.

Two different libraries were then constructed using a DNA pool of 42 single and double mutants isolated on the FcRn receptor. A first library was obtained using pol β (1.9×10^7 clones, Mut2.1)

and a second library with pol η (1×10^6 clones, Mut2.2). Finally, these two libraries were proportionally mixed to obtain the final library, called Mut2, i.e. 2×10^7 different clones.

A new library was obtained using pol β on the wild-type Fc gene, with a diversity of 4.7×10^7 clones.

Finally, the Mut1 and Mut2 libraries and the new library were proportionally mixed to obtain the final library, called Mut3, i.e. 7.8×10^7 different clones. Finally, the Mut3 library contains 3×10^7 phase mutated clones with an average of 2.2 mutated amino acids per Fc.

1.4. Construction of the Mut4sel library

A library of Fc variants was constructed from 36 clones with improved binding for C1q and/or CD16aV, selected from the Mut3 library (1.6 amino acids mutated per Fc on average). The Mut4 library was obtained using an equimolar mixture of pol β and pol η polymerases (in the pol β replication buffer). The Mut4 library has a diversity of 1.3×10^7 clones, with 77% of clones in phase and an average of 2.6 amino acids mutated per Fc.

This library was cloned into the pMG93 vector, which is a selection vector developed for Fc, and which allows selection of the coding sequences (ORFs) on ampicillin medium by virtue of a fusion with the □-lactamase gene. This construct was transformed into the non-suppressive bacteria HB2151, thereby making it possible to eliminate the TAG codons, which are recognized as stop codons in this strain. The clones of this library were plated out on agar plates containing low concentrations of ampicillin, at a low density, so as to carry out the selection of the ORFs, the viable clones then being recovered and frozen. Plating out on 500 Petri dishes (120x120) made it possible to cover the entire Mut4 library. The library thus selected was denoted Mut4sel and contained 92% of in phase clones, i.e. 1×10^7 clones mutated in phase, with an average of 2.5 amino acids mutated per Fc. The Mut4sel library was then

subcloned into the pMG58 phagemid, in XL1-Blue bacteria, in order to allow selection by "phage display" with respect to the targets.

1.5. Construction of the Mut5 library

A library of Fc variants was constructed from 42 clones with improved binding for C1q and/or one of the FcgR receptors selected from the Mut4sel library (2.4 amino acids mutated per Fc on average). The Mut5 library was obtained using pol β polymerase alone so as to obtain a low mutation rate. Finally, the Mut5 library obtained contains 1.2×10^7 clones with 95% of in phase clones with 3.1 amino acids mutated per Fc on average. The good quality of this library did not require the selection of ORFs as carried out for the Mut4 library.

2. Phage display expression of Fc libraries and selection of improved variants

During the selection steps, the Mut3, Mut4sel and Mut5 libraries were expressed on the surface of M13 bacteriophage using standard procedures (Smith GP, Science 228: 1315 (985)). E. coli XL1-Blue bacteria, containing the library to be expressed, cloned into the pMG58 vector, were cultured in 60 ml of 2YT medium supplemented with 100 µg/ml of ampicillin, 15 µg/ml of tetracycline and 1% (w/v) glucose at 30°C. The cells were then infected with M13 helper phage (M13K07, Biolabs, bacteria/phage ratio = 1/3) at 37°C for 20 min and Fc-phage production was continued overnight at 26°C, at 230 rpm, 2YT/ampicillin/glucose with 0.5 mM IPTG and 50 µg of kanamycin/ml. The following day, the phages were precipitated with PEG6000 using standard protocols, resuspended in 1 ml of PBS buffer at pH 7.4, and titrated by infecting XL1-Blue cells.

2.1 Recombinant proteins used:

Complement C1q is commercially available (Calbiochem).

CD16a is an activator receptor that exhibits a V/F polymorphism at position 158 on the Fc-binding site. The affinity is better for CD16aV.

CD16aV is commercially available (R&D Systems).

CD16aF was produced by PX'Therapeutics.

CD32a is an activator receptor that exhibits an H/R polymorphism at position 131, on the Fc-binding site. The affinity is better for CD32aH.

CD32aR is commercially available (R&D Systems).

CD32aH was produced by PX'Therapeutics. CD32b is an inhibitory receptor that has poorer affinity for IgG1 than CD32aR. It is commercially available (R&D Systems).

2.2. Solid phase selections:

For the solid phase selections, Fc-phages diluted in PBS / 5% skimmed milk / 0.1% Tween 20 were incubated in 8 wells of Maxisorp plates ($1-4\times10^{11}$ phages / well in 100 µl final volume) precoated with 500 ng/well of CD16aV, biotinylated CD16aV, biotinylated CD16aF, biotinylated C1q, biotinylated CD32aR or biotinylated CD32b, and blocked with 5% skimmed milk in PBS. After incubation for 2 hours at 37°C, the wells were washed 8 times with PBS/0.1% Tween 20 and twice with PBS. For the selections on CD32aR and CD32b, counter-selection rounds were also carried out: before the step of binding on the Maxisorp-plate-immobilized target, the Fc-phages were similarly preincubated on 8 wells with the competing receptor. Only the Fc-phages not bound to the first target were then transferred to the wells containing the second target as described above. The selected phages were then eluted by infection with XL1-Blue bacteria in exponential growth phase (2×150 µl/well, 20 min. at 37°C without shaking). The infected bacteria were then plated out on solid 2YT/ampicillin/glucose medium. The following day,

the cells were resuspended in 2YT medium with 15% glycerol, frozen and stored at -80°C until the next round of selection.

2.3. Liquid phase selections:

For the liquid phase selections, 4×10^{11} phages were first incubated with biotinylated CD16aV (250 nM), biotinylated CD16aF (1000 nM), or biotinylated C1q (250 nM) for 1 hour at ambient temperature with gentle shaking. Magnetic beads coated with streptavidin (Dynal) previously blocked with 5% skimmed milk in PBS were then added to the phages for 30 minutes at ambient temperature. The phage-bead complexes were washed 10 times with PBS/0.1% Tween 20 using a magnet. The phage-bead complexes were then used to infect 5 ml of exponentially growing XL1-Blue bacteria, which were plated out on solid 2YT/ampicillin/glucose medium. The following day, the cells were resuspended in 2YT medium with 15% glycerol, frozen and stored at -80°C until the next round of selection.

2.4. Selections from libraries:

Mut3 library:

For the liquid phase selections, 6 rounds were carried out on 3 biotinylated targets: CD16aV, CD16aF and C1q (clones denoted DL6A, DL6B and QL6A). For the solid phase selection rounds, 6 rounds were carried out on 3 targets: CD16aV, biotinylated CD16aV and biotinylated C1q (clones denoted DS6A, DS6B and QS6A).

Mut4sel library:

The selection rounds were carried out in solid phase only: 3 rounds of selection on biotinylated CD16aV, 3 rounds on biotinylated C1q, 3 rounds on biotinylated CD32aR (+/depletion on CD32b for the 3rd round) and 3 rounds on biotinylated CD32b (+/- depletion on CD32aR for the 3rd round), (clones noted A3A, G3A, J3A/B and K3A/B).

Mut5 library:

The selection rounds were carried out as before: 3 rounds of selection on biotinylated CD16aF, 3 rounds on biotinylated C1q, 3 rounds on biotinylated CD32aR, 3 rounds on biotinylated CD32b, 2 rounds on biotinylated CD32aR and 2 rounds with depletion on biotinylated CD32b, and 2 rounds on biotinylated CD32b and 2 rounds of depletion on biotinylated CD32aR (clones denoted N3A, O3A, P3A, Q3A, P4B and Q4B).

