

EFFECTORLESS IGG1 FC VARIANTS

The present invention relates to IgG1 Fc variants, molecules comprising the same, use of such IgG1 variants to decrease antibody effector functions and method to decrease antibody effector functions by introducing mutations into the IgG1 Fc.

1. BACKGROUND OF THE INVENTION

Monoclonal antibodies as well as Fc fusion proteins are very promising biopharmaceutical agents and the market for these compounds has increased significantly within the recent years. Reasons for the success of these molecules are their high specificity, safety and long half-lives in the circulation compared to small chemical compounds.

Antibodies are composed of two different protein chains, a light chain (LC) and a heavy chain (HC). One LC is covalently linked to one HC and the HCs are on their parts covalently linked by disulfide bonds. The LC is composed of a variable domain (VL) and a constant domain (CL), whereas the HC consist of a variable domain (VH) and several constant domains (e.g., CH1, CH2 and CH3 domains for IgG).

Antibodies are multifunctional proteins. Functionally, an antibody can be divided into two Fab (Fragment antigen binding) regions and one Fc (Fragment crystallizable) region. Each Fab region consists of four domains: VL, CL, VH and CH1. The Fc region comprises the remaining constant domains (CH2 and CH3 domains for IgG). While antigens are bound by Fab regions, effector functions of antibodies are mediated by the Fc region. The effector functions of antibodies include antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP) and complement-dependent cytotoxicity (CDC). These effects are mediated via binding of the antibody Fc region to Fc gamma receptors (FcγRs) and the complement protein C1q, respectively. These antibody effector functions are important for the efficacy of natural antibodies as well as some therapeutic antibodies, e.g., in oncology. On the other hand, the effector functions are undesired for many other engineered therapeutic antibodies, e.g. antibodies with anti-inflammatory effects (such as antibodies blocking pro-inflammatory molecules). Such antibodies are often designed to act mainly or only by blocking their specific targets via binding of the Fab fragment, while any effector function of the Fc region elicits an interfering and potentially harmful immune reaction, at the extreme a “cytokine storm”.

On the other hand, the Fc region markedly prolongs half-life of antibodies and Fc fusion proteins in the circulation via a “recycling” mechanism mediated by the neonatal Fc-receptor (FcRn). Moreover, the Fc region contributes to the overall stability of the molecule and another important and desired function of the Fc-domain is its strong and specific binding to protein A. Binding of antibodies and Fc fusion proteins to immobilized protein A is usually the first purification step during the purification of antibodies and Fc fusion proteins. Accordingly, a simple removal of the Fc region from the antibody molecule would have severe drawbacks.

Therefore, several variants Fc regions have been developed to reduce antibody effector functions while maintaining the positive effects of the Fc region (half-life prolongation, protein A binding, stabilization). These approaches include the aglycosylated variants and variants with point mutations (Wang et al., Protein Cell. 2018;9(1):63-73).

A common variant with reduced effector function is the so-called IgG1 LALA variant, which contains the two mutations L234A and L235A. However, the IgG1 LALA variant shows some residual effector functions, in particular residual CDC, which can be undesirable for certain applications. Further mutations have been introduced in the IgG1 LALA variant to abolish those residual effector functions, e.g. the additional mutations P329G and P329A (Schlothauer et al., Protein Eng Des Sel. 2016 Oct;29(10):457-466). While it was reported that the introduction of P329G in IgG1 LALA abolishes the antibody effector functions completely, P329A was linked to some residual effector functions (Schlothauer et al., Protein Eng Des Sel. 2016 Oct;29(10):457-466).

As different conditions can require different backbones, e.g. in case of anti-drug-antibodies against particular parts of the antibody backbone, there is the need for IgG1 Fc variants which have as least as little residual effector functions as IgG1 LALA P329G.

The present invention meets this need by providing the IgG1 LALA GASS variant, which comprises the mutations L234A, L235A, A327G, P329A, A330S, and P331S, and the use of this variant to reduce antibody effector functions.

2. SUMMARY OF THE INVENTION

The present invention provides an IgG1 Fc variant polypeptide, wherein said IgG1 Fc variant polypeptide comprises the mutations L234A, L235A, A327G, P329A, A330S, and P331S,

according to EU numbering, wherein the IgG1 Fc variant polypeptide does not comprise SEQ ID NO:28.

In some embodiments, the IgG1 Fc variant polypeptide does not consist of SEQ ID NO:31.

In some embodiments, the IgG1 Fc variant polypeptide is a human IgG1 Fc variant polypeptide.

In some embodiments, the IgG1 Fc variant polypeptide does not consist of SEQ ID NO:31 and is a human IgG1 Fc variant polypeptide

In some embodiments, the IgG1 Fc variant polypeptide comprises SEQ ID NO:31.

The present invention also provides a molecule comprising two IgG1 Fc variant polypeptides according to any of the previous embodiments described before.

In some embodiments, said two IgG1 Fc variant polypeptides are identical or are different.

In some embodiments, the molecule has not more antibody effector functions compared to the identical molecule comprising, instead of the IgG1 Fc variant polypeptides of any of the previous embodiments, two IgG1 Fc variant polypeptides comprising the mutations L234A, L235A and P329G.

In some embodiments, the molecule is an antibody.

The present invention also provides the use of an IgG1 Fc variant polypeptide, comprising the mutations L234A, L235A, A327G, P329A, A330S, and P331S, according to EU numbering, to reduce one or more antibody effector functions.

The present invention also provides the use of a molecule which comprises two IgG1 Fc variant polypeptides, comprising the mutations L234A, L235A, A327G, P329A, A330S, and P331S, according to EU numbering, to reduce one or more antibody effector functions.

In some embodiments, one or more antibody effector functions are reduced compared to an identical IgG1 Fc variant polypeptide comprising the mutations L234A and L235, but not comprising the mutations A327G, P329A, A330S, and P331S.

In some embodiments, the reduction of one or more antibody effector functions comprises the reduction of one or more of complement dependent cytotoxicity (CDC), antibody-dependent cellular phagocytosis (ADCP), and antibody-dependent cellular cytotoxicity (ADCC).

In some embodiments, the reduction of one or more antibody effector functions comprises the reduction of CDC, ADCP, and ADCC.

In some embodiments, the IgG1 Fc variant polypeptide or the molecule displays no antibody effector function as measured by the CDC, ADCC, and ADCC assays as described in examples 2-5.

The present invention also provides a method of reducing one or more antibody effector functions of a molecule which comprises an IgG1 Fc polypeptide, comprising introducing the mutations L234A, L235A, A327G, P329A, A330S, and P331S into said IgG1 Fc polypeptide.

In some embodiments, said one or more reduced antibody effector functions comprise one or more of CDC, ADCC, and ADCC.

3. SHORT DESCRIPTION OF THE FIGURES

Figure 1: Sequences of the CH2 domains of IgG1, IgG4 and IgG1 LALA GASS.

The figure shows an alignment of the CH2 domain sequences of IgG1 wildtype (wt), IgG4 wt and an IgG1 Fc variant of the present invention (named "IgG1 LALA GASS") which comprises the mutations L234A, L235A, A327G, P329A, A330S, and P331S. The numbering is according to the EU numbering system (see, e.g., Edelman, G.M. et al., Proc. Natl. Acad. USA, 63, 78-85, 1969). Highlighted are the mutations L234A, L235A (in IgG1 LALA GASS) as well as the amino acids forming the CH2-FG-loop (in IgG1 wt and IgG4 wt) and the mutations A327G, P329A, A330S, and P331S (in IgG1 LALA GASS).

Figure 2: CDC assay for the IgG1 Fc variants LALA N297A and LALA P329A.

CDC was determined by measuring specific lysis conferred by anti-TNF antibodies comprising different IgG1 Fc variants. Plotted is the specific lysis (in %) against the employed antibody concentration (ng/mL, logarithmic scale). Tested were Ab1 (IgG1 wt, circle symbols), Ab7.1 (IgG1 LALA N297A, square symbols) and Ab7.2 (IgG1 LALA P329A, triangle symbols).

Figure 3: CDC assay for IgG1 LALA GASS and other IgG1/IgG4 variants.

CDC of the IgG1 Fc variant LALA GASS (see also Fig. 1) and other IgG1 or IgG4 Fc variants was measured in a cytotoxicity assay. Cell viability (in %) is plotted against antibody concentration (nM, logarithmic scale). Tested were the anti-TNF antibodies Ab1 (IgG1 wt), Ab2 (IgG1 LALA), Ab3 (IgG4 PE), Ab4 (IgG4 P-FALA), Ab5 (IgG1 NNAS), Ab6 (IgG1 LALA P329G), and Ab8 (IgG1 LALA GASS), as well as three negative controls of antibodies not binding to

TNF (Ab9 (anti-HER2 IgG1 wt), Ab16 (NIST IgG1 wt), anti-lysozyme IgG1 wt antibody). Dashed lines indicate determination of an EC₅₀ value.

Figure 4: High-sensitivity ADCP assay for IgG1 LALA GASS and other IgG1/IgG4 variants. ADCP of the IgG1 Fc variant LALA GASS (see also Fig. 1) and other IgG1 or IgG4 Fc variants was measured in two luciferase reporter assays. Determined was activation via FcγRIIa H131 (A) and FcγRI (B), fold luciferase induction is plotted against antibody concentration (nM, logarithmic scale). Tested were the anti-TNF antibodies Ab1 (IgG1 wt), Ab2 (IgG1 LALA), Ab3 (IgG4 PE), Ab4 (IgG4 P-FALA), Ab5 (IgG1 NNAS), Ab6 (IgG1 LALA P329G), and Ab8 (IgG1 LALA GASS), as well as three negative controls of antibodies not binding to TNF (Ab9 (anti-HER2 IgG1 wt), Ab16 (NIST IgG1 wt), anti-lysozyme IgG1 wt antibody). Dashed lines indicate determination of an EC₅₀ value.

Figure 5: High-sensitivity ADCC assay for IgG1 Fc variant LALA GASS and other IgG1/IgG4 Fc variants. ADCC of the IgG1 Fc variant LALA GASS (see also Fig. 1) and other IgG1 or IgG4 Fc variants was measured in a luciferase reporter assay. Determined was activation via FcγRIIIa V158, fold luciferase induction is plotted against antibody concentration (nM, logarithmic scale). Tested were the anti-TNF antibodies Ab1 (IgG1 wt), Ab2 (IgG1 LALA), Ab3 (IgG4 PE), Ab4 (IgG4 P-FALA), Ab5 (IgG1 NNAS), Ab6 (IgG1 LALA P329G), and Ab8 (IgG1 LALA GASS), as well as three negative controls of antibodies not binding to TNF (Ab9 (anti-HER2 IgG1 wt), Ab16 (NIST IgG1 wt), anti-lysozyme IgG1 wt antibody). Dashed lines indicate determination of an EC₅₀ value.

Figure 6: Pharmacokinetics of IgG1 LALA GASS. Pharmacokinetics of antibodies comprising the IgG1 Fc variant LALA GASS were compared to the same antibodies comprising instead IgG1 wt or the IgG1 Fc variant LALA. Antibody concentration (ng/mL) in the blood of mice is plotted against time after injection. Fig. 6A shows the results for the anti-TNF antibodies Ab1 (IgG1 wt, triangle), Ab2 (IgG1 LALA, diamonds), and Ab8 (IgG1 LALA GASS, circles). Fig. 6B shows the results for the anti-HER2 antibodies Ab9 (IgG1 wt, triangle), Ab10 (IgG1 LALA, diamonds), and Ab15 (IgG1 LALA GASS, circles).

