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(54) Title: STABILISED HUMAN FC

(57) Abstract: The invention refers to a method of preparing a stability engineered human IgG Fc mutant by engineering at least one of the point mutations as listed in Table 1 or a combination of such point mutations, the stability engineered Fc mutant and an Fc library comprising a repertoire of stability engineered Fc mutants

STABILISED HUMAN FC

The invention refers to a method of preparing a stability engineered human IgG Fc mutant and respective mutants.

Background

Monoclonal antibodies have been widely used as therapeutic binding agents. The basic antibody structure will be explained here using as an example an intact IgG1 immunoglobulin.

Two identical heavy (H) and two identical light (L) chains combine to form the Y-shaped antibody molecule. The heavy chains each have four domains. The amino terminal variable domains (VH) are at the tips of the Y. In the case of IgG, IgD and IgA, these are followed by three constant domains: CH1, CH2, and the carboxy-terminal CH3, at the base of the Y's stem. In the case of IgM and IgE there are four different constant domains. A short stretch, the switch, connects the heavy chain variable and constant regions. The hinge connects CH2 and CH3 (the Fc fragment) to the remainder of the antibody (the Fab fragments). One Fc and two identical Fab fragments can be produced by proteolytic cleavage of the hinge in an intact antibody molecule. The light chains are constructed of two domains, variable (VL) and constant (CL), separated by a switch.

Disulfide bonds in the hinge region connect the two heavy chains. The light chains are coupled to the heavy chains by additional disulfide bonds. Asn-linked carbohydrate moieties are attached at different positions in constant domains depending on the class of immunoglobulin. For human IgG1 two disulfide bonds in the hinge region, between Cys226 and Cys229 pairs, unite the two heavy chains. The light chains are coupled to the heavy chains by two additional disulfide bonds, between the Cys following Ser221 in the CH1 domain and Cys214 in the CL domain. Carbohydrate moieties are attached to Asn297 of each CH2, generating a pronounced bulge in the stem of the Y. The numbers here are given according to the Kabat numbering scheme.

These features have profound functional consequences. The variable regions of both the heavy and light chains (VH) and (VL) lie at the "tips" of the Y, where they are positioned to react with antigen. This tip of the molecule is the side on which the N-terminus of the amino acid sequence is located. The stem of the Y projects in a way to efficiently mediate effector functions such as the activation of complement and

interaction with Fc receptors, or ADCC and ADCP. Its CH2 and CH3 domains bulge to facilitate interaction with effector proteins. The C-terminus of the amino acid sequence is located on the opposite side of the tip, which can be termed "bottom" of the Y.

Two types of light chain, termed lambda (λ) and kappa (κ), are found in antibodies. A given immunoglobulin either has kappa chains or lambda chains, never one of each. No functional difference has been found between antibodies having lambda or kappa light chains.

Each domain in an antibody molecule has a similar structure of two beta sheets packed tightly against each other in a compressed antiparallel beta barrel. This conserved structure is termed the immunoglobulin fold. The immunoglobulin fold of constant domains contains a 3-stranded sheet packed against a 4-stranded sheet. The fold is stabilised by hydrogen bonding between the beta strands of each sheet, by hydrophobic bonding between residues of opposite sheets in the interior, and by a disulfide bond between the sheets. The 3-stranded sheet comprises strands C, F, and G, and the 4-stranded sheet has strands A, B, E, and D. The letters A through G denote the sequential positions of the beta strands along the amino acid sequence of the immunoglobulin fold.

The fold of variable domains has 9 beta strands arranged in two sheets of 4 and 5 strands. The 5-stranded sheet is structurally homologous to the 3-stranded sheet of constant domains, but contains the extra strands C' and C". The remainder of the strands (A, B, C, D, E, F, G) have the same topology and similar structure as their counterparts in constant domain immunoglobulin folds. A disulfide bond links strands B and F in opposite sheets, as in constant domains.

The variable domains of both light and heavy immunoglobulin chains contain three hypervariable loops, or complementarity-determining regions (CDRs). The three CDRs of a V domain (CDR1, CDR2, CDR3) cluster at one end of the beta barrel. The CDRs are loops that connect beta strands B-C, C'-C", and F-G of the immunoglobulin fold. The residues in the CDRs vary from one immunoglobulin molecule to the next, imparting antigen specificity to each antibody.

The VL and VH domains at the tips of antibody molecules are closely packed such that the 6 CDRs (3 on each domain) cooperate in constructing a surface (or cavity) for antigen-specific binding. The natural antigen binding site of an antibody thus

is composed of the loops which connect strands B-C, C'-C", and F-G of the light chain variable domain and strands B-C, C'-C", and F-G of the heavy chain variable domain.

The loops, which are not CDR-loops in a native immunoglobulin, apart from the antigen-binding pocket, which is determined by the CDR loops and optionally adjacent loops within the CDR loop region that contribute to the antigen-binding pocket, do not have antigen binding or epitope binding specificity, but contribute to the correct folding of the entire immunoglobulin molecule, and are therefore called structural loops for the purpose of this invention.

Prior art documents show that the immunoglobulin scaffold has been employed so far for the purpose of manipulating the existing antigen binding site, thereby introducing novel binding properties. In most cases the CDR regions have been engineered for various antigen binding, in other words, in the case of the immunoglobulin fold, the natural antigen binding site has been modified in order to change its binding affinity or specificity. A vast body of literature exists which describes different formats of such manipulated immunoglobulins, frequently expressed in the form of single-chain Fv fragments (scFv) or Fab fragments, either displayed on the surface of phage particles or solubly expressed in various prokaryotic or eukaryotic expression systems. Various immunoglobulin libraries have been proposed in the art to obtain specific immunoglobulin binders. However, the scaffolds used for preparing such libraries were limited, because of possible deterioration of the framework when engineering the antigen-binding pocket.

The CH3 domain of IgG has been subject to different engineering strategies. With the aim to develop a method for the robust production of bispecific antibodies, Atwell S et al. (J Mol Biol. 1997 Jul 4; 270(1):26-35) have developed a heterodimeric CH3 system by mutating residues located in the interface between the two CH3 domains. While the formation of a heterodimer could be shown, the constructs were significantly compromised in stability compared to the wildtype protein.

Another reason for mutating a constant domain of an antibody can be to modify its interaction strength with an Fc receptor, as e.g. described by Stavenhagen JB et al. (Adv Enzyme Regul. 2008;48:152-64. Epub 2007 Dec).

Further modifications for modulating Fc effector function and/ or half-life of the molecule are described in WO2005047327A2, WO2005063815A2, WO2004035752A2, WO2006053301A2, US20070003546A1, WO2006076594A2,

WO2006105338A2, US20060235208A1, WO2006053301A2, WO2006076594A2, WO2006020114A2, WO0042072A2, US20050037000A1, WO2006088494A2 and WO2004063351A1.

Demarest SJ et al. (J Mol Biol. 2004 Jan 2;335(1):41-8) have performed residue frequency analysis on CH3 domains from different types of immunglobulins and different species and were successful in creating mutants of a single bovine CH3 domain of IgG1 that had its T_m increased by 10° in one case of a triple mutant.

Oganesyan Vaheh et al. (Molecular Immunology (2008) 45(7) 1872-1882) disclose a CH2 domain with point mutations resulting in an increase in human IgG1 binding to human FcgammaRIIIA, however with reduced thermostability.

Dall' Acqua et al. (Biochemistry (1998) 37(26) 9266-9273) identify domain interface amino acid residues of the CH3 domain contributing to the stability of CH3 domain homodimers.

There is a need to provide stable immunoglobulins for preparing respective libraries. It is, thus, the object of the invention to provide an improved immunoglobulin as a scaffold for antibody engineering. The objective of the invention particularly refers to engineering human IgG1 Fc domains with increased stability, in order to subsequently use these stabilised domains as basic scaffolds for Fc libraries. Specifically it would be highly desirable that the overall structure of selected binding mutants is as close as possible to the wildtype structure, which represents a superior scaffold to carry mutations in structural loops, leading to engineered binding sites.

The object is solved by the subject matter as claimed.

Summary of the Invention

According to the invention there is provided a method of preparing a stability engineered human IgG Fc mutant by engineering at least one of the point mutations as listed in Table 1. Preferably a combination of such point mutations is employed to enhance the effect of the individual point mutations on thermostability.

Herein and further below any such preferred combination of mutations mentioned in the context of the mutations of Table 1, preferably is with each other or with any further mutations. In a preferred method said combination is with one or more of the mutations as listed in Table 2.

According to a specific embodiment, the method according to the invention comprises engineering at least one of the point mutations as listed in Table 2, i.e. in addition to at least one of the point mutations as listed in Table 1.

Specific embodiments according to the invention refer to the Fc molecule or fragment that comprises at least the exemplified point mutations or combinations of point mutations as listed in Tables 3 or 5.

Specifically an Fc molecule is engineered to provide a Fc mutant comprising at least one of the mutations selected from the group consisting of:

- L351M;
- L368M;
- V369I;
- V397I, L398M;
- Y349F;
- Q347M, Y349F;
- K360N;
- V369I;
- K370L;
- N390R, S400D;
- N390E, S400R;
- A378V;
- D249R, R255L;
- V282K;
- E258M;
- V305A, T307P;
- E258K;
- K274Q, N276T, Y278F;
- K246N;
- V305E, T307P;
- N276T, Y278F;
- T250I;
- Y278F;
- T250I, K246N;
- K370L, T250V;

- K370L, V305A, T307P;
- K370L, K246N;
- K370L, K246N, T250I;
- K246N, T250I, V305A, T307P;
- T250V, V305A, T307P;
- T250I, V305A, T307P;
- K370L, T250V, V305A, T307P;
- K370L, T250V, K246N;
- K370L, T250I, V305A, T307P, S383N;
- T250V, V305A, T307P, K246N;
- K370L, T250V, V305A, T307P, K246N;
- K370L, T250V, V305A, T307P, K246N, K274Q;
- K370L, T250V, V305A, T307P, K246N, N276T;
- K370L, T250V, V305A, T307P, K246N, K274Q, N276T;
- K370L, T250I, V305A, T307P;
- K370L, N390R, S400D;
- T250V, N390R, S400D;
- K370L, T250V, K246N, N390R, S400D;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D;
- K370L, T250V, V305A, T307P, K246N, K274Q, N390R, S400D;
- K370L, T250V, V305A, T307P, K246N, K274Q, N276T, N390R, S400D;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D, Q438K;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D, Q438R;

- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D, Q438K, Q347E;

- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D, Q438R, Q347E;

- Q418L;

- K360Q, G446C;

- K360Q, Q438R;

- K360Q, Q418L, Q438R;

- Q347E;

- K360Q;

- Q438R;

- K392R;

- S400F;

- K360Q, Q418L;

- K360E;

- Q438K;

- Q347E, Q418L;

- T437I;

- S424T;

- N421D; and

-S354P.

wherein the numbering is according to the Kabat numbering scheme.

The Fc according to the invention preferably is stability engineered by site directed mutagenesis to incorporate the claimed stabilising mutations. The nucleotide or amino acid sequence is preferably mutated by insertion or substitution.

Preferably the Fc mutant is selected for its functional scaffold structure as determined by binding to an effector molecule, like CD16a, CD64, FcRn, CD32, C1q and Protein A. Herein the binding to Protein A serves as a surrogate for binding to an effector molecule.

The preferred Fc mutant has a Tm value, which is higher than the wild type. For the purpose of the invention, the increase of any individual Tm value regarding the Fc molecule, including T_m values of a CH2 and/or CH3 domain, such as T_m1, T_m2 and/or T_m3, is considered a T_m value, which is higher than the wild type.

According to a specific aspect the Fc molecule is further engineered to incorporate a new antigen binding site into the structural loop region, either before engineering the thermostabilising mutations, concomitantly or afterwards.

The Fc according to the invention preferably is engineered to incorporate a new antigen binding site by randomisation.

According to a specific embodiment the invention refers to stability engineered human IgG Fc mutant having at least one of the point mutations as listed in Table 1 or a combination of such point mutations.

The preferred Fc according to the invention comprises a combination with one or more of the mutations as listed in Table 2.

A further preferred Fc according to the invention comprises at least one of the mutations as listed in Table 3 or 5.

Specifically the Fc according to the invention comprises at least one of the mutations selected from the group consisting of:

- L351M;
- L368M;
- V369I;
- V397I, L398M;
- Y349F;
- Q347M, Y349F;
- K360N;
- V369I;
- K370L;
- N390R, S400D;
- N390E, S400R;
- A378V;
- D249R, R255L;
- V282K;
- E258M;
- V305A, T307P;
- E258K;
- K274Q, N276T, Y278F;
- K246N;

- V305E, T307P;
- N276T, Y278F;
- T250I;
- Y278F;
- T250I, K246N;
- K370L, T250V;
- K370L, V305A, T307P;
- K370L, K246N;
- K370L, K246N, T250I;
- K246N, T250I, V305A, T307P;
- T250V, V305A, T307P;
- T250I, V305A, T307P;
- K370L, T250V, V305A, T307P;
- K370L, T250V, K246N;
- K370L, T250I, V305A, T307P, S383N;
- T250V, V305A, T307P, K246N;
- K370L, T250V, V305A, T307P, K246N;
- K370L, T250V, V305A, T307P, K246N, K274Q;
- K370L, T250V, V305A, T307P, K246N, N276T;
- K370L, T250V, V305A, T307P, K246N, K274Q, N276T;
- K370L, T250I, V305A, T307P;
- K370L, N390R, S400D;
- T250V, N390R, S400D;
- K370L, T250V, K246N, N390R, S400D;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D;
- K370L, T250V, V305A, T307P, K246N, K274Q, N390R, S400D;
- K370L, T250V, V305A, T307P, K246N, K274Q, N276T, N390R, S400D;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R;

- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D, Q438K;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D, Q438R;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D, Q438K, Q347E;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D, Q438R, Q347E;
- Q418L;
- K360Q, G446C;
- K360Q, Q438R;
- K360Q, Q418L, Q438R;
- Q347E;
- K360Q;
- Q438R;
- K392R;
- S400F;
- K360Q, Q418L;
- K360E;
- Q438K;
- Q347E, Q418L;
- T437I;
- S424T;
- N421D; and
-S354P.

wherein the numbering is according to the Kabat numbering scheme.

Specifically the Fc according to the invention comprises at least one of the following combinations of point mutations

- V305A and T250V;
- V305A and T250V, and further K274Q and/or N276T;
- K360Q and G446C;

- K360Q and Q438R;
- K360Q and Q418L;
- K360Q, Q418L and Q438R;
- K370L and T250V;
- K370L and T307P;
- K370L and T307P, and further K246N and/or S400D and/or N390R,

wherein the numbering is according to the Kabat numbering scheme.

The Fc according to the invention preferably comprises an antigen binding site in the structural loop region.

The Fc according to the invention preferably is provided as a single Fc or antigen-binding Fc ("Fcab") or as part of an antibody or Fc construct, such as a full-length antibody or combinations with at least one constant domain and/ or variable domain of an antibody or other binding moieties, such as a receptor. Any kind of binding moiety may be combined with the Fc according to the invention to provide a ligand binding Fc construct.

A preferred Fc according to the invention comprises a constant domain contributing to the antigen-binding function of the modular antibody, such as a constant domain which forms at least part of an antigen binding site.

Specifically preferred is an Fc, which has a homodimeric structure, e.g. a CH2-CH3 homodimer of polypeptide chains, optionally linked by a hinge region or linker peptide.

According to a further aspect there is provided an Fc library comprising a repertoire of stability engineered Fc mutants according to the invention.

The Fc according to the invention is preferably used to provide for a novel scaffold for producing a library of variants, such as antigen-binding variants having different binding properties. The preferred Fc library according to the invention thus comprises Fc variants having different antigen binding properties.

The preferred library of Fc variants is mutagenised to obtain a randomised amino acid sequence within a loop region. A preferred method according to the invention provides for mutating a constant domain, which contributes to antigen-binding, such as a constant domain which forms at least part of an antigen binding site.

Preferably at least 35% of the Fc variants of such library have a functional scaffold structure as determined by binding to an effector molecule, like CD16a, CD64 and FcRn.

According to a specific aspect a thermostabilised or otherwise stabilised mutant of a human IgG Fc is used to engineer an Fc or an Fc library according to the invention. It has proven that at least one of the three observed thermal transitions (T_m1, T_m2, T_m3) is improved with the Fc according to the invention, as compared to the value obtained with wildtype Fc sequences, irrespective of the existence of an antigen-binding site in the structural loop region or not.

In addition to the stabilising mutations of the invention further engineering of the Fc is feasible, e.g. by artificial disulfide bridges. Therefore, intrachain or interchain disulfide bridges may be engineered by mutating a constant domain of the Fc according to the invention to introduce new Cys residues or any other thiol forming amino acid or amino acid analogue to form artificial disulfide bridges.