The selection is then made as previously described.

2.5. Pooled cloning in the pMGM05 vector:

The clones selected at the end of the selection rounds were then transferred directly as a mixture (approximately 10^4 clones per condition) into the eukaryotic vector pMGM05-CD20 (pCEP4 Invitrogen), which contains the same cloning sites as the pMG58 phagemid for the Fc fragment (BamHI and NotI) and the VH variable chain of the anti-CD20 antibody. This construction leads to the mutation of two amino acids in the Fc (aa224 and 225, HT changed to GS) and the addition of the EFAAA sequence at the C-terminal of the Fc, but allows a very large number of clones to be tested very rapidly. Initially, it was verified that these mutations do not modify the binding of IgG-WT to the various receptors. Subsequently, the positive controls were cloned into this system in order to validate it:

- IgG1-S239D, I332E, originating from the Xencor anti-CD19 antibody XmAb5574 (C1): positive control for CD16a;
- IgG1-G236A, originating from Xencor (C4): positive control for CD32aH/R;
- IgG1-K326W, E333S, originating from Abgenix/Genentech (C3): positive control for C1q; and
- IgG1-S267E, L328F, originating from the Xencor anti-CD19 antibody XmAb5574 (C5): positive control for CD32b.

The DNA of about 100 clones isolated per round of selection was sequenced by PCR on colonies. After bioinformatic analyses, the clones containing new mutations were frozen at -80°C in XL1-Blue bacteria and the sequences were included in our database. Thus, 158 clones were isolated from the Mut3 library, 371 clones from the Mut4sel library and 171 clones from the Mut5 library.

2.6. Production of variants in HEK293 cells:

The anti-CD20 light chain was inserted into a vector pCEP4 identical to the vector used for the heavy chain, denoted pMGM01-CDC20 (pCEP4 Invitrogen). FreeStyle™ HEK293-F cells (Invitrogen), cultured in 24-well plates, were cotransfected with the pMGM01-CD20 and pMGM05-CD20 vectors (Fc-WT and variants) in equimolar amounts (250 ng/ml) with a FreeStyle™ MAX reagent (1 µl/ml) using standard protocols (Invitrogen). The cells were cultured in suspension in serum-free medium for 7 days post-transfection and the supernatants (1 ml) containing IgGs were harvested after centrifugation of the cells at 100 g for 10 min. The IgGs secreted into the supernatants were quantified using an ELISA assay on recombinant protein L (Pierce), with a purified anti-CD20 antibody produced in 293-F cells used as standard. The supernatants and the standard antibodies, serially diluted in PBS/0.05% Tween-20, were tested on Maxisorp immunoplates (Nunc) precoated with 0.25 µg protein L/well and blocked with 5% skimmed milk in PBS. After incubation for 1 hour at 37°C, the wells were washed three times with PBS/0.05% Tween-20. Bound IgG variants were detected with an F(ab')2 fragment of goat anti-human HRP IgG (specific for the y chain) (Sigma). The IgG variants produced were quantified (1-4 µg/ml) using the standard curve.

2.7. ELISA assays of IgG variants produced in the supernatants of 293-F cells:

The IgG variants were tested for their binding to human complement C1q and several human FcRs by ELISA. Maxisorp immunoplates were coated with 0.5 µg complement C1q/well, 0.05

µg CD32aH/well, 0.2 µg CD16aF/well or 0.1 µg CD16aV/well in PBS. Immobilizing nickel chelating plates (Nunc) were coated with 0.1 µg CD32aR/well or 0.4 µg CD32b/well in 0.01 M KCl. After coating overnight at 4°C, the plates were washed twice with PBS/0.05% Tween-20 and saturated with PBS/4% BSA for 2 hours at 37°C. In parallel, the supernatants were diluted in PBS to a final concentration of 0.5 µg IgG/ml and mixed with F(ab')2 of goat anti-human HRP IgG at the same concentration for 2 hours at ambient temperature. The F(ab')2-aggregated IgGs are then incubated with gentle stirring for 1 hour at 30°C on saturated ELISA plates without dilution for C1q, CD16aF, CD32aR and CD32b (i.e. IgG at 0.5 µg/ml), diluted in PBS at 0.25 µg/ml for CD16aV and CD32aH. The plates are then developed with TMB (Pierce) and the absorbance read at 450 nm.

Selections on the Mut3 library:

By virtue of this ELISA assay, the variants selected by phage display were tested for their binding to complement C1q and to the various receptors. They were tested in comparison with the wild-type Fc (Fc-WT) and the positive controls. 36 clones positive on CD16aV and/or C1q were thus selected on the Mut3 library and used to construct the Mut4 library.

Selections on the Mut4sel library:

The ELISA assays carried out on the 371 isolated clones made it possible to identify 116 clones with a ratio greater than 2 for at least one FcyR and 17 clones with a ratio greater than 3 for C1q alone, thereby corresponding to 133 clones of interest (Table 1).

Table 1: 133 isolated clones of interest

Name of the mutant	**Mutations**	**ELISA assay results**			
		C1q	**CD16aV**	**CD32aR**	**CD32b**
K3B-01	N315D, N361H, P396L	3.46	2.01	7.65	7.30

Name of the mutant	Mutations	ELISA assay results			
		C1q	CD16aV	CD32aR	CD32b
A3A-105	G316D, K326E	5.18	2.48	4.50	7.13
G3A-59	N315D, T350A, P396L	4.05	2.22	7.95	6.19
J3B-81	V284L, V323I, P352S, A378V, Y436H	4.32	2.52	13.17	6.12
J3A-123	V266M, P352S, A378V	0.77	0.35	7.76	5.93
J3B-118	P396L, N421T	3.88	2.58	9.00	4.99
A3A-46	T350A, P396L	2.19	2.40	6.17	4.79
J3A-129	T350A, G385R, P396L	1.33	2.84	6.56	4.33
QL4A-55	N315D, P396L	2.06	2.00	3.41	4.27
A3A-27	K326T, H435R	5.81	2.21	3.41	4.10
A3A-123	N315D, P396L, K447N	4.30	2.70	4.53	4.08
J3A-109	N315D, P396L, N434K	1.75	2.46	6.01	4.07
J3B-124	P396L	2.25	2.52	7.78	3.86
A3A-79	E283G, A378T, V397M	1.62	1.35	3.07	3.83
G3A-69	K326T, V397M, F423L, A431V	3.30	2.51	5.38	3.43
A3A-178	P396L, F423S	4.03	1.73	3.55	3.37
DS3A-39	V284L, A378V	2.76	3.18	3.48	3.30
J3A-89	V284L, N325S, A378V	0.80	0.51	4.50	3.29
DS3A-09	A378V	1.30	3.03	2.70	3.21
J3A-120	N315D, P343S, P396L	1.66	2.32	4.71	3.16
K3A-35	K290E, S375R, F405V	1.89	1.64	3.93	3.12
DL3A-58	S375R	1.85	2.25	3.95	3.11
A3A-50	V284L, K322E, A378V	0.67	1.50	5.08	3.10
DL4A-146	P352S, A378V	1.56	2.31	2.35	3.10
A3A-91	K340E, A378V, N389S, N421T	2.39	1.88	2.67	3.03
DS3A-93	F243I, P352S, A378V	1.29	1.71	4.19	2.97