4. DESCRIPTION OF THE INVENTION

The present invention provides IgG1 Fc variant polypeptides comprising the mutations L234A, L235A, A327G, P329A, A330S, and P331S, molecules comprising said IgG1 Fc variant polypeptides and their use to reduce antibody effector functions.

Advantages of the present invention

Compared to known IgG1 Fc variants, like the IgG1 Fc variant LALA, the IgG1 Fc variants of the present invention have the advantage that they do completely abolish antibody effector functions, i.e. they do not elicit CDC, ADCP or ADCC.

This lack of antibody effector functions can be partially contributed to the mutation at position 329. Position 329 is located in the FG-loop of the immunoglobulin fold of the CH2 domain. The proline at position 329 has been shown to be crucial for the interaction of the Fc with FcγRs and for the binding to complement factor C1q which provides an explanation for reduced antibody effector functions for variants which harbor mutations at position 329. However, not all mutations at position 329 have the same effect. While it was reported that the introduction of P329G in IgG1 LALA abolishes the antibody effector functions completely, P329A was linked to some residual effector functions (Schlothauer et al., Protein Eng Des Sel. 2016 Oct;29(10):457-466). The present invention overcomes those shortcomings of the P329A Fc variant by introducing the additional mutations A327G, A330S, and P331S in the IgG1 Fc variant LALA. The resulting IgG1 Fc variant "LALA GASS" showed no residual effector functions.

The IgG1 Fc variants of the present invention provide further advantageous properties. In contrast to IgG1 Fc variants which harbor single mutations in the FG loop (like P329G and P329A), IgG1 Fc variants of the present invention are considered to be less prone to immunogenicity because their FG-loop resembles the FG-loop of the natural occurring IgG4.

Furthermore, the IgG1 Fc variants of the present invention comprise IgG1 Fc regions. Compared to IgG4 Fc regions, IgG1 has advantages regarding conformational and colloidal stability, making IgG1 the most popular IgG1 Fc region for therapeutic antibodies.

Moreover, it has been shown that molecules comprising the IgG1 Fc variants of the present invention show no reduction in pharmacokinetics compared to IgG1 wt or the IgG1 Fc variant LALA.

Thus, the present invention provides novel stable IgG1 Fc variants, and molecules comprising the same, with no residual antibody effector functions and good pharmacokinetics.

4.1 Definitions

The term “IgG1 Fc” or “IgG1 Fc region”, as used herein interchangeably, is the constant region of an IgG1 antibody, but excluding CH1 and CL domains. Thus, IgG1 Fc is a protein complex which is formed by portions of two IgG1 heavy chain polypeptides and consists of two CH2 domains, two CH3 domains and the hinge region between CH1 and CH2 domains. The hinge region comprises disulfide bridges which link the two IgG1 heavy chain polypeptides. Although the exact boundaries of the Fc region may vary, typical boundaries for the human IgG1 Fc region are from residue C226 to the C-terminus of the IgG1 heavy chain or from P230 to the C-terminus of the IgG1 heavy chain, wherein the numbering is according to the EU numbering. “IgG1 Fc” or “IgG1 Fc region” may refer to the IgG1 Fc region in isolation, or in the context of a molecule comprising the Fc, for example an antibody.

The term “IgG1 Fc polypeptide”, as used herein, is a polypeptide which is capable of forming an IgG1 Fc, together with another IgG1 Fc polypeptide. The two IgG1 Fc polypeptides forming an IgG1 Fc can be either identical or different. An IgG1 Fc polypeptide comprises a CH2 domain, a CH3 domain and an amino acid sequence which can form a hinge region. An IgG1 Fc polypeptide can additionally comprise other domains or moieties.

An “IgG1 wildtype Fc polypeptide” or “IgG1 wt Fc polypeptide” is a IgG1 Fc polypeptide comprising the naturally occurring sequence of SEQ ID NO:30. The term “wildtype” is used correspondingly in other terms, for instance an “IgG1 wildtype Fc” is an IgG1 Fc formed by two IgG1 Fc polypeptides each comprising the sequence of SEQ ID NO:30.

An “IgG1 Fc variant polypeptide” is an IgG1 Fc polypeptide which comprises an amino acid sequence which differs from that of the sequence of the IgG1 wildtype (wt) Fc polypeptide (SEQ ID NO:30) by virtue of at least one amino acid substitution, also called “mutation”. The terms “amino acid” substitution and “mutation” are used herein interchangeably. Fig. 1 shows how an IgG1 Fc variant polypeptide of the present invention (IgG1 LALA GASS) is aligned with IgG1 wt Fc polypeptide to determine the number of mutations (6 mutations in the case of Fig. 1).

It should be noted that the IgG1 Fc variant polypeptides of the present invention share higher sequence identity with IgG1 wt Fc polypeptide than with other IgG wt Fc polypeptide, i.e. the

IgG1 Fc variant polypeptide of the present invention has higher sequence identity to the IgG1 wt Fc polypeptide of SEQ ID NO:30 than to the IgG2 wt Fc polypeptide, the IgG3 wt Fc polypeptide and the IgG4 wt Fc polypeptide. When the IgG1 wt Fc polypeptide of the present invention is a human IgG1 wt Fc polypeptide, it has higher sequence identity to the IgG1 wt Fc polypeptide of SEQ ID NO:30 than to the human IgG2 wt Fc polypeptide, the human IgG3 wt Fc polypeptide and the human IgG4 wt Fc polypeptide.

The term "antibody effector function" or "effector function" as used herein refers to a function contributed by an Fc region of an antibody, such as IgG. Such function can be effected by, for example, binding of an Fc region to an Fc receptor on an immune cell with phagocytic or cytolytic activity or by binding of an Fc region to components of the complement system. Antibody effector functions are ADCC, ADCP and CDC.

The term "antibody-dependent cell-mediated cytotoxicity" (ADCC) refers to a cell-mediated reaction in which nonspecific cytotoxic cells that express FcRs (e.g. Natural Killer (NK) cells, neutrophils, and macrophages) recognize bound antibody on a target cell and subsequently cause lysis of the target cell. The primary cells for mediating ADCC, NK cells, express FcγRIII only. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch, and Kinet, *Annu. Rev. Immunol* 9 (1991) 457-492.

The term "antibody-dependent cellular phagocytosis" (ADCP) refers to a process by which antibody-coated cells are internalized, either in whole or in part, by phagocytic immune cells (e.g., macrophages, neutrophils and dendritic cells) that bind to an immunoglobulin Fc region. Unlike antibody-dependent cell-mediated cytotoxicity (ADCC), which is mediated primarily through FcγRIIIa expressed on NK cells, ADCP can be mediated by monocytes, macrophages, neutrophil and dendritic cells mainly via FcγRIIa (CD32a) and FcγRI (CD64).

The term "complement-dependent cytotoxicity" (CDC) refers to an immune response in which target cells are lysed through activation and recruitment of the complement cascade to the targeted cell surface. CDC is mediated by binding of the protein C1q to the antibody Fc region. C1q together with two serine proteases, C1r and C1s, forms the complex C1, the first component of the complement dependent cytotoxicity (CDC) pathway.

Use of an IgG1 Fc variant polypeptide "to reduce" antibody effector functions means that said IgG1 Fc variant polypeptide is incorporated in a molecule and the molecule is exposed to a setting where antibody effector functions can potentially occur. The use is achieved if the antibody effector functions are reduced or completely abolished for the molecule comprising

said IgG1 Fc variant polypeptide, compared to the same molecule comprising instead an IgG1 wt Fc polypeptide or an IgG1 Fc variant LALA polypeptide.

Use of a molecule comprising an IgG1 Fc variant polypeptide “to reduce” antibody effector functions, as used herein, means that said molecule is exposed to a setting where antibody effector functions can potentially occur. The use is achieved if the antibody effector functions are reduced or completely abolished for the said molecule comprising an IgG1 Fc variant polypeptide, compared to the same molecule comprising instead an IgG1 wt Fc polypeptide or an IgG1 Fc variant LALA polypeptide. Said reduction of antibody effector function can be determined using the assays described in this disclosure as examples 2-5.

Throughout this application, the numbering of antibodies or antibody domains is according to EU numbering as established in Edelman, G.M. et al., Proc. Natl. Acad. USA, 63, 78-85, 1969, unless indicated otherwise. To provide a reference to other numbering systems, the mutations L234A, L235A, A327G, P329A, A330S, and P331S, according to EU numbering, are L247A, L248A, A346G, P348A, A349S, P350S, according to Kabat numbering.

The expression “does not consist of SEQ ID NO:31” means that the IgG1 Fc variant polypeptide is not just SEQ ID NO:31, without comprising any other amino acids. This feature does not mean that the IgG1 Fc variant polypeptide does not comprise SEQ ID:31 as part of its sequence, provided that the IgG1 Fc variant polypeptide further comprise additional amino acids.

For sake of brevity, the designations “IgG1 Fc variant LALA”, “IgG1 Fc LALA” and “IgG1 LALA” (and analog designations for other IgG Fc variants, e.g. “IgG1 Fc variant LALA GASS”/“IgG1 Fc LALA GASS”/“IgG1 LALA GASS”) are used interchangeably throughout this application. Similarly, “IgG1 wt” is used interchangeably with “IgG1 wt Fc region”.

4.2 Embodiments of the present invention

4.2.1 Mutations in the IgG1 Fc variant polypeptides

The present invention provides IgG1 Fc variant polypeptides and molecules comprising said IgG1 Fc variant polypeptides. The IgG1 Fc variant polypeptides comprise mutations compared to the IgG1 wt Fc polypeptide (SEQ ID NO:30). Those mutations comprise the 6 mutations L234A, L235A, A327G, P329A, A330S, and P331S, according to EU numbering. The IgG1 Fc variant polypeptide of the present invention can comprise further mutations. In some embodiments, the IgG1 Fc variant polypeptide comprises from 6 to 15 mutations compared to

the IgG1 wt Fc region polypeptide. In some embodiments, the IgG1 Fc variant polypeptide comprises from 6 to 12 mutations compared to the IgG1 wt Fc region polypeptide. In some embodiments, the IgG1 Fc variant polypeptide comprises from 6 to 10 mutations compared to the IgG1 wt Fc region polypeptide. In some embodiments, the IgG1 Fc variant polypeptide comprises 6 or 7 mutations compared to the IgG1 wt Fc region polypeptide. In some embodiments, the IgG1 Fc variant polypeptide comprises 6 mutations compared to the IgG1 wt Fc region polypeptide.

In some embodiments, the IgG1 Fc variant polypeptide possess at least about 90% sequence identity, at least about 95% sequence identity or at least about 97% sequence identity with the IgG1 wt Fc polypeptide of SEQ ID NO:30.

The IgG1 Fc variant polypeptides of the present invention do not comprise SEQ ID NO:28, which is the heavy chain of a Fab fragment disclosed in WO2022003169.

As SEQ ID NO:28 is a subsequence of SEQ ID NO:29, the IgG1 Fc variant polypeptides of the present invention do also not comprise SEQ ID NO:29 which is a heavy chain disclosed in WO2022003169.

In some embodiments, the IgG1 Fc variant polypeptide does not consist of SEQ ID NO:31. In some embodiments, the IgG1 Fc variant polypeptide comprises SEQ ID NO:31 and comprises one or more additional amino acids.

In some embodiments, the IgG1 Fc variant polypeptide is a human IgG1 Fc variant polypeptide, meaning that it comprises a variant of the human IgG1 wt Fc sequence (SEQ ID NO:30). In some embodiment, the IgG1 Fc variant polypeptide is a rabbit IgG1 Fc variant polypeptide, a guinea pig IgG1 Fc variant polypeptide, or a monkey IgG1 Fc variant polypeptide.