The invention further provides for a method of engineering a constant CH2 and/or CH3 domain by at least one of the point mutations of Table 1 or a combination of such point mutations to increase thermostability of a multidomain modular antibody, specifically when compared to the multidomain modular antibody without such point mutations.

The invention further provides for a method of engineering a constant CH2 and/or CH3 domain by at least one of the point mutations of Table 1 or a combination of such point mutations to improve antigen-binding of a multidomain modular antibody, specifically when compared to the multidomain modular antibody without such point mutations.

Specifically the invention further provides for a method of stability engineering in the framework of a constant domain to increase thermostability and for improving antigen-binding properties of a multidomain modular antibody, which comprises the Fc according to the invention and is composed of at least four antibody domains.

Figures

Figure 1 shows the sequence of human Fc including the hinge region, numbered by Kabat EU (SEQ ID No. 1). Positions of mutation as listed in Tables 1 and 2 are marked bold and underlined)

Figure 2 shows the sequence of the CH2 (SEQ ID No. 2) and CH3 (SEQ ID No. 3) domains of human Fc, numbered according to the IMGT. Positions of mutation as listed in Tables 1 and 2 are marked bold and underlined)

Detailed Description of the Invention

Definitions

Specific terms as used throughout the specification have the following meaning.

The term “antigen” or “target” as used according to the present invention shall in particular include all antigens and target molecules capable of being recognised by a binding site of an antibody. Specifically preferred antigens as targeted by the Fc according to the invention are those antigens or molecules, which have already been proven to be or are capable of being immunologically or therapeutically relevant, especially those, for which a clinical efficacy has been tested.

The term specifically comprises molecules selected from the group consisting of allergens, tumor associated antigens, self antigens including cell surface receptors, enzymes, Fc-receptors, FcRn, HSA, IgG, interleukins or cytokines, proteins of the complement system, transport proteins, serum molecules, bacterial antigens, fungal antigens, protozoan antigen and viral antigens, also molecules responsible for transmissible spongiform encephalitis (TSE), such as prions, infective or not, and markers or molecules that relate to inflammatory conditions, such as pro-inflammatory factors, multiple sclerosis or Alzheimer’s disease, or else haptens.

The antigen is either recognized as a whole target molecule or as a fragment of such molecule, especially substructures of targets, generally referred to as epitopes (e.g. B-cell epitopes, T-cell epitopes). Epitopes are understood to be immunologically relevant, i.e. are recognisable by natural or monoclonal antibodies. Therefore, the term “epitope” as used herein according to the present invention shall mean a molecular structure which may completely make up a specific binding partner or be part of a specific binding partner to a binding site of an antibody. The term epitope may also refer to haptens. Chemically, an epitope may either be composed of a carbohydrate, a peptide, a fatty acid, an organic, biochemical or inorganic substance or derivatives thereof and any combinations thereof. If an epitope is a polypeptide, it will usually include at least 3 amino acids, preferably 8 to 50 amino acids, and more preferably between about 10 to 20 amino acids in the peptide. There is no critical upper limit to the length of the peptide, which could comprise nearly the full length of a polypeptide

sequence of a protein. Epitopes can be either linear or conformational epitopes. A linear epitope is comprised of a single segment of a primary sequence of a polypeptide chain. Linear epitopes can be contiguous or overlapping. Conformational epitopes are comprised of amino acids brought together by folding of the polypeptide to form a tertiary structure and the amino acids of the epitope are not necessarily adjacent to one another in the linear sequence. Specifically, epitopes are at least part of diagnostically relevant molecules, i.e. the absence or presence of an epitope in a sample is qualitatively or quantitatively correlated to either a disease or to the health status of a patient or to a process status in manufacturing or to environmental and food status. Epitopes may also be at least part of therapeutically relevant molecules, i.e. molecules which can be targeted by the specific binding domain which changes the course of the disease.

The term “effector molecule” as used for the purpose of the invention shall mean a ligand mediating effector functions. Exemplary effector ligands are Fc receptors or Fc receptor-like molecules interfering with immunoglobulins. An Fc receptor is a protein found on the surface of certain cells - including natural killer cells, macrophages, neutrophils, and mast cells - that contribute to the protective functions of the immune system. There are several different types of Fc receptors, which are classified based on the type of antibody that they recognize; those that bind the most common class of antibody, IgG, are called Fc-gamma receptors (FcγR). Equivalent to an effector ligand and thus incorporated into the definition is any surrogate ligand that recognizes the same or similar binding site within the Fc, such as Protein A.

The family of FcγRs includes several members: FcγRI (CD64), FcγRIIA (CD32a), FcγRIIB (CD32b), FcγRIIIA (CD16a), FcγRIIIB (CD16b). Another Fc receptor, the neonatal Fc receptor (FcRn) also binds IgG and is involved in preservation and half-life of this antibody. Among the effector molecules there are also complement proteins, such as C1q. Optionally the binding to Protein A serves as a surrogate of an effector molecule, Protein A is thus included in the term “effector molecule”.

The term "expression system" refers to nucleic acid molecules containing a desired coding sequence and control sequences in operable linkage, so that hosts transformed or transfected with these sequences are capable of producing the encoded proteins. In order to effect transformation, the expression system may be

included on a vector; however, the relevant DNA may then also be integrated into the host chromosome. Alternatively, an expression system can be used for in vitro transcription/translation.

The term “Fc” as used according to the invention refers to the tail region of an antibody that interacts with cell surface receptors called Fc receptors and some proteins of the complement system. This property allows antibodies to activate the immune system. In IgG the Fc region is composed of two identical protein fragments, derived from the second (CH2) and third (CH3) constant domains of the antibody's two heavy chains. The Fc may be glycosylated or not, depending on the expression system. The term shall include Fc mutants or Fc variants, specifically variants with different antigen- or receptor-binding properties, e.g. antigen binding Fc (Fcab) and Fc constructs. Specifically, the term Fc shall also include full-length antibodies. The term is understood to include functionally active variants or functional homologues.

The term “Fc construct” as used herein shall mean any molecule comprising an Fc, in particular a full-length antibody or a modular antibody comprising the Fc and other peptides, polypeptides or protein domains, such as a receptor or antigen-binding domain, e.g. an immunoglobulin variable domain, or any other binding moiety.

The term “foreign” in the context of amino acids shall mean a newly introduced amino acid in an amino acid sequence, which is usually naturally occurring, but foreign to the site of modification, e.g. by insertion or a substitute of a naturally occurring amino acid. The stabilising point mutations according to the inventiontypically introduce foreign amino acids.

The term “framework” or “framework region” shall refer to those conserved regions of an antibody or antibody domain that are located outside the CDR loop region of an antibody domain including the structural loop regions. The framework region usually comprises or consists of a beta-sheet region of an immunoglobulin domain. Typically, the mutations as provided according to the invention are in a framework region, where they do not sterically hinder any antigen-binding site. Thus, it is understood that the framework region of an Fc according to the invention typically is aside from antigen-binding sequences.

The term “immunoglobulin” as used according to the present invention is defined as polypeptides or proteins that may exhibit mono- or bi- or multi-specific, or mono-, bi- or multivalent binding properties, preferably at least two, more preferred at

least three specific binding sites for epitopes of e.g. antigens, effector molecules or proteins either of pathogen origin or of human structure, like self-antigens including cell-associated or serum proteins. The term immunoglobulin as used according to the invention also includes functional fragments of an antibody, such as Fc, Fab, scFv, single chains of pairs of immunoglobulin domains, like single chain dimers of CH1/CL domains, Fv, or dimers such as VH/VL, CH1/CL, CH2/CH2, CH3/CH3, or other derivatives or combinations of the immunoglobulins. The definition further includes domains of the heavy and light chains of the variable region (such as dAb, Fd, Vl, Vk, Vh, VHH) and the constant region or individual domains of an intact antibody such as CH1, CH2, CH3, CH4, Cl and Ck, as well as mini-domains consisting of at least two beta-strands of an immunoglobulin domain connected by a structural loop, or recombined antibody domains, such as strand-exchange engineered domains (SEEDbodies), like those interdigitating beta-strand segments of human IgG and IgA CH3 domains. The term is understood to include functionally active variants or functional homologues.

"Modular antibodies" as used according to the invention are defined as antigen-binding molecules, like antibodies, composed of at least one polypeptide module or protein domain, preferably in the natural form. The term "modular antibodies" includes antigen-binding molecules that are either immunoglobulins, fragments thereof or constructs comprising immunoglobulins and other domains exhibiting modular formats and antigen-binding properties similar to immunoglobulins or antibodies, which can be used as antigen-binding scaffolds, preferably based on human proteins.

The term "multidomain modular antibody" as used according to the invention refers to a modular antibody comprising at least two modular antibodies and domains, respectively, preferably at least four antibody domains, e.g. the four domains of an Fc molecule.

It is understood that the terms "modular antibody", "immunoglobulin", "Fc" include a derivative thereof as well. A derivative is any variant or combination with one or more peptides, polypeptides or protein domains, such as antibody domains and/ or a fusion protein in which any domain or minidomain of the Fc of the invention may be bound or fused at any position with one or more other proteins (such as other modular antibodies, immunoglobulins, ligands, scaffold proteins, enzymes, toxins and the like). A derivative of the Fc of the invention may also be obtained by association or binding

to other substances by various chemical techniques such as covalent coupling, electrostatic interaction, disulphide bonding etc. The other substances bound to the Fc may be lipids, carbohydrates, nucleic acids, organic and inorganic molecules or any combination thereof (e.g. PEG, prodrugs or drugs). A derivative would also comprise an Fc with the homologous amino acid sequence, which may contain non-natural or chemically modified amino acids. Further derivatives are provided as fragments of the molecules, containing at least the Fc part of an antibody.

"Percent (%) amino acid sequence identity" with respect to the polypeptide sequences identified herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the specific polypeptide sequence, after aligning the sequence and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

The term “point mutations” as used herein shall refer to a single base substitution, wherein a single base nucleotide is replaced with another nucleotide of the genetic material, DNA or RNA.

The term “randomisation” or “randomised” sequence as used herein refers to specific modifications of the nucleic acid or amino acid sequences at predetermined positions, which result from random insertion or exchange or deletion of amino acids, or nucleid acid encoding the exchanged amino acids. Therefore a selection of amino acids or the whole range of natural or synthetic amino acids may be used. The preferred mutagenesis refers to such randomisation techniques, where the amino acid sequence of a peptide or polypeptide is mutated in at least one position, thus a randomised sequence is obtained.

“Scaffold” shall mean a temporary framework either natural or artificial used to support the molecular structure of a polypeptide in the construction of variants or a repertoire of the polypeptide. It is usually a modular system of polypeptide domains that maintains the tertiary structure or the function of the parent molecule. Exemplary scaffolds are fragments of modular antibodies, such as Fc, which may be mutagenized to produce variants within said scaffold, to obtain a library. The functional structure of a

scaffold is referred to as the “functional scaffold structure”, which can be proven by binding to an effector molecule, e.g. an Fc receptor to an Fc molecule, which is also called scaffold ligand for the purpose of determining the functional scaffold structure.

As used herein, the term "specifically binds" or “specific binding” refers to a binding reaction which is determinative of the cognate ligand of interest in a heterogeneous population of molecules. Thus, under designated conditions (e.g. immunoassay conditions), the antigen-binding Fc according to the invention binds to its particular target and does not bind in a significant amount to other molecules present in a sample. The specific binding means that binding is selective in terms of target identity, high, medium or low binding affinity or avidity, as selected. Selective binding is usually achieved if the binding constant or binding dynamics is at least 10 fold different, preferably the difference is at least 100 fold, and more preferred a least 1000 fold.

A “structural loop” or “non-CDR-loop” according to the present invention is to be understood in the following manner: modular antibodies, immunoglobulins or Fc are made of domains with a so called immunoglobulin fold. In essence, antiparallel beta sheets are connected by loops to form a compressed antiparallel beta barrel. Loop regions of constant domains or loop regions of variable domains that are apart from the CDR loop region, i.e. non-CDR loops, are called structural loops. In the variable region, some of the loops of the domains contribute essentially to the specificity of the antibody, i.e. the binding to an antigen by the natural binding site of an antibody. These loops are called CDR-loops. The CDR loops are located within the CDR loop region, which may in some cases also include the variable framework region (called “VFR”) adjacent to the CDR loops. It is known that VFRs may contribute to the antigen binding pocket of an antibody, which generally is mainly determined by the CDR loops. Thus, those VFRs are considered as part of the CDR loop region, and would not be appropriately used for engineering new additional antigen binding sites. Contrary to those VFRs within the CDR loop region or located proximal to the CDR loops, other VFRs of variable domains would be particularly suitable for engineering an additional antigen binding site. Those are the structural loops of the VFRs located opposite to the CDR loop region, or at the C-terminal side of a variable immunoglobulin domain. Other structural loops are located at the N-terminal or C-terminal side of a constant immunoglobulin domain.

The term “variable binding region” sometimes called “CDR region” as used herein refers to varying structures capable of binding interactions with antigens. Molecules with a variable binding region can be used as such or integrated within a larger protein, thus forming a specific region of such protein with binding function. The varying structures can be derived from natural repertoires of binding proteins such as immunoglobulins or receptors. The varying structures can as well be produced by randomisation techniques, in particular those described herein. These include mutagenized CDR or non-CDR regions, loop regions of immunoglobulin variable domains or constant domains.

Fc molecules with different modifications at specific sites are also referred to as “variants”. Variants of a scaffold, specifically Fc variants, are preferably grouped to form libraries of binding agents, which can be used for selecting members of the library with predetermined functions. In accordance therewith, a loop region of a binding agent comprising positions within one or more loops potentially contributing to a binding site, is preferably mutated or modified to produce libraries, preferably by randomisation, such as random, semi-random or, in particular, by site-directed random mutagenesis methods, in particular to delete, exchange or introduce randomly generated inserts into loops, preferably into structural loops. Alternatively preferred is the use of combinatorial approaches. Any of the known mutagenesis methods may be employed, among them cassette mutagenesis. These methods may be used to make amino acid modifications at desired positions of the Fc of the present invention. In some cases positions are chosen randomly, e.g. with either any of the possible amino acids or a selection of preferred amino acids to randomise loop sequences, or amino acid changes are made using simplistic rules. For example all residues may be mutated preferably to specific amino acids, such as alanine, referred to as amino acid or alanine scanning. Such methods may be coupled with more sophisticated engineering approaches that employ selection methods to screen higher levels of sequence diversity. The term “variant” specifically shall refer to functionally active variants.

The term "functionally equivalent variant" or “functionally active variant” of a molecule, such as the Fc as used herein, means a sequence resulting from modification of this sequence by insertion, deletion or substitution of one or more amino acids or nucleotides within the sequence or at either or both of the distal ends of

the sequence, and which modification does not affect (in particular impair) the activity of this sequence. In the case of a binding site having specificity to a selected target antigen, the functionally active variant of a molecule would still have the predetermined binding specificity, though this could be changed, e.g. to change the fine specificity to a specific epitope, the affinity, the avidity, the Kon or Koff rate, etc. In a preferred embodiment the functionally active variant a) is a biologically active fragment of the molecule, the functionally active fragment comprising at least 50% of the sequence of the molecule, preferably at least 70%, more preferably at least 80%, still more preferably at least 90%, even more preferably at least 95% and most preferably at least 97%, 98% or 99%; b) is derived from the molecule by at least one amino acid substitution, addition and/or deletion, wherein the functionally active variant has a sequence identity to the molecule or part of it, such as a Fc domain, of at least 50%, preferably at least 60%, more preferably at least 70%, more preferably at least 80%, still more preferably at least 90%, even more preferably at least 95% and most preferably at least 97%, 98% or 99%; and/or c) consists of the molecule or a functionally active variant thereof and additionally at least one amino acid or nucleotide heterologous to the polypeptide or the nucleotide sequence, preferably wherein the functionally active Fc variant is derived from or identical to any of the naturally occurring variants of human IgG Fc (SEQ ID No. 1):

tcppcpapellggpsvflfppkpkdtlmisrtpevtcvvvdvshedpevkfnwyvdgvevhnaktkpreeqynstyrvv
svltvlhqdwlngkeykckvsnkalpapiektiskakgqprepqvytlppsrdeltknqvsltclvkgfypsdiavewesn
gqpennykttppvldsdgsfflyskltvdksrwqqgnvfscsvmhealhnhytqkslslspgk

Functionally active variants may be obtained by changing the sequence as defined above and are characterized by having a biological activity similar to that displayed by the respective sequence, including the ability to bind an antigen and/or Fc effector molecules, or at least maintaining the favourable thermostability properties.

The functionally active variant may be obtained by sequence alterations in the polypeptide or the nucleotide sequence, wherein the sequence alterations retains a function of the unaltered polypeptide or the nucleotide sequence, when used in combination of the invention. Such sequence alterations can include, but are not limited to, (conservative) substitutions, additions, deletions, mutations and insertions.