Name of the mutant	Mutations	ELISA assay results			
		C1q	CD16aV	CD32aR	CD32b
A3A-184	K334N, P352S, V397M	1.44	2.90	3.06	2.91
A3A-112	T307P, N389T, V397M, S442F	3.17	1.74	2.39	2.91
A3A-132	K248E, N315D, P396L	5.03	2.12	3.70	2.79
DS3B-92	V284L, Y349H, A378V	2.52	1.98	2.57	2.79
QL3A-61	N286I, A378V	1.33	2.07	2.20	2.77
J3B-115	T359A, S383R, V397M	1.72	1.69	5.17	2.77
A3A-173	K248E, K334R, A378V	OVER (>30)	1.63	2.96	2.76
A3A-164	A378V, K447N	1.64	2.41	2.82	2.71
G3A-118	N286I, P352S, A378V	1.21	2.45	4.83	2.68
QS5A-66	T394P	2.64	1.81	2.05	2.67
DL4A-55	A378V, N421T	1.20	1.87	2.34	2.66
G3A-176	V259A, V284L, A378V, N421T	1.05	1.68	2.10	2.61
G3A-09	A378T, V397M, K447N	1.58	2.23	3.12	2.45
DL3 A-131	A378T, V397M	1.20	1.72	2.30	2.40
J3A-10	T307P, P396L	5.18	2.74	5.71	2.39
J3B-49	N315D, T393A, P396L	1.42	1.77	5.17	2.30
K3A-41	V302A, P352L, L398P	1.11	0.77	2.17	2.30
DL4A-54	P352L, Q418P	1.35	1.49	1.58	2.30
A3A-06	K248E, A378T, V397M	4.79	1.59	1.78	2.29
G3A-154	A378T, V397M, F423P, S442P	2.51	2.11	4.14	2.28
A3A-12	V363I, V397M	3.02	1.56	2.99	2.27
J3A-14	K326T	1.83	1.43	4.25	2.26
G3A-05	V323I, S383N	2.21	1.33	3.37	2.25
J3B-138	K334R, T394P	4.41	1.09	5.34	2.24
QL2A-11	V302A	0.97	0.68	1.52	2.22

Name of the mutant	Mutations	ELISA assay results			
		C1q	CD16aV	CD32aR	CD32b
K3B-32	S254F, A378T, V397M	5.80	1.42	3.72	2.22
G3A-88	K320E, T394P, G402D	1.32	1.88	4.94	2.21
K3B-30	I253F, K326T, F423L, A431V	7.34	1.58	5.99	2.20
A3A-176	V284A, A378V, A431T	3.32	1.82	2.01	2.20
K3A-59	N315H, Y319H, V323I	2.88	0.59	3.33	2.17
J3B-120	A378V, L398P	1.29	1.67	2.94	2.17
QL4B-10	S383R, V397M	1.40	1.35	1.95	2.08
J3B-89	K290E, V308I, A327T, S383N	0.50	0.14	0.30	2.05
G3A-83	C226Y, A378V, N421T	0.90	2.26	2.00	2.04
G3A-165	V323I, T393I	1.33	1.52	4.15	2.04
J3B-135	K248E, S383R, V397M, N434S	6.36	1.51	3.99	2.04
A3A-137	K290E, V323I, F405L	1.91	1.65	3.02	2.03
K3B-94	R255W, A287T, P352S, A378V	4.66	1.52	5.08	2.02
A3A-30	V284L, T350A	2.09	1.47	2.47	2.01
K3A-07	N315D, A378V	1.30	1.76	2.26	2.01
G3A-164	T394P, N434S	1.72	1.19	2.29	1.99
J3A-16	N286Y, P352S, A378V	1.46	1.83	3.54	1.98
G3A-43	A231V, A378V	1.13	2.44	3.57	1.96
A3A-09	V308G, V323I	3.65	1.23	2.26	1.95
G3A-106	S254F, V284L, A378V	1.57	1.95	3.96	1.94
J3A-08	K274R, A378V, N421T	1.17	1.53	3.47	1.94
QL3A-20	V397M	2.49	1.96	2.60	1.93
G3A-108	T394P, K414R	1.50	1.54	3.67	1.91
G3A-163	V323I, M428T	1.06	1.05	2.41	1.89
A3A-125	T394P, K447N	1.94	1.83	2.48	1.84

Name of the mutant	Mutations	ELISA assay results			
		C1q	CD16aV	CD32aR	CD32b
J3B-109	E345G, V397M	1.18	1.36	2.37	1.83
DS3B-33	K326T, F423L, A431V	5.29	1.31	1.73	1.80
QL4A-28	F243L, P247T, Q362R, G402D, S440N	0.74	1.65	2.88	1.80
J3B-101	Y278H, N315D, P396L	1.18	1.64	2.35	1.78
A3A-140	D312G, A378T, V397M	1.47	1.39	2.23	1.76
G3A-25	V262A, V305A, A378V	1.76	1.56	2.01	1.70
G3A-45	K246R, A378V	1.15	2.46	2.76	1.68
QS6A-78	V323I	1.41	1.31	2.03	1.68
K3B-91	V308I, K326T, F423L, A431V	4.84	1.13	2.71	1.65
G3A-103	K248E, A378V	2.54	2.00	1.96	1.64
G3A-07	N276S, T394P, K439Q	2.27	1.13	2.17	1.59
A3A-17	K290E, E380G	1.64	2.02	1.91	1.59
J3B-16	S267G, A378T, V397M	0.85	0.57	4.95	1.58
J3B-23	N286I, A378V, F423Y	1.13	1.81	3.33	1.57
J3B-68	K320E, T350A	0.82	1.57	3.30	1.55
A3A-37	A231T, K290E, S383N, F423L	1.41	1.36	2.02	1.53
J3A-49	K320R, A378T, V397M	0.60	0.45	2.15	1.52
A3A-31	K334R, L410R	6.23	1.36	1.57	1.50
A3A-41	V323I, P352L, L398P	2.00	1.46	2.53	1.47
K3A-36	K248E, K320M	2.81	1.69	2.34	1.42
J3A-06	A378T, V397M, V412M	1.60	1.65	2.74	1.41
G3A-139	T307N, V323I	1.30	1.10	2.57	1.41
G3A-98	S375R, N434S	1.14	1.56	3.02	1.40
K3B-80	V284L, T366A, A378V	2.58	1.25	2.27	1.39
J3B-74	P230S, N389S, T394P	0.94	0.65	1.81	1.39

Name of the mutant	Mutations	ELISA assay results			
		C1q	CD16aV	CD32aR	CD32b
K3B-41	Y349S, V397M	1.82	1.27	2.11	1.38
G3A-13	K248E, A339T, T350A, S440N	2.10	1.28	2.89	1.37
J3B-44	K246E, K274E, V397M	1.59	1.05	2.38	1.37
K3B-43	A231P, K290E, S383N	1.66	1.43	2.15	1.36
J3B-107	T307P, A378T, N389T	1.50	1.32	2.05	1.36
K3B-33	K334R, K392R, S440G	4.87	0.77	0.97	1.33
K3A-11	K246E, K290E, T307P, N389T	1.73	1.65	2.00	1.33
K3B-49	D376G, S383R, V397M	2.54	1.34	2.57	1.33
G3A-95	I336T, A378V	1.05	2.25	1.82	1.31
K3B-90	V308A, K334R, A378V, K447N	1.32	1.30	3.72	1.27
A3A-146	K290E, R355Q, S383N	1.49	1.38	2.15	1.26
A3A-11	S254F, K447N	4.78	1.18	1.02	1.22
G3A-31	K248E, K334Q	3.60	1.52	1.06	1.21
QL2A-16	K248E, N421T	5.30	0.79	0.77	1.19
J3A-43	K248E, P352L, Q418P	2.96	1.23	2.13	1.19
J3A-28	E333G, A378T, V397M	1.89	1.83	1.73	1.18
A3A-07	L365P, T366S, A378T	1.20	1.94	1.17	1.18
A3A-96	K248E, L365P, A378T	4.09	1.43	0.70	1.12
K3B-87	V323I, F405L	1.56	0.93	2.36	1.10
G3A-148	K248E, S375R	3.79	1.25	1.64	1.08
K3B-34	K334N, T394P, S408N, K414N	0.62	1.18	2.25	1.02
K3B-89	L309M, A378V	1.98	1.42	2.07	1.02
G3A-49	T307A, E380G	3.66	0.91	0.82	1.02
G3A-159	E382G, L432P, Q438DEL	3.00	0.91	1.13	0.98
G3A-28	T307P, H435R	3.49	1.06	0.95	0.96

