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(54) **Title:** MODULATION OF COMPLEMENT-DEPENDENT CYTOTOXICITY THROUGH MODIFICATIONS OF THE C-TERMINUS OF ANTIBODY HEAVY CHAINS

(57) **Abstract:** Antibody variants having decreased or increased ability to mediate CDC due to modifications at the C-terminus of their heavy chains are described. Methods of generating such antibodies, as well as nucleotide constructs and host cells suitable for the production of said antibodies are also described.



MODULATION OF COMPLEMENT-DEPENDENT CYTOTOXICITY THROUGH MODIFICATIONS OF THE C-TERMINUS OF ANTIBODY HEAVY CHAINS

FIELD OF THE INVENTION

5 The present invention relates to antibodies and their ability to induce complement-dependent cytotoxicity (CDC). Antibody variants are provided that have altered ability to mediate CDC due to modifications at the C-terminus of their heavy chains. Furthermore, the invention provides methods of generating such antibodies, and nucleotide constructs and host cells suitable for the production of said antibodies.

BACKGROUND OF THE INVENTION

Monoclonal antibodies have in recent years become successful therapeutic molecules, in particular for the treatment of cancer and autoimmune diseases. Effector functions mediated by the Fc region of antibodies, such as antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) are often important mechanisms for the clinical efficacy of monoclonal antibodies.

Antibody-induced CDC is mediated through the proteins of the classical complement cascade. This cascade is triggered by binding of the complement protein C1q to the antibody. C1q is composed of a bundle of six heterotrimeric subunits having globular heads and collagen-like tails. Binding of C1q to the Fc region of an antibody is known to involve the CH2 region of the antibody (Duncan and Winter (1988) Nature 332:738). Furthermore, sugar moieties on the Fc region are known to influence the binding of C1q (Raju (2008) Curr Opin Immunol 20:471).

25 Monoclonal antibodies are complex molecules that can undergo many types of enzymatic and non-enzymatic post-translational modifications, including formation of disulfide bonds, glycosylation, glycation, N-terminal glutamine cyclization, C-terminal lysine processing (removal), deamidation, isomerization, oxidation and peptide bond cleavage (for a review of these modifications, see by Liu et al. (2008) J Pharm Sci 97: 2426).

Removal of C-terminal lysines by carboxypeptidases from the heavy chain is a commonly observed antibody modification, both upon recombinant expression of antibodies in mammalian cells, as well as *in vivo* in human serum (Cai et al. (2010) Biotechnol. Bioeng. Sep 9). Removal is often partial, resulting in a mixed population of antibodies with zero (K0), one (K1) or two (K2) C-terminal lysines (i.e., in the

case of K2, one C-terminal lysine in each heavy chain of the antibody). In particular, B cell hybridomas produce mixtures of K0, K1 and K2 molecules (Dick et al. (2008) Biotech. Bioeng. 100:1132).

WO 2009027471 describes that deletion of the codon for the C-terminal lysine of a heavy chain of an antibody can result in higher antibody titers upon expression in Chinese Hamster Ovary (CHO) cells.

WO 2008006554 describes that an antibody preparation produced in Δ xyl-t/ Δ fuc-t moss cells comprises an N-glycan structure free of fucose and xylose and lacks C-terminal lysine residues. The ability of this preparation to mediate ADCC was less inhibited by serum than a preparation of the same antibody produced in murine Sp2/0 cells. Complement-mediated lytic activity, on the other hand, was reduced. Removal of the C-terminal lysine residue was proposed to increase ADCC, although the effect of this modification alone was not tested.

The general belief in the art has been that C-terminal lysines have little or no effect on antibody function, see e.g. the literature review by Cai et al. (2010) *supra*, which concluded that no activity had been attributed to C-terminal lysines, or Harris (2005) Dev Biol (Basel) 122:127 which stated that the presence or absence of heavy chain Lys residues had no effect on antigen binding, and was not likely to influence Fc effector functions, clearance or any other biological property.

Antes et al. (2007) J Chromatogr. B 852:250 described testing the effect of C-terminal lysines on CDC. Antibody preparations were generated in Sp2/0 cells containing a significant subpopulation of C-terminal lysine-containing antibody molecules. Proteolytic removal of the C-terminal lysines had no effect on the ability of these antibody preparations to mediate CDC. The authors concluded, e.g., that both antibody variants – clipped and unclipped – elicited the same potency in a complement dependent cytotoxicity (CDC) assay demonstrating that lysine clipping of IGN311 does not impair Fc-mediated effector functions.

SUMMARY OF THE INVENTION

It has now surprisingly been found that lysines and other charged amino acid residues at the C-terminus of the heavy chains of an antibody do in fact have a major impact on their ability to mediate CDC.

Without being bound by any specific theory, it is hypothesized that this was not detected previously, because pure preparations of the K0, K1 and K2 forms were not compared.

The observation, described in the Examples herein, of the relationship between the presence of C-terminal charged residues and potency to mediate CDC provides the basis for novel antibody products having altered CDC properties as well as novel antibody production methods.

5 In a first aspect, the present invention provides a method for producing an antibody in a host cell, such as an antibody having increased CDC, wherein said antibody comprises at least a heavy chain, said method comprising the following steps:

- 10 a) providing a nucleotide construct encoding said heavy chain, wherein said construct does not encode a lysine residue at the C-terminus of said heavy chain,
- b) expressing said nucleotide construct in a host cell, with the proviso that said host cell is not a CHO cell or a moss cell,
- and
- c) recovering said antibody from a cell culture of said host cell.

15 In another aspect, the invention relates to a method for increasing the ability of an antibody to mediate CDC, said method comprising the step of removing a C-terminal lysine residue from the heavy chains of an antibody.

The product of the above methods is a population of antibody molecules having an increased ability to mediate CDC as compared to corresponding antibody
20 molecules containing a C-terminal lysine residue. Such antibodies are useful for a number of purposes, e.g. therapeutic purposes, where killing of target cells is desired. This may, for example, be desired in the treatment of cancer.

In a further aspect, the invention provides an antibody variant comprising a
25 heavy chain comprising at least one charged amino acid residue in the C-terminal region, wherein said charged amino acid residue is not susceptible to proteolytic removal.

This antibody variant has a decreased ability to mediate CDC as compared to a corresponding antibody molecule lacking one or both C-terminal lysine residues.
30 Such antibodies are useful for applications, e.g. therapeutic applications, where only silencing of a target is desired. This can be advantageous in, e.g., the treatment of autoimmune diseases or inflammation.

In further aspects, the invention relates to methods, nucleotide constructs and host cells for producing antibody variants having at least one charged amino acid

residue in the C-terminal region of the heavy chain, wherein said charged amino acid residue is not susceptible to proteolytic removal.

In a further aspect, the invention relates to a mammalian cell which has been genetically modified to eliminate the activity of carboxypeptidases capable of removing C-terminal lysines from heavy chains.

In even further aspects, the invention provides compositions of antibodies which have been modified to strengthen intermolecular interactions between antibody molecules in order to strengthen their ability to mediate effector functions, such as CDC. In one such aspect, the invention relates to an antibody mixture comprising a subpopulation of antibody variant molecules comprising heavy chains having at least one positively-charged amino acid residue in the C-terminal region and a subpopulation of antibody variant molecules comprising heavy chains having at least one negatively-charged amino acid residue in the C-terminal region, wherein said positively-charged and negatively-charged amino acid residues are not susceptible to proteolytic removal.

In another such aspect, the invention provides an antibody comprising a heavy chain having a variant C-terminal region, comprising one or more amino acid modifications that favour intermolecular C-terminal interactions between antibody molecules.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: CIEX profiles, cIEF and SDS-PAGE analysis of C-terminal lysine isoforms of anti-CD20 and anti-CD38 antibodies.

CIEX profiles of anti-CD20 (a) and anti-CD38 (b) antibodies. cIEF profiles of unfractionated antibody (unfr.), and collected isoforms, containing zero, one or two C-terminal lysines per IgG molecule (K0, K1, K2), of anti-CD20 (c) and anti-CD38 (d) antibodies. SDS PAGE analysis of unfractionated antibodies and collected isoforms of anti-CD20 (e) and anti-CD38 (f) antibodies. Samples were analyzed untreated (-) or after carboxypeptidase B treatment (+) (c-f).

Figure 2 – Binding curves and CDC induction by C-terminal lysine isoforms of anti-CD20 and anti-CD38 antibodies.

Binding to Daudi cells of unfractionated antibodies (unfr.) and collected K2 isoforms of anti-CD20 and anti-CD38 antibodies with and without carboxypeptidase B (CPB) treatment, as determined by FACS analysis (upper panel). Data shown are mean fluorescence intensities (MFI). Induction of CDC of Daudi cells by unfractionated

antibodies and collected K2 isoforms of anti-CD20 and anti-CD38 antibodies with and without carboxypeptidase B treatment, as measured using propidium iodide staining method (lower panel). Data shown are percentages lysis.

Figure 3 – Efficacy of C1q utilization by C-terminal lysine isoforms of anti-CD38 antibody.

Induction of CDC of Daudi cells by unfractionated and collected K2 isoform of anti-CD38 antibody with and without carboxypeptidase B (CPB) treatment, as measured using propidium iodide staining method. Cells were incubated with a fixed antibody concentration (10 µg/mL). Titrated amounts of C1q were added in the assay as complement source. Data shown are mean percentages of lysis ± S.E.M of three experiments.

Figure 4 – cIEF analysis, binding and CDC induction by anti-CD38 antibody and mutants.

cIEF analysis of anti-CD38 antibody and mutants (a). Binding of anti-CD38 antibody and mutants to Daudi cells as analyzed by FACS analysis (b). Data shown are mean fluorescence intensities (MFI). Induction of CDC of Daudi cells by anti-CD38 antibody and mutants as measured by PI staining method (c). Data shown are percentages lysis.

Figure 5 – CDC induction by mixtures of anti-CD38 antibody mutants.

K0 and K4 (a) or K0 and E2 (b) mutants were mixed in different ratios, as indicated, and induction of CDC of Daudi cells was measured by propidium iodide staining method. Total IgG concentration was 10 µg/mL. Induction of CDC by K4 mutant, E2 mutant or a 1:1 mixture of both (c). Total IgG concentration was titrated from 10 µg/mL to 0.016 µg/mL. K0 is shown as positive control. Induction of CDC by E2:K4 mutant mixtures in different ratios, at two total IgG concentrations (10 µg/mL and 0.25 µg/mL) (d). Data shown (a-d) are percentages (maximal) lysis.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

The term “immunoglobulin” refers to a class of structurally related glycoproteins consisting of two pairs of polypeptide chains, one pair of light (L) low molecular weight chains and one pair of heavy (H) chains, all four inter-connected by disulfide bonds. The structure of immunoglobulins has been well characterized. See for instance Fundamental Immunology Ch. 7 (Paul, W., ed., 2nd ed. Raven Press, N.Y. (1989)). Briefly, each heavy chain typically is comprised of a heavy chain variable

region (abbreviated herein as VH) and a heavy chain constant region. The heavy chain constant region typically is comprised of three domains, CH1, CH2, and CH3. The heavy chains are inter-connected via disulfide bonds in the so-called "hinge region". Each light chain typically is comprised of a light chain variable region (abbreviated herein as VL) and a light chain constant region. The light chain constant region typically is comprised of one domain, CL. Typically, the numbering of amino acid residues in the constant region is performed according to the EU-index as described in Kabat et al., *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991). The VH and VL regions may be further subdivided into regions of hypervariability (or hypervariable regions which may be hypervariable in sequence and/or form of structurally defined loops), also termed complementarity determining regions (CDRs), interspersed with regions that are more conserved, termed framework regions (FRs). Each VH and VL is typically composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4 (see also Chothia and Lesk J. Mol. Biol. 196, 901 917 (1987)).