The variant of the polypeptide or the nucleotide sequence is functionally active in the context of the present invention, if the activity of the composition of the invention including the variant (but not the original) amounts to at least 10%, preferably at least 25%, more preferably at least 50%, even more preferably at least 70%, still more preferably at least 80%, especially at least 90%, particularly at least 95%, most preferably at least 99% of the activity of the Fc of the invention including the polypeptide or the nucleotide sequence without sequence alteration (i.e. the original polypeptide or the nucleotide sequence).

In one preferred embodiment of the invention, the functionally active variant of the Fc according to the invention is essentially identical to the polypeptide or the nucleotide sequence described above, but differs from the polypeptide or the nucleotide sequence, respectively, in that it is derived from a homologous sequence of a different species. These are referred to as naturally occurring variants.

Therefore, the term "functionally active variant" also includes naturally occurring allelic variants, as well as mutants or any other non-naturally occurring variants. As is known in the art, an allelic variant is an alternate form of a (poly)peptide that is characterized as having a substitution, deletion, or addition of one or more amino acids that does essentially not alter the biological function of the polypeptide.

In a preferred embodiment, the functionally active variant derived from an Fc molecule according to the invention by amino acid exchanges, deletions or insertions may also conserve, or more preferably improve, the activity or structural stability.

Conservative substitutions are those that take place within a family of amino acids that are related in their side chains and chemical properties. Examples of such families are amino acids with basic side chains, with acidic side chains, with non-polar aliphatic side chains, with non-polar aromatic side chains, with uncharged polar side chains, with small side chains, with large side chains etc.

In another embodiment of the invention the polypeptide or the nucleotide sequence as defined above may be modified by a variety of chemical techniques to produce derivatives having essentially the same activity (as defined above for fragments and variants) as the modified modular antibody, and optionally having other desirable properties.

All numbering of the amino acid sequences of the Fc according to the invention is according to the Kabat EU index. The IMGT numbering scheme is provided where

indicated (IMGT, the international ImMunoGeneTics, Lefranc et al., 1999, Nucleic Acids Res. 27: 209-212).

Sites of introducing one or more appropriate point mutations according to the invention, herein called stability mutations and stabilising mutants, respectively, are shown in Table 1.

Table 1: Stability mutations of human IgG Fc

	IMGT numbering
K246N	10
D249R	13
E258K	20
E258M	20
Y278F	42
V282K	45.1
V305A	89
T307P	91
Q347E	3
Q347M	3
Y349F	5
L351M	7
S354P	10
K360E	16
K360N	16
K360Q	16
L368M	24
V369I	25
K370L	26
A378V	36
S383N	43
N390E	77
N390R	77
K392R	79
V397I	84
L398M	84.1
S400D	84.3
S400F	84.3
S400R	84.3
Q418L	97
N421D	100
S424T	103
T437I	117
Q438R	118
Q438K	118
G446C	129

In addition, it is preferred to combine at least one of the point mutations of Table 1 with each other or with at least one of the point mutations of Table 2, herein called combinatory mutations, to even improve the thermostability.

Table 2: Combinatory mutations of human IgG Fc

	IMGT numbering
T250I	14
T250V	14
R255L	17
E258R	20
H268Q	30
K274Q	36
N276T	40
V305A	89
V305E	89
S324T	107
S324Y	107
A339P	124
A378L	36
A378M	36
A378Y	36
E382Q	42

Though the numbering in column 1 of Tables 1 and 2 refers to a human IgG1 Fc molecule, the analogous positions of other antibody domains, e.g. of different antibody class or different origin, like a mammalian species other than human, or a mutant or variant antibody domain, may be chosen (using the IMGT numbering scheme which is given in column 2 of Tables 1 and 2) for the purpose of stability engineering an Fc molecule.

According to a specific embodiment the Fc according to the invention is an immunoglobulin of human origin, and may be employed for various purposes, in particular in pharmaceutical compositions. Of course, the Fc according to the invention may also be part of a humanized or chimeric immunoglobulin.

A preferred Fc according to the invention, which is a human Fc, is derived from immunoglobulin IgG type, specifically preferred IgG1, IgG2, IgG3 and IgG4 type immunoglobulins or derivatives thereof.

Preferably the Fc according to the invention is glycosylated. More preferably the glycosylation is a mammalian or plant glycosylation, such as a human, yeast or moss glycosylation, including humanised glycosylation, such as humanised yeast or humanised plant glycosylation.

Therefore, the invention relates to selected point mutations, which have in common the surprisingly increased thermostability of an Fc molecule. It was surprising to find out that a single amino acid substitution would result in a thermostabilised Fc and in the increase of the thermostability as compared to a wildtype Fc. This allows the maintenance of other Fc properties to the best extent, and in particular to use the Fc as a scaffold to provide respective variants, e.g. through mutagenesis within the structural loop region of the Fc.

The point mutations of the present invention are preferably introduced aside from an antigen or receptor binding site of the Fc. Thus, the biological activity or any antigen-binding property would not be hindered by such stability engineering.

An exemplary Fc is characterised in that said at least one framework region comprises at least one of the stabilising mutations besides an antigen-binding region, such as the loop region, which may be part of or comprise a binding site. Preferably the framework is mutated to incorporate at least one of the stabilising mutations in such a way, that a binding site could be engineered within the loop region or, if already present, the binding site, represented e.g. by a receptor, peptide or other binding moiety, a CDR loop region or a structural region, would be essentially maintained, e.g. with a loss of affinity (Kd) in binding an antigen, which is not more than 10^{-2}M, preferably not more than 10^{-1}M.

Stability engineering is thus preferred at a position within the framework region of said domain, in particular the beta-sheet region or structural loop regions, which are not used to create an antigen binding site, including loops on the opposite side of the domain as to where the binding site is engineered, such as selected from the group consisting of following amino acid positions:

Sheet A: 1-15.1

Sheet B: 16-26

Sheet C: 39-45.1

Sheet D: 77-84

Sheet E: 85.1-96

Sheet F: 96.2-104

Sheet G: 118-129

The stabilising mutations of the present invention may be engineered within an antibody domain, e.g. to stabilise the beta-sheet structure, or witin a chain of domains to stabilise the chain or Fc dimer structure (intrachain or interchain stabilisation), to constrain the structure of the Fc according to the invention and support its interaction with potential binding partners.

The stability mutations turned out to follow the concept of an optimised sequence for the individual Fc domains, deviating from the wildtype sequence. However, the design of the optimised structure could not easily be derived from rational design models. It turned out that many point mutations would not contribute to the increase of thermostabilty as a single mutation. In contrast, each of the stabilising mutations of Table 1 surprisingly turned out to individually result in the thermo-stabilisation, thus, was qualified as stabilising mutation.

Basis for the selection of point mutations was mainly supported by the method of *in silico* prediction based on homology modelling and energy calculations as descreibed by Schymkowitz, J. W. et al. (Proc Natl Acad Sci U S A 102, 10147-52 (2005)) and Kiel, C. et al. (J Mol Biol 348, 759-75 (2005)). Here it was essential to optimize the crystal structure of the Fc, including the CH2—CH3 chains, identifying those residues that have bad torsion angles, van der Waals clashes, or total energies and belong to the complex interface. First a selected position was mutated to alanine and the side-chain energies of the neighbour residues were annotated. Then the alanine was mutated to the selected amino acid and the side-chain energies of the same neighbour residues re-calculated. Those that exhibit an energy difference were mutated to themselves to see if another rotamer will be more favourable. According to an additional function all side-chains were slightly moved in order to eliminate small steric clashes. The advantage of this was that it quickly eliminates small local clashes and gains time by decreasing the number of real rotamer searches. From this, a new structure with the lowest energy was selected for the protein design.

To select the residues to be mutated for stabilisation a strategy of multiple sequence comparison of the target CH2-CH3 sequence was further used. All residues that are not conserved in the alignment were selected for mutagenesis to the variants found in the other genomes. A further optimization scan was employed to select for

residues that would stabilise the Fc molecule. In contrast to designing single domains using prior art techniques, only a few point mutations were found to increase the thermostability of the Fc molecule.

For example, i*n silico*-guided mutagenesis of the Fc fragment was applied with the aim to generate a more robust scaffold for further loop engineering applications. Single mutations and combinations of mutations were introduced by site-directed mutagenesis. The Fc comprising the selected point mutations was prepared by standard molecular biology methods and expressed in a suitable expression system, e.g. in a *Pichia pastoris* expression system. Alternatively, expression in CHO cells or in HEK cells is preferred. Mutant proteins were purified from culture supernatants by standard techniques, e.g. using Protein A affinity chromatography.

Biophysical analysis included differential scanning calorimetry (DSC), circular dichroism spectrometry (CD) and the verification of the presence of wildtype like effector functions, e.g. by binding to Protein A, CD16a, and FcRn. The introduction of the single point mutations e.g. improved the thermal stability of the Fc fragment by 4.5°C. The combination of stabilising mutations even showed a synergistic or at least an additive effect on thermal stability. A shift in the Tm values of the CH2 domain by approx. 9°C and for the CH3 domain by approx. 6°C could be gained by the combination of specific point mutations. Moreover, the manipulations had no significant effects neither on the functional scaffold structure of the Fc fragment nor on the binding to generic ligands.

Stability of the Fc can be analysed by an array of spectroscopic techniques, such as electronic (UV-VIS) spectroscopy, electronic (ECD) and vibrational (VCD) circular dichroism spectroscopy, fluorescence spectroscopy and Fourier Transform Infrared (FTIR) spectroscopy, and by differential scanning calorimetry. These complementary methods cover a wide range of structural features in proteins, primarily secondary structure but provide in addition valuable information about tertiary structural elements including location and mobility of aromatic amino acids and disulfide bridges. These techniques are very sensitive to structural changes mediated by (i) mutagenesis, (ii) physical (e.g. temperature) and chemical (e.g. chaotropic agents and/or pH) impacts, and/or (iii) interaction of proteins with ligands/binder. The methods are used to study temperature dependent stability and denaturation as well as chemically induced unfolding by urea and guanidinium hydrochloride (GuHCl) and

provide important thermodynamic and mechanistic data about domain stability, the pathway of unfolding and the susceptibility of the mutant domains for aggregation.

The thermostability was determined by temperature mediated denaturation by DSC. The resulting data included the melting temperature, T_m, enthalpy, $\Delta H(T_m)$, and heat capacity increment, ΔC_p, and can be used to determine protein stability function, $\Delta G(T)$. The key assumption of these approaches is that the protein unfolding is (up to a certain point) a thermodynamically reversible (that is, an equilibrium) transition.

According to a preferred embodiment the stabilising mutation or a combination of such mutation is engineered into an Fc molecule to increase the thermostability value T_m1 and/or T_m2 and/or T_m3 by at least 0,5°C, preferably at least 1°C, more preferred at least 2°C, at least 3°C, at least 4°C, even more preferred at least 5°C as compared to wildtype Fc. The highest thermostability was obtained with those stabilising mutations that brought about the increase of each of T_m1, T_m2 and T_m3.

It was surprising that the Fc according to the invention could be prepared with a thermal stability of at least 77°C, preferably at least 78°C, more preferably at least 79°C, more preferably at least 80°C, more preferably at least 81°C, or at least 82°C, or at least 83°C, or at least 84°C, or at least 85°C, or at least 86°C, or at least 87°C, or at least 88°C, or at least 89°C, or at least 90°C, even more than 90°C, possibly up to 100°C. In an antigen binding Fc molecule, which usually would have a lower thermostability than the wildtype, a respective increase of thermostability can be shown by the stabilising mutations according to the invention.

The heat capacity (or specific heat) of any substance (usually designated Cp at constant pressure) reflects the ability of the substance to absorb heat energy without increase in temperature. At temperatures well below the onset of thermal unfolding, the Cp, excess heat capacity, simply reflects the difference in heat capacity between the protein and the solvent it has displaced. Since water has a high heat capacity compared to most organic substances, including proteins, the apparent Cp in this region will normally be negative. As the protein begins to unfold, the Cp increases as more heat energy is taken up in denaturing the protein, reaching a peak at approximately the mid-point temperature (Tm) of the process. The calorimetric enthalpy (ΔHcal) is the total integrated area under the thermogram peak which represents the total heat energy uptake by the sample undergoing the transition. This heat uptake is an absolute measure of the absolute enthalpy of the process. The van't

Hoff enthalpy (ΔHVH) is another estimate of the enthalpy of the transition, based on an assumed model for the process. Here the area under the Cp peak at any temperature, divided by the total area, is used as a measure of the fraction or extent of unfolding that has occurred at that temperature.

There are two ways in which the van't Hoff enthalpy might differ from the calorimetric enthalpy in protein unfolding. If the unfolding transition is not two-state, but involves one or more intermediate steps, then the transition will appear broader than anticipated, and the ΔHVH will be less than ΔHcal. Alternatively, if the protein unfolds cooperatively as a dimer, or higher oligomer, then the transition will be sharper than anticipated for the two-state transition of a monomer, and the ΔHVH will be correspondingly greater than ΔHcal.

The molecular interpretation of H (enthalpy, or heat content is the total energy (including pressure/volume work terms) taken up in raising the system to temperature T whilst keeping the pressure constant. This will include the energy associated with all the atomic and molecular motions - translation, rotation, vibration, etc. - together with energy taken up in changes in inter- and intra-molecular interactions ("bonds"). The absolute entropy (S) is described in terms of "molecular disorder" or the multiplicity of ways in which the molecules in a system can take up energy without increasing temperature. Chemical stability and thermodynamic equilibrium represent a balance between two opposing tendencies: firstly the natural trend for systems to move to lower energies (decrease H), and secondly the equally natural tendency at the molecular level for molecules to explore the multiplicity of states available (higher S) under the influence of disruptive thermal motions. This is represented by the Gibbs free energy change (ΔG) expression: $\Delta G = \Delta H - T.\Delta S$ which indicates how much work must be done to bring about the desired change.

Changes in protein thermostability arising from single- or multi-site mutations are reflected in the change in Gibbs free energy (DeltaDeltaG, ΔΔG) of thermal denaturation between the mutant and the wildtype protein .Destabilizing mutations are characterized by a $\Delta\Delta G<0$ and stabilizing mutations are characterized by $\Delta\Delta G>0$.

Oligomerization and aggregation of engineered domains can be followed by multi-angle light scattering (MALS) and attenuated total reflectance (ATR) FTIR spectroscopy. MALS allows assessment of molar masses and sizes of the domains in solution and studying of protein interactions in dependence of concentration and

physicochemical conditions, e.g. ionic strength. Thermal and chemical denaturation of antibody fragments can be irreversible due to aggregation of the heat-unfolded domains. If protein unfolding and aggregation are concomitant, the unfolding process is inherently irreversible. If aggregation occurs after completion of the unfolding transition, it does not necessarily preclude equilibrium thermodynamic analysis by the spectroscopic techniques described above. In any case it is important to discriminate between these possibilities in order to arrive at the right conclusions about stability of the respective engineered antibody domains. ATR-FTIR spectroscopy is a very useful tool to analyse the formation of highly scattering aggregates and to determine their secondary structure. It allows spectral recording of nearly any sample in contact with the surface of the internal reflection element, regardless of transparency. This approach allows the determination and differentiation between different forms of aggregates.

Routinely qualitative and quantitative analysis of recombinant antibody domain-glycan is performed by HPLC, MALDI and LC-ESI-MS.

The above mentioned spectroscopic techniques (electronic UV-VIS, ECD, fluorescence and FTIR) can be applied in real-time monitoring of ligand binding to engineered domains.

Based on the obtained results, mutant Fc molecules according to the invention were designed that contain at least one or combine two, three, or more of these mutations, and the resulting proteins underwent the same analysis and preferred combinatorial mutants were identified.

Once an Fc mutant was obtained which displays significantly enhanced thermal and chemical stability as compared to the wildtype Fc, it was tested whether the gain in stability is also seen in the context of mutations in structural loops, in other words in an Fcab molecule or in one or preferably several binders selected from an Fcab library. In addition, it was verified that properties of binding the scaffold ligands Protein A, and effector molecules, such as CD16a, CD64 and FcRn to Fc could be maintained. Thus, the correct folding and functional scaffold structure of the Fc was essentially maintained.

The preferred Fcab libraries will contain a high percentage of well-folded clones, thus increasing the efficiency of identifying high quality binders from the libraries, and finally providing better folded, more stable Fcabs. Such a library derived from a

thermostabilised basic scaffold can show a higher proportion of well-folded Fcabs as compared to libraries based on the wildtype Fc scaffold. The number (percentage) of positive clones can be used as a measure for the overall quality of the library members. Those Fc libraries are specifically preferred when at least 35% of the Fc variants have a functional scaffold structure, e.g. as determined through binding to an effector molecule, preferably at least 45%, further preferred at least 55%, at least 60%, at least 70%, at least 80%, at least 90%, even at least 95%. Thereby the chances of selecting well-folded target-specific Fcabs can be significantly increased.