Name of the mutant	Mutations	ELISA assay results			
		C1q	CD16aV	CD32aR	CD32b
K3B-78	H310R, P352L, Q418P	1.20	0.84	2.04	0.96
G3A-22	P352S, E382G, L432P, Y436N	3.11	0.95	0.62	0.94
G3A-56	K248E, N421T, K447N	3.25	0.71	1.03	0.89
G3A-145	E430G, K447N	4.34	1.01	0.81	0.88
A3A-21	S267N, N384D, N389S, P396L	4.32	1.00	0.56	0.88
G3A-33	K248E, S267N, I336M, P352L, P396L	5.67	0.79	0.39	0.76
G3A-51	V240I, K246E, P353L	3.90	0.95	0.51	0.64

Among these clones, 47 improved clones were used to construct the Mut5 library. From these results, 18 new variants were constructed by site-directed mutagenesis so as to cumulate mutations of interest (Table 2).

Table 2: 18 new variants constructed and compared to reference variants

NA = not determined

Name of the mutant	Mutations	ELISA assay results					
		C1q	CD16aF	CD16aV	CD32aH	CD32aR	CD32b
A3A-105	G316D, K326E	5.18	1.31	2.48	1.50	4.50	7.13
A3A-105A	G316D, K326E, T394P	15.33	1.09	1.45	0.66	5.60	1.21
A3A-105B	G316D, K326E, P396L	14.30	2.25	3.52	3.92	12.64	4.16
A3A-105C	G316D, K326E, V397M	9.61	2.90	1.86	1.81	11.00	2.22
A3A-105D	G316D, K326E, A378V	13.32	2.46	3.68	1.39	8.20	5.96

NA = not determined

Name of the mutant	**Mutations**	**ELISA assay results**					
		C1q	**CD16aF**	**CD16aV**	**CD32aH**	**CD32aR**	**CD32b**
A3A-184	K334N, P352S, V397M	1.44	1.71	2.90	1.34	3.06	2.91
A3A-184A	K334N, P352S, A378V, V397M	1.75	3.40	4.74	3.25	5.03	2.32
DS3A-09	A378V	1.30	3.15	3.03	2.90	2.70	3.21
DS3A-09A	A378V, T394P	1.24	2.29	2.83	2.26	2.00	1.26
DS3A-09B	A378V, P396L	NA	1.83	2.43	NA	NA	NA
DS3A-09C	A378V, V397M	NA	1.48	2.25	NA	NA	NA
G3A-43	A231V, A378V	1.13	1.79	2.44	1.42	3.57	1.96
G3A-43A	A231V, A378V, T394P	NA	1.13	1.65	NA	NA	NA
G3A-43B	A231V, A378V, P396L	NA	1.85	2.42	NA	NA	NA
G3A-43C	A231V, A378V, V397M	NA	1.81	2.68	NA	NA	NA
G3A-45	K246R, A378V	1.15	1.92	2.46	1.38	2.76	1.68
G3A-45A	K246R, A378V, T394P	NA	1.34	2.34	NA	NA	NA
G3A-45B	K246R, A378V, P396L	NA	1.37	1.75	NA	NA	NA
G3A-45C	K246R, A378V, V397M	NA	1.51	2.21	NA	NA	NA
G3A-95	I336T, A378V	1.05	1.29	2.25	1.17	1.82	1.31
G3A-95A	I336T, A378V, T394P	NA	1.50	1.90	NA	NA	NA
G3A-95B	I336T, A378V, P396L	NA	2.00	2.54	NA	NA	NA
G3A-95C	I336T, A378V, V397M	NA	1.55	2.01	NA	NA	NA

NA = not determined

Name of the mutant	Mutations	ELISA assay results					
		C1q	CD16aF	CD16aV	CD32aH	CD32aR	CD32b
J3B-118	P396L, N421T	3.88	1.26	2.58	NA	9.00	4.99
J3B-118A	A378V, P396L, N421T	NA	1.76	3.37	NA	NA	NA
QL3A-20	V397M	2.49	0.62	1.96	1.09	2.60	1.93
QS5A-66	T394P	2.64	NA	1.81	NA	2.05	2.67

Finally, from these 151 variants tested, 26 variants of interest were selected to be produced on a larger scale and studied more precisely (Table 3).

Table 3: 26 new variants selected

NA = not determined

Name of the mutant	Mutations	ELISA assay results					
		C1q	CD16aF	CD16aV	CD32aH	CD32aR	CD32b
A3A-105	G316D, K326E	5.18	1.31	2.48	1.50	4.50	7.13
A3A-105D	G316D, K326E, A378V	13.32	2.46	3.68	1.39	8.20	5.96
A3A-14	S298N, A378V	0.53	0.96	0.85	0.22	0.35	0.88
A3A-173	K248E, K334R, A378V	NA	NA	1.63	NA	2.96	2.76
A3A-184	K334N, P352S, V397M	1.44	1.71	2.90	1.34	3.06	2.91
A3A-184A	K334N, P352S, A378V, V397M	1.75	3.40	4.74	3.25	5.03	2.32
A3A-31	K334R, L410R	6.23	NA	1.36	NA	1.57	1.50
DL4A-54	P352L, Q418P	1.35	1.18	1.49	1.09	1.58	2.30
G3A-103	K248E, A378V	2.54	1.17	2.00	0.83	1.96	1.64

NA = not determined

Name of the mutant	Mutations	ELISA assay results					
		C1q	CD16aF	CD16aV	CD32aH	CD32aR	CD32b
G3A-139	T307N, V323I	1.30	0.81	1.10	0.65	2.57	1.41
G3A-43	A231V, A378V	1.13	1.79	2.44	1.42	3.57	1.96
G3A-45	K246R, A378V	1.15	1.92	2.46	1.38	2.76	1.68
G3A-88	K320E, T394P, G402D	1.32	NA	1.88	NA	4.94	2.21
G3A-95	I336T, A378V	1.05	1.29	2.25	1.17	1.82	1.31
J3A-06	A378T, V397M, V412M	1.60	1.24	1.65	1.58	2.74	1.41
J3A-14	K326T	1.83	0.52	1.43	0.99	4.25	2.26
J3A-16	N286Y, P352S, A378V	1.46	1.35	1.83	1.77	3.54	1.98
J3A-28	E333G, A378T, V397M	1.89	1.41	1.83	1.39	1.73	1.18
J3B-115	T359A, S383R, V397M	1.72	1.32	1.69	1.42	5.17	2.77
J3B-118	P396L, N421T	3.88	1.26	2.58	NA	9.00	4.99
J3B-118A	A378V, P396L, N421T	NA	1.76	3.37	NA	NA	NA
J3B-16	S267G, A378T, V397M	0.85	0.59	0.57	0.42	4.95	1.58
J3B-23	N286I, A378V, F423Y	1.13	1.91	1.81	1.57	3.33	1.57
K3B-01	N315D, N361H, P396L	3.46	0.98	2.01	2.10	7.65	7.30
K3B-90	V308A, K334R, A378V, K447N	1.32	1.06	1.30	1.40	3.72	1.27
QL2A-16	K248E, N421T	5.30	0.69	0.79	0.33	0.77	1.19

Selections on the Mut5 library:

The ELISA assays carried out on the 171 isolated clones made it possible to identify 87 clones with a ratio greater than 2 for at least one of the FcγRs tested or C1q complement (Table 4).