The term "antibody" (Ab) in the context of the present invention refers to an immunoglobulin molecule, a fragment of an immunoglobulin molecule, or a derivative of either thereof, which has the ability to specifically bind to an antigen under typical physiological conditions with a half life of significant periods of time, such as at least about 30 minutes, at least about 45 minutes, at least about one hour, at least about two hours, at least about four hours, at least about eight hours, at least about 12 hours, about 24 hours or more, about 48 hours or more, about three, four, five, six, seven or more days, etc., or any other relevant functionally-defined period (such as a time sufficient to induce, promote, enhance, and/or modulate a physiological response associated with antibody binding to the antigen and/or time sufficient for the antibody to recruit an effector activity). The variable regions of the heavy and light chains of the immunoglobulin molecule contain a binding domain that interacts with an antigen. The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (such as effector cells) and components of the complement system such as C1q, the first component in the classical pathway of complement activation. An antibody may also be a bispecific antibody, diabody or similar molecule. The term "bispecific antibody" refers to antibody having specificities

for at least two different, typically non-overlapping, epitopes. As indicated above, the term antibody herein, unless otherwise stated or clearly contradicted by the context, includes fragments of an antibody that retain the ability to specifically bind to the antigen. Such fragments may be provided by any known technique, such as enzymatic cleavage, peptide synthesis and recombinant expression techniques. It has been shown that the antigen-binding function of an antibody may be performed by fragments of a full-length antibody. It also should be understood that the term antibody, unless specified otherwise, also includes polyclonal antibodies, monoclonal antibodies and antibody-like polypeptides, such as chimeric antibodies and humanized antibodies. An antibody as generated can possess any isotype. In one embodiment of the invention, the antibody is isolated and/or purified.

As used herein, "isotype" refers to the immunoglobulin class (for instance IgG1, IgG2, IgG3, IgG4, IgD, IgA, IgE, or IgM) that is encoded by heavy chain constant region genes.

Unless stated otherwise, the term "C-terminal lysine residue" refers to the lysine residue at the C-terminus of the heavy chain (i.e. the C-terminus of the CH3 region).

Unless stated otherwise, the term "C-terminal region", refers to the C-terminal region of the heavy chain of an antibody, consisting of the six most C-terminal amino acids of the heavy chain polypeptide.

The term "full-length antibody" when used herein, refers to an antibody which contains all heavy and light chain constant and variable domains that are normally found in an antibody of that isotype.

The term "human antibody", as used herein, is intended to include antibodies having variable and constant regions derived from human germline immunoglobulin sequences. The human antibodies of the invention may include amino acid residues not encoded by human germline immunoglobulin sequences (e.g., mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*). However, the term "human antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

When used herein, the term "heavy-chain antibody" refers to an antibody which consists only of two heavy chains and lacks the two light chains usually found in antibodies. Heavy-chain antibodies, which naturally occur in e.g. camelids, can

bind antigens despite having only VH domains, see e.g. Hamers-Casterman (1993) Nature 363:446.

As used herein, the term "binding", in the context of the binding of an antibody to a predetermined antigen, typically is a binding with an affinity corresponding to a K_D of about 10^{-6} M or less, e.g. 10^{-7} M or less, such as about 10^{-8} M or less, such as about 10^{-9} M or less, about 10^{-10} M or less, or about 10^{-11} M or even less, when determined by for instance surface plasmon resonance (SPR) technology in a BIAcore 3000 instrument, using the antigen as the ligand and the antibody as the analyte, and binds to the predetermined antigen with an affinity corresponding to a K_D that is at least ten-fold lower, such as at least 100-fold lower, for instance at least 1,000-fold lower, such as at least 10,000-fold lower, for instance at least 100,000-fold lower than its affinity for binding to a non-specific antigen (e.g., BSA, casein), other than the predetermined antigen or a closely-related antigen. The amount with which the affinity is lower is dependent on the K_D of the antibody, so that when the K_D of the antibody is very low (that is, the antibody is highly specific), then the amount with which the affinity for the antigen is lower than the affinity for a non-specific antigen may be at least 10,000-fold. The term " K_D " (M), as used herein, refers to the dissociation equilibrium constant of a particular antibody-antigen interaction.

An "isolated antibody," as used herein, denotes that the material has been removed from its original environment (e.g., the natural environment if it is naturally occurring or the host cell if it is recombinantly expressed). It is also advantageous that the antibodies be in purified form. The term "purified" does not require absolute purity; rather, it is intended as a relative definition, indicating an increase of the antibody concentration relative to the concentration of contaminants in a composition as compared to the starting material.

The term "host cell", as used herein, is intended to refer to a cell into which a recombinant nucleotide construct or an expression vector has been introduced, e.g. a nucleotide construct or an expression vector encoding an antibody of the invention. Recombinant host cells include, for example, transfectomas, such as CHO cells, HEK293 cells, NS0 cells or lymphocytic cells, or plant cells, fungal cells, such as yeast cells or prokaryotic cells.

Further aspects and embodiments of the invention

As described above, in a first aspect, the invention relates to methods for increasing the ability of an antibody to mediate CDC by reducing the number of charged amino acid residues in the C-terminal region of the heavy chain. Antibody products having increased ability to mediate CDC can be obtained by post-translational proteolytic removal of the charged residue from the heavy chain or by recombinant production of the antibody from a nucleotide construct lacking codons for charged amino acids, such as lysines, in the region encoding the C-terminus.

Thus, in a first aspect, the invention provides a method for producing an antibody in a host cell, wherein said antibody comprises at least a heavy chain, said method comprising the following steps:

- a) providing a nucleotide construct encoding said heavy chain, wherein said construct does not encode a lysine residue at the C-terminus of said heavy chain,
- b) expressing said nucleotide construct in a host cell, with the proviso that said host cell is not a CHO cell or a moss cell,
- and
- c) recovering said antibody from a cell culture of said host cell.

Typically, the heavy chain comprises a constant region comprising at least a CH3 domain, at least a CH2 and a CH3 domain, or all of a CH1, a CH2 and a CH3 domain. In one embodiment, the heavy chain comprises at least a CH2 and a CH3 domain.

In some embodiments, the antibody is a heavy-chain antibody. In most embodiments, however, the antibody will also contain a light chain and thus said host cell further expresses a light-chain-encoding construct, either on the same or a different vector.

Host cells suitable for the recombinant expression of antibodies are well-known in the art. In a one embodiment, said host cell is a cell which is capable of Asn-linked glycosylation of proteins, e.g. a eukaryotic cell, such as a mammalian cell, e.g. a human cell. In a further embodiment, said host cell is a non-human cell which is genetically engineered to produce glycoproteins having human-like or human glycosylation. Examples of such cells are genetically-modified *Pichia pastoris* [Hamilton et al., Science 301 (2003) 1244-1246; Potgieter et al., J. Biotechnology

139 (2009) 318-325] and genetically-modified *Lemna minor* [Cox et al., *Nature Biotechnology* 12 (2006) 1591-1597].

In one embodiment, said host cell is not capable of efficiently removing C-terminal lysine residues from antibody heavy chains. For example, Table 2 in Liu et al. (2008) *J Pharm Sci* 97: 2426 (incorporated herein by reference) lists a number of such antibody production systems, e.g. Sp2/0, NS0 or transgenic mammary gland (goat), wherein only partial removal of C-terminal lysines is obtained.

More specifically, such a host cell may be a cell which, when expressing a C-terminal lysine-containing construct, would produce an antibody preparation wherein more than 10% of the antibody molecules is in the K2 isoform, e.g. wherein more the 30% is in the K2 isoform. Table 1 gives expected amounts of K0, K1 and K2 as a function of C-terminal cleavage. Thus, phrased in another manner, a preferred host cell is a host cell in which more than 30% of the heavy chains remain uncleaved at the C-terminus, e.g. wherein more than 60% of the heavy chains remain uncleaved at the C-terminus of the heavy chain.

Table 1

% uncleaved	% K0 in batch	% K1 in batch	% K2 in batch
10	81	18	1
20	64	32	4
30	49	42	9
40	36	48	16
50	25	50	25
60	16	48	36
70	9	42	49
80	4	32	64
90	1	18	81
100	0	0	100

In a further embodiment of the invention, said host cell is selected from the group consisting of:

- (i) a yeast cell, e.g. a *Pichia pastoris* or *Saccharomyces cerevisiae*, *Hansenula polymorpha* and *Ogataea minuta* and
- (ii) a filamentous fungal cell, such as *Aspergillus awamori*, *Aspergillus niger*, *Aspergillus oryzae*, *Trichoderma reesi* and
- 5 (iii) a plant cell, such as an *Arabidopsis* cell, *Lemna minor*, *Nicotiana benthamiana* (tobacco), oilseed crop plant (*Brassica napus*), soybean, rice, maize (*Zea mays*) or carrot cells, and
- (iv) an NS0, Sp2/0 or PER.C6 cell.

Recombinant production of antibodies in yeasts and filamentous fungi has e.g. been
 10 described and reviewed by Joosten et al. (2003) *Microbial Cell Factories* 2:1 and
 Gasser and Mattanovich (2007) *Biotech Lett* 29:201. For production of antibodies in
 plant cells, see e.g. Cox et al. (2006) *Nat Biotechnol* 24:1591, Decker and Reski
 (2007) *Curr Opin Biotechnol* 18:393, Giritch et al. (2006) *Proc Natl Acad Sc USA*
 103:14701 or Hood et al. (2002) *Curr Opin Biotechnol* 13:630. Antibody expression
 15 in NS0 cell lines has, for example, been described by Dempsey et al. (2003)
Biotechnol. Prog. 19:175. Jones et al. (2003) *Biotechnol Prog* 19:163, inter alia,
 have described antibody production in human PER.C6 cells. For antibody production
 in Sp2/0 cells, see e.g. Yang et al. (2007) *Biotechnol Bioeng.* 98:141.

In a further embodiment, wherein said heavy-chain-encoding construct does
 20 not encode a lysine or an arginine at the C-terminus of said heavy chain, preferably
 said heavy-chain-encoding construct does not encode a charged amino acid residue
 at the C-terminus of said heavy chain. More preferably, said heavy-chain-encoding
 construct does not contain any codon for a charged amino acid residue among the
 codon coding for the six most C-terminal amino acids.

25 In one embodiment, the antibody is a human antibody comprising a human
 heavy chain. The antibody can be of any isotype, including, but not limited to, IgG1,
 IgG2, IgG3, IgG4, IgD, IgA, IgE, and IgM. In one embodiment, the antibody is of an
 IgG isotype, such as an isotype selected from IgG1, IgG2, IgG3, and optionally IgG4.
 Specific wild-type heavy-chain constant sequences are provided below in SEQ ID
 30 NOS:1-5. However, different allotypes of each isotype are known in the art, and are
 described in Jefferies and Lefranc (2009), mAbs 1:4, 1-7, hereby incorporated by
 reference in its entirety.

In a further embodiment of the above method of the invention, the nucleotide construct provided in step a) is derived from, or designed based on, an original heavy-chain sequence having a codon for a C-terminal lysine residue. For example, the original heavy-chain sequence may comprise a C-terminal sequence which is residues 325-330 of SEQ ID NOS:1 (IgG1m (az) allotype) or 5 (IgG1m(f) allotype), or residues 321 to 326 of SEQ ID NO:4, as set forth below. Thus, said nucleotide construct may comprise a deletion or a substitution of the codon for the C-terminal lysine (k) residue compared to said original heavy-chain sequence. Accordingly, in one embodiment, the nucleotide construct comprises a C-terminal sequence which comprises or consists of residues 325-329 of SEQ ID NOS:1 or 5. In one embodiment, the nucleotide construct comprises a C-terminal sequence which comprises or consists of residues 321 to 325 of SEQ ID NO:4.

In one embodiment, said nucleotide construct encodes a heavy chain comprising a constant region sequence which, other than the C-terminal modification, comprises at least a CH3 domain, at least CH2 and CH3 domains, or CH1, CH2 and CH3 domains of the IgG1 isotype, optionally the IgG1m (f) allotype. In one embodiment, said nucleotide construct encodes a heavy chain comprising a constant region sequence which comprises at least a CH3 domain, at least CH2 and CH3 domains, or CH1, CH2 and CH3 domains of the IgG2 isotype, other than the C-terminal modification. In one embodiment, said nucleotide construct encodes a heavy chain comprising a constant region sequence which comprises at least a CH3 domain, at least CH2 and CH3 domains, or CH1, CH2 and CH3 domains of the IgG3 isotype, other than the C-terminal modification. In one embodiment, said nucleotide construct encodes a heavy chain comprising a constant region sequence which comprises at least a CH3 domain, at least CH2 and CH3 domains, or CH1, CH2 and CH3 domains of the IgG4 isotype, other than the C-terminal modification. In one embodiment, the heavy chain comprises a sequence selected from SEQ ID NOS:1-5, apart from the C-terminal lysine residue.