4.2.2 Further domains/moieties of the IgG1 Fc variant polypeptides

In some embodiments, the IgG1 Fc variant polypeptide further comprises one or more other domains or moieties, in addition to the CH2 domain, the CH3 domain, and the amino acid sequence which can form a hinge region. In some embodiments, the IgG1 Fc variant polypeptide further comprises a CH1 domain. In some embodiments, the IgG1 Fc variant polypeptide further comprises a CH1 and a VH domain. In some embodiments, the IgG1 Fc variant polypeptide further comprises a CH1 and a VH domain linked to a VL and CL domain, forming a single-chain fragment variable (scFv). In some embodiments, the IgG1 Fc variant polypeptide further comprises a CH1 domain and two VH domains. In some embodiments, the IgG1 Fc variant polypeptide further comprises a CH1 domain and two VH domains, forming

the heavy chain of a CODV. In some embodiments, the IgG1 Fc variant polypeptide further comprises an immunoglobulin single variable domain (ISVD). In some embodiments, the IgG1 Fc variant polypeptide further comprises a VHH. In some embodiments, the IgG1 Fc variant polypeptide further comprises a Nanobody® ISVD. In some embodiments, the IgG1 Fc variant polypeptide further comprises 2 Nanobody® ISVDs. In some embodiments, the IgG1 Fc variant polypeptide further comprises 3 Nanobody® ISVDs. In some embodiments, the IgG1 Fc variant polypeptide further comprises a VNAR.

The term “immunoglobulin single variable domain” (ISVD), interchangeably used with “single variable domain”, defines immunoglobulin molecules wherein the antigen binding site is present on, and formed by, a single immunoglobulin domain. This sets immunoglobulin single variable domains apart from “conventional” immunoglobulins (e.g. monoclonal antibodies) or their fragments (such as Fab, Fab', F(ab')₂, scFv, di-scFv), wherein two immunoglobulin domains, in particular two variable domains, interact to form an antigen binding site. Typically, in conventional immunoglobulins, a heavy chain variable domain (VH) and a light chain variable domain (VL) interact to form an antigen binding site. In this case, the complementarity determining regions (CDRs) of both VH and VL will contribute to the antigen binding site, i.e. a total of 6 CDRs will be involved in antigen binding site formation. In view of the above definition, the antigen-binding domain of a conventional 4-chain antibody (such as an IgG, IgM, IgA, IgD or IgE molecule; known in the art) or of a Fab fragment, a F(ab')₂ fragment, an Fv fragment such as a disulfide linked Fv or a scFv fragment, or a diabody (all known in the art) derived from such conventional 4-chain antibody, would normally not be regarded as an immunoglobulin single variable domain, as, in these cases, binding to the respective epitope of an antigen would normally not occur by one (single) immunoglobulin domain but by a pair of (associating) immunoglobulin domains such as light and heavy chain variable domains, i.e., by a VH-VL pair of immunoglobulin domains, which jointly bind to an epitope of the respective antigen.

In contrast, immunoglobulin single variable domains are capable of specifically binding to an epitope of the antigen without pairing with an additional immunoglobulin variable domain. The binding site of an immunoglobulin single variable domain is formed by a single VH, a single VHH or single VL domain. Hence, the antigen binding site of an immunoglobulin single variable domain is formed by no more than three CDRs.

As such, the single variable domain may be a light chain variable domain sequence (e.g., a VL-sequence) or a suitable fragment thereof; or a heavy chain variable domain sequence (e.g., a

VH-sequence or VHH sequence) or a suitable fragment thereof; as long as it is capable of forming a single antigen binding unit (i.e., a functional antigen binding unit that essentially consists of the single variable domain, such that the single antigen binding domain does not need to interact with another variable domain to form a functional antigen binding unit). An immunoglobulin single variable domain (ISVD) can for example be a heavy chain ISVD, such as a VH, VHH, including a camelized VH or humanized VHH. In one embodiment, it is a VHH, including a camelized VH or humanized VHH. Heavy chain ISVDs can be derived from a conventional four-chain antibody or from a heavy chain antibody.

For example, the immunoglobulin single variable domain may be a (single) domain antibody (or an amino acid sequence that is suitable for use as a single domain antibody), a "dAb" or dAb (or an amino acid sequence that is suitable for use as a dAb) or a Nanobody® ISVD; other single variable domains, or any suitable fragment of any one thereof.

In particular, the immunoglobulin single variable domain may be a Nanobody® ISVD (such as a VHH, including a humanized VHH or camelized VH) or a suitable fragment thereof. (Note: Nanobody® is a registered trademark of Ablynx N.V.)

In some embodiments, the IgG1 Fc variant polypeptide comprises an antigen-binding moiety on its N-terminus and an antigen-binding moiety on its C-terminus. In some embodiments, both antigen-binding moieties are Fab fragments. In some embodiments, one antigen-binding moiety is a Fab fragment and the other antigen binding moiety is an scFv. In some embodiments, one antigen-binding moiety is a Fab fragment and the other antigen binding moiety is an ISVD. In some embodiments, both antigen binding moieties are scFvs. In some embodiments, one antigen-binding moiety is an scFv and the other antigen binding moiety is an ISVD. In some embodiments, both antigen binding moieties are ISVDs.

In some embodiments, the IgG1 Fc variant comprises or is fused to another moiety. In some embodiments, the moiety is a molecule which exerts a biological function by interacting with a biological structure (e.g. a receptor for said molecule). In some embodiments, the moiety is a peptide. In some embodiments, the moiety is a receptor agonist. In some embodiments, the moiety is a receptor antagonist. In some embodiments, the moiety is an enzyme. In some embodiments, the moiety is a cytokine. In some embodiments, the moiety is a chemokine. In some embodiments, the moiety is an interferon. In some embodiments, the moiety is an interleukin. In some embodiments, the moiety is a lymphokine. In some embodiments, the moiety is a tumor necrosis factor. In some embodiments, the moiety is a toxin. In some

embodiments, the moiety is hormone. In some embodiments, the moiety is a growth factor. In some embodiments, the moiety is GLP1 or insulin.

4.2.3 Molecules of the present invention

The present invention provides molecules which comprise two IgG1 Fc variant polypeptides, at least one of them comprising the mutations L234A, L235A, A327G, P329A, A330S, and P331S, according to EU numbering, wherein the IgG1 Fc variant polypeptide does not comprise SEQ ID NO:28.

In some embodiments, the molecule comprises one IgG1 Fc variant polypeptide and one IgG1 wt Fc polypeptide. The IgG1 Fc variant polypeptide can be any of the IgG1 Fc variant polypeptides peptides described above, i.e. of the IgG1 Fc variant polypeptides comprising the 6 mutations L234A, L235A, A327G, P329A, A330S, and P331S, according to EU numbering, wherein the polypeptide, wherein the IgG1 Fc variant polypeptide does not comprise SEQ ID NO:28. The IgG1 wt Fc polypeptide is a polypeptide comprising SEQ ID NO:30.

In some embodiments, the molecule comprises two IgG1 Fc variant polypeptides. In some embodiments, both IgG1 Fc variant polypeptides are IgG1 Fc variant polypeptides comprising the 6 mutations L234A, L235A, A327G, P329A, A330S, and P331S, according to EU numbering, wherein the IgG1 Fc variant polypeptides do not comprise SEQ ID NO:28. The two IgG1 Fc variant polypeptides can be any of the IgG1 Fc variant polypeptides peptides described above. The two IgG1 Fc variant polypeptides can be identical or can be different. In some embodiments, the two IgG1 Fc variant polypeptides are identical. In some embodiments, the two IgG1 Fc variant polypeptides are different.

In some embodiments, the two IgG1 Fc variant polypeptides are identical and both further comprise one or more other domains or moieties, in addition to the CH2 domain, the CH3 domain, and the amino acid sequence which can form a hinge region. In some embodiments, the two IgG1 Fc variant polypeptides are identical and further comprise a CH1 domain. In some embodiments, the two IgG1 Fc variant polypeptides are identical and further comprise a CH1 and a VH domain. In some embodiments, the two IgG1 Fc variant polypeptides are identical and further comprise a CH1 and a VH domain linked to a VL and CL domain, forming an scFv. In some embodiments, the two IgG1 Fc variant polypeptides are identical and further comprise a CH1 domain and two VH domains. In some embodiments, the two IgG1 Fc variant polypeptides are identical and further comprise a CH1 domain and two VH domains, forming the heavy chain of a CODV. In some embodiments, the two IgG1 Fc variant polypeptides are

identical and further comprise an immunoglobulin single variable domain (ISVD). In some embodiments, the two IgG1 Fc variant polypeptides are identical and further comprise a VHH. In some embodiments, the two IgG1 Fc variant polypeptides are identical and further comprise a Nanobody® ISVD. In some embodiments, the two IgG1 Fc variant polypeptides are identical and further comprise 2 Nanobody® ISVDs. In some embodiments, the two IgG1 Fc variant polypeptides are identical and further comprise 3 Nanobody® ISVDs. In some embodiments, the two IgG1 Fc variant polypeptides are identical and further comprise a VNAR.

In some embodiments, the two IgG1 Fc variant polypeptides are different and one or both of them further comprise one or more other domains or moieties, in addition to the CH2 domain, the CH3 domain, and the amino acid sequence which can form a hinge region.

In some embodiments, the two IgG1 Fc variant polypeptides are different and both further comprise a CH1 domain. In some embodiments, the two IgG1 Fc variant polypeptides are different and both further comprise a CH1 and a VH domain, wherein those two VH domains are different. In some embodiments, the two IgG1 Fc variant polypeptides are different and one further comprises a CH1 and a VH domain while the other further comprises a CH1 and a VH domain linked to a VL and CL domain, forming an scFv. In some embodiments, the two IgG1 Fc variant polypeptides are different and one further comprises a CH1 and a VH domain while the other further comprises a CH1 domain and two VH domains, forming the heavy chain of a CODV. In some embodiments, the two IgG1 Fc variant polypeptides are different and one further comprises a CH1 and a VH domain while the other further comprises an ISVD. In some embodiments, the two IgG1 Fc variant polypeptides are different and one further comprises a CH1 and a VH domain while the other further comprises a VHH. In some embodiments, the two IgG1 Fc variant polypeptides are different and one further comprises a CH1 and a VH domain while the other further comprises an Nanobody® ISVDs. In some embodiments, the two IgG1 Fc variant polypeptides are different and one further comprises a CH1 and a VH domain while the other further comprises a VNAR. In some embodiments, the two IgG1 Fc variant polypeptides are different and one further comprises a CH1 and a VH domain while the other does not comprise an antigen-binding moiety. In some embodiments, the two IgG1 Fc variant polypeptides are different and one further comprises a CH1 and a VH domain while the other consists of SEQ ID NO:31.

In some embodiments, the two IgG1 Fc variant polypeptides are different and both further comprise an scFv, wherein those two scFvs are different. In some embodiments, the two IgG1

Fc variant polypeptides are different and one further comprises an scFv while the other further comprises a CH1 domain and two VH domains, forming the heavy chain of a CODV. In some embodiments, the two IgG1 Fc variant polypeptides are different and one further comprises an scFv while the other further comprises an ISVD. In some embodiments, the two IgG1 Fc variant polypeptides are different and one further comprises an scFv while the other further comprises a VHH. In some embodiments, the two IgG1 Fc variant polypeptides are different and one further comprises an scFv while the other further comprises an Nanobody® ISVD. In some embodiments, the two IgG1 Fc variant polypeptides are different and one further comprises an scFv while the other further comprises a VNAR. In some embodiments, the two IgG1 Fc variant polypeptides are different and one further comprises an scFv while the other does not comprise an antigen-binding moiety. In some embodiments, the two IgG1 Fc variant polypeptides are different and one further comprises an scFv while the other consists of SEQ ID NO:31.