Table 4: 87 clones with ratio greater than 2

Name of the mutant	**Mutations**	**ELISA assay results**					
		C1q	**CD16aF**	**CD16aV**	**CD32aH**	**CD32aR**	**CD32b**
P3A-01	K334N, S383R	0.93	1.34	2.05	1.10	2.00	1.57
P3A-17	K334N, Y373D, A378V	0.88	1.46	1.63	1.35	2.17	1.55
P3A-18	K248E, N286Y, K334R, A378V, K447N	24.77	0.94	1.62	1.07	1.24	1.13
P3A-23	N361H, P396L	2.70	1.75	2.49	2.53	3.33	1.76
P3A-30	K288E, K334N, P352S, V397M	1.18	1.35	4.33	2.14	1.79	1.41
P3A-31	K248E, T359A, S383R, V397M	5.80	0.80	2.05	1.80	1.62	1.51
P3A-50	T307P, A378V	1.11	1.55	2.89	2.20	1.85	1.44
P3A-56	G316D	1.74	1.07	1.90	2.02	1.45	1.39
P3A-70	K274R, A378V, V397M	1.13	1.93	4.05	3.54	4.89	3.20
P3A-74	V302A, V397M	0.92	0.72	0.63	1.47	2.43	1.32
P3A-94	L309P, T359A, S383R, V397M	1.02	1.32	2.11	2.54	1.79	1.32
N3A-111	V302A, K334N, S375R	1.36	0.82	1.18	1.20	2.73	2.33
N3A-113	E283G, Y349S, P396L	6.55	1.30	1.73	2.17	5.19	2.94
N3A-114	K320E, S375R, F405V	0.69	1.03	1.12	0.92	2.11	1.60

Name of the mutant	Mutations	ELISA assay results					
		C1q	CD16aF	CD16aV	CD32aH	CD32aR	CD32b
N3A-117	K334N	1.32	1.61	2.28	1.31	1.93	1.98
N3A-123	K326E	3.15	1.03	2.21	1.27	7.71	3.53
N3A-132	S375R, N389S	1.71	0.99	1.10	1.41	3.50	2.35
N3A-133	K334N, P352S, K447N	0.77	1.02	2.23	0.88	1.25	1.40
N3A-138	S298T, K334R, K370E	3.56	0.90	0.68	0.59	0.73	1.17
N3A-14	T307N, A378V, K414R	1.00	1.70	2.83	2.50	2.49	1.74
N3A-141	T350A, V379A, G385R, P396L	2.82	1.30	1.79	2.36	5.60	5.70
N3A-145	S267G, K290E, E293G, E380G, V397M	0.99	0.70	0.38	0.31	2.45	1.33
N3A-150	S267G, K334N, P352S, V397M	0.86	0.76	0.68	0.33	4.82	1.78
N3A-161	K248E, N315D, A378V	8.59	1.28	2.49	1.12	1.23	1.32
N3A-175	S375R, F405V	0.94	1.16	2.47	1.99	1.30	1.10
N3A-177	V282A, K334R,	5.95	1.15	1.28	1.68	3.22	1.68
	T394P						
N3A-190	T307P, T394P	2.46	1.35	2.46	2.93	3.33	1.94
N3A-27	K290E, A378V, K392R, S440G	1.67	2.25	3.77	2.05	2.59	1.49

Name of the mutant	Mutations	ELISA assay results					
		C1q	CD16aF	CD16aV	CD32aH	CD32aR	CD32b
N3A-28	T366A, A378V	1.04	1.77	2.89	2.26	1.84	1.36
N3A-32	K326T, K334N, P352S, N421T	1.61	2.47	4.35	1.59	3.24	2.04
N3A-52	K326T, S383R, V397M	3.70	1.11	2.07	1.92	5.40	2.41
N3A-58	V284L, K334R, A378V, K447N	5.37	1.02	1.67	2.01	4.00	1.67
N3A-59	N315D, T366A, G385R, P396L	1.08	1.25	1.66	1.75	2.73	1.56
N3A-61	V308A, K334R, A378V	2.07	1.10	1.57	1.11	2.87	2.67
N3A-74	K288R, T394P	1.38	1.22	1.51	1.58	2.15	1.73
N3A-85	K334R, V397M	4.96	1.11	1.45	1.89	3.75	2.77
N3A-87	K290E, K320E, T350A, P396L	0.91	2.15	2.85	3.12	5.94	4.63
N3A-93	A378T, P396L	1.63	1.84	2.21	2.55	3.86	3.06
O3A-04	A231V, T359A, S383R, V397M	1.00	1.16	1.21	1.69	2.35	1.09
O3A-05	K290E, T366A, A378V	0.99	2.79	3.12	2.07	3.34	1.34
O3A-10	Y300H, T394P	0.98	0.76	0.90	1.81	2.25	1.17
O3A-11	R255W, A287T, P352S, A378V, N421T	1.85	1.01	2.10	2.20	3.15	1.55
O3A-16	K334R, A378V, K447N	4.30	1.71	2.51	2.43	3.98	1.59
O3A-17	I336V, T359A, S383R, V397M	0.85	1.79	2.70	2.30	1.73	0.93
O3A-22	K334R, P396L, H435R	23.55	1.68	2.16	2.75	4.79	1.62

Name of the mutant	Mutations	ELISA assay results					
		C1q	CD16aF	CD16aV	CD32aH	CD32aR	CD32b
O3A-24	K326T, K447N	2.12	1.23	2.27	1.55	2.94	1.40
O3A-25	V308A, K334R, A378T, V397M	11.12	1.40	2.07	2.04	4.16	1.96
O3A-34	A231V, Y349S, V397M	0.73	1.40	1.90	2.34	2.62	1.28
O3A-38	K248E, N286Y, Q418P	2.17	1.59	1.16	0.99	0.69	0.57
O3A-40	V308A, A378T, V397M, V412M	0.82	1.65	2.15	2.09	2.64	1.03
O3A-42	S304N, A378V	1.04	2.16	0.53	0.87	0.87	1.77
O3A-44	A378V, K439R	1.95	2.44	4.40	3.20	3.13	1.81
O3A-45	D270N, K334R, L410R	3.32	1.57	0.61	0.56	1.19	1.36
O3A-46	V302A, A378V	0.94	1.15	1.75	2.36	2.91	1.37
O3A-50	K334R, A378V, N421T	3.62	1.28	2.49	2.15	4.02	2.30
O3A-57	T359A, V397M	1.50	1.18	2.27	2.23	2.78	1.59
O3A-66	A378T, T394P	1.10	1.42	2.42	2.36	2.45	1.76
O3A-67	K248E, N361H	2.05	0.89	1.36	1.06	1.07	1.76
O3A-69	K290E, A378V	1.44	2.64	4.04	2.62	4.11	2.06
O3A-80	K274R, T394P, G402D, K447N	1.26	1.26	1.81	2.16	2.32	1.28
O3A-81	K274R, A378T, V397M	1.36	1.20	2.39	2.06	3.08	1.58
O3A-86	K248E, K290E, N361H, P396L	24.34	2.49	4.89	2.86	4.19	2.57
O3A-93	K290E, T394P	2.07	1.45	3.53	2.54	3.36	1.78
P4B-89	T307P, T366A, A378V	0.63	2.99	4.15	0.70	1.19	0.87