A number of Fc variants with different antigen-binding properties can be selected from these stability-enhanced libraries, and resulting clones can be characterized individually for their binding properties as well as for their structural and stability properties as described above.

Based on the obtained results, structural and stability properties for a series of Fc stabilised scaffolds and a number of different binding clones isolated from libraries on the basis of these improved scaffolds were obtained, second generation libraries can then be constructed, aiming to find the best combination and synergy of the stabilising mutations. The analysis and characterization of these new libraries and of clones selected from them follow the same paths as described above.

As an alternative to rational design the stabilising point mutations could be identified by random mutagenesis and *in vitro* directed evolution techniques, by applying a high-throughput screening strategy which correlates sequence with structure and stability. Still the outcome of such screening is unforeseeable. It was therefore surprising that the single point mutations as described before were found to be responsible for the increase in thermostability.

The Fc according to the invention can be used as isolated Fc or as combination molecules, such as Fc constructs, e.g. through recombination, fusion or conjugation techniques with other peptides or polypeptides. Combinations with peptides, polypeptides and/ or antibody domains are preferably obtained by recombination techniques, but also by binding through adsorption, electrostatic interactions or the like, or else through conjugation or chemical binding with or without a linker. The preferred linker sequence is either a natural linker sequence or functionally suitable artificial sequence.

The peptides used for combination purposes are preferably at least 5 amino acids long, more preferably at least 10 or even at least 50 or 100 amino acids long, and preferably constitute at least partially the loop region of an antibody domain. The preferred binding characteristics relate to predefined epitope binding, affinity and avidity.

In general the Fc according to the invention may be used as a building block to molecularly combine other antibody domains or biologically active substances or molecules. It is preferred to molecularly combine at least one antibody binding to the specific partner via the variable or non-variable sequences, like structural loops, with at least one other binding molecule which can be an antibody, antibody fragment, a soluble receptor, a ligand or another antibody domain, or a binding moiety thereof. Other combinations refer to proteinaceous molecules, nucleic acids, lipids, organic molecules and carbohydrates.

The preferred format is an Fc, which is homodimeric, e.g. composed of two heavy chains, consisting of CH2-CH3 antibody domains, with or without a covalent bond or a hinge region.

In an Fc construct as provided according to the invention, the Fc is preferably combined with at least two further antibody domains, more preferred at least 3 or 4 domains, such as to obtain full length antibodies or modular antibodies containing at least the Fc molecule combined with at least one further constant and/or at least one variable domain. Formats based on the combination with at least one pair of modular antibody domains, such as four antibody domains, are particularly preferred.

A constant domain is an immunoglobulin fold unit of the constant part of an immunoglobulin molecule, also referred to as a domain of the constant region (e.g. CH1, CH2, CH3, CH4, Ckappa, Clambda).

A variable domain is an immunoglobulin fold unit of the variable part of an immunoglobulin, also referred to as a domain of the variable region (e.g. Vh, Vkappa, Vlambda, Vd).

The preferred size of the Fc according to the invention is at least 50kD. Fc constructs may have a higher molecular weight, e.g. up to 150kD or even higher, depending on the glycosylation or any additional conjugation of pharmacologically active substances, like toxins or peptides.

The Fc according to the invention preferably comprises a binding site to act as a binding agent or binding partner, either as antigen-binding Fc fragment (Fcab) through modifications of the amino acid sequence or as conjugates or fusions to receptors, peptides or other antigen-binding modules.

For the purposes of this invention, the term “binding agent” or “ligand” refers to a member of a binding pair, in particular binding polypeptides having the potential of serving as a binding domain for a binding partner. Examples of binding partners include pairs of binding agents with functional interactions, such as receptor binding to ligands, antibody binding to antigen or receptors, a drug binding to a target, and enzyme binding to a substrate.

Binding partners are agents that specifically bind to one another, usually through non-covalent interactions. Examples of binding partners include pairs of binding agents with functional interactions, such as receptor binding to ligands, antibody binding to antigen, a drug binding to a target, and enzyme binding to a substrate. Binding partners have found use in many therapeutic, diagnostic, analytical and industrial applications. Most prominent binding pairs are antibodies or immunoglobulins, fragments or derivatives thereof. In most cases the binding of such binding agents is required to mediate a biological effect or a function, a “functional interaction”.

The Fc according to the invention preferably comprises at least one antigen-binding site within the variable and/or the framework region of a variable and/or a constant domain, either formed by respective binding moiteties, such as CDR loops or by randomisation, e.g. within the structural loop region. Binding sites to one or more antigens may be presented by the CDR-region or any other binding structure, or be introduced into a structural loop region of an antibody domain, either of a variable or constant domain structure. Thus, the Fc according to the present invention optionally exerts one or more binding regions to antigens, including binding sites binding specifically to an epitope of an antigen and binding sites potentially mediating effector function. The antigens as used for testing the binding properties of the binding sites may be naturally occurring molecules or chemically synthesized molecules or recombinant molecules, either in solution or in suspension, e.g. located on or in particles such as solid phases, on or in cells or on viral surfaces. It is preferred that the binding of the Fc to an antigen is determined when the antigen is still adhered or

bound to molecules and structures in the natural context. Thereby it is possible to identify and obtain those modified modular antibodies that are best suitable for the purpose of diagnostic or therapeutic use.

The stabilised modular antibody according to the invention is particularly useful as a scaffold for mutagenesis to introduce new binding sites. Among the preferred mutagenesis methods available for the generation of large random mutant libraries, error prone PCR is the most frequently used, and numerous protocols and kits are available. The total random approach may be complemented by a semi-random approach, which has been described in the literature as “massive mutagenesis” (Saboulard D et al. Biotechniques. 2005 Sep; 39(3):363-8), and which in essence is site-directed mutagenesis using multiple mutagenic primers simultaneously.

A preferred method according to the invention refers to a randomly modified nucleic acid molecule coding for an immunoglobulin, immunoglobulin domain or a part thereof which comprises at least one nucleotide repeating unit within a structural loop coding region having the sequence 5'-NNS-3', 5’-NNN-3’, 5’- NNB-3’ or 5’- NNK-3’. In some embodiments the modified nucleic acid comprises nucleotide codons selected from the group of TMT, WMT, BMT, RMC, RMG, MRT, SRC, KMT, RST, YMT, MKC, RSA, RRC, NNK, NNN, NNS or any combination thereof (the coding is according to IUPAC).

The modification of the nucleic acid molecule may be performed by introducing synthetic oligonuleotides into a larger segment of nucleic acid or by de novo synthesis of a complete nucleic acid molecule. Synthesis of nucleic acid may be performed with tri-nucleotide building blocks which would reduce the number of nonsense sequence combinations if a subset of amino acids is to be encoded (e.g. Yanez et al. Nucleic Acids Res. (2004) 32:e158; Virnekas et al. Nucleic Acids Res. (1994) 22:5600-5607).

The randomly modified nucleic acid molecule may comprise the above identified repeating units, which code for all known naturally occurring amino acids or a subset thereof. Those libraries that contain modified sequences wherein a specific subset of amino acids are used for modification purposes are called “focused” libraries. The member of such libraries have an increased probability of an amino acid of such a subset at the modified position, which is at least two times higher than usual, preferably at least 3 times or even at least 4 times higher. Such libraries have also a limited or lower number of library members, so that the number of actual library

members reaches the number of theoretical library members. In some cases the number of library members of a focused library is not less than 10^3 times the theoretical number, preferably not less than 10^2 times, most preferably not less than 10 times.

It is preferred to modify at least one loop region of an Fc according to the invention, which results in a substitution, deletion and/or insertion of one or more nucleotides or amino acids, preferably a point mutation, or even the exchange of whole loops, more preferred the change of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15, up to 30 amino acids. Thereby the modified sequence comprises amino acids not included in the conserved regions of the loops, the newly introduced amino acids being naturally occurring, but foreign to the site of modification, or substitutes of naturally occurring amino acids.

However, the maximum number of amino acids inserted into a loop region of a binding agent preferably may not exceed the number of 30, preferably 25, more preferably 20 amino acids at a maximum. The substitution and the insertion of the amino acids occurs preferably randomly or semi-randomly using all possible amino acids or a selection of preferred amino acids for randomisation purposes, by methods known in the art and as disclosed in the present patent application.

The site of modification may be at a specific single loop or a loop region, in particular a structural loop or a structural loop region. A loop region usually is composed of at least two, preferably at least 3 or at least 4 loops that are adjacent to each other, and which may contribute to the binding of an antigen through forming an antigen binding site or antigen binding pocket. It is preferred that the one or more sites of modification are located within the area of 10 amino acids, more preferably within 20, 30, 40, 50, 60, 70, 80, 90 up to 100 amino acids, in particular within a structural region to form a surface or pocket where the antigen can sterically access the loop regions.

In this regard the preferred modifications are engineered in the loop regions of CH1, CH2, CH3 and CH4, in particular in the range of amino acids 7 to 21, amino acids 25 to 39, amino acids 41 to 81, amino acids 83 to 85, amino acids 89 to 103 and amino acids 106 to 117.

In another preferred embodiment a modification in the structural loop region comprising amino acids 92 to 98 is combined with a modification in the structural loop region comprising amino acids 8 to 20.

The above identified amino acid regions of the respective immunoglobulins comprise loop regions to be modified. Preferably, a modification in the structural loop region comprising amino acids 92 to 98 is combined with a modification in one or more of the other structural loops.

In a preferred embodiment a modification in the structural loop region comprising amino acids 92 to 98 is combined with a modification in the structural loop region comprising amino acids 41 to 45.2.

Most preferably each of the structural loops comprising amino acids 92 to 98, amino acids 41 to 45.2 and amino acids 8 to 20 contain at least one amino acid modification.

In another preferred embodiment each of the structural loops comprising amino acids 92 to 98, amino acids 41 to 45.2, and amino acids 8 to 20 contain at least one amino acid modification.

According to another preferred embodiment the amino acid residues in the area of positions 15 to 17, 29 to 34, 41 to 45.2, 84 to 85, 92 to 100, and/or 108 to 115 of CH3 are modified.

The preferred modifications of Igk-C and Igl-C of human origin are engineered in the loop regions in the area of amino acids 8 to 20, amino acids 26 to 36, amino acids 41 to 82, amino acids 83 to 88, amino acids 92 to 100, amino acids 107 to 124 and amino acids 123 to 126.

The preferred modifications of loop regions of Igk-C and Igl-C of murine origin are engineered at sites in the area of amino acids 8 to 20, amino acids 26 to 36, amino acids 43 to 79, amino acids 83 to 85, amino acids 90 to 101, amino acids 108 to 116 and amino acids 122 to 126.

According to a specific embodiment, an immunoglobulin comprising the Fc according to the invention may contain a modification within the variable domain, which is selected from the group of VH, Vkappa, Vlambda, VHH and combinations thereof. More specifically, they comprise at least one modification within amino acids 7 to 22, amino acids 39 to 55, amino acids 66 to 79, amino acids 77 to 89 or amino acids 89 to

104, where the numbering of the amino acid position of the domains is that of the IMGT.

In a specific embodiment, the immunoglobulin preferably used is characterised in that the loop regions of VH or Vkappa or Vlambda of human origin comprise at least one modification within amino acids 7 to 22, amino acids 43 to 51, amino acids 67 to 77, amino acids 77 to 88, and amino acids 89 to 104, most preferably amino acid positions 12 to 17, amino acid positions 45 to 50, amino acid positions 68 to 77, amino acids 79 to 88, and amino acid positions 92 to 99, where the numbering of the amino acid position of the domains is that of the IMGT.

The structural loop regions of the variable domain of the immunoglobulin of human origin selected for modification purposes are preferably located in the area of amino acids 8 to 20, amino acids 44 to 50, amino acids 67 to 76, amino acids 78 to 87, and amino acids 89 to 101.

According to a preferred embodiment the structural loop regions of the variable domain of the immunoglobulin of murine origin selected for modification purposes are preferably located in the area of amino acids 6 to 20, amino acids 43 to 52, amino acids 67 to 79, amino acids 79 to 87, and amino acids 91 to 100.

The Fc according to the invention is particularly useful as a stable scaffold for a library preparation. It is understood that the term “Fc library” always includes libraries of Fc or Fc constructs, fusion proteins, genetic packages or nucleic acids encoding such variants of an Fc or Fc construct, which are understood as members of a library.

The term “fusion protein” or “chimeric fusion protein” as used for the purpose of the invention shall mean the molecule composed of a genetic package, at least part of an outer surface structure, such as a coat protein, optionally a linker sequence, and a binding agent. The fusion protein is encoded by a vector with the gene of the binding agent and information to display a copy of the binding agent at the surface of the genetic package.

Another important aspect of the invention for producing Fc libraries is that each potential binding domain remains physically associated with the particular DNA or RNA molecule which encodes it, and in addition, a the fusion proteins oligomerize at the surface of a genetic package to present the binding polypeptide in the native and functional oligomeric structure. Once successful binding domains are identified, one may readily obtain the gene for expression, recombination or further engineering

purposes. The form that this association takes is a replicable genetic package, such as a virus, cell or spore which replicates and expresses the binding domain-encoding gene, and transports the binding domain to its outer surface. Another form is an in-vitro replicable genetic package such as ribosomes that link coding RNA with the translated protein. In ribosome display the genetic material is replicated by enzymatic amplification with polymerases.

Those cells or viruses or nucleic acid bearing the binding agents which recognize the target molecule are isolated and, if necessary, amplified. The preferred expression system for the fusion proteins is a non-suppressor host cell, which would be sensitive to a stop codon, such as an amber stop codon, and would thus stop translation thereafter. In the absence of such a stop codon such non-suppressor host cells, preferably E.coli, are preferably used. In the presence of such a stop codon supressor host cells would be used.

Preferably in the method of this invention the vector or plasmid of the genetic package is under tight control of the transcription regulatory element, and the culturing conditions are adjusted so that the amount or number of vector or phagemid particles displaying less than two copies of the fusion protein on the surface of the particle is less than about 20%. More preferably, the amount of vector or phagemid particles displaying less than two copies of the fusion protein is less than 10% the amount of particles displaying one or more copies of the fusion protein. Most preferably the amount is less than 1%.

The expression vector preferably used according to the invention is capable of expressing a binding polypeptide, and may be produced as follows: First a binding polypeptide gene library is synthesized by introducing a plurality of polynucleotides encoding different binding sequences. The plurality of polynucleotides may be synthesized in an appropriate amount to be joined in operable combination into a vector that can be propagated to express a fusion protein of said binding polypeptide. Alternatively the plurality of olynucleotides can also be amplified by polymerase chain reaction to obtain enough material for expression. However, this would only be advantageous if the binding polypeptide would be encoded by a large polynucleotide sequence, e.g. longer than 200 base pairs or sometimes longer than 300 base pairs. Thus, a diverse synthetic library is preferably formed, ready for selecting from said diverse library at least one expression vector capable of producing binding

polypeptides having the desired preselected function and binding property, such as specificity.

Various alternatives are available for the manufacture of genes encoding the randomised library. It is possible to produce the DNA by a completely synthetic approach, in which the sequence is divided into overlapping fragments which are subsequently prepared as synthetic oligonucleotides. These oligonucleotides are mixed together, and annealed to each other by first heating to ca. 100°C and then slowly cooling down to ambient temperature. After this annealing step, the synthetically assembled gene can be either cloned directly, or it can be amplified by PCR prior to cloning.

Alternatively, other methods for site directed mutagenesis can be employed for generation of the library insert, such as the Kunkel method (Kunkel TA. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc Natl Acad Sci U S A. 1985 Jan;82(2):488-92) or the DpnI method (Weiner MP, Costa GL, Schoettlin W, Cline J, Mathur E, Bauer JC. Site-directed mutagenesis of double-stranded DNA by the polymerase chain reaction. Gene. 1994 Dec 30; 151(1-2):119-23.).

For various purposes, it may be advantageous to introduce silent mutations into the sequence encoding the library insert. For example, restriction sites can be introduced which facilitate cloning or modular exchange of parts of the sequence. Another example for the introduction of silent mutations is the ability to “mark” libraries, that means to give them a specific codon at a selected position, allowing them (or selected clones derived from them) e.g. to be recognized during subsequent steps, in which for example different libraries with different characteristics can be mixed together and used as a mixture in the selection procedure.

As is well-known in the art, there is a variety of display and selection technologies that may be used for the identification and isolation of proteins with certain binding characteristics and affinities, including, for example, display technologies such as cellular and non-cellular methods, in particular mobilized display systems. Among the cellular systems the phage display, virus display, yeast or other eukaryotic cell display, such as mammalian or insect cell display, may be used. Mobilized systems are relating to display systems in the soluble form, such as in vitro display systems, among them ribosome display, mRNA display or nucleic acid display.