In an even further embodiment, said nucleotide construct, other than the C-terminal modification, encodes a heavy chain of the IgG1 or IgG2 isotype, i.e. has a constant region sequence which is identical in sequence to the IgG1 or IgG2 constant region, except for the C-terminal modification. In one embodiment, said nucleotide construct, other than the C-terminal modification, encodes a heavy chain of the IgG1 or IgG3 isotype, i.e. has a constant region sequence which is identical in sequence to the IgG1 or IgG3 constant region, except for the C-terminal modification. In a further

embodiment, the C-terminal codon of said nucleotide construct encodes a Pro or a Gly residue, e.g. the -Pro-Gly-Lys sequence at the C-terminus of an IgG1 or IgG2 heavy chain would be truncated to -Pro-Gly or -Pro only.

In a further aspect, the invention relates to an antibody obtained or obtainable by the method of the invention described above.

In a yet further aspect, the invention relates to a host cell which is not capable of efficiently removing C-terminal lysine residues from antibody heavy chains, said host cell comprising a nucleotide construct encoding a heavy chain lacking a C-terminal lysine. Preferably, said host cell is not moss or CHO. More preferably, said host cell is of one of the types specified above under (i) to (iv).

In an even further aspect, the invention relates to a method for increasing the ability of an antibody to mediate CDC, said method comprising the step of removing a C-terminal lysine residue from the heavy chains of an antibody. In a preferred embodiment, the lysine residue is removed by enzymatic cleavage, e.g. using a carboxypeptidase, such as carboxypeptidase B or carboxypeptidase N (Cia et al. (2010) *Supra*). In one embodiment, said antibody is purified prior to removal of the C-terminal lysine residue.

As mentioned above, some cell types, e.g. hybridomas, produce heterogenous populations of antibody molecules, wherein some molecules contain one or two C-terminal lysines and others do not. As it is clear from the present work that C-terminal lysines affect the ability of antibodies to mediate CDC, such C-terminal heterogeneity may be undesired if different antibodies from a plurality of samples are to be compared, as there may be differences between different hybridomas with respect to the degree of C-terminal processing. To eliminate such differences, it may be advantageous to remove the C-terminal lysines from antibody samples prior to comparative testing in order to be able to better compare properties related to the binding properties of the antibodies.

Accordingly, in a further aspect, the invention relates to a method for testing a plurality of antibody-producing cell cultures, e.g. hybridoma cell cultures, for a desired property, said method comprising treating samples of said cultures to remove the C-terminal lysines as described above and testing the treated samples for the desired property. In some embodiments, it may be advantageous to remove cells and/or purify antibody from the culture sample before removal of the C-terminal lysines. In a preferred embodiment, the desired property to be tested is cell lysis or cell killing.

As explained above, in a further aspect, the invention relates to an antibody variant comprising a heavy chain having at least one charged amino acid residue in the C-terminal region, such as one, two, three, four or more charged amino acids, wherein said charged amino acid residue is not susceptible to proteolytic removal.

Examples of charged amino acids residues include those having a positive charge, such as lysine (K or Lys), arginine (R or Arg) and histidine (H or His), and those having a negative charge, such as glutamic acid (E or Glu) and aspartic acid (D or Asp). In one embodiment, the charged amino acid residue is selected from lysine, arginine and histidine, such as from lysine and arginine, such as lysine. In one embodiment, the charged amino acid residue is selected from glutamic acid and aspartic acid, such as glutamine.

Such variants typically have reduced ability to mediate CDC as compared to a corresponding antibody lacking the charged residue. Such variants may e.g. be useful for therapeutic uses wherein reduced CDC is desired, e.g. uses wherein silencing of a target antigen, without killing of the target cell, is desired.

In some embodiments, the antibody is a heavy-chain antibody. In most embodiments, however, the antibody will further comprise a light chain.

In a one embodiment, said charged amino acid residue is not susceptible to removal by proteases of mammalian origin, preferably not susceptible to removal by proteases of CHO, HEK-293, PER.C6, NS0 or Sp2/0 origin, more preferably not susceptible to removal by proteases, such as carboxypeptidases, active in the secretory pathway of CHO, HEK-293, PER.C6, NS0 or Sp2/0 cells.

In a further embodiment, said charged amino acid residue is not susceptible to removal by human proteases, preferably not susceptible to removal by human proteases that can act on proteins in circulation, e.g. in blood.

In one embodiment, said charged amino acid is amongst the six most C-terminal amino acid residues, e.g. amongst the five most C-terminal amino acid residues of said heavy chain, such as amongst the four most C-terminal amino acid residues of said heavy chain, e.g. amongst the three most C-terminal amino acid residues of said heavy chain, such as amongst the two most C-terminal amino acid residues of said heavy chain, e.g. wherein said charged amino acid is the most C-terminal amino acid residue of said heavy chain.

In a further embodiment, said heavy chain comprises the CH3 sequence set forth in SEQ ID NO:1, 2, 3, 4 or 5 herein below, modified such that said charged

amino acid is situated amongst the most six most C-terminal amino acid residues, i.e. from position 325 to 330, from position 321 to 326, from position 372 to 377, from 321 to 326 or from position 325 to 330, respectively.

- 5 SEQ ID NO: 1: The amino acid sequence of the IgG1 constant region (accession number P01857; IgGm(za) allotype) (the CH3 sequence is underlined)

1 astkgpsvfp lapsskstsg gtaalgclvk dyfpepvtvs wnsgaltsgv
 51 htfpavlqss glyslssvvt vpssslgtqt yicnvnhkps ntkvdkkvep
 101 kscdkthtcp pcpapellgg psvflfppkp kdtlmisrtp evtcvvvdvs
 10 151 hedpevkfnw yvdgvevhna ktkpreeqyn styrvvsvlt vlhqdwlngk
 201 eykckvsnka lpapiektis kakgqprepq v tylpps rde ltknqvsltc
 251 lvkgfypsdi avewesnggp ennykttppv ldsdgsffly skltvdksrw
 301 qggnvfscsv mhealhnhyt qkslslspgk

- 15 SEQ ID NO: 2: The amino acid sequence of the IgG2 constant region (accession number P01859) (the CH3 sequence is underlined)

1 astkgpsvfp lapcsrstse staalgclvk dyfpepvtvs wnsgaltsgv
 51 htfpavlqss glyslssvvt vpssnfgtqt ytcnvdhkps ntkvdktver
 101 kccvecppcp appvagpsvf lfppkpkdtl misrtpevtc vvvvdvshedp
 20 151 evqfnwyvdg vevhnaktkp reeqfnstfr vsvlvtvvhq dwlngkeykc
 201 kvsnkglpap iektisktkg qprepqv tyl ppsreemtkn qvsltclvkq
 251 fypsdiavew esngqpenny kttppmlsd qsfflysklt vdksrwqqqn
 301 vfscsvmhea lhnhytqksl slspgk

- 25 SEQ ID NO: 3: The amino acid sequence of the IgG3 constant region (accession number P1860) (the CH3 sequence is underlined)

1 astkgpsvfp lapcsrstsg gtaalgclvk dyfpepvtvs wnsgaltsgv
 51 htfpavlqss glyslssvvt vpssslgtqt ytcnvnhkps ntkvdkrvel
 101 ktplgdtht cprcpepksc dtpppcprcp epkscdtppp cprcpepksc
 30 151 dtpppcprcp apellggs v flfppkpkdt lmisrtpevt cvvvdvshed
 201 pevqfkwyvd gvevhnaktk preeqynstf rvvsvltvlh qdwlngkeyk
 251 ckvsnk alpa piektisktk qqprepqv tyl ppsreemtk nqvsltclvk
 301 gfypsdiave wessqpenn ynttpmlsd dgsfflyskl tvdksrwqqq
 351 nifscsvmhe alhnrtqks lslspgk

- 35 SEQ ID NO:4: The amino acid sequence of the IgG4 constant region (the CH3 sequence is underlined)

astkgpsvfp lapcsrstse staalgclvk dyfpepvtvs wnsgaltsgv
 htfpavlqss glyslssvvt vpssslgtkt ytcnvdhkps ntkvdkrves

kygppcpscp apeflggpsv flfppkpkdt lmisrtpevt cvvvdvsqed
 pevqfnwyvd gvevhnaktk preeqfnsty rvvsvltvlh qdwlngkeyk
 ckvsnkglps siektiskak ggprepgvyt lppsqeemtk ngvsltclvk
gfypsdiave wesnggpenn ykttppvlds dgsfflysr1 tvdksrwqeg
 5 nvfscsvmhe alhnhytqks ls1slgk

SEQ ID NO: 5: The amino acid sequence of the IgG1m(f) allotype constant region
 (the CH3 sequence is underlined)

1 astkgpsvfp lapsskstsg gtaalgclvk dyfpepvtvs wmsgaltsgv
 10 51 htfpavlqss glyslssvvt vpssslgtqt yicnvnhkps ntkvdkrvep
 101 kscdkthtcp pcpapellgg psvflfppkp kdtlmisrtp evtcvvvdvs
 151 hedpevkfnw yvdgvevhna ktkpreeqyn styrvsvlt vlhqdwlngk
 201 eykckvsnka lpapiektis kakggprepg vytlppsree mtkngvsltc
 251 lvkgfypsdi avewesnggp ennykttppv ldsdgsffly skltvdksrw
 15 301 gggnvfscsv mhealhnhyt qks1slspgk

In a further embodiment, said heavy chain comprises the entire constant region sequence set forth in SEQ ID NO:1, 2, 3, 4 or 5 and said charged amino acid is situated amongst the six most C-terminal amino acid residues, i.e. from position
 20 325 to 330, from position 321 to 326 or from position 372 to 377, respectively.

In one embodiment, said charged amino acid is a positively charged amino acid residue, preferably a lysine residue. As C-terminal lysines are normally susceptible to proteolytic removal, said variant will contain one or more amino acid modifications, such as additions or substitutions, at a position situated C-terminally
 25 of said lysine. For example, in one embodiment, said charged amino acid residue is not susceptible to proteolytic removal due to the presence of a proline residue situated C-terminally of said charged amino acid residue, wherein said proline residue is preferably placed immediately C-terminal of said charged amino acid residue, more preferably wherein said charged amino acid residue and said proline residue are the two most C-terminal amino acid residues of said heavy chain. For
 30 example, the antibody heavy chain may be an IgG1, IgG2, IgG3 or IgG4 heavy chain to which a proline residue has been added at the C-terminus.

In another embodiment, said charged amino acid is a negatively-charged amino acid residue, preferably a glutamic acid residue. Negatively-charged C-
 35 terminal residues are normally not susceptible to proteolytic removal upon expression in mammalian cells and thus, in some embodiments, such a variant does not contain further amino acid modifications.

In a further embodiment, said heavy chain comprises two or more charged amino acid residues in the C-terminal region, such as two, three, four or five charged amino acid residues, preferably having either all a positive charge or all a negative charge, optionally comprising further amino acid modifications C-terminally of said charged residues, e.g. a proline residue situated C-terminally of said charged residues.

In some embodiments, the specified amino acid modifications are the result of amino acid substitutions. In other embodiments, the specified amino acid modifications are the result of C-terminal amino acid additions.

In one embodiment, said heavy chain comprises a C-terminal sequence selected from pro-gly-glu (PGE), pro-gly-lys-pro (PGKP; SEQ ID NO:6), pro-gly-lys-lys-pro (PGKKP; SEQ ID NO:7), and pro-gly-lys-lys-lys-pro (PGKKKP; SEQ ID NO:8).

In one embodiment, said antibody is a human antibody, preferably a human IgG1 or a human IgG3 antibody.

In another embodiment, said antibody is not conjugated at the C-terminus, e.g. not conjugated to another molecule, such as a toxin or label. In a further embodiment, said antibody is not conjugated at the C-terminus, but is conjugated at another site of the molecule, e.g. the antibody may, at the other site, be linked to a compound selected from the group consisting of: a toxin (including a radioisotope) a prodrug or a drug. Such compounds may make killing of target cells more effective, e.g. in cancer therapy. The resulting antibody is thus an immunoconjugate.

In a further aspect, the invention relates to an antibody of the invention as described above for use as a medicament, in particular for use as a medicament for the treatment of diseases or disorders, wherein silencing (i.e. inhibition) of a target antigen is desired, without killing of the target cell. Examples of such diseases and disorders include autoimmune diseases and inflammation.

In a further aspect, the invention relates to a nucleotide construct encoding a heavy chain having a charged amino acid residue in the C-terminal region, wherein said charged amino acid residue is not susceptible to proteolytic removal. In a further embodiment, said nucleotide construct has any one or more of the further features described above for the antibody of the invention.