Name of the mutant	Mutations	ELISA assay results					
		C1q	CD16aF	CD16aV	CD32aH	CD32aR	CD32b
Q4B-08	V284L, K290E, A378V	2.43	1.75	3.47	2.69	3.58	1.63
Q4B-15	K274R, A378V	1.26	1.04	2.69	2.61	1.75	1.03
Q4B-18	I377T, A378V, F423Y	1.21	1.08	2.82	1.96	1.37	1.07
Q4B-34	K248E, K290M, V308A, P352S	4.47	0.63	0.56	0.38	0.70	0.75
Q4B-59	T350A, A378T, V397I	1.32	2.22	2.01	2.76	2.52	1.51
Q4B-61	K320E, T394P, V397M	0.87	1.10	0.98	1.60	2.43	1.08
Q4B-68	T307P, A378V, T394P	3.33	1.53	4.12	4.01	3.69	1.03
Q4B-91	K334R, A378V	2.81	1.14	2.35	2.61	2.46	0.94
Q3A-01	P352L, A378V	1.13	1.14	1.53	2.04	1.81	0.87
Q3A-39	N286I, P396L, N421T	2.80	1.63	1.99	3.52	3.09	1.02
Q3A-58	S267G, V397M	0.73	1.30	0.34	0.40	2.87	1.02
Q3A-85	P396L, N421T, K447N	3.80	1.65	2.17	3.67	3.50	1.13
O3A-103	K290E, K320T, A378V	2.11	1.22	2.08	2.76	2.41	1.50
O3A-117	K334R, T394P, N421S	0.98	1.05	1.71	2.29	3.05	1.37
O3A-119	K334N, V397M	1.53	1.04	1.97	1.45	2.28	1.18
O3A-126	P227S, V284L, A378V	4.58	1.31	2.93	2.85	4.47	1.29

Name of the mutant	Mutations	ELISA assay results					
		C1q	CD16aF	CD16aV	CD32aH	CD32aR	CD32b
O3A-127	V302A, K334R, T366A, S383R, V397M	1.53	1.00	1.24	2.85	3.56	1.37
O3A-131	C261R, A378T, V397M, V412M	2.25	1.03	1.72	2.00	2.27	1.15
O3A-137	K248E, T350A	0.84	0.66	0.43	0.68	2.06	0.97
O3A-172	K320E, T394P	8.19	0.88	1.46	2.01	2.52	1.06
O3A-179	R255Q, G385R	0.81	0.74	0.83	0.57	2.86	1.30
O3A-186	T307P, V397M	1.09	1.41	2.32	2.17	3.97	1.48
O3A-99	N276S, N286I, T359A, S383R, V397M	2.14	1.32	2.27	2.84	3.09	1.42

Among these variants, 10 variants of interest were selected (Table 5).

Table 5: 10 variants

Name of the mutant	Mutations	ELISA assay results					
		C1q	CD16aF	CD16aV	CD32aH	CD32aR	CD32b
O3A-05	K290E, T366A, A378V	0.99	2.79	3.12	2.07	3.34	1.34
P4B-89	T307P, T366A, A378V	0.63	2.99	4.15	0.70	1.19	0.87
O3A-86	K248E, K290E, N361H, P396L	24,34	2.49	4.89	2.86	4.19	2.57

Name of the mutant	Mutations	ELISA assay results					
		C1q	CD16aF	CD16aV	CD32aH	CD32aR	CD32b
N3A-32	K326T, K334N, P352S, N421T	1.61	2.47	4.35	1.59	3.24	2.04
O3A-44	A378V, K439R	1.95	2.44	4.40	3.20	3.13	1.81
N3A-27	K290E, A378V, K392R, S440G	1.67	2.25	3.77	2.05	2.59	1.49
N3A-87	K290E, K320E, T350A, P396L	0.91	2.15	2.85	3.12	5.94	4.63
Q4B-68	T307P, A378V, T394P	3.33	1.53	4.12	4.01	3.69	1.03
N3A-58	V284L, K334R, A378V, K447N	5.37	1.02	1.67	2.01	4.00	1.67
Q3A-39	N286I, P396L, N421T	2.80	1.63	1.99	3.52	3.09	1.02

3. Production and purification of variants of interest

The IgG variants are obtained by direct mutagenesis in pCEP4-WT-H-CD20. The IgG controls, i.e. C1, C3, C4, C5 and wild-type (WT), are produced with the G1m3 allotype (comprising 3 mutations compared to G1m1,17: (K214R/)D356E/L358M).

The 26 IgG variants derived from the Mut4sel library, and also the wild-type, were produced with the G1m1,17 allotype. They are produced by incubation for 6-7 days in batches (250-300 ml) of 293-E cells (Freestyle Invitrogen) in F17 medium.

Centrifugation and filtration are then carried out.

Purification is carried out on protein A Hi-Trap, and elution with a 25 mM citrate buffer, pH=3.0, neutralization and dialysis in TBS or PBS are carried out before sterilization.

10 mg of each IgG control, i.e. C1, C3, C4, C5 and wild-type (WTG1m3 and WTGlm1,17) are obtained, and also 2-3 mg of 26 IgG variants.

Their characterization shows that the molecular weight is maintained, and that glycosylation profiles are similar for all variants.

4. Tests of variants of interest

4.1. Tests for binding on FcRn

Jurkat-FcRn cells are incubated at pH=6.0 with IgG variants at various concentrations (0 to 1000 µg/ml) and Rituximab-Alexa.

Flow cytometry is performed on the bound Rituximab-Alexa.

The results show no loss of binding to FcRn for all IgG variants.

4.2 Antigen-binding tests

Raji cells are incubated with the IgG variants at 1µg/ml for 15 minutes at 4°C.

The bound IgGs are revealed by binding with an anti-human IgG-PE secondary antibody (for 15 minutes at 4°C).

The results show that the antigen recognition is not altered by the various mutations present on the Fc.

All the IgG variants bind to CD20 on the cells, in a manner similar to the IgG-WT control.

4.3 CD16aV/F-binding ELISA assays

The purified antibodies were tested by ELISA for their binding to CD16F and CD16aV according to a protocol identical to that

described in 2.6, diluting the antibodies to various concentrations.

4.4. ADCC activity assays

NK cells are incubated with Raji target cells expressing CD20, in the presence of various concentrations of IgG variants (0.005 to 5000 ng/ml).

The level of intracellular LDH released by the lysed target cells is measured.

Human NK cells were purified from peripheral blood of healthy voluntary donors by the negative depletion technique developed by Miltenyi. The ADCC assay comprises incubation of NK cells with Raji target cells expressing the CD20 antigen, in the presence of various concentrations of anti-CD20 antibodies. After incubation for 16 hours, the cytotoxicity induced by the anti-CD20 antibodies was measured by quantifying the intracellular lactate dehydrogenase (LDH) released by the lysed target cells in the cell supernatants. The specific lysis results are expressed as a percentage of lysis as a function of the antibody concentration. The values of EC50 (antibody concentration inducing 50% of the maximum lysis induced by IgG-WT) and Emax (maximum lysis percentage) were calculated using the GraphPad Prism software.