Preferably the library is a yeast library and the yeast host cell exhibits at the surface of the cell the Fc and Fc variants. Yeast display offers a number of attractive features: The eukaryotic transcription and translation machinery is very well suited for expression of the Fc, and the use of flow cytometry allows high-throughput quantitative analysis of individual clones in real-time, using scaffold ligands.

The yeast host cell is preferably selected from the genera Saccharomyces, Pichia, Hansenula, Schizisaccharomyces, Kluyveromyces, Yarrowia and Candida. Most preferred the host cell is Saccharomyces cerevisiae.

It is possible to use the engineered proteins to produce molecules which are monospecific, bispecific, trispecific, and may even carry more specificities. By the invention it is be possible to provide a stable framework of an Fc for a multispecific binding agent.

The preferred method of producing the Fc according to the invention refers to engineering an Fc that is binding specifically to at least one first epitope and comprising modifications in each of at least two structural loop regions, and determining the specific binding of said at least two loop regions to at least one second epitope, wherein the unmodified structural loop region (non-CDR region) does not specifically bind to said at least one second epitope. Thus, an Fc or antigen-binding structure specific for a first antigen may be improved by adding another valency or specificity against a second antigen, which specificity may be identical, either targeting different epitopes or the same epitope, to increase valency or to obtain bi-, oligo- or multispecific molecules.

The quality of the Fc library may be proven by the functional scaffold structure. The scaffold ligand can be selected from the group consisting of an effector molecule, FcRn, Protein A, Protein G, Protein L and CDR target. As an example, the effector molecule can be selected from the group consisting of CD64, CD32, CD16 and Fc receptors.

Libraries according to the invention preferably comprise at least 10^2 library members, more preferred at least 10^3, more preferred at least 10^4, more preferred at least 10^5, more preferred at least 10^6 library members, more preferred at least 10^7, more preferred at least 10^8, more preferred at least 10^9, more preferred at least 10^{10}, more preferred at least 10^{11}, up to 10^{12} members of a library.

According to the invention it is also provided a pool of preselected independent clones, which is e.g. affinity maturated, which pool comprises preferably at least 10, more preferably at least 10^2, more preferably at least 10^3, more preferably at least 10^4, even more than 10^5 independent clones. Those libraries, which contain the pre-selected pools, are preferred sources to select the high affinity antigen binding Fc according to the invention.

Preferred Fc according to the invention are binding said target antigen with a high affinity, in particular with a high on and/or a low off rate, or a high avidity of binding. Usually a binder is considered a high affinity binder with a Kd of less than10^{-9} M. Medium affinity binders with a Kd of less than 10^{-6} M up to 10^{-9} M may be provided according to the invention as well, preferably in conjunction with an affinity maturation process.

Affinity maturation is the process by which antibodies with increased affinity for antigen are produced. With structural changes of an antibody, including amino acid mutagenesis or as a consequence of somatic mutation in immunoglobulin gene segments, variants of a binding site to an antigen are produced and selected for greater affinities. Affinity matured Fc may exhibit a several logfold greater affinity than a parent antibody. Single parent antibodies may be subject to affinity maturation. Alternatively pools of Fc with similar binding affinity to the target antigen may be considered as parent structures that are varied to obtain affinity matured single antibodies or affinity matured pools of such antibodies.

The preferred affinity maturated variant of an Fc according to the invention exhibits at least a 10 fold increase in affinity of binding, preferably at least a 100 fold increase. The affinity maturation may be employed in the course of the selection campaigns employing respective libraries of parent molecules, either with Fc having medium binding affinity to obtain a preferred modular antibody of the invention having a high specific target binding property of a Kd<10^{-8} M and/or a potency of EC50<10^{-8} M. The binding potency or affinity may be even more increased by affinity maturation of the Fc according to the invention to obtain the high values corresponding to a Kd or EC50 of less than 10^{-9} M, preferably less than 10^{-10} M or even less than 10^{-11} M, most preferred in the picomolar range.

The EC50, sometimes called IC50, also called 50% saturation concentration, is a measure for the binding potency of a modular antibody. It is the molar concentration

of a binder, which produces 50% of the maximum possible binding at equilibrium or under saturation. The potency of an antagonist is usually defined by its IC50 value. This can be calculated for a given antagonist by determining the concentration of antagonist needed to elicit half saturation of the maximum binding of an agonist. Elucidating an IC50 value is useful for comparing the potency of antibodies or antibody variants with similar efficacies; however the dose-response curves produced by both drug antagonists must be similar. The lower the IC50, the greater the potency of the antagonist, and the lower the concentration of drug that is required to inhibit the maximum biological response, like effector function or cytotoxic activity. Lower concentrations of drugs may also be associated with fewer side effects.

Usually the affinity of an antibody correlates well with the IC50. The affinity of an antagonist for its binding site (K_i), is understood as its ability to bind to a receptor, which determines the duration of binding and respective agonist activity. Measures to increase the affinity by affinity maturation usually also increase the potency of binding, resulting in the respective reduction of IC50 values in the same range of the Kd values.

The IC50 and Kd values may be determined using the saturation binding assays well-known in the art.

It is preferred to employ those Fc molecules according to the invention that contain native structures interacting with effector molecules or immune cells, thus providing for ADCC, CDC or ADPC. Those native structures either remain unchanged or are preferably modulated for an increased effector function. Binding sites for e.g. Fc receptors are described to be located in a CH2 and/or CH3 domain region, and may be mutagenized by well-known techniques.

ADCC, antibody-dependent cell-mediated cytotoxicity is the killing of antibody-coated target cells by cells with Fc receptors that recognize the constant region of the bound antibody. Most ADCC is mediated by NK cells that have the Fc receptor FcgammaRIII or CD16 on their surface. Typical assays employ target cells, like Ramos cells, incubated with serially diluted antibody prior to the addition of freshly isolated effector cells. The ADCC assay is then further incubated for several hours and % cytotoxicity detected. Usually the Target: Effector ratio is about 1:16, but may be 1:1 up to 1:50.

Complement-dependent cytotoxicity (CDC) is a mechanism of killing cells in which antibody bound to the target cell surface fixes complement, which results in

assembly of the membrane attack complex that punches holes in the target cell membrane resulting in subsequent cell lysis. The commonly used CDC assay follows the same procedure as for ADCC determination, however, with complement containing serum instead of effector cells.

The Fc according to the invention preferably has a cytotoxic activity as determined by either of ADCC and CDC assay, preferably in a way to provide a significant increase in the percentage of cytolysis as compared to a control. The absolute percentage increase preferably is higher than 5%, more preferably higher than 10%, even more preferred higher than 20%.

The antibody-dependent cellular phagocytosis, ADCP sometimes called ADPC, is usually investigated side by side with cytolysis of cultured human cells. Phagocytosis by phagocytes, usually human monocytes or monocyte-derived macrophages, as mediated by an antibody can be determined as follows. Purified monocytes may be cultured with cytokines to enhance expression of FcγRs or to induce differentiation into macrophages. ADCP and ADCC assays are then performed with target cells. Phagocytosis is determined as the percentage of positive cells measured by flow cytometry. The positive ADCP activity is proven with a significant uptake of the antibody-antigen complex by the phagocytes. The absolute percentage preferably is higher than 5%, more preferably higher than 10%, even more preferred higher than 20%.

In a typical assay PBMC or monoycytes or monocyte derived macrophages are resuspended in RF2 medium (RPMI 1640 supplemented with 2% FCS) in 96-well plates at a concentration of 1 x 10^5 viable cells in 100 ml/well. Appropriate target cells, expressing the target antigen, e.g. Her2/neu antigen and SKBR3 cells, are stained with PKH2 green fluorescence dye. Subsequently 1 x 10^4 PKH2-labeled target cells and a test Fc or Fc construct or respective control are added to the well of PBMC's in different concentrations (e.g. 1-100 µg/ml) and incubated in a final volume of 200 ml at 37°C for 24 h. Following the incubation, PBMCs or monoycytes or monocyte derived macrophages and target cells are harvested with EDTA-PBS and transferred to 96-well V-bottomed plates. The plates are centrifuged and the supernatant is aspirated. Cells are counterstained with a 100-ml mixture of RPE-conjugated anti-CD11b, anti-CD14, and human IgG, mixed and incubated for 60 min on ice. The cells are washed and fixed with 2% formaldehyde-PBS. Two-color flow cytometric analysis is performed with e.g. a FACS Calibur under optimal gating. PKH2-labeled target cells (green) are

detected in the FL-1 channel (emission wavelength, 530 nm) and RPE-labeled PBMC or monoycytes or monocyte derived macrophages (red) are detected in the FL-2 channel (emission wavelength, 575 nm). Residual target cells are defined as cells that are $PKH2^+/RPE^-$ Dual-labeled cells ($PKH2^+/RPE^-$) are considered to represent phagocytosis of targets by PBMC or monoycytes or monocyte derived macrophages. Phagocytosis of target cells is calculated with the following equation: percent phagocytosis = 100 x [(percent dual positive)/(percent dual positive + percent residual targets)].

The Fc according to the invention may specifically bind to any kind of binding molecules or structures, in particular to antigens, proteinaceous molecules, proteins, peptides, polypeptides, nucleic acids, glycans, carbohydrates, lipids, organic molecules, in particular small organic molecules, anorganic molecules, or combinations or fusions thereof, including PEG, prodrugs or drugs. The preferred Fc according to the invention may comprise at least two loops or loop regions whereby each of the loops or loop regions may specifically bind to different molecules or epitopes.

According to a further preferred embodiment the target antigen is selected from those antigens presented by cells, e.g. cellular targets, like epithelial cells, cells of solid tumors, infected cells, blood cells, antigen-presenting cells and mononuclear cells.

Preferably a target antigen is selected from cell surface antigens, including receptors, in particular from the group consisting of erbB receptor tyrosine kinases (such as EGFR, HER2 including Her2neu, HER3 and HER4, in particular those epitopes of the extracellular domains of such receptors, e.g. the 4D5 epitope). In addition further antigens may be targeted, e.g. molecules of the TNF-receptor superfamily, such as Apo-1 receptor, TNFR1, TNFR2, nerve growth factor receptor NGFR, CD40, CD40-Ligand, OX40, TACI, BCMA, BAFF-receptor, T-cell surface molecules, T-cell receptors, T-cell antigen, Apo-3, DR4, DR5, DR6, decoy receptors, such as DcR1, DcR2, CAR1, HVEM, GITR, ZTNFR-5, NTR-1, TNFL1, IGFR-1, c-Met, but not limited to these molecules, B-cell surface antigens, such as CD10, CD19, CD20, CD21, CD22, DC-SIGN, antigens or markers of solid tumors or hematologic cancer cells, cells of lymphoma or leukaemia, other blood cells including blood platelets, but not limited to these molecules.

According to a further preferred embodiment a target antigen is selected from those antigens presented by cells, like epithelial cells or cells of solid tumors. Those

target antigens expressed or overexpressed by cells are preferably targeted, which are selected from the group consisting of tumor associated antigens, in particular EpCAM, tumor-associated glycoprotein-72 (TAG-72), tumor-associated antigen CA 125, Prostate specific membrane antigen (PSMA), High molecular weight melanoma-associated antigen (HMW-MAA), tumor-associated antigen expressing Lewis y related carbohydrate, Carcinoembryonic antigen (CEA), CEACAM5, HMFG PEM, mucin MUC1, MUC18 and cytokeratin tumor-associated antigen, CD44 and its splice variants, bacterial antigens, viral antigens, allergens, allergy related molecules IgE, cKIT and Fc-epsilon-receptor I, IRp60, IL-5 receptor, CCR3, red blood cell receptor (CR1), human serum albumin, mouse serum albumin, rat serum albumin, Fc receptors, like neonatal Fc-gamma-receptor FcRn, Fc-gamma-receptors Fc-gamma RI, Fc-gamma-RII, Fc-gamma RIII, Fc-alpha-receptors, Fc-epsilon-receptors, fluorescein, lysozyme, toll-like receptor 9, erythropoietin, CD2, CD3, CD3E, CD4, CD11, CD11a, CD14, CD16, CD18, CD19, CD20, CD22, CD23, CD25, CD28, CD29, CD30, CD32, CD33 (p67 protein), CD38, CD40, CD40L, CD52, CD54, CD56, CD64, CD80, CD147, GD3, IL-1, IL-1R, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-6R, IL-8, IL-12, IL-15, IL-17, IL-18, IL-23, LIF, OSM, interferon alpha, interferon beta, interferon gamma; TNF-alpha, TNFbeta2, TNFalpha, TNFalphabeta, TNF-R1, TNF-RII, FasL, CD27L, CD30L, 4-1BBL, TRAIL, RANKL, TWEAK, APRIL, BAFF, LIGHT, VEG1, OX40L, TRAIL Receptor-1, A1 Adenosine Receptor, Lymphotoxin Beta Receptor, TACI, BAFF-R, EPO; LFA-3, ICAM-1, ICAM-3, integrin beta1, integrin beta2, integrin alpha4/beta7, integrin alpha2, integrin alpha3, integrin alpha4, integrin alpha5, integrin alpha6, integrin alphav, alphaVbeta3 integrin, FGFR-3, Keratinocyte Growth Factor, GM-CSF, M-CSF, RANKL, VLA-1, VLA-4, L-selectin, anti-Id, E-selectin, HLA, HLA-DR, CTLA-4, T cell receptor, B7-1, B7-2, VNRintegrin, TGFbeta1, TGFbeta2, eotaxin1, BLyS (B-lymphocyte Stimulator), complement C5, IgE, IgA, IgD, IgM, IgG, factor VII, CBL, NCA 90, EGFR (ErbB-1), Her2/neu (ErbB-2), Her3 (ErbB-3), Her4 (ErbB4), Tissue Factor, VEGF, VEGFR, endothelin receptor, VLA-4, carbohydrates such as blood group antigens and related carbohydrates, Galili-Glycosylation, Gastrin, Gastrin receptors, tumor associated carbohydrates, Hapten NP-cap or NIP-cap, T cell receptor alpha/beta, E-selectin, P-glycoprotein, MRP3, MRP5, glutathione-S-transferase pi (multi drug resistance proteins), alpha-granule membrane protein(GMP) 140, digoxin, placental alkaline phosphatase (PLAP) and testicular PLAP-like alkaline phosphatase,

transferrin receptor, Heparanase I, human cardiac myosin, Glycoprotein IIb/IIIa (GPIIb/IIIa), human cytomegalovirus (HCMV) gH envelope glycoprotein, HIV gp120, HCMV, respiratory syncytial virus RSV F, RSVF Fgp, VNRintegrin, Hep B gp120, CMV, gpIIbIIIa, HIV IIIB gp120 V3 loop, respiratory syncytial virus (RSV) Fgp, Herpes simplex virus (HSV) gD glycoprotein, HSV gB glycoprotein, HCMV gB envelope glycoprotein, Clostridium perfringens toxin and fragments thereof.

The Fc according to the invention is preferably conjugated to a label or reporter molecule, selected from the group consisting of organic molecules, enzyme labels, radioactive labels, colored labels, fluorescent labels, chromogenic labels, luminescent labels, haptens, digoxigenin, biotin, metal complexes, metals, colloidal gold and mixtures thereof. Modified Fc conjugated to labels or reporter molecules may be used, for instance, in assay systems or diagnostic methods.

The Fc according to the invention may be conjugated to other molecules which allow the simple detection of said conjugate in, for instance, binding assays (e.g. ELISA) and binding studies.

Methods for production and screening of antibody or Fc variants are well-known in the art. General methods for antibody molecular biology, expression, purification, and screening are also well-known in the art. In a preferred embodiment, Fc variants are screened using one or more cell-based or in vivo assays. For such assays, purified or unpurified modified Fc are typically added exogenously such that cells are exposed to individual Fc or pools of Fc belonging to a library. These assays are typically, but not always, based on the function of the immunoglobulin; that is, the ability of the antibody to bind to its target and mediate some biochemical event, for example effector function, ligand/receptor binding inhibition, apoptosis, and the like. Such assays often involve monitoring the response of cells to the antibody, for example cell survival, cell death, change in cellular morphology, or transcriptional activation such as cellular expression of a natural gene or reporter gene. For example, such assays may measure the ability of Fc variants to elicit ADCC, ADCP, or CDC. For some assays additional cells or components, that is in addition to the target cells, may need to be added, for example example serum complement, or effector cells such as peripheral blood monocytes (PBMCs), NK cells, macrophages, and the like. Such additional cells may be from any organism, preferably humans, mice, rat, rabbit, and monkey. Fc molecules may cause apoptosis of certain cell lines expressing the target, or they may mediate attack on

target cells by immune cells which have been added to the assay. Methods for monitoring cell death or viability are known in the art, and include the use of dyes, immunochemical, cytochemical, and radioactive reagents. For example, caspase staining assays may enable apoptosis to be measured, and uptake or release of radioactive substrates or fluorescent dyes such as alamar blue may enable cell growth or activation to be monitored.