In a further aspect, the invention relates to a host cell capable of producing an antibody variant of the invention. Such a host cell may be a cell that has been transformed or transfected with a nucleotide construct of the invention or a host cell which has been genetically modified to eliminate the activity of carboxypeptidases

capable of removing C-terminal lysines from heavy chains. Examples of host cells include CHO, HEK-293, PER.C6, NS0 and Sp2/0.

In an even further aspect, the invention relates to a method for producing an antibody variant according to the invention comprising culturing a host cell according to the invention and recovering said antibody from the cell culture. In one embodiment, said host cell is a CHO or HEK cell comprising a nucleotide construct encoding a heavy chain with a negatively-charged amino acid residue at the C-terminus or a proline residue preceded by a positively-charged amino acid residue at the C-terminus.

In one aspect, the invention relates to a method for decreasing the ability of an antibody to mediate complement-dependent cytotoxicity, said method comprising the step of adding at least one charged amino acid residue, a proline residue, or both to the C-terminal region of the heavy-chains of an antibody. In one embodiment, a positively charged amino acid is inserted N-terminal to a proline residue already present in the C-terminal region. In one embodiment, a proline residue is added C-terminal to a lysine residue already present in the C-terminal region. In one embodiment, a positively charged amino acid and a proline is added to the C-terminal, wherein the positively charged amino acid is added N-terminal to the proline residue. In such embodiments, the positively charged amino acid can be selected from lysine, arginine and histidine, such as lysine or arginine, such as lysine.

In one embodiment, a positively charged amino acid is added to the C-terminal region. In such an embodiment, the positively charged amino acid can be selected from glutamic acid and aspartic acid.

In one embodiment, the method comprises adding one or more further charged amino acids to the C-terminal region.

As explained above, in a further aspect, the invention relates to an antibody mixture comprising a subpopulation of antibody variant molecules comprising heavy chains having a positively-charged amino acid residue in the C-terminal region and a subpopulation of antibody variant molecules comprising heavy chains having a negatively-charged amino acid residue C-terminal region, wherein said positively-charged and negatively-charged amino acid residues are not susceptible to proteolytic removal.

Without being bound any specific theory, it is hypothesized that electrostatic interactions between oppositely-charged residues reinforce intermolecular antibody interactions and thus facilitate the activation of the complement system.

5 In one embodiment, each of said subpopulations constitutes at least 10%, such as at least 20% of said mixture, e.g. at least 30% of said mixture.

In another embodiment, the antibody molecules within each of the two subpopulations comprise two identical heavy chains. In a further aspect, the invention relates to the antibody mixture according to the invention for use as a medicament, preferably for use in the treatment of cancer.

10 As explained above, in a further aspect, the invention relates to an antibody comprising a heavy chain having a variant C-terminal region, said variant C-terminal region comprising one or more amino acid modifications that favour intermolecular C-terminal interactions between antibody molecules. Without being bound by any specific theory, it is believed that strengthened intermolecular interactions between
15 antibody molecules facilitate the activation of the complement system and thus result in an increased ability to mediate CDC compared to an antibody product lacking said amino acid modifications.

In one embodiment, said antibody comprises two non-identical heavy chains. In a particularly interesting embodiment hereof, one heavy chain of the antibody
20 comprises a negatively-charged amino acid residue in the C-terminal region and the other heavy chain of the same antibody comprises a positively-charged charged amino acid residue in the C-terminal region, wherein said charged amino acid residues are not susceptible to proteolytic removal. Such an antibody will comprise a positively-charged heavy chain and a negatively-charged heavy chain and, thus,
25 through alignment of oppositely-charged heavy chains between antibody molecules, form a multimeric complex containing strong intermolecular antibody-antibody interactions favoring CDC activation.

In one embodiment, such an antibody is a monospecific antibody. In another embodiment, it is a bispecific antibody.

30 In a further aspect, the invention relates to the above-described antibody of the invention for use as a medicament, preferably for use in the treatment of cancer.

In an even further aspect, the invention relates to a method for producing such an antibody comprising culturing a host cell comprising nucleotide constructs encoding said two different heavy chains and recovering said antibody from the cell
35 culture of said host cell. Such a host cell may e.g. be a CHO or HEK cell.

As described above, the present invention inter alia relates to modifications of antibodies either to remove or add and/or protect charged amino acid residues at the C-terminus of the heavy chain. Antibodies to be used as starting material of the present invention before modification may e.g. be produced by the hybridoma method first described by Kohler et al., *Nature* 256, 495 (1975), or may be produced by recombinant DNA methods. Monoclonal antibodies may also be isolated from phage antibody libraries using the techniques described in, for example, Clackson et al., *Nature* 352, 624 628 (1991) and Marks et al., *J. Mol. Biol.* 222, 581 597 (1991). Monoclonal antibodies may be obtained from any suitable source. Thus, for example, monoclonal antibodies may be obtained from hybridomas prepared from murine splenic B cells obtained from mice immunized with an antigen of interest, for instance in the form of cells expressing the antigen on the surface, or a nucleic acid encoding an antigen of interest. Monoclonal antibodies may also be obtained from hybridomas derived from antibody-expressing cells of immunized humans or non-human mammals such as rats, dogs, primates, etc.

Antibodies to be used as starting material of the present invention may be e.g. chimeric or humanized antibodies. In another embodiment, the antibody is a human antibody. Human monoclonal antibodies may be generated using transgenic or transchromosomal mice, e.g. HuMAb mice, carrying parts of the human immune system rather than the mouse system. The HuMAb mouse contains a human immunoglobulin gene minilocus that encodes unrearranged human heavy (μ and γ) and κ light chain immunoglobulin sequences, together with targeted mutations that inactivate the endogenous μ and κ chain loci (Lonberg, N. et al., *Nature* 368, 856 859 (1994)). Accordingly, the mice exhibit reduced expression of mouse IgM or κ and in response to immunization, the introduced human heavy and light chain transgenes undergo class switching and somatic mutation to generate high affinity human IgG, κ monoclonal antibodies (Lonberg, N. et al. (1994), supra; reviewed in Lonberg, N. *Handbook of Experimental Pharmacology* 113, 49 101 (1994) , Lonberg, N. and Huszar, D., *Intern. Rev. Immunol.* Vol. 13 65 93 (1995) and Harding, F. and Lonberg, N. *Ann. N.Y. Acad. Sci* 764 536 546 (1995)). The preparation of HuMAb mice is described in detail in Taylor, L. et al., *Nucleic Acids Research* 20, 6287 6295 (1992), Chen, J. et al., *International Immunology* 5, 647 656 (1993), Tuailon et al., *J. Immunol.* 152, 2912 2920 (1994), Taylor, L. et al., *International Immunology* 6, 579 591 (1994), Fishwild, D. et al., *Nature Biotechnology* 14, 845 851 (1996). See

also US 5,545,806, US 5,569,825, US 5,625,126, US 5,633,425, US 5,789,650, US 5,877,397, US 5,661,016, US 5,814,318, US 5,874,299, US 5,770,429, US 5,545,807, WO 98/24884, WO 94/25585, WO 93/1227, WO 92/22645, WO 92/03918 and WO 01/09187. Splenocytes from these transgenic mice may be used
5 to generate hybridomas that secrete human monoclonal antibodies according to well known techniques.

Further, human antibodies of the present invention or antibodies of the present invention from other species may be identified through display-type technologies, including, without limitation, phage display, retroviral display,
10 ribosomal display, mammalian display, and other techniques, using techniques well known in the art, and the resulting molecules may be subjected to additional maturation, such as affinity maturation, as such techniques are well known in the art.

15 Target diseases and antigens

Antibodies of the invention, lacking C-terminal lysine or other charged amino acid residues, may be used for many different purposes, such as purposes where increased cell killing via CDC is desired. Examples of suitable diseases include, without limitation, various forms of cancer. Examples of suitable antigen targets for
20 such antibodies include, without limitation, tumor erbB1 (EGFR), erbB2 (HER2), erbB3, erbB4, MUC-1, CD4, CD19, CD20, CD25, CD32, CD37, CD38, CD74, CD138, CXCR5, c-Met, HERV-envelope protein, periostin, Bigh3, SPARC, BCR, CD79, EGFvIII, IGF α , L1-CAM, AXL, Tissue Factor (TF), EpCAM and MRP3. Preferred antigens include CD20, HER2, EGFR, CD38, IGF α , CD25 and CD32. Exemplary
25 antibodies include HuMAb 7D8, HuMAb 2F2 (described in WO2004035607) and HuMAb 005 (described in WO 2006099875).

Antibodies of the invention containing C-terminal lysine or other charged amino acid residues may, for example, be used in applications where CDC is not favored as a mode-of-action, e.g. in the treatment of autoimmune disease or
30 inflammation. Examples of suitable antigen targets include, without limitation TNF- α , IL-1, VEGF, IL-6 and IL-8.

Compositions and uses

In a further main aspect, the invention relates to a pharmaceutical composition comprising an antibody according to the invention as described herein and a pharmaceutically-acceptable carrier.

5 The pharmaceutical compositions may be formulated in accordance with conventional techniques such as those disclosed in Remington: The Science and Practice of Pharmacy, 19th Edition, Gennaro, Ed., Mack Publishing Co., Easton, PA, 1995. A pharmaceutical composition of the present invention may e.g. include diluents, fillers, salts, buffers, detergents (e. g., a non-ionic detergent, such as
10 Tween-20 or Tween-80), stabilizers (e. g., sugars or protein-free amino acids), preservatives, isotonicity agents, antioxidants, tissue fixatives, solubilizers, and/or other materials suitable for inclusion in a pharmaceutical composition. Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions of the present invention include water, saline,
15 phosphate-buffered saline, ethanol, dextrose, polyols (such as glycerol, propylene glycol, polyethylene glycol).

The pharmaceutical composition may be administered by any suitable route and mode. In one embodiment, a pharmaceutical composition of the present invention is administered parenterally. "Administered parenterally" as used herein
20 means modes of administration other than enteral and topical administration, usually by injection, and include epidermal, intravenous, intramuscular, intra-arterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, intratendinous, transtracheal, subcutaneous, subcuticular, intra-articular, subcapsular, subarachnoid, intraspinal, intracranial, intrathoracic, epidural and
25 intrasternal injection and infusion.

The efficient dosages and the dosage regimens for the antibody depend on the disease or condition to be treated and may be determined by the persons skilled in the art. An exemplary, non-limiting range for a therapeutically effective amount of an antibody of the present invention is about 0.1-100 mg/kg, such as about 0.1-50
30 mg/kg, for example about 0.1-20 mg/kg, such as about 0.1-10 mg/kg, for instance about 0.5, about such as 0.3, about 1, about 3, about 5, or about 8 mg/kg.

Antibodies, of the present invention may also be administered in combination therapy, i.e., combined with other therapeutic agents relevant for the disease or condition to be treated. Accordingly, in one embodiment, the antibody-containing
35 medicament is for combination with one or more further therapeutic agents, such as

a cytotoxic, chemotherapeutic or anti-angiogenic agents. Such combined administration may be simultaneous, separate or sequential. In a further embodiment, the present invention provides a method for treating or preventing disease, such as cancer, which method comprises administration to a subject in need thereof of a therapeutically effective amount of an antibody of the present invention, in combination with radiotherapy and/or surgery.