In some embodiments, the two IgG1 Fc variant polypeptides are different and both further comprise a CH1 domain and two VH domains, forming the heavy chain of a CODV, wherein those two CODV heavy chains are different. In some embodiments, the two IgG1 Fc variant polypeptides are different and one further comprises a CH1 domain and two VH domains, forming the heavy chain of a CODV, while the other further comprises an ISVD. In some embodiments, the two IgG1 Fc variant polypeptides are different and one further comprises a CH1 domain and two VH domains, forming the heavy chain of a CODV, while the other further comprises a VHH. In some embodiments, the two IgG1 Fc variant polypeptides are different and one further comprises a CH1 domain and two VH domains, forming the heavy chain of a CODV, while the other further comprises an Nanobody® ISVD. In some embodiments, the two IgG1 Fc variant polypeptides are different and one further comprises a CH1 domain and two VH domains, forming the heavy chain of a CODV, while the other further comprises a VNAR. In some embodiments, the two IgG1 Fc variant polypeptides are different and one further comprises a CH1 domain and two VH domains, forming the heavy chain of a CODV, while the other does not comprise an antigen-binding moiety. In some embodiments, the two IgG1 Fc variant polypeptides are different and one further comprises a CH1 domain and two VH domains, forming the heavy chain of a CODV, while the other consists of SEQ ID NO:31.

In some embodiments, the two IgG1 Fc variant polypeptides are different and both further comprise an ISVD, wherein those two ISVDs are different. In some embodiments, one or two of those ISVDs are VHHs. In some embodiment, one or two of those ISVDs are Nanobody® ISVDs. In some embodiments, one or two of those ISVDs are VNARs. In some embodiments, the two IgG1 Fc variant polypeptides are different and one further comprises an ISVD while

the other does not comprise a antigen-binding moiety. In some embodiments, the two IgG1 Fc variant polypeptides are different and one further comprises an ISVD while the other consists of SEQ ID NO:31.

4.2.4 Antibodies

In some embodiments, the molecule of the present invention is an antibody. This means that the molecule comprises at least one antigen-binding moiety, such as a Fab, a scFv or an ISVD, e.g. a VHH, a Nanobody® ISVD or a VNAR. An antibody of the present invention comprises two IgG1 Fc variant polypeptide, wherein one or both IgG1 Fc variant polypeptide comprises the mutations L234A, L235A, A327G, P329A, A330S, and P331S, wherein the IgG1 Fc variant polypeptide does not comprise SEQ ID NO:28.

In some embodiments, an antibody of the present invention has the same polypeptide chain architecture as a wt IgG1, i.e. it consists of two heavy chains and two light chains. The two heavy chains are formed by the two IgG1 Fc variant polypeptides, wherein both IgG1 Fc variant polypeptides comprise a VH domain and a CH1 domain located N-terminally from the portion of the polypeptide which form the Fc region (hinge, CH2, CH3).

In some embodiments, an antibody of the present invention has the same polypeptide chain architecture as a wt IgG1 which lacks one of its Fab fragment, i.e. it consists of two heavy chains and one light chains. The two heavy chains are formed by the two IgG1 Fc variant polypeptides, wherein one IgG1 Fc variant polypeptide comprises a VH domain and a CH1 domain located N-terminally from the portion of the polypeptide which form the Fc region (hinge, CH2, CH3), while the other IgG1 Fc variant polypeptide does not comprise a VH domain or CH1 domain, but consists e.g. of SEQ ID NO:31.

4.2.5 Reduction of antibody effector functions

The IgG1 Fc variants and molecules of the present invention have reduced antibody effector functions compared to molecules comprising IgG1 wt Fc or IgG1 Fc variant LALA, instead of an IgG1 Fc variant of the present inventions. This means that one or more of the antibody effector functions CDC, ADCP and ADCC are reduced. In some embodiments, CDC, ADCP and ADCC are reduced.

In some embodiments, the molecules of the present invention have also reduced antibody effector functions compared to molecules comprising the IgG1 Fc variant LALA P329A, instead of an IgG1 Fc variant of the present inventions. This means that one or more of the

antibody effector functions CDC, ADCP and ADCC are reduced. In some embodiments, CDC is reduced. In some embodiments, CDC, ADCP and ADCC are reduced.

In some embodiments, the molecules of the present invention have not more antibody effector functions compared to molecules comprising the IgG1 Fc variant LALA P329G, instead of an IgG1 Fc variant of the present inventions. This means that all antibody effector functions CDC, ADCP and ADCC are either the same as for the IgG1 Fc variant LALA P329G or reduced. In some embodiments, CDC, ADCP and ADCC are the same. In some embodiments, one or more of CDC, ADCP and ADCC are reduced. In some embodiments, CDC, ADCP and ADCC are reduced.

Whether reduction of one or more antibody effector functions occurs can be determined according to the assays described in example 2 and 3 for CDC, example 4 for ADCP and example 5 for ADCC.

4.2.6 Use for reduction of antibody effector functions

The present invention provides the use of an IgG1 Fc variant polypeptide comprising the mutations L234A, L235A, A327G, P329A, A330S, and P331S, or a molecule comprising said Ig1 Fc variant polypeptide, to reduce one or more antibody effector functions. In such a use of the present invention, antibody effector functions are reduced compared to molecules comprising IgG1 wt Fc, instead of an IgG1 Fc variant comprising the mutations L234A, L235A, A327G, P329A, A330S, and P331S. In some embodiments, antibody effector functions are also reduced compared to molecules comprising the IgG1 Fc variant LALA, instead of an IgG1 Fc variant comprising the mutations L234A, L235A, A327G, P329A, A330S, and P331S.

In some embodiments the use comprises the reduction of one or more of CDC, ADCP, and ADCC. In some embodiments the use comprises the reduction of CDC, ADCP, and ADCC. In some embodiments the use comprises the reduction of CDC and ADCP. In some embodiments the use comprises the reduction of CDC and ADCC. In some embodiments the use comprises the reduction of ADCP, and ADCC. In some embodiments the use comprises the reduction of CDC. In some embodiments the use comprises the reduction of ADCP. In some embodiments the use comprises the reduction of ADCC.

Whether reduction of one or more antibody effector functions occurs can be determined according to the assays described in example 2 and 3 for CDC, example 4 for ADCP and example 5 for ADCC.

4.2.7 Methods of reducing one or more antibody effector functions

The present invention provides methods of reducing one or more antibody effector functions of a molecule which comprises an IgG1 Fc polypeptide, comprising introducing the mutations L234A, L235A, A327G, P329A, A330S, and P331S into said IgG1 Fc polypeptide. The introduction of the mutations can be done by modifying a nucleic acid encoding the IgG1 Fc polypeptide using commonly known methods. Reduction of antibody effector functions means that antibody effector functions are reduced compared to the molecules without the mutations L234A, L235A, A327G, P329A, A330S, and P331S, i.e. having the IgG1 wt amino acids at the respective positions.

In some embodiments the reduction comprises the reduction of one or more of CDC, ADCP, and ADCC. In some embodiments the use comprises the reduction of CDC, ADCP, and ADCC. In some embodiments the use comprises the reduction of CDC and ADCP. In some embodiments the use comprises the reduction of CDC and ADCC. In some embodiments the use comprises the reduction of ADCP, and ADCC. In some embodiments the use comprises the reduction of CDC. In some embodiments the use comprises the reduction of ADCP. In some embodiments the use comprises the reduction of ADCC.

Whether reduction of one or more antibody effector functions occurs can be determined according to the assays described in example 2 and 3 for CDC, example 4 for ADCP and example 5 for ADCC.

4.2.8 Polynucleotides, vectors, cell

In another aspect, the present invention relates to one or more polynucleotides encoding the IgG1 Fc variant polypeptides and molecules of the present invention. This refers to all embodiments described above. The one or more polynucleotides may also encode the one or more additional polypeptides comprised in a complex with the molecule of the present invention. In one embodiment, the one or more polynucleotides encode an antibody or antibody-like structure. In one embodiment, the one or more polynucleotides are isolated.

In another aspect, the present invention relates to one or more expression vectors comprising the one or more polynucleotides according to the previous aspect of the invention. The term "vector" as used herein refers to any molecule (e.g., nucleic acid, plasmid, or virus) that is used to transfer coding information to a host cell. The term "vector" includes a nucleic acid molecule that is capable of transporting another nucleic acid to which it has been fused. One type of vector is a "plasmid," which refers to a circular double-stranded DNA molecule into which additional DNA segments may be inserted. Another type of vector is a viral vector, wherein

additional DNA segments may be inserted into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell and thereby are replicated along with the host genome. In addition, certain vectors are capable of directing the expression of genes they comprise. Such vectors are referred to herein as "expression vectors".

In another aspect, the present invention relates to a cell comprising the one or more polynucleotides or the one more expression vectors according to the previous aspects of the invention. A wide variety of cell expression systems can be used to express said polynucleotides including the use of prokaryotic and eukaryotic cells, such as bacterial cells (e.g. *E. coli*), yeast cells, insect cells or mammalian cells (e.g. mouse cells, rat cells, human cells etc.). For this purpose, a cell is transformed or transfected with said polynucleotide(s) or expression vector(s) such that the polynucleotide(s) of the invention are expressed in the cell and, in one embodiment, secreted into the medium in which the cells are cultured, from where the expression product can be recovered.

5. EXAMPLES

Molecules of the present invention comprise an IgG1 Fc variant comprising the mutations L234A, L235A, A327G, P329A, A330S, and P331S, according to EU numbering. Those mutations are depicted in Fig. 1, the respective IgG1 Fc variant is named "LALA GASS". In the following examples, molecules comprising the IgG1 Fc variant LALA GASS were produced, characterized and compared to IgG1 wt and other IgG Fc variants.

Example 1: Protein expression, purification and characterization

Protein expression

Antibodies were expressed in transiently transfected HEK293 cells. DNA coding for the different variants were cloned into an expression vector under a CMV promoter and a leader sequence directing the proteins into the culture supernatant. For expression in HEK293 cells sequence SEQ ID 001 was used as leader sequence. For large scale expression, cells were grown in non-baffled shake flasks (Corning) at 110 rpm, 37°C and 8% CO₂ in Freestyle F17 medium (Gibco) supplemented with 6 mM glutamine and 0.02 % Pluronic F-68 (Gibco). At the time of

transfection, the cell density was approximately 1.2×10^6 cells/mL. Cell densities were determined with an automated cell counter (Nucleocounter NC-200, Chemometec, Allerød, Denmark). Before transfection, DNA was mixed with linear polyethyleneimine (PEI) at a ratio of 1:3 in Opti-MEM I-medium (Thermo Fisher Scientific). The transfection mixture was further incubated 10 min at room temperature and then added to the cell cultures. Cultivation was continued for 6 days. For large scale purification, cells were separated from the supernatants by centrifugation and the cell pellets were discarded. The $0.22 \mu\text{m}$ filtered supernatant was loaded on a protein A column (MabSelect Sure resin, GE Healthcare) equilibrated in phosphate buffered saline (PBS, Gibco). Mabs were eluted with 0.1 M citrate buffer, pH 3.0. Subsequently buffer was exchanged to PBS (Gibco) on a Sephadex G25 desalting column (GE Healthcare). Further purification was done using a Superdex 200 gel filtration column (GE healthcare) equilibrated in 10 mM histidine, 150 mM NaCl, pH 6.0. Corresponding fractions were pooled, concentrated, and stored until usage at 4°C or -80°C .

Table 1 provides an overview of the generated antibody variants and their respective sequences.

Table 1: Produced antibody variants.