The results are presented in Tables 6 and 7.

Table 6: ADCC assay results

Mutant	**G3A43**	**G3A45**	**J3B-118**	**J3B16**	**A3A-184**	**WT**
EC50: Antibody concentration (ng/ml) giving 50% lysis of an internal control	1.03	4.38	1.27	> 5545.36	2.35	12.34
[WT]/[antibody] ratio	11.98	2.82	9.72	<0.0022	5.25	1

Table 7a: ADCC assay results

Mutant	**EC50: Antibody concentration (ng/ml) giving 50% maximum lysis of an internal control**	**[WT]/[antibody] ratio**
G3A-103	0.31	33.90
A3A-184A	0.34	30.91
J3B-23	0.57	18.44
J3A-28	0.57	18.44
K3B-01	0.69	15.23
A3A-14	0.82	12.82
G3A-95	0.97	10.84
J3B-118A	0.97	10.84
J3A-16	1.27	8.28
J3A-06	1.27	8.28
A3A-105D	1.27	8.28
G3A-139	2.80	3.75
QL2A-16	4.36	2.41
J3A-14	7.39	1.42
G3A-88	8.07	1.30
WT	10.51	1
K3B-87	16.33	0.64
DL4A-54	>5000	<0.002
A3A-90	>5000	<0.002

Mutant	EC50: Antibody concentration (ng/ml) giving 50% maximum lysis of an internal control	[WT]/[antibody] ratio
A3A-34	>5000	<0.002

Table 7b: ADCC assay results

Mutant	EC50: Antibody concentration (ng/ml) giving 50% maximum lysis of an internal control	[WT]/[antibody] ratio
WT	0.45	1
A3A-173	1.38	0.33
K3B-90	0.75	0.60
A3A-105	1.374	0.33
A3A-31	1.839	0.24
J3B-115	0.57	0.79

Table 7c: ADCC test results (EC45 instead of EC50)

Mutant	EC45: Antibody concentration (ng/ml) giving 45% maximum lysis of an internal control	[WT]/[antibody] ratio
WT	1.60	1
O3A-44	2.6	0.62
N3A-27	18.7	0.86
N3A-58	4.4	0.36
O3A-86	4.4	0.36
N3A-87	1.0	1
P4B-89	3.6	0.44
O3A-05	0.2	8

Mutant	EC45: Antibody concentration (ng/ml) giving 45% maximum lysis of an internal control	[WT]/[antibody] ratio
N3A-32	2.9	0.55
Q3A-39	0.3	5.33
Q4B-68	1.7	0.94

The best variants are A3A-184A and G3A-103.

4.5. CDC activity assays

Raji target cells expressing the CD20 antigen were incubated with various concentrations of anti-CD20 antibodies (0 to 5000 ng/ml) in the presence of rabbit serum as complement source (Cedarlane, 1/10 dilution). After incubation for 1 hour at 37°C, the level of LDH released into the supernatant by the lysed target cells was measured chromogenically (cytotoxicity detection kit from Roche Applied Sciences) and used to quantify antibody-mediated complement-dependent cytotoxicity. The results are expressed as a lysis percentage. The EC50 (amount of antibody that induces 50% maximum lysis) and Emax (maximum lysis percentage) were calculated using the GraphPad Prism software.

The results are presented in Tables 8 and 9.

Table 8: CDC assay results

Antibody	Antibody concentration (ng/ml) giving 50% lysis of an internal control	[WT]/[antibody] ratio
A3A-173	16.01	16.36
K3B-90	23.12	11.33
J3B-118	38.69	6.77
A3A-105	64.76	4.05
A3A-31	108.37	2.42

Antibody	**Antibody concentration (ng/ml) giving 50% lysis of an internal control**	**[WT]/[antibody] ratio**
J3B-115	145.44	1.80
G3A-43	168.49	1.55
G3A-45	195.20	1.34
A3A-184	210.10	1.25
J3B-16	210.10	1.25
WT	261.97	1

Table 9a: CDC assay results

Antibody	**Antibody concentration (ng/ml) giving 50% lysis of an internal control**	**[WT]/[antibody] ratio**
J3B-118A	21.564	10.98
J3A-28	31.004	7.64
G3A-103	33.339	7.10
A3A-105D	38.550	6.14
QL2A-16	51.544	4.59
K3B-01	59.600	3.97
J3A-06	64.089	6.70
J3A-14	64.089	6.70
A3A-184A	74.107	3.20
G3A-139	85.691	2.76
J3B-23	99.085	2.39
K3B-87	106.548	2.22
A3A-34	123.202	1.92
J3A-16	132.481	1.79
DL4A-54	164.727	1.44
G3A-95	177.134	1.34
WT	236.837	1
G3A-88	273.856	0.86

Antibody	Antibody concentration (ng/ml) giving 50% lysis of an internal control	[WT]/[antibody] ratio
A3A-14	340.513	0.70
A3A-90	566.098	0.42

Table 9b: CDC assay results

Antibody	Antibody concentration (ng/ml) giving 50% lysis of an internal control	[WT]/[antibody] ratio
WT	133	1
O3A-44	129	1.03
N3A-27	179	0.74
N3A-58	36	3.69
O3A-86	44	3.02
N3A-87	106	1.25
P4B-89	115	1.16
O3A-05	91	1.46
N3A-32	66	2.02
Q3A-39	61	2.18
Q4B-68	126	1.06

Table 10 below shows the results obtained with certain variants, classified by subgroup.

Table 10

				ELISA ratio (purified Abs)			
Name	**Mutations**	**ADCC**	**CDC**	**CD32aH**	**CD32aR**	**CD32b**	**CD64**
G3A-103	K248E, A378V	33.9	7.1	0.5	0.9	1.2	1.2
J3A-28	E333G, A378T, V397M	18.4	7.6	1.1	1.8	1.6	1.3

				ELISA ratio (purified Abs)			
Name	**Mutations**	**ADCC**	**CDC**	**CD32aH**	**CD32aR**	**CD32b**	**CD64**
J3B-118A	P396L, N421T, A378V	10.8	11.0	2.7	1.8	3.7	1.4
J3B-118	P396L, N421T	9.7	12.2	1.0	1.1	1.0	1.6
A3A-105D	G316D, K326E, A378V	8.3	6.1	1.4	2.6	2.0	1.5
A3A-14	5298N,A378V	12.8	0.7	1.1	0.8	0.9	1.6
G3A-95	I336T, A378V	10.8	1.3	0.7	0.9	1.0	1.3
A3A-184A	K334N, P352S, V397M, A378V	30.9	3.2	1.8	4.3	3.1	1.5
J3B-23	N286I, A378V, F423Y	18.4	2.4	NA	NA	NA	NA
K3B-01	N315D, N361H, P396L	15.2	4.0	5.7	1.0	1.0	2.3
G3A-43	A231V, A378V	12.0	2.8	5.3	3.6	2.7	1.4
J3A-06	A378T, V397M, V412M	8.3	3.7	0.84	2.0	2.4	1.3
J3A-16	N286Y, P352S, A378V	8.3	1.8	2.61	1.7	1.7	1.3
O3A-05	K290E, T366A, A378V	8.0	1.5	4.02	3.3	4.5	1.2
Q3A-39	N286I, P396L, N421T	5.3	2.2	4.83	2.3	3.8	1.5
A3A-184	K334N, P352S, V397M	5.3	2.2	1.33	3.2	3.3	1.4
K3B-90	V308A, K334R, A378V, K447N	0.6	20.4	1.20	1.2	1.1	1.5
A3A-173	K248E, K334R, A378V	0.3	29.5	0.95	1.3	1.3	1.2
J3A-14	K326T	1.4	3.7	1.86	1.3	1.5	2.3
G3A-88	K320E, T394P, G402D	1.3	0.9	2.94	3.7	3.9	2.4
N3A-87	K290E, K320E, T350A, P396L	1.0	1.3	3.92	3.7	9.0	1.5
J3B-115	T359A, S383R, V397M	0.8	3.2	4.65	5.1	4.2	1.5