EXAMPLES

Example 1: Cation exchange chromatography (CIEX) profiles, capillary isoelectric focusing (cIEF) and sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of anti-CD20 and anti-CD38 C-terminal lysine variants

Anti-CD20 antibody (2F2, described in WO 2004035607 (Genmab)) and anti-CD38 antibody (005, described in WO 2006099875 (Genmab)) were isolated from hybridoma supernatants and subjected to preparative cation exchange chromatography (CIEX). CIEX was performed on an AKTA Purifier system using a ProPac® WCX 10 (9 mm x 250 mm) preparative column. Mobile phases A and B were 10 mM sodium phosphate (pH 7.2) and 25 mM sodium chloride in 10 mM sodium phosphate (pH 7.0). Antibodies were dialyzed O/N prior to injection. Linear gradients from 0% to 12% B in 60 min (anti-CD20) and from 8% to 13% B in 25 min (anti-CD38) were used. Flow rate was 4 mL/min for both antibody separations, and concentrations were determined by absorbance at 280 nm. For each antibody, six consecutive injections were performed and the individual K0, K1 and K2 isoforms (containing zero, one or two heavy-chain C-terminal lysines per antibody) were pooled, concentrated (Sartorius, Vivaspinn, 10,000 Da MWCO) and separation buffer was exchanged for PBS buffer. Pooled fractions were biochemically analyzed by cIEF and SDS PAGE without treatment or after carboxypeptidase B (CPB) treatment (500 µL [450 µg/mL] antibody in 20 mM sodium phosphate [pH 7.2] was mixed with 10 µL of 0.05 IU/µL CPB [Calbiochem] and incubated at 37°C for 4 h). Samples were stored at -80°C until further use. For cIEF analysis, antibody samples were diluted to 5 µg/mL and 20 µL was loaded onto a precast cIEF FocusGel 6-11 24S (ETC, Kirchentellinsfurt, Germany) with high pI Kit and pH 7.65 and 10.0 markers (Amersham, Piscataway, USA). Gels were pre-focused at 500 V, 30 mA and 10 W for 30 min, followed by focusing at 1500 V, 18 mA and 20 W for 90 min and 2000 V, 15 mA and 25 W for 30 min. Gels were fixed in 20% (w/v) trifluoroacetic acid at 30°C

for 45 min. Detection of the bands was performed using ammoniacal silver staining procedure recommended by the FocusGel supplier. cIEF gels were digitally imaged using the GeneGenius Imaging System (Synoptics, Cambridge, UK). All other reagents and devices used for cIEF were obtained from GE Healthcare (Uppsala, Sweden). Non-reduced SDS-PAGE was performed on 4-12% NuPAGE Bis-Tris SDS-PAGE gels (Invitrogen, Breda, The Netherlands). SDS-PAGE gels were digitally imaged using the GeneGenius Imaging System (Synoptics, Cambridge, UK).

Figure 1 shows the CIEF profiles for anti-CD20 (a) and anti-CD38 (b) antibodies. cIEF analysis (Figures 1c and 1d) clearly shows that three IgG charge variants, containing zero, one or two C-terminal lysines per IgG molecule (K0, K1 and K2) are present in unfractionated anti-CD20 and anti-CD38 antibody preparations. Variants could be separated by CIEF as shown in the cIEF profiles of the collected isoforms. SDS PAGE analysis (Figure 1e and 1f) shows that structural integrity was maintained after CIEF fractionation. Only the K0 isoform was present after carboxypeptidase B (CPB) treatment of unfractionated anti-CD20 and anti-CD38 preparations, as well as in collected isoforms (Figures 1c and 1d).

Example 2: Binding capacity and induction of complement-mediated cytotoxicity (CDC) by C-terminal lysine isoforms of anti-CD20 and anti-CD38 antibodies

Antibody preparations and collected isoforms, with or without carboxypeptidase B (CPB) treatment, were obtained by preparative CIEF as described supra. Binding of antibody samples to Daudi cells, which express both CD20 and CD38, was analyzed by FACS analysis. 10^5 cells were incubated in 50 μ L in polystyrene 96-well round bottom plates (Greiner bio-one 650101) with serial dilutions of antibody preparations, ranging from 0.04 μ g/mL to 10 μ g/mL, in RPMI1640/0.2% BSA at 4°C for 30 min. After washing twice in RPMI/0.2% BSA, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human IgG (F0056, Dako, Glostrup, Denmark) at 4°C for 30 min. Cells were washed twice in RPMI/0.2% BSA, resuspended in RPMI/0.2% BSA, and analyzed on a FACS Calibur (BD Biosciences). Binding curves were analyzed using non-linear regression (sigmoidal dose response with variable slope) using GraphPad Prism V5.01 software (GraphPad Software, San Diego, CA, USA). To test induction of CDC, Daudi cells (2×10^6 cells/mL) were incubated with serial dilutions of antibody preparations at RT for 15 min. Normal human serum (NHS; M0008, Sanquin, Amsterdam, The Netherlands)

was added as a source of complement (final concentration 20% [v/v]). After incubation in round-bottom 96-well plates (Nunc, Rochester, NY) at 37°C for 45 min, the reaction was stopped by placing the samples on ice. Cell lysis was determined by FACS analysis using propidium iodide (PI, Sigma Aldrich, Zwijndrecht, The Netherlands) staining method. % lysis was determined as follows: % lysis = (number of PI pos cells/total number of cells) x 100%.

	Unfract.	Unfract. + CPB	K2	K2 + CPB
anti-CD20	0.84	0.82	1.8	1.0
anti-CD38	0.12	0.13	0.20	0.16

Table 1 – Induction of CDC by anti-CD20 and anti-CD38 antibodies.

Data shown are EC₅₀ values [µg/mL] for induction of CDC of Daudi cells by unfractionated and collected K2 isoforms, with and without carboxypeptidase B (CPB) treatment, of anti-CD20 and anti-CD38 antibodies.

Figure 2 upper left panel shows that binding to Daudi cells of unfractionated and collected K2 isoforms of anti-CD20 antibody with and without carboxypeptidase B treatment are comparable. The upper right panel shows that the same is true for anti-CD38. The lower left panel and Table 1 show that induction of CDC is less efficient (~ factor 2 less) for the collected K2 isoforms of anti-CD20 antibody. Capacity to induce CDC was restored after carboxypeptidase B treatment of the collected K2 isoforms of anti-CD20. The lower right panel and Table 1 show that the same is true for anti-CD38 antibody. Carboxypeptidase B treatment partially restored the capacity to induce CDC of the K2 isoforms. These data suggest that the presence of C terminal lysines negatively influences the capacity of an antibody to induce CDC.

Carboxypeptidase B treatment did not affect the capacity of either unfractionated anti-CD20 or anti-CD38 antibody to induce CDC. The unfractionated antibody preparations do contain C-terminal lysine isoforms (K1 and K2), as shown in Figure 1. However, the fraction of C-terminal lysine containing isoforms in the unfractionated antibody preparations is probably too small to influence the capacity to induce CDC.

Example 3: Efficacy of C1q utilization by unfractionated and collected K2 isoforms of anti-CD38 antibody

Antibody preparations and collected K2 isoforms, with or without carboxypeptidase B (CPB) treatment, were obtained as described supra. To test efficacy of C1q

utilization, Daudi cells (2×10^6 cells/mL) were incubated in RPMI1640 medium, supplemented with 10% fetal bovine serum, with a fixed concentration (10 μ g/mL) of antibody preparations at RT for 15 min. C1q-depleted serum (Quidel, San Diego, CA) supplemented with 1 mM $MgCl_2$ and 1 mM $CaCl_2$ and low concentrations of C1q (Complement Technologies, Tyler, TX) was added as a source of complement (final concentration 50% [v/v]). The CDC assay was performed as described supra.

	Unfract.	Unfract. + CPB	K2	K2 + CPB
anti-CD38	1.3	0.8	4.8	0.7

Table 2 – Efficacy of C1q utilization by unfractionated and collected K2 isoforms of anti-CD38 antibody.

Data shown are EC_{50} values [μ g/mL] for C1q requirement for induction of CDC of Daudi cells by unfractionated and collected K2 isoforms, with and without CPB treatment, of anti-CD38 antibody.

Figure 3 and Table 2 show that the amount of C1q required to induce CDC of Daudi cells was highly increased ($> 3.5x$) for the collected K2 isoforms of anti-CD38. Efficacy of C1q use was completely restored by carboxypeptidase B treatment. Carboxypeptidase B treatment of unfractionated anti-CD38 antibody only slightly ($< 2x$) improved the efficacy of C1q utilization.

Example 4: Binding and CDC induction by HEK-produced anti-CD38 antibody and mutants containing one, two or three C-terminal lysines or a C-terminal glutamic acid per heavy chain

To further verify that indeed the presence of C-terminal lysines was responsible for the decreased efficacy of CDC induction by the collected K2 isoform compared with the unfractionated antibody preparation, mutants were constructed containing zero, one, two or three C-terminal lysines in each heavy chain. Furthermore, a mutant with a negative charge at the C-terminus (glutamic acid; E) was constructed. Mutants are described in the table below. Amino acid P445 in EU-numbering corresponds to the proline at position 328 in SEQ ID NOS:1 and 5 (IgG1m (za) and IgG1m(f) allotype Fc-sequences, respectively).

C-terminal sequence (using EU numbering)	Abbreviation	Charge	pI value of mutated protein
445-PGE-447	E2	-	8.2
445-PG-446	K0	0	8.5

445-PGKP-448	K2	+	8.8
445-PGKKP-449	K4	++	9.0
445-PGKKKP-450	K6	+++	9.1

Table 3 – Mutants of anti-CD38 antibody.

pI values were determined by cIEF analysis (Figure 4a).

Mammalian expression vectors for the expression of anti-CD38 antibody 005 (described in WO 2006099875 (Genmab)) were constructed by cloning the coding regions for the human IgG1 (allotype f) heavy and the kappa light chain of the antibody in pcDNA3.3 (Invitrogen). PCR was used to introduce the C-terminal heavy-chain extensions –KP, –KKP, –KKKP and –E in the heavy-chain expression vector. Proline was introduced to prevent cleavage of the added C-terminal lysine(s). All anti-CD38 antibody mutants were produced, under serum-free conditions, using Freestyle medium (Invitrogen, Carlsbad, CA) by transiently co-transfecting relevant heavy and light chain expression vectors in HEK293F cells (Invitrogen) using 293fectin (Invitrogen), according to the manufacturer's instructions. Antibodies were purified by Protein A affinity chromatography (MabSelect SuRe, GE Healthcare, Uppsala, Sweden), dialyzed O/N to PBS and filter-sterilized over 0.2 µm dead-end filters. Concentrations of purified C-terminal IgG1 variants were determined by absorbance at 280 nm. Anti-CD38 antibody and anti-CD38 antibody mutants were analyzed by cIEF, binding to Daudi cells was analyzed by FACS analysis and induction of CDC was tested in a CDC assay with Daudi cells, all assays were performed as described supra.

	anti-CD38	K0	K2	K4	K6	E2
EC ₅₀	0.21	0.21	0.29	N.D. ^a	0.28	0.26
Max. lysis	97	95	65	9	25	26

a) could not be determined.

Table 4 – CDC induction by anti-CD38 antibody and mutants.

Data shown are EC₅₀ values (µg/mL) and percentage lysis induced at the highest concentration of antibody tested (4 µg/mL).

Figure 4a shows that all antibody mutants migrated at the pI calculated based on the amino acids introduced, indicating that the mutants are stable and that the additional C-terminal amino acids were not cleaved. Figure 4b shows that binding of

anti-CD38 antibody to Daudi cells was not affected by the introduction of C-terminal lysines or the glutamic acid and was comparable for all mutants. Figure 4c and Table 4 show that induction of CDC of Daudi cells was similar for anti-CD38 antibody and PG mutant. Introduction of one C-terminal lysine, which has a positive charge, per heavy chain, decreased the capacity to induce CDC, reflected in a lower percentage of maximal lysis (~ 30% decrease) induced. Introduction of two C-terminal lysines (KK) per heavy chain totally abolished the capacity to induce CDC. The mutant containing three C terminal lysines per heavy chain was slightly more efficient in induction of CDC than the mutant containing two C-terminal lysines. The mutant containing C-terminal glutamic acid was inefficient in inducing CDC as well.

Example 5: CDC induction by mixtures of anti-CD38 antibody mutants

To investigate whether charge repulsion between IgG molecules is responsible for the reduced CDC activity of anti-CD38 mutants carrying lysine or glutamic acid at the C-terminus, mutants with different charges at the C-terminus were mixed. The CDC assay was performed as described supra.

Figure 5a shows that adding K4 mutant to K0, while keeping the total IgG concentration constant, decreased the efficacy of CDC induction, compared with that of K0 alone. Figure 5b shows that the same effect was observed for addition of the E2 mutant. These experiments are in good agreement with our previous findings and confirm that mutants of anti-CD38 with charged residues at their C-terminus have strongly reduced CDC activity. Figure 5c shows that mixing the negatively charged E2 mutant with the positively charged K4 mutant almost fully restored efficacy of CDC induction, to the level of CDC induced by K0. Similar results were obtained for a mixture of E2 mutant and K2 mutant (data not shown). Figure 5d shows that maximal lysis was obtained at an E2:K4 ratio of approximately 7:3.

The data suggest that interaction between different IgG molecules, and more specifically Fc-Fc interaction, plays an important role in the induction of CDC.