Antibody	Target	IgG Fc variant	Mutations in Fc region (EU numbering)	SEQ ID NO Heavy chain	SEQ ID NO Light chain
Ab1	Anti-TNF	IgG1 Fc wildtype (wt)	none	SEQ ID NO:5	SEQ ID NO:2
Ab2	Anti-TNF	IgG1 Fc variant LALA	L234A, L235A	SEQ ID NO:6	SEQ ID NO:2
Ab3	Anti-TNF	IgG4 Fc variant PE	S228P/L235E	SEQ ID NO:7	SEQ ID NO:2
Ab4	Anti-TNF	IgG4 Fc variant P-FALA	S228P/F234A/L235A	SEQ ID NO:8	SEQ ID NO:2
Ab5	Anti-TNF	IgG1 Fc variant NNAS	S298N/T299A/Y300S	SEQ ID NO:9	SEQ ID NO:2
Ab6	Anti-TNF	IgG1 Fc variant LALA P329G	L234A, L235A, P329G	SEQ ID NO:10	SEQ ID NO:2
Ab7.1	Anti-TNF	IgG1 Fc variant LALA N297A	L234A, L235A, N297A	SEQ ID NO:26	SEQ ID NO:2

Ab7.2	Anti-TNF	IgG1 Fc variant LALA P329A	L234A, L235A, P329A	SEQ ID NO:27	SEQ ID NO:2
Ab8	Anti-TNF	IgG1 Fc variant LALA GASS	L234A, L235A, A327G, P329A, A330S, P331S	SEQ ID NO:11	SEQ ID NO:2
Ab9	Anti-HER2	IgG1 Fc wildtype (wt)	none	SEQ ID NO:12	SEQ ID NO:3
Ab10	Anti-HER2	IgG1 Fc variant LALA	L234A, L235A	SEQ ID NO:13	SEQ ID NO:3
Ab11	Anti-HER2	IgG4 Fc variant PE	S228P/L235E	SEQ ID NO:14	SEQ ID NO:3
Ab12	Anti-HER2	IgG4 Fc variant P-FALA	S228P/F234A/L235A	SEQ ID NO:15	SEQ ID NO:3
Ab13	Anti-HER2	IgG1 Fc variant NNAS	S298N/T299A/Y300S	SEQ ID NO:16	SEQ ID NO:3
Ab14	Anti-HER2	IgG1 Fc variant LALA P329G	L234A, L235A, P329G	SEQ ID NO:17	SEQ ID NO:3
Ab15	Anti-HER2	IgG1 Fc variant LALA GASS	L234A, L235A, A327G, P329A, A330S, P331S	SEQ ID NO:18	SEQ ID NO:3
Ab16	Unknown (NIST antibody)	IgG1 Fc wildtype (wt)	none	SEQ ID NO:19	SEQ ID NO:4
Ab17	Unknown (NIST antibody)	IgG1 Fc variant LALA	L234A, L235A	SEQ ID NO:20	SEQ ID NO:4
Ab18	Unknown (NIST antibody)	IgG4 Fc variant PE	S228P/L235E	SEQ ID NO:21	SEQ ID NO:4
Ab19	Unknown (NIST antibody)	IgG4 Fc variant P-FALA	S228P/F234A/L235A	SEQ ID NO:22	SEQ ID NO:4
Ab20	Unknown (NIST antibody)	IgG1 Fc variant NNAS	S298N/T299A/Y300S	SEQ ID NO:23	SEQ ID NO:4
Ab21	Unknown (NIST antibody)	IgG1 Fc variant LALA P329G	L234A, L235A, P329G	SEQ ID NO:24	SEQ ID NO:4

Ab22	Unknown (NIST antibody)	IgG1 Fc variant LALA GASS	L234A, L235A, A327G, P329A, A330S, P331S	SEQ ID NO:25	SEQ ID NO:4
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Protein characterization

Analytical size-exclusion chromatography (SEC)

Analytical SEC was performed using a BioSECcurity instrument (PSS Polymer) with an AdvanceBio 300 column (4.6 mm x 300 mm) and an AdvanceBio 300 guard column (Agilent Technologies). The analysis was performed at 25 °C with a flow rate of 0.5 ml/min using 2x concentrated D-PBS buffer (Thermo Fisher Scientific) with detection at 280 nm after loading 10 µl of protein sample (at 1 mg/ml) onto the column. Data evaluation was performed using Chromeleon 7 software (Thermo Fisher Scientific). For estimation of the molecular weight, the SEC column was calibrated with the AdvanceBio SEC 300Å Protein Standard (Agilent Technologies). Purity was calculated by dividing the area of the main peak by the combined area of all peaks.

Capillary gel electrophoresis (cGE)

The cGE was conducted according to the Protein Clear HR Assay guide provided by Perkin Elmer. Shortly: To prepare the chip, all assay components were allowed to equilibrate to room temperature. Protein Clear HR Gel matrix was partly mixed with Protein Clear HR Dye solution and filtered before adding to rinsed chip wells according to the manufacturer's instructions. For assay calibration the provided assay control VeriMAb was diluted in non-reducing sample buffer, denatured (70°C for 10 min) and subsequently diluted with water. The indicated volumes of VeriMAb assay control, Protein Clear HR Ladder and wash buffer were transferred into corresponding tubes and placed in LabChip GXII Touch instrument (Perkin Elmer). Calibration process was finished successfully before sample measurement. The samples were normalized to 0.5 mg/mL in water and 5 µL of each sample were added to 18 µL of non-reducing (8.75 mM f.c. Iodoacetamide) and reducing sample buffer (50 mM f.c. Dithiothreitol) in a PCR plate. The samples were denatured at 70°C for 10 min on a Thermostat C Thermoblock and subsequently diluted with 35 µL water before it was measured with the LabChip GXII Touch instrument (Perkin Elmer). Data analysis was performed using the LabChip Reviewer Software V5.8.84.0 (Perkin Elmer). All peaks with $\geq 0.85\%$ of relative peak

area were integrated. Purity was calculated by dividing the area of the main peak by the combined area of all peaks.

Table 2: Characterization of the antibodies comprising IgG Fc variants.

Antibody	Target	IgG Fc variant	Expression yield (mg/L)	SEC purity (%)	cGE purity (%) (non-reducing)
Ab1	Anti-TNF	IgG1 Fc wildtype (wt)	124,2	99,8	100,0
Ab2	Anti-TNF	IgG1 Fc variant LALA	80,7	99,8	100,0
Ab3	Anti-TNF	IgG4 Fc variant PE	107,8	99,8	98,6
Ab4	Anti-TNF	IgG4 Fc variant P-FALA	167,5	100,0	98,7
Ab5	Anti-TNF	IgG1 Fc variant NNAS	136,1	99,6	99,0
Ab6	Anti-TNF	IgG1 Fc variant LALA P329G	110,6	99,2	98,9
Ab8	Anti-TNF	IgG1 Fc variant LALA GASS	51,5	99,2	100,0
Ab9	Anti-HER2	IgG1 Fc wildtype (wt)	161,6	99,6	99,1
Ab10	Anti-HER2	IgG1 Fc variant LALA	117,6	99,6	99,1
Ab11	Anti-HER2	IgG4 Fc variant PE	187,3	99,8	98,2
Ab12	Anti-HER2	IgG4 Fc variant P-FALA	182,7	99,8	98,4
Ab13	Anti-HER2	IgG1 Fc variant NNAS	134,2	98,3	100,0
Ab14	Anti-HER2	IgG1 Fc variant	140,5	98,7	98,9

		LALA P329G			
Ab15	Anti-HER2	IgG1 Fc variant LALA GASS	147,7	99,1	99,0
Ab16	Unknown (NIST antibody)	IgG1 Fc wildtype (wt)	127,4	99,4	99,1
Ab17	Unknown (NIST antibody)	IgG1 Fc variant LALA	133,5	98,9	98,9
Ab18	Unknown (NIST antibody)	IgG4 Fc variant PE	183,0	98,5	98,5
Ab19	Unknown (NIST antibody)	IgG4 Fc variant P- FALA	139,8	99,5	98,3
Ab20	Unknown (NIST antibody)	IgG1 Fc variant NNAS	132,5	99,1	99,0
Ab21	Unknown (NIST antibody)	IgG1 Fc variant LALA P329G	63,4	99,1	98,9
Ab22	Unknown (NIST antibody)	IgG1 Fc variant LALA GASS	141,6	98,8	99,0

The antibodies Ab10, Ab14, and Ab15 were further analyzed to evaluate antibody target binding and stability.

Binding affinity determination

Binding affinities and kinetics were measured by Surface plasmon resonance (SPR) on a Biacore T200 instrument (Cytiva). For the affinity capture of the anti-HER2 protein samples, an anti-human Fc antibody (human antibody capture kit, Cytiva) was immobilized on a series S CM5 sensor chip (Cytiva) to approximately 10,000 RU. The anti-HER2 antibodies were diluted into HBS-EP+ assay buffer (Cytiva) to 0.15 µg/mL and injected in the sample flow cell (90 seconds, 10 µL/min), whereas the reference flow cell was used without captured antibody. After Fc affinity capture, the human HER2 antigen (Mat no 10004-H08H, Sino Biological) diluted into HBS-EP+ buffer, was injected in a 1:2 dilution series from 0.19 nM to 100 nM over the reference and sample flow cells. For the antigen inject the flow rate was adjusted to 30 µL/min with an association time of 240 seconds and a dissociation time of 1200 seconds. At

the end of each cycle an inject of regeneration solution (3M MgCl₂) was performed for 1 min at 30 μ L/min in order to remove antibody and antigen from the chip surface. Binding kinetics data were evaluated with a 1:1 binding model using the Biacore 8K Evaluation Software version (Cytiva).

Accelerated stress stability

To monitor changes in the chemical stability of the antibody samples, accelerated stress stability studies were performed. For the accelerated stress stability, the samples were buffer exchanged at room temperature by dialysis into either 10 mM sodium acetate buffer pH 5.0, or 10 mM sodium phosphate buffer pH 8.0 using a robotic system (BRAVO, Agilent). The concentration of the buffer exchanged samples was adjusted to 1 mg/mL and the samples were incubated for up to 21 days at 40 °C in an incubator. Control samples and samples after stress in the respective buffers were frozen at -80 °C before the analyses.

Determination of active fraction (see below) and SEC analysis (see above) were performed before (D0) and after (D21) exposure of the molecules to accelerated stress conditions.

Active fraction determination

Analysis of the active fraction of stressed antibodies was performed by SPR using the same Fc capture assay set up as described above for the affinity determination. Here, the association and dissociation times for human HER2 (Mat no 10004-H08H, Sino Biological) were set to 240 and 300 seconds, respectively. The active fraction of the samples was calculated as the ratio of binding signal for human HER2 and the Fc capture signal of the antibody, normalized to the active fraction of the unstressed control samples. Mean values from three experiments were calculated. The active fraction was determined at before (D0) and after (D21) the exposure of the molecules to accelerated stress conditions.

Table 3: Binding and stability of antibodies comprising IgG Fc variants.

Antibody	IgG Fc variant	Binding affinity to HER2			Active fraction (%)	
		K _a [1/Ms]	K _d [1/s]	K _D [nM]	D0	D21
Ab10	IgG1 Fc variant LALA	2.46 E+05	2.29 E-04	0.93	99.5	95.7
Ab14	IgG1 Fc variant LALA P329G	2.60 E+05	2.25 E-04	0.87	97.5	95.4
Ab15	IgG1 Fc variant LALA GASS	2.56 E+05	2.22 E-04	0.87	101.8	100.7

Antibody	IgG Fc variant	Chemical stability by SEC					
		% Monomer		%HMW		%LMW	
		D0	D21	D0	D21	D0	D21
Ab10	IgG1 Fc variant LALA	99.6	98.1	0.0	0.0	0.4	1.9
Ab14	IgG1 Fc variant LALA P329G	99.0	97.7	0.6	0.5	0.4	1.8
Ab15	IgG1 Fc variant LALA GASS	99.1	97.7	0.4	0.4	0.5	1.9

Example 2: CDC assay of IgG1 Fc variants with additional mutations

It is known that the IgG1 Fc variant LALA comprises some residual antibody effector functions. Thus, it was tested in a CDC assay whether the additional mutations N297A and P329A can completely abolish those effector functions. For this purpose, anti-TNF antibodies comprising IgG1 Fc variant LALA N297A (Ab7.1) or IgG1 Fc variant LALA P329A (Ab7.2) were tested in a complement dependent cytotoxicity assay as described in example 3 below. An anti-TNF antibody with IgG1 wt (Ab1) served as positive control.