In a preferred embodiment, the DELFIART EuTDA-based cytotoxicity assay (Perkin Elmer, MA) may be used. Alternatively, dead or damaged target cells may be monitored by measuring the release of one or more natural intracellular components, for example lactate dehydrogenase.

Transcriptional activation may also serve as a method for assaying function in cell-based assays. In this case, response may be monitored by assaying for natural genes or immunoglobulins which may be upregulated, for example the release of certain interleukins may be measured, or alternatively readout may be via a reporter construct. Cell-based assays may also involve the measure of morphological changes of cells as a response to the presence of an Fc according to the invention. Cell types for such assays may be prokaryotic or eukaryotic, and a variety of cell lines that are known in the art may be employed. Alternatively, cell-based screens are performed using cells that have been transformed or transfected with nucleic acids encoding the variants. That is, Fc variants are not added exogenously to the cells. For example, in one embodiment, the cell-based screen utilizes cell surface display. A fusion partner can be employed that enables display of modified Fc on the surface of cells (Wittrup, 2001, Curr Opin Biotechnol, 12:395-399).

The Fc of the invention is preferably provided as a recombinant protein expressed by a host cell, e.g. by expression in the periplasmic space of E. coli or by expression as a secreted protein in a eukaryotic expression system such as yeast or mammalian, e.g. by CHO, HEK or human production host cell lines.

In a preferred embodiment, the immunogenicity of the Fc according to the invention may be determined experimentally using one or more cell-based assays. In a preferred embodiment, ex vivo T-cell activation assays are used to experimentally quantitate immunogenicity. In this method, antigen presenting cells and naive T cells from matched donors are challenged with an Fc of interest one or more times. Then, T cell activation can be detected using a number of methods, for example by

monitoring production of cytokines or measuring uptake of tritiated thymidine. In the most preferred embodiment, interferon gamma production is monitored using Elispot assays.

The biological properties of the Fc according to the invention may be characterized ex vivo in cell, tissue, and whole organism experiments. As is known in the art, drugs are often tested in vivo in animals, including but not limited to mice, rats, rabbits, dogs, cats, pigs, and monkeys, in order to measure a drug's efficacy for treatment against a disease or disease model, or to measure a drug's pharmacokinetics, pharmacodynamics, toxicity, and other properties. The animals may be referred to as disease models. Therapeutics are often tested in mice, including but not limited to nude mice, SCID mice, xenograft mice, and transgenic mice (including knockins and knockouts). Such experimentation may provide meaningful data for determination of the potential of the antibody to be used as a therapeutic with the appropriate half-life, effector function, apoptotic activity, cytotoxic or cytolytic activity. Any organism, preferably mammals, may be used for testing. For example because of their genetic similarity to humans, primates, monkeys can be suitable therapeutic models, and thus may be used to test the efficacy, toxicity, pharmacokinetics, pharmacodynamics, half-life, or other property of the modular antibody according to the invention. Tests of the substances in humans are ultimately required for approval as drugs, and thus of course these experiments are contemplated. Thus the Fc of the present invention may be tested in humans to determine their therapeutic efficacy, toxicity, immunogenicity, pharmacokinetics, and/or other clinical properties. Especially those Fc variants according to the invention that bind to single cell or a cellular complex through at least two binding motifs, preferably binding of at least three structures cross-linking target cells, would be considered effective in effector activity or preapoptotic or apoptotic activity upon cell targeting and cross-linking. Multivalent binding provides a relatively large association of binding partners, also called cross-linking, which is a prerequisite for apoptosis and cell death.

The Fc of the present invention may find use in a wide range of antibody products. In one embodiment the Fc of the present invention is used for therapy or prophylaxis, e.g. as an active or passive immunotherapy, for preparative, industrial or analytic use, as a diagnostic, an industrial compound or a research reagent, preferably a therapeutic. The Fc may specifically find use in an antibody composition that is

monoclonal or polyclonal. In a preferred embodiment, the Fc of the present invention is used to capture or kill target cells that bear the target antigen, for example cancer cells.

In an alternate embodiment, the Fc of the present invention is used to block, antagonize, or agonize the target antigen, for example by antagonizing a cytokine or cytokine receptor.

In an alternately preferred embodiment, the Fc of the present invention is used to block, antagonize, or agonize growth factors or growth factor receptors and thereby mediate killing the target cells that bear or need the target antigen.

In an alternately preferred embodiment, the Fc of the present invention is used to block, antagonize, or agonize enzymes and substrate of enzymes.

In a preferred embodiment, a Fc of the present invention is administered to a patient to treat a specific disorder. A "patient" for the purposes of the present invention includes both humans and other animals, preferably mammals and most preferably humans. By "specific disorder" herein is meant a disorder that may be ameliorated by the administration of a pharmaceutical composition comprising an Fc of the present invention.

In one embodiment, an Fc according to the present invention is the only therapeutically active agent administered to a patient. Alternatively, the Fc according the present invention is administered in combination with one or more other therapeutic agents, including but not limited to cytotoxic agents, chemotherapeutic agents, cytokines, growth inhibitory agents, anti-hormonal agents, kinase inhibitors, anti-angiogenic agents, cardioprotectants, or other therapeutic agents. The Fc may be administered concomitantly with one or more other therapeutic regimens. For example, an Fc of the present invention may be administered to the patient along with chemotherapy, radiation therapy, or both chemotherapy and radiation therapy. In one embodiment, the Fc of the present invention may be administered in conjunction with one or more antibodies. In accordance with another embodiment of the invention, the Fc of the present invention and one or more other anti-cancer therapies is employed to treat cancer cells ex vivo. It is contemplated that such ex vivo treatment may be useful in bone marrow transplantation and particularly, autologous bone marrow transplantation. It is of course contemplated that the Fc of the invention can be employed in combination with still other therapeutic techniques such as surgery.

A variety of other therapeutic agents may find use for administration with the Fc of the present invention. In one embodiment, the Fc is administered with an anti-angiogenic agent, which is a compound that blocks, or interferes to some degree, the development of blood vessels. The anti-angiogenic factor may, for instance, be a small molecule or a protein, for example an antibody, Fc fusion molecule, or cytokine, that binds to a growth factor or growth factor receptor involved in promoting angiogenesis. The preferred anti-angiogenic factor herein is an antibody that binds to Vascular Endothelial Growth Factor (VEGF). In an alternate embodiment, the Fc is administered with a therapeutic agent that induces or enhances adaptive immune response, for example an antibody that targets CTLA-4. In an alternate embodiment, the Fc is administered with a tyrosine kinase inhibitor, which is a molecule that inhibits to some extent tyrosine kinase activity of a tyrosine kinase. In an alternate embodiment, the Fc of the present invention is administered with a cytokine. By "cytokine" as used herein is meant a generic term for proteins released by one cell population that act on another cell as intercellular mediators including chemokines.

Pharmaceutical compositions are contemplated wherein the Fc of the present invention and one or more therapeutically active agents are formulated. Stable formulations of the Fc of the present invention are prepared for storage by mixing said Fc having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilisers, in the form of lyophilized formulations or aqueous solutions. The formulations to be used for in vivo administration are typically sterile. This is readily accomplished by filtration through sterile filtration membranes or other methods. The Fc and other therapeutically active agents disclosed herein may also be formulated as immunoliposomes, and/or entrapped in microcapsules.

Administration of the pharmaceutical composition comprising an Fc of the present invention, preferably in the form of a sterile aqueous solution, may be done in a variety of ways, including, but not limited to, orally, subcutaneously, intravenously, intranasally, intraotically, transdermally, mucosal, topically (e.g., gels, salves, lotions, creams, etc.), intraperitoneally, intramuscularly, intrapulmonary (e.g., AERx^{TM} inhalable technology commercially available from Aradigm, or Inhance^{TM} pulmonary delivery system commercially available from Inhale Therapeutics), vaginally, parenterally, rectally, or intraocularly.

The subject matter of the following definitions are embodiments of the present invention:

1. Method of preparing stability engineered human IgG Fc mutant by engineering at least one of the point mutations as listed in Table 1.

2. Method according to definition 1, which further comprises engineering at least one of the point mutations as listed in Table 2.

3. Method according to definition 1 or 2, wherein an Fc mutant is engineered comprising at least one of the mutations as listed in Table 3 or 5.

4. Method according to definition 1 or 2, wherein an Fc mutant is engineered comprising at least one of the mutations selected from the group consisting of:

- L351M;
- L368M;
- V369I;
- V397I, L398M;
- Y349F;
- Q347M, Y349F;
- K360N;
- V369I;
- K370L;
- N390R, S400D;
- N390E, S400R;
- A378V;
- D249R, R255L;
- V282K;
- E258M;
- V305A, T307P;
- E258K;
- K274Q, N276T, Y278F;
- K246N;
- V305E, T307P;
- N276T, Y278F;
- T250I;
- Y278F;

- T250I, K246N;
- K370L, T250V;
- K370L, V305A, T307P;
- K370L, K246N;
- K370L, K246N, T250I;
- K246N, T250I, V305A, T307P;
- T250V, V305A, T307P;
- T250I, V305A, T307P;
- K370L, T250V, V305A, T307P;
- K370L, T250V, K246N;
- K370L, T250I, V305A, T307P, S383N;
- T250V, V305A, T307P, K246N;
- K370L, T250V, V305A, T307P, K246N;
- K370L, T250V, V305A, T307P, K246N, K274Q;
- K370L, T250V, V305A, T307P, K246N, N276T;
- K370L, T250V, V305A, T307P, K246N, K274Q, N276T;
- K370L, T250I, V305A, T307P;
- K370L, N390R, S400D;
- T250V, N390R, S400D;
- K370L, T250V, K246N, N390R, S400D;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D;
- K370L, T250V, V305A, T307P, K246N, K274Q, N390R, S400D;
- K370L, T250V, V305A, T307P, K246N, K274Q, N276T, N390R, S400D;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D, Q438K;

- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D, Q438R;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D, Q438K, Q347E;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D, Q438R, Q347E;
- Q418L;
- K360Q, G446C;
- K360Q, Q438R;
- K360Q, Q418L, Q438R;
- Q347E;
- K360Q;
- Q438R;
- K392R;
- S400F;
- K360Q, Q418L;
- K360E;
- Q438K;
- Q347E, Q418L;
- T437I;
- S424T;
- N421D; and
-S354P.

wherein the numbering is according to the Kabat numbering scheme.

5. Method according to any of definitions 1 to 4, wherein said Fc mutant is selected for its functional scaffold structure as determined by binding to an effector molecule, like CD16a, CD64, FcRn, CD32, C1q and Protein A.

6. Method according to any of definitions 1 to 5, wherein said Fc mutant has a Tm value, which is higher than the wild type.

7. Method according to any of definitions 1 to 6, which further comprises engineering a new antigen binding site into the structural loop region.

8. Method according to any of definitions 1 to 6, wherein an Fc mutant is engineered to incorporate a new antigen binding site into the structural loop region.

9. Stability engineered human IgG Fc mutant comprising at least one of the point mutation(s) as listed in Table 1.

10. Fc according to definition 9, which further comprises at least one of the point mutations as listed in Table 2.

11. Fc according to definition 9 or 10, comprising at least one of the mutations as listed in Table 3 or 5.

12. Fc according to definition 9 or 10, comprising at least one of the mutations selected from the group consisting of:

- L351M;
- L368M;
- V369I;
- V397I, L398M;
- Y349F;
- Q347M, Y349F;
- K360N;
- V369I;
- K370L;
- N390R, S400D;
- N390E, S400R;
- A378V;
- D249R, R255L;
- V282K;
- E258M;
- V305A, T307P;
- E258K;
- K274Q, N276T, Y278F;
- K246N;
- V305E, T307P;
- N276T, Y278F;
- T250I;
- Y278F;
- T250I, K246N;
- K370L, T250V;

- K370L, V305A, T307P;
- K370L, K246N;
- K370L, K246N, T250I;
- K246N, T250I, V305A, T307P;
- T250V, V305A, T307P;
- T250I, V305A, T307P;
- K370L, T250V, V305A, T307P;
- K370L, T250V, K246N;
- K370L, T250I, V305A, T307P, S383N;
- T250V, V305A, T307P, K246N;
- K370L, T250V, V305A, T307P, K246N;
- K370L, T250V, V305A, T307P, K246N, K274Q;
- K370L, T250V, V305A, T307P, K246N, N276T;
- K370L, T250V, V305A, T307P, K246N, K274Q, N276T;
- K370L, T250I, V305A, T307P;
- K370L, N390R, S400D;
- T250V, N390R, S400D;
- K370L, T250V, K246N, N390R, S400D;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D;
- K370L, T250V, V305A, T307P, K246N, K274Q, N390R, S400D;
- K370L, T250V, V305A, T307P, K246N, K274Q, N276T, N390R, S400D;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D, Q438K;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D, Q438R;

- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D, Q438K, Q347E;

- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D, Q438R, Q347E;

- Q418L;

- K360Q, G446C;

- K360Q, Q438R;

- K360Q, Q418L, Q438R;

- Q347E;

- K360Q;

- Q438R;

- K392R;

- S400F;

- K360Q, Q418L;

- K360E;

- Q438K;

- Q347E, Q418L;

- T437I;

- S424T;

- N421D; and

-S354P.

wherein the numbering is according to the Kabat numbering scheme.

13. Fc according to any of definitions 9 to 10, comprising at least one of the combination of point mutations selected from the group consisting of:

- V305A and T250V;

- V305A and T250V, and further K274Q and/or N276T;

- K360Q and G446C;

- K360Q and Q438R;

- K360Q and Q418L;

- K360Q, Q418L and Q438R;

- K370L and T250V;

- K370L and T307P;

- K370L and T307P, and further K246N and/or S400D and/or N390R,

wherein the numbering is according to the Kabat numbering scheme.

14. Fc according to any of definitions 9 to 13, comprising an antigen binding site in the structural loop region.

15. Fc library comprising a repertoire of stability engineered Fc mutants according to any of definitions 9 to 14.

16. Library according to definition 15, wherein the Fc variants have different antigen binding properties.

17. Library according to definition 15 or 16, wherein at least 35% of the Fc variants have a functional scaffold structure as determined by binding to an effector molecule, like CD16a, CD64 and FcRn.

18. Use of a thermostabilised mutant of a human IgG Fc to engineer an Fc library.

19. Method of engineering a constant CH2 and/or CH3 domain by at least one of the point mutations of Table 1 to increase thermostability of a multidomain modular antibody as compared to the multidomain modular antibody without such point mutations.

20. Method of engineering a constant CH2 and/or CH3 domain by at least one of the point mutations of Table 1 to improve antigen-binding of a multidomain modular antibody as compared to the multidomain modular antibody without such point mutations.

21. Method according to definition 19 or 20, which further comprises engineering at least one of the point mutations as listed in Table 2.

22. Method according to any of definitions 19 to 21, wherein said constant CH2 and/or CH3 domain is engineered comprising at least one of the mutations as listed in Table 3 or 5.

The foregoing description will be more fully understood with reference to the following examples. Such examples are, however, merely representative of methods of practicing one or more embodiments of the present invention and should not be read as limiting the scope of invention.

Examples

Example 1: Stabilised wildtype Fc

Using the design principles described above, point mutations as listed in Table 1 and combinatory mutations that combine at least one of the point mutations of Table 1 with each other or with at least one of the point mutations of Table 2 were designed.

The gene coding for wildtype Fc of human IgG1, starting with amino acid residue 225 (Kabat EU index numbering) and ending with amino acid residue 447 (see for example GenBank entry BAC86226.1) was cloned into the *Pichia pastoris* expression vector pPICZalphaA (Invitrogen, Carlsbad, CA). This vector allows highlevel, methanol inducible expression of the gene of interest in *Pichia*, and can be used in various Pichia strains including X-33 and GS115. pPICZalphaA contains, amongst other elements, a 5′ fragment containing the AOX1 promoter for tightly regulated, methanol-induced expression of the gene of interest, the 3' AOX1 transcription termination region and a Zeocin resistance gene for selection in both *E. coli* and *Pichia.*

The genes coding for the mutant Fc proteins were prepared by site directed mutagenesis of the gene coding for wildtype Fc using standard procedures. In particular, the QuikChange Site Directed Mutagenesis Kit or the QuikChange Multi Site Directed Mutagenesis Kit (Stratagene) were used according to the instructions provided by the manufacturer. The mutant sequences were verified by DNA sequencing, and the plasmids were tranformed in *Pichia pastoris* X33 using standard procedures. Several single *Pichia* transformants were grown in small scale cultures, expression of the recombinant Fc was induced by addition of 1% methanol to the growth medium, and the culture was further shaken at 30° C for one to five days. The amount of produced Fc was analysed by SDS-PAGE, and the highest producing clone was selected for larger scale production, which followed essentially the same standard protocol as just described. The expressed Fc (wildtype or mutant) was purified from the culture supernatant by Protein A affinity chromatography using HiTrap™ Protein A HP columns (GE Healthcare Life Sciences) according to the manufacturers instructions. Eluted protein was extensively dialysed against PBS, and analysed by SDS-PAGE for integrity and purity. The protein concentration was determined by UV spectroscopy using an absorption coefficient at 280 nm of 1.397 for wildtype Fc dimer.