Example 6: Identification of IgG1 mutations stimulating Fc:Fc interaction-mediated antibody oligomerization detected by a CDC assay

A library of mutations was generated, including those focused at the C-terminal of the CH3 domain. Mutations were introduced into the IgG1 Fc-region of anti-CD38 antibody 005 using the Quikchange site-directed mutagenesis kit (Stratagene, US). Briefly, for each desired mutation position, a forward and a reverse primer encoding

a degenerate codon at the desired location were used to replicate full length plasmid DNA template. The resulting DNA mixtures were digested using *DpnI* to remove source plasmid DNA and used to transform *E. coli*. Resulting colonies were pooled, cultured and plasmid DNA was isolated from these pools and retransformed into *E. coli* to obtain clonal colonies. Mutant plasmid DNA isolated from resulting colonies was checked by DNA sequencing (LGC genomics, Berlin, Germany). Expression cassettes were amplified from plasmid DNA by PCR and DNA mixtures containing both a mutant heavy and a wild type light chain of anti-CD38 antibody 005 were transiently transfected to Freestyle HEK293F cells (Invitrogen, US) using 293fectin (Invitrogen, US) essentially as described by the manufacturer. Supernatants of transfected cells containing antibody mutants were collected.

CDC assays were performed as follows. 0.1×10^6 Daudi or Wien 133 cells were pre-incubated in round-bottom 96-well plates with 1.0 $\mu\text{g/mL}$ unpurified antibodies in a total volume of 100 μL for 15 min on a shaker at RT. Next, 30 μL normal human serum was added as a source of complement (30% final concentration) and incubated in a 37°C incubator for 45 min. The reaction was stopped by putting the plates on ice. 10 μL propidium iodide was added and cell lysis was determined by FACS.

Mutations in the CH3 C-terminal region incorporated in the anti-CD38 antibody were tested for their ability to induce CDC of Daudi cells. The lytic effect of the mutant antibody was compared to that of wild type antibody, for which lysis was set to 100%. The cut-off for inhibition was set to $\leq 66\%$ lysis. The results are shown in Table 5.

Mutations incorporated in the anti-CD38 antibody 005 were tested for their ability to enhance oligomerization as determined by CDC on Wien 133 cells. Wild type antibody is not able to induce CDC on Wien 133 cells. Mutants displaying $\geq 10\%$ cell lysis were scored as enhancing. The results are shown in Table 6.

Table 5 shows that introduction of a negative charge at the C-terminus for mutations P445D and P445E, which decrease CDC when compared to the IgG1 wildtype, in line with expectations. Similarly, introduction of a positive charge, i.e. P445H and P445K, decreases CDC in line with expectations. The mutation K447I, however, apparently decreases CDC, whereas removal of the C-terminally positively charged K was expecting to have a positive impact on CDC. In addition, replacement of G446 and K447 with arginine seems to have a small positive effect on CDC, contrary to expectations. However, this assumes that the C-terminal arginine is, in

fact, present on the mutants. In both cases, the arginine mutants may actually have been C-terminally cleaved, resulting in mutants lacking C-terminal charge (the theoretic c-terminal sequence of the K447R mutant is PGR, which may be cleaved to PG, and the theoretic c-terminal sequence of the G446R mutant is PRK, which may be cleaved to P).

Residue	A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
P445	75	94	25	22	95	60	41		43	77							65	84		
G446					72				56	63	83				84			57	88	
K447						92		28		70			71		80			77	96	

Table 5 – CDC induction by mutants – Daudi cells.

Percentage lysis of Daudi cells in the presence of 1.0 ug/ml anti-CD38 antibody point mutants. Wildtype antibody lysed 66% of cells under these conditions.

Residue	A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
P445	3	5	2	2	4	2	4		4	3							2	3		
G446					3				4	5	4				7			3	7	
K447						3		4		3			3		2			2	6	

Table 6 – CDC induction by mutants – Wien 133 cells.

Percentage lysis of Wien 133 cells in the presence of 1.0 ug/ml anti-CD38 antibody point mutants. Wildtype antibody lysed 3% of cells under these conditions.

CLAIMS

1. A method for producing an antibody in a host cell, wherein said antibody comprises at least a heavy chain, said method comprising the following steps:

5

a) providing a nucleotide construct encoding said heavy chain, wherein said construct does not encode a lysine residue at the C-terminus of said heavy chain,

10

b) expressing said nucleotide construct in a host cell, with the proviso that said host cell is not a CHO cell or a moss cell,

and

15

c) recovering said antibody from a cell culture of said host cell.

2. The method according to claim 1, wherein said host cell further expresses a light-chain-encoding construct.

20

3. The method according to claim 1 or 2, wherein said host cell is capable of Asn-linked glycosylation of proteins.

4. The method according to any one of the preceding claims 1 to 3, wherein said host cell is a non-human cell which is genetically engineered to produce glycoproteins having human-like or human glycosylation.

25

5. The method according to any one of the preceding claims, wherein said host cell is a host cell which is not capable of efficiently removing C-terminal lysine residues from antibody heavy chains.

30

6. The method according to claim 5, wherein said host cell is a host cell which, when expressing a C-terminal-lysine-containing construct, would produce an antibody preparation wherein more than 10% of the antibody molecules is in the K2 isoform, e.g. wherein more the 30% is in the K2 isoform.

7. The method according to any one of the preceding claims, wherein said host cell is selected from the group consisting of:

- a) a yeast cell, e.g. a *Pichia pastoris* or *Saccharomyces cerevisiae*, *Hansenula polymorpha* and *Ogataea minuta*,
- b) a filamentous fungal cell, such as *Aspergillus awamori*, *Aspergillus niger*, *Aspergillus oryzae*, *Trichoderma reesi*,
- 5 c) a plant cell, such as an *Arabidopsis* cell, *Lemna minor*, *Nicotiana benthamiana* (tobacco), oilseed crop plant (*Brassica napus*), soybean, rice, maize (*Zea mays*) or carrot cells,
- d) an NS0 cell, an Sp2/0 cell, and a PER.C6 cell.

8. The method according to any one of the preceding claims, wherein the
10 nucleotide construct provided in step a) is derived from, or designed based on, an original heavy-chain sequence having a codon for a C-terminal lysine residue, e.g. wherein said nucleotide construct comprises a deletion or a substitution of the codon for the C-terminal lysine residue compared to said original heavy-chain sequence.

9. The method according to any one of the preceding claims, wherein said
15 heavy-chain-encoding construct does not encode a lysine or an arginine at the C-terminus of said heavy chain, preferably wherein said heavy-chain-encoding construct does not encode a charged amino acid residue at the C-terminus of said heavy chain.

10. The method according to any one of the preceding claims, wherein said
20 nucleotide construct, other than the C-terminal modification, encodes a heavy chain of the IgG1 or IgG2 isotype.

11. The method according to any one of the preceding claims, wherein the C-terminal codon of said nucleotide construct encodes a Pro or a Gly residue.

12. An isolated antibody obtained or obtainable by the method of any one of the
25 preceding claims.

13. A host cell which is not capable of efficiently removing C-terminal lysine residues from antibody heavy chains, said host cell comprising a nucleotide construct encoding a heavy chain lacking a C-terminal lysine.

14. The host cell according to claim 13, wherein said host cell is of one of the types specified in claim 7.

15. A method for increasing the ability of an antibody to mediate complement-dependent cytotoxicity, said method comprising the step of removing a C-terminal lysine residue from the heavy-chains of an antibody.

16. The method according to claim 15, wherein the lysine residue is removed by enzymatic cleavage, e.g. using a carboxypeptidase.

17. The method according to claim 15 or 16, wherein said antibody is purified prior to removal of the C-terminal lysine residue.

18. The method according to any one of claim 15 to 17, wherein said antibody is of the IgG1 or IgG2 isotype.

19. A method for testing a plurality of antibody-producing cell cultures, e.g. hybridoma cell cultures, for a desired property, said method comprising treating samples of cultures of said hybridomas with the method according to any one of claims 15 to 18 and testing the treated samples for the desired property.

20. The method according to claim 19, wherein the desired property is cell lysis or cell killing.

21. An isolated antibody variant comprising a heavy chain having at least one charged amino acid residue in the C-terminal region, wherein said charged amino acid residue is not susceptible to proteolytic removal.

22. The antibody variant according to claim 21 further comprising a light chain.

23. The antibody variant according to any one of the preceding claims 21 to 22, wherein the antibody has decreased ability to mediate complement-dependent cytotoxicity compared to an antibody lacking said charged amino acid residue.

24. The antibody variant according to any one of the preceding claims 21 to 23, wherein said charged amino acid residue is not susceptible to removal by proteases of mammalian origin, preferably not susceptible to removal by proteases of CHO, HEK-293, PER.C6, NS0 or Sp2/0 origin, more preferably not susceptible to removal

by proteases active in the secretory pathway of CHO, HEK-293, PER.C6, NS0 or Sp2/0 cells.

25. The antibody variant according to any one of the preceding claims 21 to 24, wherein said charged amino acid residue is not susceptible to removal by human
5 proteases, preferably not susceptible to removal by human proteases that can act on proteins in circulation.

26. The antibody variant according to any one of the preceding claims 21 to 25, wherein said charged amino acid is amongst the six most C-terminal amino acid residues, e.g. amongst the five most C-terminal amino acid residues of said heavy
10 chain, such as amongst the four most C-terminal amino acid residues of said heavy chain, e.g. amongst the three most C-terminal amino acid residues of said heavy chain, such as amongst the two most C-terminal amino acid residues of said heavy chain, e.g. wherein said charged amino acid is the most C-terminal amino acid residue of said heavy chain.

15 27. The antibody variant according to any one of the preceding claims 21 to 26, wherein said heavy chain comprises the CH3 sequence set forth in SEQ ID NO:1, 2, 3 or 5 and said charge amino acid is situated between positions 325-330, 321-326, 372-377 and 325-330, respectively.

20 28. The antibody variant according to any one of the preceding claims 21 to 27, wherein said heavy chain comprises the entire constant region sequence set forth in SEQ ID NO:1, 2, 3 or 5 and said charge amino acid is situated between positions 325-330, 321-326, 372-377 and 325-330, respectively.

25 29. The antibody variant according to any one of the preceding claims 21 to 28, wherein said charged amino acid is a positively charged amino acid residue, preferably a lysine residue.

30. The antibody variant according to any one of the preceding claims 21 to 28, wherein said charged amino acid is a negatively charged amino acid residue, preferably a glutamic acid residue.

30 31. The antibody variant according to any one of the preceding claims 21 to 30, wherein said charged amino acid residue is not susceptible to proteolytic removal

due to amino acid modifications at positions situated C-terminally of said charged amino acid residue.

32. The antibody variant according to any one of the preceding claims 21 to 31, wherein said charged amino acid residue is not susceptible to proteolytic removal due to the presence of a proline residue situated C-terminally of said charged amino acid residue, preferably wherein said proline residue is placed immediately C-terminal of said charged amino acid residue, more preferably wherein said charged amino acid residue and said proline residue are the two most C-terminal amino acid residues of said heavy chain.

33. The antibody variant according to any one of the preceding claims 21 to 32, wherein said heavy chain comprises two or more charged amino acid residues in the C-terminal region, such as two, three, four or five charged amino acid residues, preferably having either all a positive charge or all a negative charge.

34. The antibody variant according to any one of the preceding claims 21 to 33, wherein the specified amino acid modifications are the result of amino acid substitutions.

35. The antibody variant according to any one of the preceding claims 21 to 34, wherein the specified amino acid modifications are the result of C-terminal amino acid additions.

36. The antibody variant according to any one of the preceding claims 21 to 35, wherein said antibody is a human antibody, preferably a human IgG1 or a human IgG3 antibody.

37. The antibody variant according to any one of the preceding claims 21 to 36, wherein said antibody heavy chain is an IgG1, IgG2, IgG3 or IgG4 heavy chain to which a proline residue has been added at the C-terminus.

38. The antibody variant according to any one of the preceding claims 21 to 37, wherein said antibody is not conjugated at the C-terminus.

39. An antibody according to any one of claims 21 to 38 for use as a medicament.

40. A nucleotide construct encoding a heavy chain having a charged amino acid residue in the C-terminal region, wherein said charged amino acid residue is not susceptible to proteolytic removal.

5 41. The nucleotide construct according to claim 40, having the further features of any one of claims 22 to 38.

42. A host cell capable of producing an antibody variant according to any one of claims 21 to 38.

43. A host cell which has been genetically modified to eliminate the activity of carboxypeptidases capable of removing C-terminal lysines from heavy chains.

10 44. The host cell of claim 43, wherein said cell is a CHO, HEK-293, PER.C6, NS0 or Sp2/0 cell.

45. A method for producing an antibody variant according to any one of claims 21 to 38 comprising culturing the host cell according to any one of claims 42 to 44 and recovering said antibody from the cell culture.