Table 11 below shows certain possible variants according to the invention:

Table 11

Name	**Mutations**	**Starting variant**	**Mutation added**
G3A-95D	S298N, I336T, A378V	G3A-95	S298N
K3B-90A	K248E, V308A, K334R, A378V, K447N	K3B-90	K248E
G3A-88A	K320E, K326T, T394P, G402D	G3A-88	K326T
N3A-87A	K290E, K320E, K326T, T350A, P396L	N3A-87	K326T
A3A-105E	K248E, G316D, K326E, A378V	A3A-105D	K248E
A3A-14A	K248E, S298N, A378V	A3A-14	K248E
A3A-184B	K248E, K334N, P352S, V397M, A378V	A3A-184	K248E
G3A-43D	K248E, A231V, A378V	G3A-43	K248E
G3A-95E	K248E, I336T, A378V	G3A-95	K248E
J3A-28A	K248E, E333G, A378T, V397M	J3A-28	K248E
J3B-118B	K248E, P396L, N421T, A378V	J3B-118A	K248E
J3B-23A	N286I, A378V, F423Y	J3B-23	K248E
K3B-01A	N315D, N361H, P396L	K3B-01	K248E
A3A-105F	G316D, K326E, E333G, A378V	A3A-105	E333G
A3A-14B	S298N, E333G, A378V	A3A-14	E333G
A3A-184C	E333G, K334N, P352S, V397M, A378V	A3A-184	E333G
G3A-103A	K248E, E333G, A378V	G3A-103	E333G

Name	**Mutations**	**Starting variant**	**Mutation added**
G3A-43E	A231V, E333G, A378V	G3A-43	E333G
G3A-95F	E333G, I336T, A378V	G3A-95	E333G
J3B-118C	E333G, P396L, N421T, A378V	J3B-118	E333G
J3B-23B	N286I, E333G, A378V, F423Y	J3B-23	E333G
K3B-01B	N315D, E333G, N361H, P396L	K3B-01	E333G
A3A-105G	G316D, K326E, A378V, F423Y	A3A-105	F423Y
A3A-14C	E333G, S298N, A378V, F423Y	A3A-14	F423Y
A3A-184D	K334N, P352S, V397M, A378V, F423Y	A3A-184	F423Y
G3A-103B	K248E, A378V, F423Y	G3A-103	F423Y
G3A-43F	A231V, A378V, F423Y	G3A-43	F423Y
G3A-95G	I336T, A378V, F423Y	G3A-95	F423Y
J3A-28B	E333G, A378T, V397M, F423Y	J3A-28	F423Y
J3B-118D	P396L, N421T, A378V, F423Y	J3B-118	F423Y
K3B-01C	N315D, N361H, P396L,F423Y	K3B-01	F423Y

Patentkrav

1. Fremgangsmåde til fremstilling af et polypeptid, der omfatter en Fc-region af humant IgG1, som er muteret i forhold til et parentalt polypeptid, og som har en funktionel aktivitet, der medieres af Fc-regionen, som er valgt blandt antistofafhængig cellulær cytotoksicitet (ADCC) og komplementafhængig cytotoksicitet (CDC), der er forhøjet i forhold til aktiviteten af det parentale polypeptid, idet det parentale polypeptid omfatter en parental Fc-region, der er udvalgt blandt sekvenserne SEQ ID NO:1, 5, 6 og 10, hvilken fremgangsmåde omfatter et trin med indføring af en kombination af mutationer i den parentale Fc-region, som er valgt blandt:
248E/378V,
396L/421T/378V,
316D/326E/378V,
298N/378V,
336T/378V,
334N/352S/397M/378V,
286I/378V/423Y,
231V/378V,
286Y/352S/378V,
290E/366A/378V,
308A/334R/378V/447N,
248E/334R/378V,
298N/336T/378V,
248E/308A/334R/378V/447N,
248E/316D/326E/378V,
248E/298N/378V,
248E/334N/352S/397M/378V,
248E/231V/378V,
248E/336T/378V,
248E/396L/421T/378V,
316D/326E/333G/378V,
298N/333G/378V,
333G/334N/352S/397M/378V,
248E/333G/378V,
231V/333G/378V,

333G/336T/378V,
333G/396L/421T/378V,
286I/333G/378V/423Y,
316D/326E/378V/423Y,
333G/298N/378V/423Y,
334N/352S/397M/378V/423Y,
248E/378V/423Y,
231V/378V/423Y,
336T/378V/423Y eller
396L/421T/378V/423Y,
hvor nummereringen er ifølge EU-indekset.

2. Fremgangsmåde ifølge krav 1, der omfatter et trin med indføring af en kombination af mutationer i den parentale Fc-region, som er valgt blandt:
248E/378V,
396L/421T/378V,
316D/326E/378V,
298N/378V,
336T/378V,
334N/352S/397M/378V,
286I/378V/423Y,
231V/378V,
286Y/352S/378V,
290E/366A/378V,
308A/334R/378V/447N eller
248E/334R/378V.

3. Fremgangsmåde ifølge et af kravene 1 til 2, kendetegnet ved, at det parentale polypeptid omfatter en parental Fc-region, som er sekvensen SEQ ID NO:1.

4. Fremgangsmåde ifølge et af kravene 1 til 3, kendetegnet ved, at polypeptidet er valgt blandt en isoleret Fc-region, en sekvens, der er afledt af en isoleret Fc-region, et antistof og et fusionsprotein, der omfatter en Fc-region.

5. Fremgangsmåde ifølge et af kravene 1 til 4, kendetegnet ved, at polypeptidet er et polypeptid, der produceres i mælk fra transgene dyr.

6. Polypeptid bestående af sekvens SEQ ID NO:1, der er muteret ifølge en af følgende kombinationer af mutationer:
248E/378V,
396L/421T/378V,
316D/326E/378V,
298N/378V,
336T/378V,
334N/352S/397M/378V,
286I/378V/423Y,
231V/378V,
286Y/352S/378V,
290E/366A/378V,
308A/334R/378V/447N,
248E/334R/378V,
298N/336T/378V,
248E/308A/334R/378V/447N,
248E/316D/326E/378V,
248E/298N/378V,
248E/334N/352S/397M/378V,
248E/231V/378V,
248E/336T/378V,
248E/396L/421T/378V,
316D/326E/333 G/378V,
298N/333G/378V,
333G/334N/352S/397M/378V,
248E/333G/378V,
231V/333G/378V,
333G/336T/378V,
333G/396L/421T/378V,
286I/333G/378V/423Y,
316D/326E/378V/423Y,
333G/298N/378V/423Y,
334N/352S/397M/378V/423Y,
248E/378V/423Y,

231V/378V/423Y,
336T/378V/423Y eller
396L/421T/378V/423Y,
hvor nummereringen er ifølge EU-indekset.

7. Farmaceutisk sammensætning, der omfatter (i) et polypeptid ifølge krav 6 og (ii) mindst én farmaceutisk acceptabel excipiens.

8. Polypeptid ifølge krav 6 til anvendelse som lægemiddel.