In the CDC assay, IgG1 wt caused lysis of up to around 50%, IgG1 Fc variant LALA N297A resulted in lysis of up to 20%, while IgG1 Fc variant LALA P329A led to a lysis of around 10% (Fig. 2B). This indicates that the IgG1 Fc variants LALA N297A and LALA P329A possess still substantial residual CDC activity.

Example 3: Complement-dependent cytotoxicity (CDC) assay

IgG Fc variants were tested to identify Fc variants without residual effector functions. For this purpose, CDC of the IgG1 Fc variants of the present invention was evaluated in the following cytotoxicity assay in comparison to other IgG Fc variants. Cytotoxicity assays measure the ability of cytotoxic compounds to cause cell damage or cell death. As in example 2, anti-TNF antibodies comprising the respective IgG Fc variants were used.

For this CDC assay 3×10^3 engineered CHO-K1 target cells expressing membrane-bound human TNF per well (CHO-TNF clone MC2-1B10 with mutation resulting in the deletion of amino acids 77 to 88 of wild-type human pro-TNF) were plated in 96-well white/clear flat bottom plates. After an incubation step for 24 hours at 37°C, 5% CO₂ and 95% rH the target cells were treated with test antibodies in triplicate wells from 187.5 nM to 0.011 nM concentration in eight

serial 1:4 dilution steps and addition of 25 μ l/per well Guinea Pig serum complement (Millipore; #234395-5ml) equilibrated with Milli-Q water as described in the manufacturer's instructions. Incubation was done for 2 hours at 37°C, 5% CO₂ and 95% rH followed by an additional 24 hours incubation step at 37°C in 5% CO₂ incubator with addition of 7.5 μ l per well Cell Proliferation Reagent WST-1 (Roche; #05 015 944 001). The samples were measured in a Tecan Spark luminescence microplate reader for quantification of cellular viability.

EC₅₀ and the E_{max} values of the compounds were calculated by applying a Biostat-Speed statistical calculation tool (BIOST@T-SPEED-LTS2.6.1). Results were obtained using the 4-parameter logistic model according to Ratkovsky and Reedy (1986, Biometrics, Vol. 42: 575-582). The adjustment was obtained by non-linear regression using the Levenberg-Marquardt algorithm in SAS v9.4 under linux software.

Results

Ab1 (IgG1 wt) and Ab2 (IgG1 Fc variant LALA) were the only active molecules in this CDC assay (Table 7, Fig. 5). Ab 1 showed the highest activity (lower EC₅₀, lower E_{max} in viability corresponding to higher cell killing). All other tested molecules were fully inactive in this assay.

Table 4: Results of the CDC assay.

Molecule	IgG Fc variant	EC ₅₀ [nM]	E _{max}
Ab1 (Anti-TNF)	IgG1 wt	0.1	23.6
Ab2 Anti-TNF)	IgG1 Fc variant LALA	0.8	44.6
Ab3 (Anti-TNF)	IgG4 Fc variant PE	-	-
Ab4 (Anti-TNF)	IgG4 Fc variant P-FALA	-	-
Ab5 (Anti-TNF)	IgG1 Fc variant NNAS	-	-
Ab6 (Anti-TNF)	IgG1 Fc variant LALA P329G	-	-
Ab8 (Anti-TNF)	IgG1 Fc variant LALA GASS	-	-
Ab9 (Anti-HER2) (negative control)	IgG1 wt	-	-
Ab16 (NIST antibody) (negative control)	IgG1 wt	-	-
Anti-Lysozyme antibody (negative control)	IgG1 wt	-	-

Example 4: Antibody-dependent cellular phagocytosis (ADCP) assays

ADCP of the IgG1 Fc variants of the present invention was evaluated in two assays, one based on FcγRIIa binding, the other based on FcγRI binding. ADCP was determined in comparison to other IgG Fc variants. As in example 2 and 3, anti-TNF antibodies comprising the respective IgG Fc variants were used.

For the high sensitivity ADCP FcγRIIa assay 1×10^4 engineered CHO-K1 target cells expressing membrane-bound human TNF (CHO-TNF clone MC2-1B10 with mutation resulting in the deletion of amino acids 77 to 88 of wild-type human pro-TNF) per well were plated in 96-well white/clear flat bottom plates. After an incubation step for 24 hours at 37°C, 5% CO₂ and 95% rH the target cells were treated with test antibodies in triplicate wells from 125 nM to 0.49 nM concentration in nine serial 1:2 dilution steps and then co-cultured with 7.5×10^4 NFAT-luc2 Jurkat effector cells per well overexpressing the human FcγRIIa H131 high sensitivity variant (Promega; #G9871). The ratio of effector versus target cells was 7.5:1. An additional incubation step was done for 24 hours at 37°C, 5% CO₂ and 95% rH followed by readout using the Bio-Glo Luciferase Assay System (Promega; #G4790) and a Tecan Spark luminescence microplate reader. Assay buffers and medium were applied according to manufacturer's instructions.

For the ADCP FcγRI assay NFAT-luc2 Jurkat effector cells overexpressing the human FcγRI were used (Promega; #GA1341) instead of the overexpressing the human FcγRIIa NFAT-luc2 Jurkat effector cells. The cells were treated with test antibodies in triplicate wells from 125 nM to 0.02 nM concentration in nine serial 1:3 dilution steps. Same effector versus target cell ratio, incubation and readout conditions were applied as described for the ADCP FcγRIIa assay.

EC₅₀ and the E_{max} values of the compounds were calculated by applying a Biostat-Speed statistical calculation tool (BIOST@T-SPEED-LTS2.6.1). Results were obtained using the 4-parameter logistic model according to Ratkovsky and Reedy (1986, Biometrics, Vol. 42: 575-582). The adjustment was obtained by non-linear regression using the Levenberg-Marquardt algorithm in SAS v9.4 under linux software.

Results

In the ADCP FcγRIIa assay, Ab1 (IgG1 wt), Ab2 (IgG1 Fc variant LALA), Ab3 (IgG4 Fc variant PE), and Ab4 (IgG4 Fc variant P-FALA) showed similar EC₅₀ data (Table 5, Fig. 4A). Emax was highest for Ab1 and Ab3, and slightly reduced for Ab2 and Ab4. All other tested molecules were fully inactive in this assay.

Table 5: Results of the high sensitivity ADCP assay for FcγRIIa.

Molecule	IgG variant	EC50 [nM]	E _{max}
Ab1 (Anti-TNF)	IgG1 wt	8.0	3.6
Ab2 Anti-TNF)	IgG1 Fc variant LALA	8.0	2.5
Ab3 (Anti-TNF)	IgG4 Fc variant PE	8.9	4.1
Ab4 (Anti-TNF)	IgG4 Fc variant P-FALA	7.6	2.2
Ab5 (Anti-TNF)	IgG1 Fc variant NNAS	-	-
Ab6 (Anti-TNF)	IgG1 Fc variant LALA P329G	-	-
Ab8 (Anti-TNF)	IgG1 Fc variant LALA GASS	-	-
Ab9 (Anti-HER2) (negative control)	IgG1 wt	-	-
Ab16 (NIST antibody) (negative control)	IgG1 wt	-	-
Anti-Lysozyme antibody (negative control)	IgG1 wt	-	-

In the ADCP FcγRI assay, Ab1 (IgG1 wt), Ab2 (IgG1 Fc variant LALA), Ab3 (IgG4 Fc variant PE), and Ab4 (IgG4 Fc variant P-FALA) and Ab5 (IgG1 Fc variant NNAS) showed similar EC₅₀ data (Table 6, Fig. 4B). Compared to Ab1, the variants Ab2, Ab3, and Ab4 showed a slightly reduced E_{max}, while E_{max} of Ab5 was strongly reduced. All other tested molecules were fully inactive in this assay. This means that only the IgG1 Fc variants LALA P329G and LALA GASS were fully silent regarding FcγRI binding in this assay.

Table 6: Results of the ADCP assay for FcγRI.

Molecule	IgG Fc variant	EC50 [nM]	E _{max}
Ab1 (Anti-TNF)	IgG1 wt	4.3	17.7
Ab2 Anti-TNF)	IgG1 Fc variant LALA	5.3	13.5
Ab3 (Anti-TNF)	IgG4 Fc variant PE	5.2	9.9
Ab4 (Anti-TNF)	IgG4 Fc variant P-FALA	4.9	10.4
Ab5 (Anti-TNF)	IgG1 Fc variant NNAS	5.2	3.2
Ab6 (Anti-TNF)	IgG1 Fc variant LALA P329G	-	-
Ab8 (Anti-TNF)	IgG1 Fc variant LALA GASS	-	-
Ab9 (Anti-HER2) (negative control)	IgG1 wt	-	-

Ab16 (NIST antibody) (negative control)	IgG1 wt	-	-
Anti-Lysozyme antibody (negative control)	IgG1 wt	-	-

To sum it up, ADCP activity of IgG1 Fc variant LALA and the two IgG4 Fc variants were only slightly reduced compared to IgG1 wt. IgG1 Fc variant NNAS showed weak residual activity in the FcγRI assay and no activity in the FcγRIIa assay. No ADCP activity was detected for the IgG1 Fc variants LALA P329G and LALA GASS.

Example 5: High-sensitivity antibody-dependent cellular cytotoxicity (ADCC) assay

ADCC of the IgG1 Fc variants of the present invention was evaluated in a high-sensitive ADCC assay in comparison to other IgG Fc variants. As in examples 2-4, anti-TNF antibodies comprising the respective IgG Fc variants were used.

The ADCC Reporter Bioassay was used which is a bioluminescent reporter assay for quantifying biological activity on pathway activation by therapeutic antibody drugs in an ADCC mechanism of action (MOA) assay. For this ADCC assay 1×10^4 engineered CHO-K1 target cells expressing membrane-bound human TNF (CHO-TNF clone MC2-1B10 with mutation resulting in the deletion of amino acids 77 to 88 of wild-type human pro-TNF) per well were plated in 96-well white/clear flat bottom plates. After an incubation step for 24 hours at 37°C, 5% CO₂ and 95% rH the target cells were treated with test antibodies in triplicate wells from 250 nM to 0.04 nM concentration in nine serial 1:3 dilution steps and then co-cultured with 7.5×10^4 NFAT-luc2 Jurkat effector cells per well overexpressing the human FcγRIIIa V158 high sensitivity variant (Promega; #G7102). The ratio of effector versus target cells was 7.5:1. An additional incubation step was done for 24 hours at 37°C, 5% CO₂ and 95% rH followed by readout using the Bio-Glo Luciferase Assay System (Promega; #G4790) and a Tecan Spark luminescence microplate reader. Assay buffers and medium were applied according to manufacturer's instructions.

EC₅₀ and the E_{max} values of the compounds were calculated by applying a Biostat-Speed statistical calculation tool (BIOSTAT@T-SPEED-LTS2.6.1). Results were obtained using the 4-parameter logistic model according to Ratkovsky and Reedy (1986, Biometrics, Vol. 42: 575-582). The adjustment was obtained by non-linear regression using the Levenberg-Marquardt algorithm in SAS v9.4 under linux software.