15 46. The method according to claim 45, wherein said host cell is a CHO or HEK cell comprising a nucleotide-construct encoding a heavy chain having a negatively-charged amino acid residue at the C-terminus or a proline residue preceded by a positively-charged amino acid residue at the C-terminus.

20 47. An antibody mixture comprising a subpopulation of antibody variant molecules comprising heavy chains having at least one positively-charged amino acid residue in the C-terminal region and a subpopulation of antibody variants molecules comprising heavy chains having at least one negatively-charged amino acid residue C-terminal region, wherein said positively-charged and negatively-charged amino acid residues are not susceptible to proteolytic removal.

25 48. The antibody mixture according to claim 47, wherein each of said subpopulations constitutes at least 10%, such as at least 20% of said mixture, e.g. at least 30% of said mixture.

49. The antibody mixture according to claim 47 or 48, wherein said antibody molecules have the properties specified in one or more of claims 22 to 38.

50. The antibody mixture according to any one of the preceding claims 47 to 49, wherein the antibody molecules within each of the two subpopulations comprise two identical heavy chains.

51. The antibody mixture according to any one of the preceding claims 47 to 50
5 for use as a medicament, preferably for use in the treatment of cancer.

52. An isolated antibody comprising a heavy chain having a variant C-terminal region, comprising one or more amino acid modifications that favour intermolecular C-terminal interactions between antibody molecules.

53. The antibody according to claim 52, wherein the antibody has increased
10 ability to mediate complement-dependent cytotoxicity compared to an antibody lacking said amino acid modifications.

54. The antibody according to claim 52, wherein said antibody comprises two non-identical heavy chains.

55. The antibody according to claim 52, wherein one heavy chain comprises a
15 negatively-charged amino acid residue C-terminal region and the other heavy chain comprises a positively-charged charged amino acid residue in the C-terminal region, wherein said charged amino acid residues are not susceptible to proteolytic removal.

56. The antibody according to any one of the preceding claims 52 to 55, wherein the antibody is a monospecific antibody.

20 57. The antibody according to any one of the preceding claims 52 to 55, wherein the antibody is a bispecific antibody.

58. The antibody according to any one of the preceding claims 52 to 57 for use as a medicament.

59. A method for producing an antibody according to any one of claims 52 to 57
25 comprising culturing a host cell comprising nucleotide constructs encoding said heavy chains and recovering said antibody from the cell culture of said host cell.