Differential Scanning Calorimetry (DSC) experiments were performed using an automated CAP VP-DSC MicroCalorimeter (MicroCal, Northhampton, MA), using 5 µM protein solution, diluted in PBS at pH 7.4 The heating rate was 1°C/min, and the heating was performed from 20°C to 110°C. Fitting was performed using the non-2-state transition model. This model provides the calorimetric (ΔH) and the van't Hoff (ΔHv) enthalpies. ΔH is the actual heat absorption during protein unfolding, whereas ΔHvH is the theoretical heat of the transition assuming a two-state model. The results obtained from these DSC measurements are summarized in Table 3 and show that for each of these stabilised mutants, at least one of the 3 observed thermal transitions (T_m1, T_m2, T_m3) is improved as compared to the value obtained with wildtype Fc.Table 3 describes the mutations within the amino acid sequence of human IgG1 Fc (Fc wild-type). The constructs comprising point mutations in the CH2 and/or CH3 domains are given and the thermostabilising effect as measured by an increase of any individual Tm value is shown, when compared to the respective Tm value of the wild-type sequence. In this table some of the listed point mutations are combined with each other or with further point mutations, meaning that there are constructs provided comprising such double or multiple point mutations relative to wildtype. The Fc wild-type is herein provided as a reference for comparison purposes, other Fc wild-type sequences (e.g. from different species, type or subtype) may be possibly used to engineer the specific thermostabilised constructs in respective positions in a similar way as readily understood by a skilled person.

Table 3

Construct	T_m1 (°C)	ΔH1 (kcal/mol)	$ΔH_v1$ (kcal/mol)	T_m2 (°C)	ΔH2 (kcal/mol)	$ΔH_v2$ (kcal/mol)	T_m3 (°C)	ΔH3 (kcal/mol)	$ΔH_v3$ (kcal/mol)
Fc wild-type	65,9	129,5	90,7	78,1	72,6	136,7	82,6	59,7	234,1
L351M	66,2	196,9	72,2	76,9	111,6	142,7			
L368M	66,8	129,3	80,5	76,8	55,2	179,5			
V369I	66,3	153,3	68,6	76,8	80,9	126,8			
V397I, L398M	66,5	216,1	67,8	76,5	91,5	179,4			
Y349F	66,7	189,1	70,3	77,4	83,2	149,7			
Q347M, Y349F	67,3	181,0	76,3	75,6	57,0	179,7			
K360N	66,5	90,5	95,0	77,1	76,9	108,6	82,5	47,0	214,5
V369I	66,1	135,6	86,7	74,9	74,8	139,1	78,9	28,9	235,6
K370L	65,2	145,1	86,7	82,7	100,9	126,5	87,4	69,8	234,0
N390R, S400D	68,3	120,7	85,7	84,3	70,6	149,3	89,6	102,5	139,8
N390E, S400R	67,0	154,7	64,0	76,3	30,4	145,2	80,1	32,0	233,9
A378V	68,6	199,4	87,0	78,9	64,9	150,5	83,6	88,1	217,6
D249R, R255L	50,4	46,5	114,5	77,2	64,8	91,6	82,9	40,5	211,8
V282K	65,4	116,7	442,8	77,2	89,7	121,9	82,7	37,6	229,8
E258M	64,5	107,9	91,7	77,4	97,4	110,7	82,8	43,8	225,7
V305A, T307P	70,3	142,5	104,0	77,3	84,7	134,0	82,3	60,5	229,1
E258K	66,6	116,1	85,1	77,2	76,1	116,0	82,5	52,1	213,8
K274Q, N276T, Y278F	67,5	98,0	87,1	78,3	75,7	128,3	82,6	60,8	227,2
K246N	68,8	145,0	88,1	77,5	80,7	132,2	82,6	43,6	246,7
V305E, T307P	64,2	95,4	122,2	77,5	83,1	111,0	82,9	66,7	219,5
N276T, Y278F	65,4	123,8	87,4	79,2	82,9	129,7	83,7	64,0	235,3
T250I	70,8	142,7	97,3	78,1	63,4	143,5	82,9	41,2	233,0
Y278F	64,9	129,0	101,6	77,0	117,5	114,5	82,7	43,3	237,5
T250I, K246N	72,7	212,1	72,3	79,4	38,4	156,7	82,9	44,7	262,7
K370L, T250V	69,6	133,2	88,3	82,8	81,9	136,6	87,6	56,5	233,8
K370L, V305A, T307P	68,8	87,7	43,4	82,5	61,9	61,2	87,3	39,7	112,2
K370L, K246N	67,2	66,5	46,0	82,7	47,3	65,9	87,4	41,3	121,3

K370L, K246N, T250I	71,4	73,5	41,6	82,8	41,8	70,2	87,5	25,7	120,2
K246N, T250I, V305A, T307P	71,0	41,9	127,4	77,0	285,6	119,0	82,7	85,7	237,7
T250V, V305A, T307P	69,7	41,9	143,0	76,6	256,0	118,4	82,8	61,2	234,6
T250I, V305A, T307P	69,1	40,8	115,8	75,9	297,5	103,9	82,6	81,1	230,0
K370L, T250V, V305A, T307P	75,9	208,6	86,0	83,2	63,4	163,8	87,5	71,4	235,5
K370L, T250V, K246N	73,9	206,9	76,5	83,1	79,8	157,4	87,6	66,3	244,5
K370L, T250I, V305A, T307P, S383N	67,5	8,9	66,9	76,0	134,7	42,6	83,9	32,3	110,6
T250V, V305A, T307P, K246N	72,4	33,9	52,6	78,2	80,6	83,5	82,8	49,5	113,6
K370L, T250V, V305A, T307P, K246N	78,8	138,6	44,9	84,7	24,4	90,4	87,6	31,7	137,3
K370L, T250V, V305A, T307P, K246N, K274Q	78,5	101,1	94,7	80,2	117,4	110,4	87,0	75,8	222,9
K370L, T250V, V305A, T307P, K246N, N276T	73,7	52,8	93,1	81,4	234,1	64,1	87,6	56,2	256,6
K370L, T250V, V305A, T307P, K246N, K274Q, N276T	76,6	89,8	65,4	82,4	166,0	144,8	87,5	74,1	215,5
K370L, T250I, V305A, T307P	74,3	135,7	125,4	82,6	82,0	124,4	87,4	72,1	239,2
K370L, N390R, S400D	64,6	129,6	83,0	83,0	93,4	126,0	88,6	59,2	243,6
T250V, N390R, S400D	65,4	119,7	95,8	83,4	79,8	128,6	87,0	71,0	241,0
K370L, T250V, K246N, N390R, S400D	73,6	148,1	86,1	83,8	64,1	155,2	88,6	74,9	241,5
K370L, T250V, V305A, T307P, K246N, N390R, S400D	78,1	180,2	90,9	84,8	42,9	158,6	88,6	74,3	248,8
K370L, T250V, V305A, T307P, K246N, K274Q, N390R, S400D	72,1	11,8	157,5	80,3	201,8	89,5	88,4	60,6	241,2
K370L, T250V, V305A, T307P, K246N, K274Q, N276T, N390R, S400D	75,8	48,3	105,3	82,5	152,0	127,1	87,1	69,4	244,8
K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q,	77,9	188,0	91,9	84,0	75,0	130,4	87,3	66,0	258,5
K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L,	77,0	172,0	95,4	85,9	68,8	173,5	91,0	96,7	224,5
K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T,	76,3	153,5	101,5	88,5	73,7	157,5	94,0	94,6	237,0
K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R,	76,1	156,0	101,5	88,3	77,0	156,0	94,1	99,2	235,5

K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D,	76,0	159,5	97,8	89,8	92,9	153,5	95,3	94,1	241,0
K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D, Q438K,	74,9	152,0	97,6	91,4	93,4	144,5	96,8	89,8	244,5
K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D, Q438R	74,7	150,5	99,5	91,2	99,2	147,0	96,2	79,6	255,5
K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D, Q438K, Q347E	74,4	149,5	99,4	87,5	83,4	150,5	91,6	82,4	238,0
K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D, Q438R, Q347E	74,1	151,0	96,8	87,0	93,7	150,5	91,1	72,6	240,5

Example 2: Stabilised Fcab H10-03-6

H10-03-6 is an Fcab that binds specifically and with high affinity to HER2/neu via a binding site that has been engineered in structural loops of the CH3 domain (Wozniak-Knopp et al.: Introducing antigen-binding sites in structural loops of immunoglobulin constant domains: Fc fragments with engineered HER2/neu-binding sites and antibody properties. Protein Eng Des Sel. 2010 Apr;23 (4):289-97. Epub 2010 Feb 11. PubMed PMID: 20150180). Relative to wildtype Fc, H10-03-6 displays reduced thermostability, with a T_m1 of 61,6 °C and a T_m2 of 65,2 °C. No T_m3 is observed with this Fcab. H10-03-6 was cloned and expressed in *Pichia pastoris* as described above. Two constructs with stabilising mutations were also cloned and expressed, namely the single point mutation T250V as well as the combinatory mutation T250V K370L. All proteins were purified and prepared for DSC measurements as described above. The results of the DSC measurements are summarized in Table 4 below. It can be seen, that both T_m1 and T_m2 has been improved in both stabilised constructs relative to the original clone H10-03-6.

Table 4:

Construct	T_m1 (°C)	ΔH1 (kcal/mol)	ΔH_v1 (kcal/mol)	T_m2 (°C)	ΔH2 (kcal/mol)	ΔH_v2 (kcal/mol)
H10-03-6	61.6	112.8	113.6	65.2	88.3	204.2
H10-03-6 T250V	66.3	147.6	123.2	69.8	71.0	259.8
H10-03-6 T250V K370L	64.7	131.2	127.0	68.1	83.8	234.3

Example 3: Isolation of stabilised wildtype Fc by in vitro directed evolution

Stabilizing point mutations in wildtype Fc were selected by a method based on the introduction of random mutations in the Fc by using error prone PCR. The gene coding for wildtype Fc was cloned in the yeast display vector pYD1 (Invitrogen, Carlsbad, CA) using the BamHI and NotI restriction sites. A library of randomly mutated Fc-genes was created following etablished protocols for error prone PCR (reviewed e.g. in McCullum EO et al.: Random mutagenesis by error-prone PCR. Methods Mol Biol. 2010; 634:103-9; Cirino PC et al.: Generating mutant libraries using error-prone PCR. Methods Mol Biol. 2003; 231: 3-9). The GeneMorph II Random Mutagenesis Kit (Stratagene, La Jolla, CA) was used in the work decribed here. The PCR primers that were used encompass the 25 bases upstream of the BamHI site and the 25 bases downstream of the NotI site of pYD1, respectively. Alternatively, primers specific for the Fc insert can also be used. This library was expressed as a yeast surface display library following the method as described by Boder and Wittrup, 1997 (Boder ET, Wittrup KD: Yeast surface display for screening combinatorial polypeptide libraries. Nat Biotechnol. 1997 Jun; 15(6):553-7). Growth of the yeast surface display library was overnight at 20°C in SD-CAA-medium (20 g/l glucose, 0.1 M KH2PO4/K2HPO4, pH 6, 10 g/l (NH4)2SO4, 0.1 g/l L-leucine, 3.4 g/l yeast nitrogen base, 10 g/l Bacto casamino acids). Subsequently the cultures were diluted with fresh medium to an OD600 of 1 and after 4 h of incubation the cells were centrifuged and resuspended in SGR-CAA (same as SD-CAA, but 20 g/l galactose and 10 g/l raffinose instead of glucose) to an OD600 of 1. Induction was done at 20°C for 18 – 20 h. The cells were then centrifuged and resuspended in PBS/BSA (0.2 g/l KCl, 0.2 g/l KH2PO4, 8 g/l NaCl, 1.15 g/l Na2HPO4 + 20 g/l bovine serum albumin) to an OD600 of 3. The suspension was aliquoted into microcentrifuge tubes and after storage for 10 min on ice the tubes were incubated at 79°C for 10 min. Time and temperature of this heat shock were chosen such that the wildtype Fc protein is at least to some extent denatured after the heat shock. Then the tubes were cooled on ice for at least 5 min. After centrifugation, the cells were stained with 5 µg/ml anti-Xpress antibody (Invitrogen, Carlsbad, CA, USA) that had been conjugated to allophycocyanin (APC) using the LYNX Rapid APC Antibody Conjugation Kit (AbD Serotec, Kidlington, UK) and with either 2 µg/ml Fluorescein Isothiocyanate Isomer 1-labelled anti-human IgG CH2 domain antibody (anti-CH2-FITC) or 1 µg/ml His-tagged CD64. In the case of

CD64-staining, the cells were subsequently washed and incubated with 1 µg/ml Alexa Fluor 488-labeled anti-His antibody. All stainings were done in PBS/BSA at 4°C for 30 min with gentle shaking. After a further washing step, the cells were resuspended in PBS/BSA and the positively staining cells were sorted on a FACS Aria cell sorter (BD, Franklin Lakes, NJ). The sorted cells were subsequently centrifuged. Since the yeast cells did not survive the incubation at 79°C, it was necessary to isolated the DNA from the dead cells, and to use this DNA to prepare a new library in fresh *S. cerevisiae* cells. DNA was isolated from the selected *S. cerevisiae* cells by using the Zymoprep Yeast Plasmid Miniprep Kit II (ZYMO RESEARCH, Orange, CA, USA). The DNA preparation was subsequently used as the template for a PCR with the same primers that had been used for error prone PCR. With the PCR product, a new library was generated by cloning into pYD1 as described above. This cycle of library generation, induction, incubation at 79 °C, FACS sorting, DNA isolation and preparation of a new library was repeated 4 times. Finally the Fc-genes that had been enriched by this method were sequenced by Sanger Dideoxy DNA sequencing. A number of Fc mutant genes that were enriched were subsequently cloned into the vector pPICZalphaA (Invitrogen, Carlsbad, CA), which is a vector for soluble expression in *Pichia pastoris*. Mutant Fc proteins were subsequently expressed, purified and characterised as described in the examples above.

The results of the DSC measurements are summarized in Table 5 below. It can be seen, that, with one exception, both T_m2 and T_m3 has been improved in all isolated constructs relative to the wildtype Fc. The exception is mutant K360N, which has an improved T_m1 relative to wildtype Fc. This mutant is of particular interest, since it has been also found by the site directed mutagenesis approach as shown in example 1.

It is also interesting to note mutant S400F, which involves the same position in the Fc sequence as two of the mutants (S400D and S400R) that were identified by the site directed mutagenesis approach as described in Example 1.

Table 5

Construct	**T_m1 (°C)**	**ΔH1 (kcal/mol)**	**ΔH_v1 (kcal/mol)**	**T_m2 (°C)**	**ΔH2 (kcal/mol)**	**ΔH_v2 (kcal/mol)**	**T_m3 (°C)**	**ΔH3 (kcal/mol)**	**ΔH_v3 (kcal/mol)**
Fc wild-type	65,9	121,5	89,3	78,1	73,2	132,9	82,6	59,0	230,4
Q418L	65,0	123,1	84,6	81,0	88,2	128,6	86,3	54,6	238,7
K360Q, G446C	64,7	139,9	75,6	83,2	124,6	105,1	90,4	47,5	205,8
K360Q, Q438R	63,7	112,2	91,6	82,8	85,8	129,9	87,2	59,2	250,1
K360Q, Q418L, Q438R	63,8	99,7	96,0	86,5	77,4	140,5	91,0	65,5	251,6
Q347E	64,9	104,2	96,8	81,6	64,3	138,0	86,4	71,2	235,9
K360Q	65,4	96,3	107,3	81,6	62,7	147,6	85,7	76,4	243,1
Q438R	64,1	109,8	86,3	79,5	70,5	125,8	83,9	47,9	235,4
K392R	64,7	104,2	78,6	79,1	72,8	122,7	84,4	46,3	237,2
S400F	65,3	108,7	85,0	80,1	66,3	126,4	85,4	52,6	231,7
K360Q, Q418L	64,9	115,1	92,5	84,6	83,1	143,6	89,6	73,2	247,0
K360E	64,9	109,0	92,2	80,4	72,4	139,6	85,1	65,1	241,6
Q438K	64,0	107,6	89,5	80,4	68,5	122,3	85,2	55,7	231,1
Q347E, Q418L	64,9	113,8	95,6	85,0	67,0	142,8	90,2	82,8	239,1
T437I	64,4	120,5	92,9	80,2	72,2	135,5	84,3	54,7	240,3
S424T	65,2	98,9	98,1	81,0	65,0	133,1	85,9	60,1	240,5
N421D	65,4	116,5	81,8	79,5	75,1	135,9	84,2	55,9	238,3
S354P	65,1	112,0	87,1	80,0	52,6	128,8	85,8	72,1	220,0

Example 4: Fc library based on a Fc scaffold comprising the Q418L and S424T mutations

Fc libraries that are more thermostable and also more tolerant to insertions in the EF loop were constructed as follows:

The gene coding for wild-type Fc was cloned in the yeast display vector pYD1 (Invitrogen, Carlsbad, CA) using standard methods. Fcab libraries with modifications in the EF loop were constructed according to established protocols (see e.g. Wozniak-Knopp et al.: Introducing antigen-binding sites in structural loops of immunoglobulin constant domains: Fc fragments with engineered HER2/neu-binding sites and antibody properties. Protein Eng Des Sel. 2010 Apr; 23(4):289-97. Epub 2010 Feb 11. PubMed PMID: 20150180).