Results

The anti-TNF antibodies with gG1 wt and with IgG1 Fc variant LALA were the only active molecules in this ADCC assay (Table 4, Fig. 3). Ab1 (IgG1 wt) showed much higher ADCC activity than Ab2 (IgG1 Fc variant LALA). All other tested molecules were fully inactive in this assay.

Table 7: Results of the high sensitivity ADCC assay.

Molecule	IgG variant	EC50 [nM]	E _{max}
Ab1 (Anti-TNF)	IgG1 wt	4.1	25.4
Ab2 Anti-TNF)	IgG1 Fc variant LALA	4.7	2.5
Ab3 (Anti-TNF)	IgG4 Fc variant PE	-	-
Ab4 (Anti-TNF)	IgG4 Fc variant P-FALA	-	-
Ab5 (Anti-TNF)	IgG1 Fc variant NNAS	-	-
Ab6 (Anti-TNF)	IgG1 Fc variant LALA P329G	-	-
Ab8 (Anti-TNF)	IgG1 Fc variant LALA GASS	-	-
Ab9 (Anti-HER2) (negative control)	IgG1 wt	-	-
Ab16 (NIST antibody) (negative control)	IgG1 wt	-	-
Anti-Lysozyme antibody (negative control)	IgG1 wt	-	-

Example 6: summary of antibody effector functions

The summarized results of the CDC assay (example 3), the ADCP FcγRIIIa assay (example 4), the ADCP FcγRI assay (example 4) and the ADCC FcγRIIIa assay (example 5) are shown in the table below. The compounds are only classified as “active” (+) or “not-active” (-), corresponding to the results in the respective assays.

It was found that IgG1 LALA showed substantial residual ADCC, ADCP, and CDC activity. The IgG4 Fc variants PE and P-FALA display some residual ADCP activity. For IgG1 Fc variant NNAS, weak residual activity was detected in the ADCP FcγRI assay. Only IgG1 Fc variant LALA P329G and IgG1 Fc variant LALA GASS did not confer any antibody effector function in any assay.

Table 8: Combined results of ADCC, ADCP, and CDC assays.

Molecule	IgG Fc variant	assay			
		CDC	ADCP FcγRIIa	ADCP FcγRI	ADCC FcγRIIIa
Ab1 (Anti-TNF)	IgG1 wt	+	+	+	+
Ab2 Anti-TNF)	IgG1 Fc variant LALA	+	+	+	+
Ab3 (Anti-TNF)	IgG4 Fc variant PE	-	+	+	-
Ab4 (Anti-TNF)	IgG4 Fc variant P-FALA	-	+	+	-
Ab5 (Anti-TNF)	IgG1 Fc variant NNAS	-	-	+	-
Ab6 (Anti-TNF)	IgG1 Fc variant LALA P329G	-	-	-	-
Ab8 (Anti-TNF)	IgG1 Fc variant LALA GASS	-	-	-	-

Example 7: Pharmacokinetics

In Vivo Pharmacokinetics

An *in vivo* pharmacokinetics (PK) study was performed using treatment naïve 8-10 week old male hFcRn Tg32 homozygous mice (The Jackson Laboratory, #014565 M01). The PK study was conducted at Charles River Labs (CRL) Worcester, MA, USA and was designed and executed within accordance of the Animal Use Protocol (AUP) and adherence to CRLs institutional animal care and use committee (IACUC) regulations. At time=0hrs all treatment groups (groups 1-6, 3 animals per group) were administered with a single intravenous dose of 5 mg/kg (1mg/mL solution and dosing volume of 5 mL/kg) of respective molecules. Serial blood samples were collected in tubes containing K2EDTA and processed to plasma at regular timepoints over a period of 504 hrs starting at 0.25 hrs post dose, with an additional terminal sample at 672 hrs for each animal to harvest plasma. Quantitative analysis of hIgG in plasma samples was performed using a targeted LC-MS/MS approach as described below. PK parameter estimates were determined from mean animal data per group using non-compartmental analysis in WinNonlin (Phoenix version 8.2) using the plasma data module with

linear up log down interpolation. Concentration values below the limit of quantitation (BLQ) were excluded from PK calculations.

Bioanalytical methods

For the quantification of targeted proteins, the samples were first immunoprecipitated using streptavidin beads and biotinylated antibody to capture the target analyte and subsequently analyzed using trypsin digestion-LC-MS/MS method by measuring surrogate peptides. For immunoprecipitation, 25 μ L plasma sample was incubated with 10 μ L of goat anti-human IgG biotinylated antibody and 10 μ L of streptavidin binding magnetic beads in 350 μ L of 1xPBST for 2 hours. At the end of incubation period the beads were washed twice with 1xPBST and once with 1X PBS before eluting in 100 μ L of 25mM HCL for 15 mins. Digestion was performed overnight at 37 °C and 750 RPM on a Thermomixer by the addition of 10 μ L of trypsin (100 μ g/mL) in presence of 25% acetonitrile in TRIS buffer containing 5 mM CaCl₂. The digestion was stopped by adding 10 μ L of 10% formic acid in water.

Sciex QTRAP 6500+ was used for analysis of surrogate peptides from digestion samples. Chromatographic separation of peptides was carried out on an Ascentis® Express Peptide ES-C18, (75x2.1 mm, 2.7 μ m) column using a gradient elution with the mobile phase A (0.1% formic acid in deionized water) and B (98: 2 Acetonitrile: DMSO) as follows: 0-0.5min 5% B; 0.5-2.5min 5%-40% B; 2.5-2.6 min 40%-90% B; 2.6-3.0min 90% B; 3.0-3.1 90%-5% B; 3.1-4.5min 5% B and stop at 4.5 min. The total running time was 4.5 minutes with an injection volume of 75 μ L. Mass transitions of 593.8/699.4 (Ab1 (IgG1 wt)), 593.8/418.2 (Ab2 (IgG1 LALA)), 593.8/418.2 (Ab8 (IgG1 LALA-GASS)), 593.8/699.4 (Ab9 (IgG1 wt)), 593.8/846.5 (Ab10 (IgG1 LALA)), and 593.8/418.2 (Ab15 (IgG1 LALA-GASS)) were used to monitor surrogate peptides with a linear range 20.0-10240 ng/mL.

The pharmacokinetics of antibodies comprising the IgG1 Fc variant LALA GASS did not differ from antibodies comprising the IgG1 wt or the IgG1 Fc variant LALA.

References

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- Ravetch and Kinet Annu. Rev. Immunol 9 (1991) 457-492

Sequences

SEQ ID NO:1 (Signal sequence, UniProt P01749, Ighv1-61, HVM05_MOUSE, Aa 1-19)

MGWSCILFLVATATGVHS

SEQ ID NO:2 (anti-TNF α , light chain)

DIQMTQSPSSLSASVGDRVTITCRASQGIRNYLAWYQQKPGKAPKLLIYAASLTQSGV
PSRFGSGSGTDFTLTISSLQPEDVATYYCQRYNRAPYTFGGGTKVEIKRTVAAPSVFI
FPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYS
LSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO:3 (anti-Her2, light chain)

DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSG
VPSRFGSGSGTDFTLTISSLQPEDFATYYCQQHYYTPPTFGGGTKVEIKRTVAAPSVFI
FPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYS
LSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO:4 (NIST, light chain)

DIQMTQSPSTLSASVGDRVTITCSASSRVGYMHWYQQKPGKAPKLLIYDTSKLASGV
PSRFGSGSGTEFTLTISSLQPDDFATYYCFQGSQYPFTFGGGTKVEIKRTVAAPSVFI
PPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSL
SSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO:5 (anti-TNF α -huIgG1, heavy chain)

EVQLVESGGGLVQPGRSLRLSCAASGFTFDDYAMHWVRQAPGKGLEWVSAITWNS
GHIDYADSVEGRFTISRDNANKSLYLQMNSLRAEDTAVYYCAKVSYLSTASSLDYW
GQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS
GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKT
HTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDG
VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTIS
KAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTT
PPVLDSDGSFFLYSKLTVDKSRWQQGNVVFSCSVMHREALHNYHTQKSLSLSPG

SEQ ID NO:6 (anti-TNF α -huIgG1(LALA), heavy chain)

EVQLVESGGGLVQPGRSLRLSCAASGFTFDDYAMHWVRQAPGKGLEWVSAITWNS
GHIDYADSVEGRFTISRDNANKSLYLQMNSLRAEDTAVYYCAKVSYLSTASSLDYW
GQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS
GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKT
HTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD

GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI
SKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKT
TPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPG

SEQ ID NO:7 (anti-TNFa-huIgG4(PE), heavy chain)

EVQLVESGGGLVQPGRSLRLSCAASGFTFDDYAMHWVRQAPGKGLEWVSAITWNS
GHIDYADSVGRFTISRDNANKNSLYLQMNSLRAEDTAVYYCAKVSYLSTASSLDYW
GQGTLLTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTS
GVHTFPAVLQSSGLYSLSSVTVPSSSLGKTITTCNVDHKPSNTKVDKRVESKYGPP
CPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVE
VHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAK
GQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPV
LDSDGSFFLYSRLTVDKSRWQEGNVFCFSVMHEALHNHYTQKSLSLSLGL

SEQ ID NO:8 (anti-TNFa-huIgG4(P-FALA), heavy chain)

EVQLVESGGGLVQPGRSLRLSCAASGFTFDDYAMHWVRQAPGKGLEWVSAITWNS
GHIDYADSVGRFTISRDNANKNSLYLQMNSLRAEDTAVYYCAKVSYLSTASSLDYW
GQGTLLTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTS
GVHTFPAVLQSSGLYSLSSVTVPSSSLGKTITTCNVDHKPSNTKVDKRVESKYGPP
CPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGV
EVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKA
KGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP
VLSDSDGSFFLYSRLTVDKSRWQEGNVFCFSVMHEALHNHYTQKSLSLSLGL

SEQ ID NO:9 (anti-TNFa-huIgG1(NNAS), heavy chain)

EVQLVESGGGLVQPGRSLRLSCAASGFTFDDYAMHWVRQAPGKGLEWVSAITWNS
GHIDYADSVGRFTISRDNANKNSLYLQMNSLRAEDTAVYYCAKVSYLSTASSLDYW
GQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS
GVHTFPAVLQSSGLYSLSSVTVPSSSLGKTQTYICNVNHKPSNTKVDKKVEPKSCDKT
HTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDG
VEVHNAKTKPREEQYNNASRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTIS
KAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
PPVLSDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPG

SEQ ID NO:10 (anti-TNFa-huIgG1(LALA) P329G, heavy chain)

EVQLVESGGGLVQPGRSLRLSCAASGFTFDDYAMHWVRQAPGKGLEWVSAITWNS
GHIDYADSVGRFTISRDNANKNSLYLQMNSLRAEDTAVYYCAKVSYLSTASSLDYW
GQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS
GVHTFPAVLQSSGLYSLSSVTVPSSSLGKTQTYICNVNHKPSNTKVDKKVEPKSCDKT
HTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD
GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTI

SKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKT
TPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPG

SEQ ID NO:11 (anti-TNF α -huIgG1(LALA-GASS), heavy chain)

EVQLVESGGGLVQPGRSLRLSCAASGFTFDDYAMHWVRQAPGKGLEWVSITWNS
GHIDYADSVGRFTISRDNANKNSLYLQMNSLRAEDTAVYYCAKVSYLSTASSLDYW
GQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS
GVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKT
HTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD
GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLASSIEKTI
SKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKT
TPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPG

SEQ ID NO:12 (anti-HER2-huIgG1, heavy chain)