60. The method according to claim 59, wherein said host cell is a CHO or HEK cell.

FIG. 1

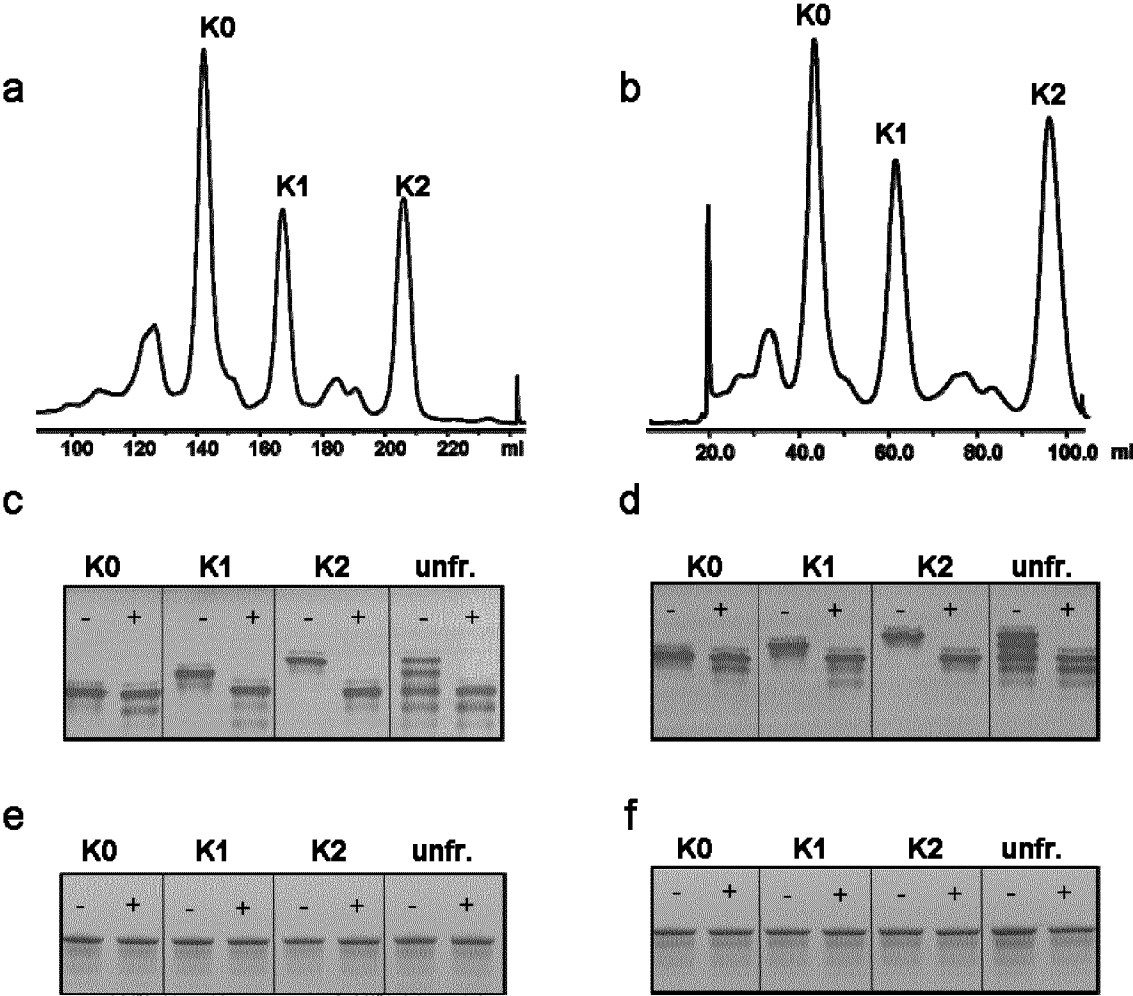


FIG. 2

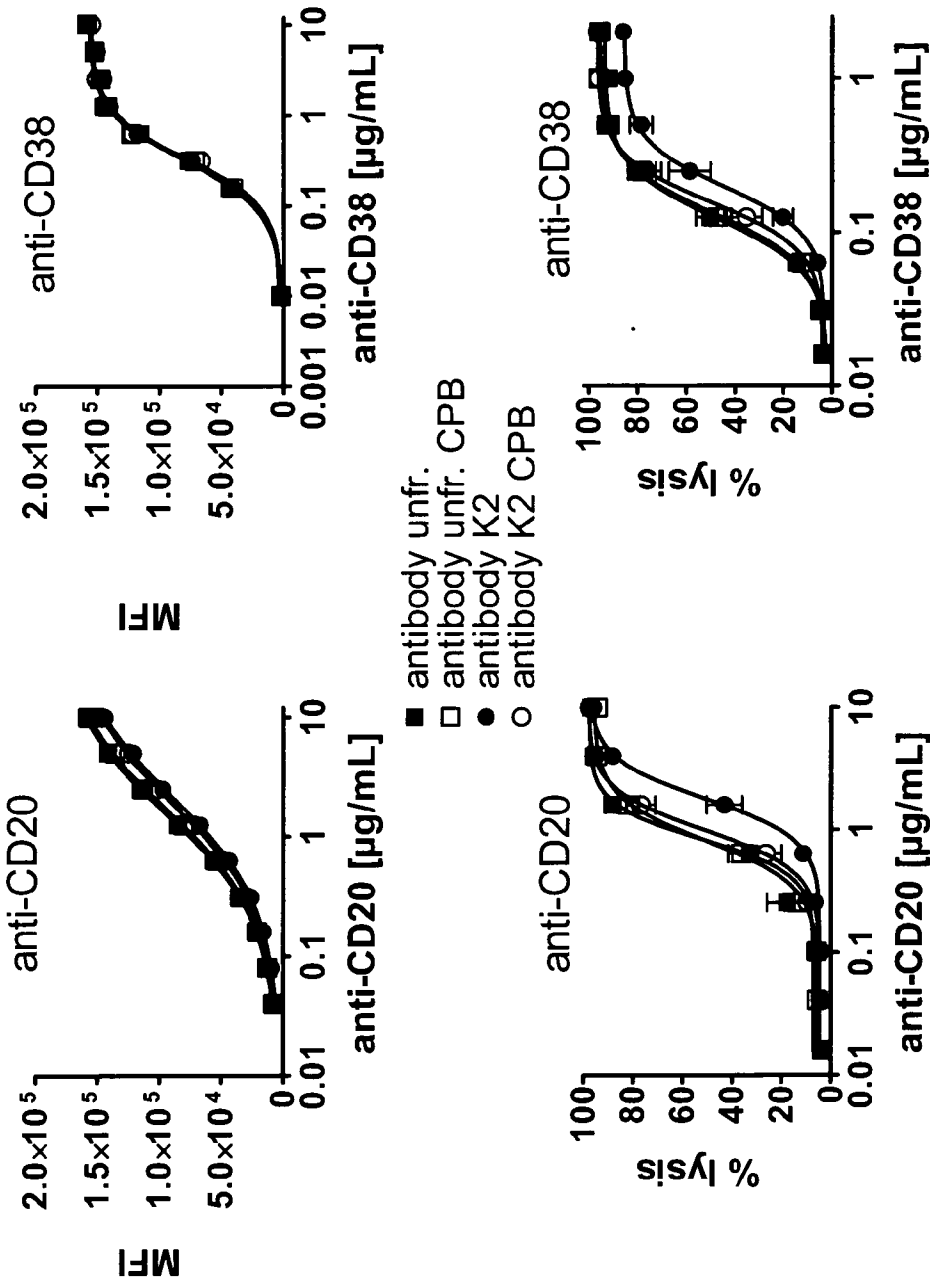


FIG. 3

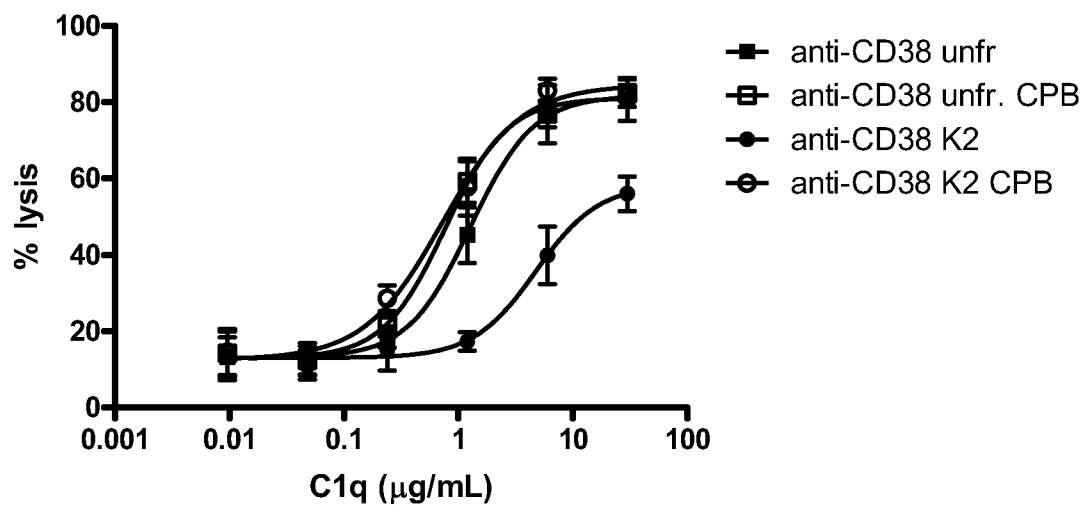


FIG. 4

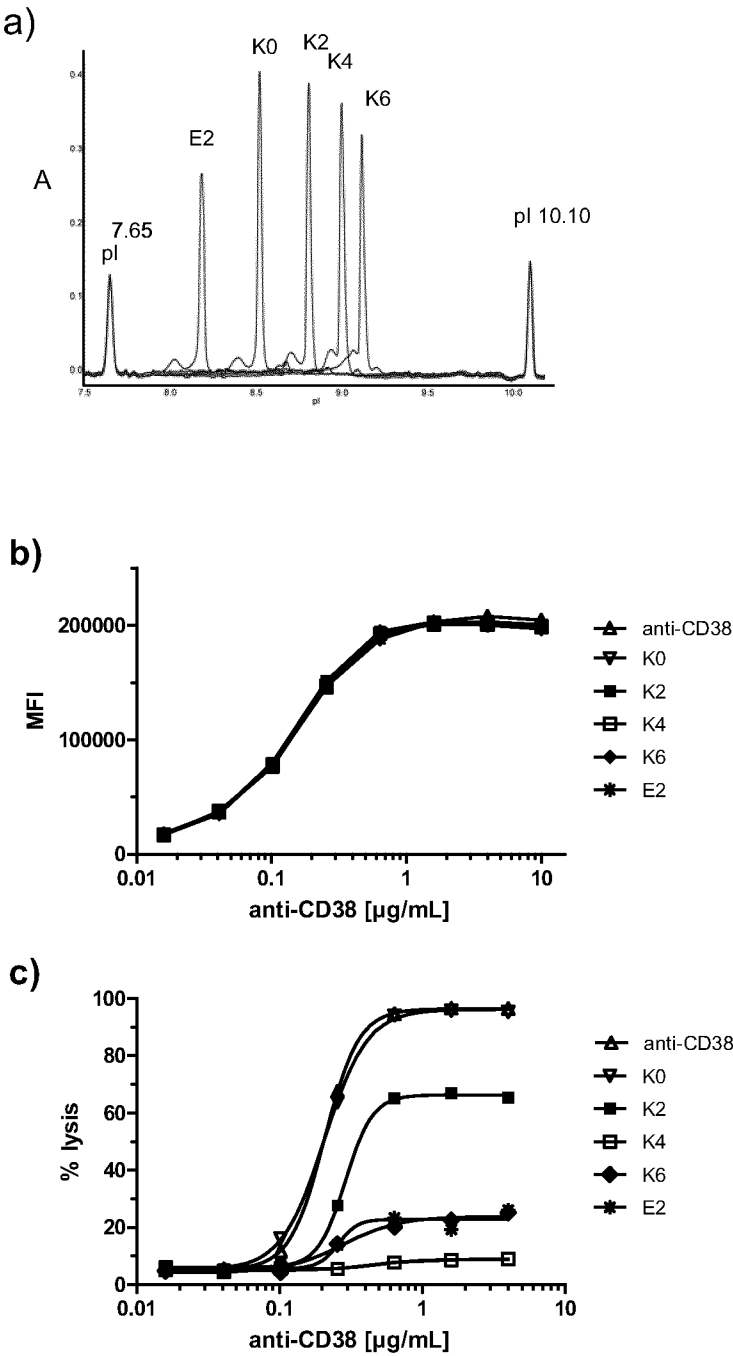
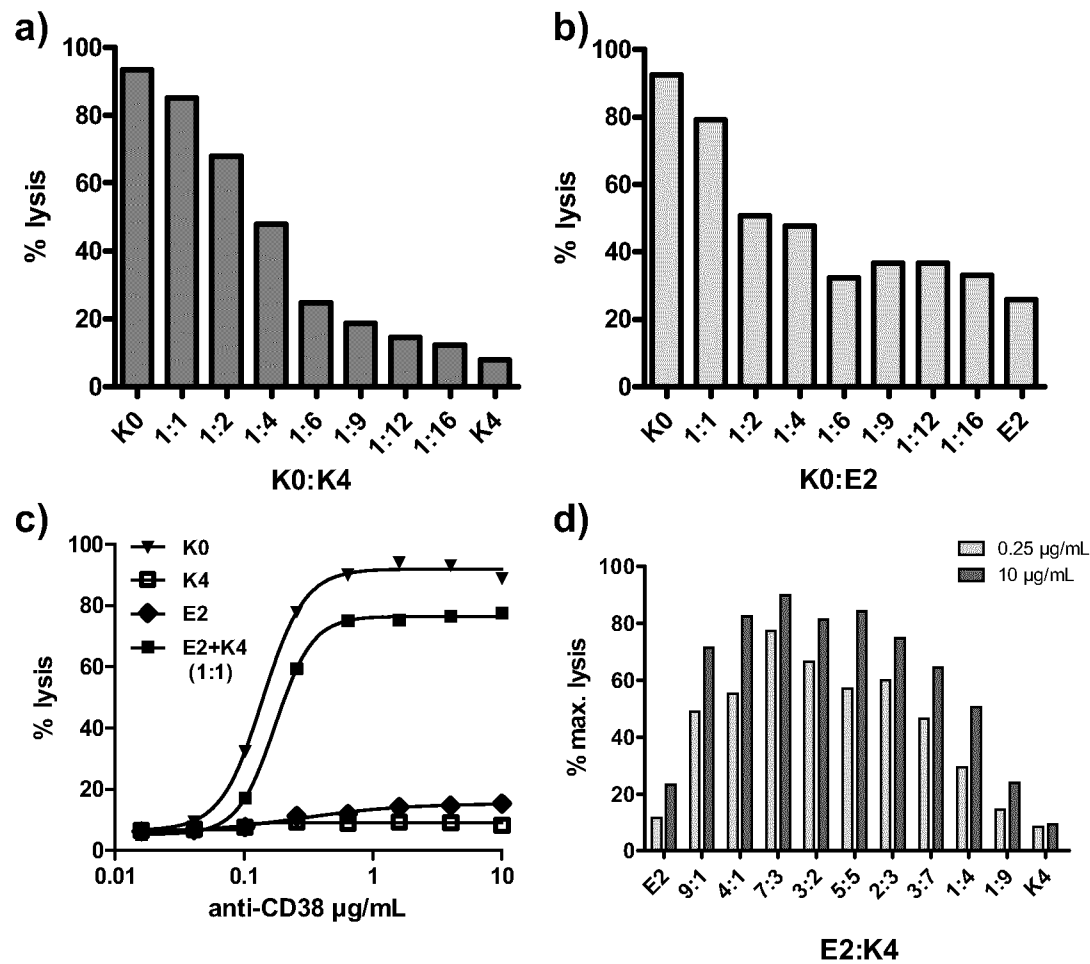


FIG. 5



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/063338

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/00
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)
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 practicable, search terms used)

EPO-Internal, WPI Data, 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	US 2010/297697 A1 (AMBROSIUS DOROTHEE [DE] ET AL) 25 November 2010 (2010-11-25)	1-3,5-14
Y	the whole document	4
X	WO 2008/006554 A2 (GREENOVATION BIOTECH GMBH [DE]; SCHUSTER MANFRED [AT]; KIRCHEIS RALF []) 17 January 2008 (2008-01-17) cited in the application page 30 - page 31	12
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Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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Date of the actual completion of the international search

21 November 2012

Date of mailing of the international search report

03/12/2012

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Pérez-Mato, Isabel

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/063338

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
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X	LINTAO WANG ET AL: "Structural Characterization of a Recombinant Monoclonal Antibody by Electrospray Time-of-Flight Mass Spectrometry", PHARMACEUTICAL RESEARCH, KLUWER ACADEMIC PUBLISHERS-PLENUM PUBLISHERS, NL, vol. 22, no. 8, 1 August 2005 (2005-08-01), pages 1338-1349, XP019370906, ISSN: 1573-904X, DOI: 10.1007/S11095-005-5267-7 the whole document	12
X	WO 2007/106707 A2 (MACROGENICS INC [US]; STAVENHAGEN JEFFREY [US]) 20 September 2007 (2007-09-20) the whole document in particular pages 52-55	12
A	STROHL ET AL: "Optimization of Fc-mediated effector functions of monoclonal antibodies", CURRENT OPINION IN BIOTECHNOLOGY, LONDON, GB, vol. 20, no. 6, 1 December 2009 (2009-12-01), pages 685-691, XP026778879, ISSN: 0958-1669, DOI: 10.1016/J.COPBIO.2009.10.011 [retrieved on 2009-11-04] the whole document in particular, figure 1.	1-14
Y	HAMILTON ET AL: "Glycosylation engineering in yeast: the advent of fully humanized yeast", CURRENT OPINION IN BIOTECHNOLOGY, LONDON, GB, vol. 18, no. 5, 24 October 2007 (2007-10-24), pages 387-392, XP022350908, ISSN: 0958-1669, DOI: 10.1016/J.COPBIO.2007.09.001 the whole document	4
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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2012/063338

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
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Y	JEFFERIS ROY: "Glycosylation as a strategy to improve antibody-based therapeutics", NATURE REVIEWS. DRUG DISCOVERY, NATURE PUBLISHING GROUP, GB, vol. 8, no. 3, 1 March 2009 (2009-03-01), pages 226-234, XP002600969, ISSN: 1474-1784, DOI: 10.1038/NRD2804 the whole document	4
Y	WO 2007/087420 A2 (RECOPHARMA AB [SE]; HOLGERSSON JAN [SE]; GUSTAVSSON ANKI [SE] RECOPHAR) 2 August 2007 (2007-08-02) the whole document	4
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A	MOORE GREGORY L ET AL: "Engineered Fc variant antibodies with enhanced ability to recruit complement and mediate effector functions", MABS, LANDES BIOSCIENCE, US, vol. 2, no. 2, 1 March 2010 (2010-03-01), pages 181-189, XP009143498, ISSN: 1942-0862, DOI: 10.4161/MABS.2.2.11158 the whole document -/--	15-20

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/063338

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	<p>& G L Moore ET AL: "Engineered Fc variant antibodies with enhanced ability to recruit complement and mediate effector functions (Supplementary Information)", mAbs, 1 March 2010 (2010-03-01), pages 1-2, XP055045074, Retrieved from the Internet: URL: http://dx.doi.org/10.4161/mabs.2.2.11158 [retrieved on 2012-11-21] the whole document</p> <p>-----</p>	
A	<p>CHOWDHURY P S ET AL: "Tailor-made antibody therapeutics", METHODS : A COMPANION TO METHODS IN ENZYMOLOGY, ACADEMIC PRESS INC., NEW YORK, NY, US, vol. 36, no. 1, 1 May 2005 (2005-05-01), pages 11-24, XP027216559, ISSN: 1046-2023 [retrieved on 2005-04-19] the whole document</p> <p>-----</p>	15-20
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A	<p>ANTES ET AL: "Analysis of lysine clipping of a humanized Lewis-Y specific IgG antibody and its relation to Fc-mediated effector function", JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL SCIENCES & APPLICATIONS, ELSEVIER, AMSTERDAM, NL, vol. 852, no. 1-2, 31 May 2007 (2007-05-31), pages 250-256, XP022099894, ISSN: 1570-0232, DOI: 10.1016/J.JCHROMB.2007.01.024 the whole document</p> <p>-----</p> <p style="text-align: center;">-/--</p>	15-20

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2012/063338

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>BRUNHOUSE R ET AL: "Isotypes of IgG: Comparison of the primary structures of three pairs of isotypes which differ in their ability to activate complement", MOLECULAR IMMUNOLOGY, PERGAMON, GB, vol. 16, no. 11, 1 November 1979 (1979-11-01), pages 907-917, XP023788873, ISSN: 0161-5890, DOI: 10.1016/0161-5890(79)90089-0 [retrieved on 1979-11-01] the whole document</p> <p>-----</p>	15-20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2012/063338

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of Item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

1-20
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-14

directed to a method of producing antibodies in a host cell, by expressing a nucleotide construct encoding the heavy chain, wherein said heavy chain does not comprise a lysine residue at its C-terminus, to the antibodies obtained by said method and to host cells comprising said nucleotide construct.

2. claims: 15-20

directed to a method to increase the ability of an antibody to mediate CDC, comprising the step of removing a C-terminal lysine residue from the antibody heavy chains and to the use of this method to test a plurality of antibody-producing cell cultures for a desired property.

3. claims: 21-42, 47-51(completely); 45, 46(partially)

directed to antibody variants comprising a heavy chain having at least one charged amino acid residue at the C-term, said charged amino acid residue not being susceptible to proteolytic removal, and to an antibody mixture comprising two subpopulations of antibody variants having, respectively, positively and negatively charged C-terminal residues, not susceptible to proteolytic removal.

4. claims: 43, 44(completely); 45, 46(partially)

directed to a host cell which has been genetically modified to eliminate the activity of carboxypeptidases capable of removing C-terminal lysines from heavy chains and to a method to produce an antibody variant using these cells.

5. claims: 52-60

directed to an antibody comprising a heavy chain having a variant C-terminal region, comprising one or more amino acid modifications that favour C-terminal interactions between antibody molecules.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2012/063338

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