The libraries had the following designs and designations:
Library1: residues 419-422 were mutated and in addition, 5 residues were inserted
Library2: same as Library1, plus introduction of the stabilising mutations Q418L and S424T

As indicated above, libraries were prepared in a modified form in which the positions Q418 und S424 were mutated to introduce the stabilizing mutations Q418L and S424T. For control, the libraries were prepared in parallel with the respective wild-type residues at positions 418 and 424. In order to show that these stabilizing mutations lead to libraries that include a higher percentage of thermostable clones as compared to the libraries with the respective wild-type residues at positions 418 and 424, the libraries were analyzed as follows:

The libraries were expressed as yeast surface display libraries following the method as described by Boder and Wittrup, 1997 (Boder ET, Wittrup KD: Yeast surface display for screening combinatorial polypeptide libraries. Nat Biotechnol. 1997 Jun; 15(6):553-7). Growth of the yeast surface display libraries was overnight at 20°C in SD-CAA-medium (20 g/l glucose, 0.1 M KH_2PO_4/K_2HPO_4, pH 6, 10 g/l $(NH_4)_2SO_4$, 0.1 g/l L-leucine, 3.4 g/l yeast nitrogen base, 10 g/l Bacto casamino acids). Subsequently the cultures were diluted with fresh medium to an OD600 of 1 and after 4 h of incubation the cells were centrifuged and resuspended in SGR-CAA (same as SD-CAA, but 20 g/l galactose and 10 g/l raffinose instead of glucose) to an OD600 of 1. Induction was done at 20°C for 18 – 20 h. The cells were then centrifuged and resuspended in PBS/BSA (0.2 g/l KCl, 0.2 g/l KH_2PO_4, 8 g/l NaCl, 1.15 g/l Na_2HPO_4 +

20 g/l bovine serum albumin) to an OD600 of 1. The suspension was aliquoted into microcentrifuge tubes and after storage for 10 min on ice the tubes were incubated at different temperatures between 50°C and 75°C for 30 min, shaking at 300 rpm. Then the tubes were cooled on ice for at least 5 min.

After centrifugation, the cells were stained with 10.8 µg/ml biotinylated anti-Xpress antibody as well as with 2 µg/ml His-tagged CD64. As secondary reagents, 2 µg/ml Streptavidin-APC and 2 µg/ml Alexa Fluor 488-labeled anti-His antibody were used. All staining steps were performed in PBS/BSA at 4°C for 30 min with gentle shaking. Washing steps were performed with PBS/BSA.

Cells were analysed for CD64 binding by FACS. Only cells that were positively staining with anti-Xpress antibody were considered. Only cells that carry a well-folded Fc that has not been denatured during the heat incubation step above are expected to stain positively with CD64. Results are given in Table 1. It can be seen that after incubation at elevated temperatures, in particular at 65°C and above, Library2 (that included the stabilising mutations Q418L and S424T) exhibited markedly higher residual binding to CD64 than Library1, indicating that the introduction of stabilising mutations counteracted the heat denaturation of library clones containing randomised regions in a structural loop.

Table 1: mean fluorescence intensity of library populations after incubation of the libraries at various temperatures.

	ice	**50°C, 30'**	**55°C, 30'**	**60°C, 30'**	**65°C, 30'**	**70°C, 30'**	**75°C, 30'**
Library1	54.9	50.9	45.4	22.8	7.92	4.71	4.44
Library2	61.5	55.5	53.1	30.2	18.7	7.18	4.81

Claims

1. Method of preparing stability engineered human IgG Fc mutant by engineering at least one of the point mutations as listed in Table 1.

2. Method according to claim 1, which further comprises engineering at least one of the point mutations as listed in Table 2.

3. Method according to claim 1 or 2, wherein an Fc mutant is engineered comprising at least one of the mutations as listed in Table 3 or 5.

4. Method according to claim 1 or 2, wherein an Fc mutant is engineered comprising at least one of the mutations selected from the group consisting of:

- L351M;
- L368M;
- V369I;
- V397I, L398M;
- Y349F;
- Q347M, Y349F;
- K360N;
- V369I;
- K370L;
- N390R, S400D;
- N390E, S400R;
- A378V;
- D249R, R255L;
- V282K;
- E258M;
- V305A, T307P;
- E258K;
- K274Q, N276T, Y278F;
- K246N;
- V305E, T307P;
- N276T, Y278F;
- T250I;
- Y278F;

- T250I, K246N;
- K370L, T250V;
- K370L, V305A, T307P;
- K370L, K246N;
- K370L, K246N, T250I;
- K246N, T250I, V305A, T307P;
- T250V, V305A, T307P;
- T250I, V305A, T307P;
- K370L, T250V, V305A, T307P;
- K370L, T250V, K246N;
- K370L, T250I, V305A, T307P, S383N;
- T250V, V305A, T307P, K246N;
- K370L, T250V, V305A, T307P, K246N;
- K370L, T250V, V305A, T307P, K246N, K274Q;
- K370L, T250V, V305A, T307P, K246N, N276T;
- K370L, T250V, V305A, T307P, K246N, K274Q, N276T;
- K370L, T250I, V305A, T307P;
- K370L, N390R, S400D;
- T250V, N390R, S400D;
- K370L, T250V, K246N, N390R, S400D;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D;
- K370L, T250V, V305A, T307P, K246N, K274Q, N390R, S400D;
- K370L, T250V, V305A, T307P, K246N, K274Q, N276T, N390R, S400D;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D, Q438K;

- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D, Q438R;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D, Q438K, Q347E;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D, Q438R, Q347E;
- Q418L;
- K360Q, G446C;
- K360Q, Q438R;
- K360Q, Q418L, Q438R;
- Q347E;
- K360Q;
- Q438R;
- K392R;
- S400F;
- K360Q, Q418L;
- K360E;
- Q438K;
- Q347E, Q418L;
- T437I;
- S424T;
- N421D; and
-S354P.

wherein the numbering is according to the Kabat numbering scheme.

5. Method according to any of claims 1 to 4, wherein said Fc mutant is selected for its functional scaffold structure as determined by binding to an effector molecule, like CD16a, CD64, FcRn, CD32, C1q and Protein A.

6. Method according to any of claims 1 to 5, wherein said Fc mutant has a Tm value, which is higher than the wild type.

7. Method according to any of claims 1 to 6, which further comprises engineering a new antigen binding site into the structural loop region.

8. Method according to any of claims 1 to 6, wherein an Fc mutant is engineered to incorporate a new antigen binding site into the structural loop region.

9. Stability engineered human IgG Fc mutant comprising at least one of the point mutation(s) as listed in Table 1.

10. Fc according to claim 9, which further comprises at least one of the point mutations as listed in Table 2.

11. Fc according to claim 9 or 10, comprising at least one of the mutations as listed in Table 3 or 5.

12. Fc according to claim 9 or 10, comprising at least one of the mutations selected from the group consisting of:

- L351M;
- L368M;
- V369I;
- V397I, L398M;
- Y349F;
- Q347M, Y349F;
- K360N;
- V369I;
- K370L;
- N390R, S400D;
- N390E, S400R;
- A378V;
- D249R, R255L;
- V282K;
- E258M;
- V305A, T307P;
- E258K;
- K274Q, N276T, Y278F;
- K246N;
- V305E, T307P;
- N276T, Y278F;
- T250I;
- Y278F;
- T250I, K246N;
- K370L, T250V;

- K370L, V305A, T307P;
- K370L, K246N;
- K370L, K246N, T250I;
- K246N, T250I, V305A, T307P;
- T250V, V305A, T307P;
- T250I, V305A, T307P;
- K370L, T250V, V305A, T307P;
- K370L, T250V, K246N;
- K370L, T250I, V305A, T307P, S383N;
- T250V, V305A, T307P, K246N;
- K370L, T250V, V305A, T307P, K246N;
- K370L, T250V, V305A, T307P, K246N, K274Q;
- K370L, T250V, V305A, T307P, K246N, N276T;
- K370L, T250V, V305A, T307P, K246N, K274Q, N276T;
- K370L, T250I, V305A, T307P;
- K370L, N390R, S400D;
- T250V, N390R, S400D;
- K370L, T250V, K246N, N390R, S400D;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D;
- K370L, T250V, V305A, T307P, K246N, K274Q, N390R, S400D;
- K370L, T250V, V305A, T307P, K246N, K274Q, N276T, N390R, S400D;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D, Q438K;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D, Q438R;

- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D, Q438K, Q347E;

- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D, Q438R, Q347E;

- Q418L;

- K360Q, G446C;

- K360Q, Q438R;

- K360Q, Q418L, Q438R;

- Q347E;

- K360Q;

- Q438R;

- K392R;

- S400F;

- K360Q, Q418L;

- K360E;

- Q438K;

- Q347E, Q418L;

- T437I;

- S424T;

- N421D; and

-S354P.

wherein the numbering is according to the Kabat numbering scheme.

13. Fc according to any of claim 9 to 10, comprising at least one of the combination of point mutations selected from the group consisting of:

- V305A and T250V;

- V305A and T250V, and further K274Q and/or N276T;

- K360Q and G446C;

- K360Q and Q438R;

- K360Q and Q418L;

- K360Q, Q418L and Q438R;

- K370L and T250V;

- K370L and T307P;

- K370L and T307P, and further K246N and/or S400D and/or N390R,

wherein the numbering is according to the Kabat numbering scheme.

14. Fc according to any of claims 9 to 13, comprising an antigen binding site in the structural loop region.

15. Fc library comprising a repertoire of stability engineered Fc mutants according to any of claims 9 to 14.

16. Library according to claim 15, wherein the Fc variants have different antigen binding properties.

17. Library according to claim 15 or 16, wherein at least 35% of the Fc variants have a functional scaffold structure as determined by binding to an effector molecule, like CD16a, CD64 and FcRn.

18. Use of a thermostabilised mutant of a human IgG Fc to engineer an Fc library.

19. Method of engineering a constant CH2 and/or CH3 domain by at least one of the point mutations of Table 1 to increase thermostability of a multidomain modular antibody as compared to the multidomain modular antibody without such point mutations.

20. Method of engineering a constant CH2 and/or CH3 domain by at least one of the point mutations of Table 1 to improve antigen-binding of a multidomain modular antibody as compared to the multidomain modular antibody without such point mutations.

21. Method according to claim 19 or 20, which further comprises engineering at least one of the point mutations as listed in Table 2.

22. Method according to any of claims 19 to 21, wherein said constant CH2 and/or CH3 domain is engineered comprising at least one of the mutations as listed in Table 3 or 5.

23. Method according to any of claims 19 to 21, wherein said constant CH2 and/or CH3 domain is engineered comprising at least one of the mutations selected from the group consisting of:

- L351M;
- L368M;
- V369I;
- V397I, L398M;
- Y349F;

- Q347M, Y349F;
- K360N;
- V369I;
- K370L;
- N390R, S400D;
- N390E, S400R;
- A378V;
- D249R, R255L;
- V282K;
- E258M;
- V305A, T307P;
- E258K;
- K274Q, N276T, Y278F;
- K246N;
- V305E, T307P;
- N276T, Y278F;
- T250I;
- Y278F;
- T250I, K246N;
- K370L, T250V;
- K370L, V305A, T307P;
- K370L, K246N;
- K370L, K246N, T250I;
- K246N, T250I, V305A, T307P;
- T250V, V305A, T307P;
- T250I, V305A, T307P;
- K370L, T250V, V305A, T307P;
- K370L, T250V, K246N;
- K370L, T250I, V305A, T307P, S383N;
- T250V, V305A, T307P, K246N;
- K370L, T250V, V305A, T307P, K246N;
- K370L, T250V, V305A, T307P, K246N, K274Q;
- K370L, T250V, V305A, T307P, K246N, N276T;

- K370L, T250V, V305A, T307P, K246N, K274Q, N276T;
- K370L, T250I, V305A, T307P;
- K370L, N390R, S400D;
- T250V, N390R, S400D;
- K370L, T250V, K246N, N390R, S400D;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D;
- K370L, T250V, V305A, T307P, K246N, K274Q, N390R, S400D;
- K370L, T250V, V305A, T307P, K246N, K274Q, N276T, N390R, S400D;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D, Q438K;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D, Q438R;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D, Q438K, Q347E;
- K370L, T250V, V305A, T307P, K246N, N390R, S400D, K360Q, Q418L, S424T, K392R, N421D, Q438R, Q347E;
- Q418L;
- K360Q, G446C;
- K360Q, Q438R;
- K360Q, Q418L, Q438R;
- Q347E;
- K360Q;
- Q438R;
- K392R;
- S400F;

- K360Q, Q418L;
- K360E;
- Q438K;
- Q347E, Q418L;
- T437I;
- S424T;
- N421D; and
-S354P.

wherein the numbering is according to the Kabat numbering scheme.

Fig 1.

```
225                 235                 245                 255                 265                 275                 285                 295
|                   |                   |                   |                   |                   |                   |                   |
tcppcpapel lggpsvflfp pkpkdtlmis rtpevtcvvv dvshedpevk fnwyvdgvev hnaktkpree qynstyrvvs

305                 315                 325                 335                 345                 355                 365                 375
|                   |                   |                   |                   |                   |                   |                   |
vltvlhqdwl ngkeykckvs nkalpapiek tiskakgqpr epqvytlpps rdeltknqvs ltclvkgfyp sdiavewesn

385                 395                 405                 415                 425                 435                 445
|                   |                   |                   |                   |                   |                   |
gqpennyktt ppvldsdgsf flyskltvdk srwqqgnvfs csvmhealhn hytqkslsls pgk
```

Fig. 2

```
                         A          AB     B            BC          C       CD
                  --------------->  ------------->            ------>
                  1        10    15  16  20           30     36 39     45
              7654321|........|....|123|...|......  ...|.....|  |.....|1234567
huIgG1CH2    (A)PELLGGPSVFLFPPKPKDTLMI.SRTPEVTCVVV DVSHEDPEVK  FNWYVDGVEVH...
huIgG1CH3      (G)QPREPQVYTLPPSRDELT...KNQVSLTCLVK GFYP..SDIA  VEWESNGQPEN...

                D           DE           E      EF  F            FG          G
             ------->               ------------> -------->              ------------>
             77     84              85          96 97            110      118        130
              |......|12345677654321|..........|12|.......  .....|..... ..|...........|
huIgG1CH2    .NAKTKPREEQYN......STYRVVSVLTVLHQDW..LNGKEYKC KVSNKA..LPAPI EKTISKAK
huIgG1CH3    .NYKTTPPVLDSD......GSFFLYSKLTVDKSRW..QQGNVFSC SVMHEA.LHNHYT QKSLSLSP...GK
```

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2011/065463

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. (means)

   [ ] on paper

   [X] in electronic form

   b. (time)

   [X] in the international application as filed

   [ ] together with the international application in electronic form

   [ ] subsequently to this Authority for the purpose of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2011/065463

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/00 A61K39/395 C07K16/32
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DEMAREST STEPHEN J ET AL: "Optimization of the antibody C(H)3 domain by residue frequency analysis of IgG sequences.", JOURNAL OF MOLECULAR BIOLOGY 2 JAN 2004 LNKD- PUBMED:14659738, vol. 335, no. 1, 2 January 2004 (2004-01-02), pages 41-48, XP002610108, ISSN: 0022-2836 cited in the application	13
Y	table 1	1-12, 14-16
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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 November 2011	22/11/2011

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Hix, Rebecca

3

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	US 2006/235208 A1 (LAZAR GREGORY A [US] ET AL) 19 October 2006 (2006-10-19) paragraphs [[0016]] - [[0017]]	1-16
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2011/065463

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