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGY
TRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQ
GTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS
GVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT
CPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVE
VHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA
KGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP
VLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPG

SEQ ID NO:13 (anti-HER2-huIgG1(LALA), heavy chain)

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGY
TRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQ
GTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS
GVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT
CPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV
EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISK
AKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
PVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPG

SEQ ID NO:14 (anti-HER2-huIgG4(PE), heavy chain)

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGY
TRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQ
GTLVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTS
GVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYTCNVNHHKPSNTKVDKRVESKYGPPCP
CPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVH
NAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQ
PREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLD
SDGSFFLYSRLTVDKSRWQEGNVFCFSVMHEALHNHYTQKSLSLSLG

SEQ ID NO:15 (anti-HER2-huIgG4(P-FALA), heavy chain)

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGY
TRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQ
GTLVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGV
HTFPAVLQSSGLYSLSSVTVPSSSLGTQTYTCNVDPKPSNTKVDKRVESKYGPPCPP
CPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVH
NAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQ
PREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPVLDS
DGSFFLYSRLTVDKSRWQEGNVFSCSVMEALHNHYTQKSLSLSLG

SEQ ID NO:16 (anti-HER2-huIgG1(NNAS), heavy chain)

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGY
TRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQ
GTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGV
HTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT
CPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVE
VHNAKTKPREEQYNNASRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA
KGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
VLDS DGSFFLYSKLTVDKSRWQQGNVFSCSVMEALHNHYTQKSLSLSPG

SEQ ID NO:17 (anti-HER2-huIgG1(LALA) P329G, heavy chain)

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGY
TRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQ
GTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGV
HTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT
CPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV
EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISK
AKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
PVLDS DGSFFLYSKLTVDKSRWQQGNVFSCSVMEALHNHYTQKSLSLSPG

SEQ ID NO:18 (anti-HER2-huIgG1(LALA-GASS), heavy chain)

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGY
TRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQ
GTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGV
HTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT
CPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV
EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLASSIEKTISK
AKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
PVLDS DGSFFLYSKLTVDKSRWQQGNVFSCSVMEALHNHYTQKSLSLSPG

SEQ ID NO:19 ((NIST8671)-huIgG1, heavy chain)

QVTLRESGPALVKPTQTLTLTCTFSGFSLSTAGMSVGWIRQPPGKALEWLADIWWDD
KKHYNPSLKDRLTISKDTSKNQVVLKVTNMDPADTATYYCARDMIFNFYFDVWGQ
GTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGV
HTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT
CPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVE
VHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA
KGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPP
VLDSGDSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG

SEQ ID NO:20 ((NIST8671)-huIgG1(LALA), heavy chain)

QVTLRESGPALVKPTQTLTLTCTFSGFSLSTAGMSVGWIRQPPGKALEWLADIWWDD
KKHYNPSLKDRLTISKDTSKNQVVLKVTNMDPADTATYYCARDMIFNFYFDVWGQ
GTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGV
HTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT
CPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGV
EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISK
AKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTP
PVLDSGDSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG

SEQ ID NO:21 ((NIST8671)-huIgG4(PE), heavy chain)

QVTLRESGPALVKPTQTLTLTCTFSGFSLSTAGMSVGWIRQPPGKALEWLADIWWDD
KKHYNPSLKDRLTISKDTSKNQVVLKVTNMDPADTATYYCARDMIFNFYFDVWGQ
GTTVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGV
HTFPAVLQSSGLYSLSSVTVPSSSLGKTYTCNVDHKPSNTKVDKRVESKYGPCCPP
CPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVDVVSQEDPEVQFNWYVDGVEVH
NAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQ
PREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDS
DGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLG

SEQ ID NO:22 ((NIST8671)-huIgG4(P-FALA), heavy chain)

QVTLRESGPALVKPTQTLTLTCTFSGFSLSTAGMSVGWIRQPPGKALEWLADIWWDD
KKHYNPSLKDRLTISKDTSKNQVVLKVTNMDPADTATYYCARDMIFNFYFDVWGQ
GTTVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGV
HTFPAVLQSSGLYSLSSVTVPSSSLGKTYTCNVDHKPSNTKVDKRVESKYGPCCPP
CPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVDVVSQEDPEVQFNWYVDGVEVH
NAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQ
PREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDS
DGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLG

SEQ ID NO:23 ((NIST8671)-huIgG1(NNAS), heavy chain)

QVTLRESGPALVKPTQTLTLCTFSGFSLSTAGMSVGWIRQPPGKALEWLADIWWDD
KKHYNPSLKDRLTISKDTSKNQVVLKVTNMDPADTATYYCARDMIFNFYFDVWGQ
GTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGV
HTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT
CPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVE
VHNAKTKPREEQYNNASRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA
KGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
VLDSGDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPG

SEQ ID NO:24 ((NIST8671)-huIgG1(LALA) P329G, heavy chain)

QVTLRESGPALVKPTQTLTLCTFSGFSLSTAGMSVGWIRQPPGKALEWLADIWWDD
KKHYNPSLKDRLTISKDTSKNQVVLKVTNMDPADTATYYCARDMIFNFYFDVWGQ
GTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGV
HTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT
CPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV
EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISK
AKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
PVLDSGDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPG

SEQ ID NO:25 ((NIST8671)-huIgG1(LALA-GASS), heavy chain)

QVTLRESGPALVKPTQTLTLCTFSGFSLSTAGMSVGWIRQPPGKALEWLADIWWDD
KKHYNPSLKDRLTISKDTSKNQVVLKVTNMDPADTATYYCARDMIFNFYFDVWGQ
GTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGV
HTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHT
CPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV
EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLASSIEKTISK
AKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
PVLDSGDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPG

SEQ ID NO: 26 (anti-TNF α -huIgG1(LALA) N297A, heavy chain)

EVQLVESGGGLVQPGRSLRLSCAASGFTFDDYAMHWVRQAPGKGLEWVSAITWNS
GHIDYADSVGRFTISRDNANKNSLYLQMNSLRAEDTAVYYCAKVSYLSTASSLDYW
GQGTLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS
GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKT
HTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD
GVEVHNAKTKPREEQYASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI
SKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKT
TPPVLDSGDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPG

SEQ ID NO: 27 (anti-TNF α -huIgG1(LALA) P329A, heavy chain)

EVQLVESGGGLVQPGRSLRLSCAASGFTFDDYAMHWVRQAPGKGLEWVSAITWNS
GHIDYADSVGRFTISRDNANKNSLYLQMNSLRAEDTAVYYCAKVSYLSTASSLDYW

GQGT LVT VSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT VSWNSGALTS
GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKV DKKVEPKSCDKT
HTCPPCPAPEAAGGPSVF LFPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD
GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALAAPIEKT
SKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKT
TPPVLDSDGSFFLYSKLTVDKSRWQQGNV FSCSVMHEALHNHYTQKSLSLSPG

SEQ ID NO:28 (anti-FGFG1-16H7 VH-CH1)

QVTLKESGPVLVKPTETLTLTCTVSGFSLNNARMGVSWIRQPPGKALEWLAHIFSND
EKSYSTSLKSRLTISKDTSKSQVVLIMTNMDPVD TATYYCARSVVTGGYY YDGMDV
WGQGT TTVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVT VSWNSGALT
SGVHTFPAVLQSSGLYSLSSVVTVPSSNFGTQTYTCNV DHKPSNTKV DKT V

SEQ ID NO: 29 (anti-FGFR1-16H7 huIgG1(LALA) GASS, heavy chain)

QVTLKESGPVLVKPTETLTLTCTVSGFSLNNARMGVSWIRQPPGKALEWLAHIFSND
EKSYSTSLKSRLTISKDTSKSQVVLIMTNMDPVD TATYYCARSVVTGGYY YDGMDV
WGQGT TTVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVT VSWNSGALT
SGVHTFPAVLQSSGLYSLSSVVTVPSSNFGTQTYTCNV DHKPSNTKV DKT VEPKSCD
KTHTCPPCPAPEAAGGPSVF LFPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV
DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLASSIEKT
ISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKT
TPPVLDSDGSFFLYSKLTVDKSRWQQGNV FSCSVMHEALHNHYTQKSLSLSPG

SEQ ID NO: 30 (IgG1 wt Fc)

EPKSCDKTHTCPPCPAPELLGGPSVFLFPKPKDTLMISRTPEVTCVVVDVSHEDPEVK
FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP
APIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPE
NNYKTTTPPVLDSDGSFFLYSKLTVDKSRWQQGNV FSCSVMHEALHNHYTQKSLSLSP
G

SEQ ID NO: 31 (huIgG1(LALA) GASS Fc)

EPKSCDKTHTCPPCPAPEAAGGPSVFLFPKPKDTLMISRTPEVTCVVVDVSHEDPEV
KFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKG
LASSIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQ
PENNYKTTTPPVLDSDGSFFLYSKLTVDKSRWQQGNV FSCSVMHEALHNHYTQKSLSL
SPG

CLAIMS

1. An IgG1 Fc variant polypeptide, wherein said IgG1 Fc variant polypeptide comprises the mutations L234A, L235A, A327G, P329A, A330S, and P331S, according to EU numbering, wherein the IgG1 Fc variant polypeptide does not comprise SEQ ID NO:28.
2. The IgG1 Fc variant polypeptide of claim 1, wherein the IgG1 Fc variant polypeptide does not consist of SEQ ID NO:31.
3. The IgG1 Fc variant polypeptide of claim 1 or 2, wherein the IgG1 Fc variant polypeptide is a human IgG1 Fc variant polypeptide.
4. The IgG1 Fc variant polypeptide of any of claims 1-3, wherein the IgG1 Fc variant polypeptide comprises SEQ ID NO:31.
5. A molecule comprising two IgG1 Fc variant polypeptides according to any of claims 1-4.
6. The molecule of claim 5, wherein said two IgG1 Fc variant polypeptides are identical or are different.
7. The molecule of claim 5 or 6, wherein the molecule has not more antibody effector functions compared to the identical molecule comprising, instead of the IgG1 Fc variant polypeptides of any of claims 1-4, two IgG1 Fc variant polypeptides comprising the mutations L234A, L235A and P329G.
8. The molecule of any of claims 5-7, wherein the molecule is an antibody.
9. Use of an IgG1 Fc variant polypeptide, comprising the mutations L234A, L235A, A327G, P329A, A330S, and P331S, according to EU numbering, to reduce one or more antibody effector functions.
10. Use of a molecule which comprises two IgG1 Fc variant polypeptides, comprising the mutations L234A, L235A, A327G, P329A, A330S, and P331S, according to EU numbering, to reduce one or more antibody effector functions.

11. The use of claim 9 or 10, wherein one or more antibody effector functions are reduced compared to an identical IgG1 Fc variant polypeptide comprising the mutations L234A and L235, but not comprising the mutations A327G, P329A, A330S, and P331S.
12. The use of any of claims 9-11, wherein the reduction of one or more antibody effector functions comprises the reduction of one or more of complement dependent cytotoxicity (CDC), antibody-dependent cellular phagocytosis (ADCP), and antibody-dependent cellular cytotoxicity (ADCC).
13. The use of any of claims 9-12, wherein the reduction of one or more antibody effector functions comprises the reduction of CDC, ADCP, and ADCC.
14. The use of any of claims 9-13, wherein the IgG1 Fc variant polypeptide or the molecule displays no antibody effector function as measured by the CDC, ADCP, and ADCC assays as described in examples 2-5.
15. A method of reducing one or more antibody effector functions of a molecule which comprises an IgG1 Fc polypeptide, comprising introducing the mutations L234A, L235A, A327G, P329A, A330S, and P331S into said IgG1 Fc polypeptide.