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(54) Title: OBINUTUZUMAB AND RITUXIMAB VARIANTS HAVING REDUCED ADCP

(57) Abstract: The present invention relates to modified antibodies. In particular, the present invention relates to recombinant monoclonal antibodies or fragments, including chimeric, primatized or humanized antibodies or fragments, having reduced effector function and altered ability to mediate cell signaling activity by a target antigen. In addition, the present invention relates to nucleic acid molecules encoding such antibodies, and vectors and host cells comprising such nucleic acid molecules. The invention further relates to methods for producing the antibodies of the invention, and to methods of using these antibodies in treatment of disease.

OBINUTUZUMAB AND RITUXIMAB VARIANTS HAVING REDUCED ADCP

FIELD OF THE INVENTION

The present invention relates to modified antibodies. In particular, the present invention relates to recombinant monoclonal antibodies or fragments, including chimeric, primatized or humanized antibodies or fragments, having reduced effector function and altered ability to mediate cell signaling activity by a target antigen. In addition, the present invention relates to nucleic acid molecules encoding such antibodies, and vectors and host cells comprising such nucleic acid molecules. The invention further relates to methods for producing the antibodies of the invention, and to methods of using these antibodies in treatment of disease.

BACKGROUND

Antibodies, also called immunoglobulins, have a basic structure comprising four polypeptide chains: two identical heavy (H) chains paired with two identical light (L) chains. Each heavy and light chain comprises a variable region (VH and VL, respectively) and a constant region (CH and CL, respectively). The CH region has 3 domains (CH1, CH2, and CH3), while the smaller CL region has only one domain (simply refered to as CL). Each VH and VL region comprises 3 complementarity determining regions (CDRs) flanked by 4 framework regions in the following order: FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4. The CDRs are the most variable part of the V region, and determine the antigen specificity of the antibody. Together, a paired VH and VL region form the antigen binding site, and bivalent antibodies have two such antigen binding sites.

The Fc region of an antibody, i.e., the terminal ends of the heavy chains of antibody spanning domains CH2, CH3 and a portion of the hinge region, is limited in variability and is involved in effecting the physiological roles played by the antibody. The effector functions attributable to the Fc region of an antibody vary with the class and subclass of antibody and include binding of the antibody via the Fc region to a specific Fc receptor ("FcR") on a cell which triggers various biological responses.

These receptors typically have an extracellular domain that mediates binding to Fc, a membrane spanning region, and an intracellular domain that may mediate some signaling

event within the cell. The Fc receptors are expressed in a variety of immune cells including monocytes, macrophages, neutrophils, dendritic cells, eosinophils, mast cells, platelets, B cells, large granular lymphocytes, Langerhans' cells, natural killer (NK) cells, and T cells. Formation of the Fc/FcγR complex recruits these effector cells to sites of bound antigen, typically resulting in signaling events within the cells and important subsequent immune responses such as release of inflammation mediators, B cell activation, endocytosis, phagocytosis, and cytotoxic attack. The ability to mediate cytotoxic and phagocytic effector functions is a potential mechanism by which antibodies destroy targeted cells. The cell-mediated reaction wherein nonspecific cytotoxic cells that express FcγRs recognize bound antibody on a target cell and subsequently cause lysis of the target cell is referred to as antibody dependent cell-mediated cytotoxicity (ADCC) (Ravetch, et al., Annu Rev Immunol 19 (2001) 275-290). The cell-mediated reaction wherein nonspecific cytotoxic cells that express FcγRs recognize bound antibody on a target cell and subsequently cause phagocytosis of the target cell is referred to as antibody dependent cell-mediated phagocytosis (ADCP). In addition, an overlapping site on the Fc region of the molecule also controls the activation of a cell independent cytotoxic function mediated by complement, otherwise known as complement dependent cytotoxicity (CDC).

For the IgG class of antibodies, ADCC and ADCP are governed by engagement of the Fc region with a family of receptors referred to as Fcγ receptors (FcγRs). In humans, this protein family comprises FcγRI (CD64); FcγRII (CD32), including isoforms FcγRIIA, FcγRIIB, and FcγRIIC; and FcγRIII (CD16), including isoforms FcγRIIIA and FcγRIIIB (Raghavan, and Bjorkman, Annu. Rev. Cell Dev. Biol. 12 (1996) 181-220; Abes, et al., Expert Reviews VOL 5(6), (2009) 735-747). FcγRs are expressed on a variety of immune cells, and formation of the Fc/FcγR complex recruits these cells to sites of bound antigen, typically resulting in signaling and subsequent immune responses such as release of inflammation mediators, B cell activation, endocytosis, phagocytosis, and cytotoxic attack. Furthermore, whereas FcγRI, FcγRIIA/c, and FcγRIIIA are activating receptors characterized by an intracellular immunoreceptor tyrosine-based activation motif (ITAM), FcγRIIB has an inhibition motif (ITIM) and is therefore inhibitory. Moreover, de Reys, et al., Blood, 81, (1993) 1792-1800 concluded that platelet activation and aggregation induced by monoclonal antibodies, like for example CD9, is initiated by antigen recognition followed by an Fc domain dependent step, which involves the FcγRII- receptor (see also: Taylor, et al., Blood 96 (2000) 4254-4260). While FcγRI binds monomelic IgG with high affinity, FcγRIII and FcγRII are low-affinity receptors, interacting with complexed or aggregated IgG.

In many circumstances, the binding and stimulation of effector functions mediated by the Fc region of immunoglobulins is highly beneficial, however, in certain instances effector function can lead to adverse effects during antibody-mediated treatment of disease. This is particularly true for those antibodies designed to deliver a drug (e.g., toxins and isotopes) to the target cell where the Fc/FcγR mediated effector functions bring healthy immune cells into the proximity of the deadly payload, resulting in depletion of normal lymphoid tissue along with the target cells. In other instances, for example, where blocking the interaction of a widely expressed receptor with its cognate ligand is the objective, recruitment of immune effector cells results in unwanted toxicity. Also, in the instance where a therapeutic antibody exhibited promiscuous binding across a number of human tissues induction of effector function leads to adverse events and toxicity. Last but not least, affinity to the FcγRII receptor can lead to platelet activation and aggregation via FcγRII receptor binding, which is a serious side-effect of such antibodies.

In addition to mediating effector functions, monoclonal antibodies can modulate cellular functions by inducing or inhibiting cell signaling pathways. For example, monoclonal antibodies have been shown to mediate antigen cross-linking, activate death receptors (e.g., by facilitating oligomerization of receptors or mimicking ligand binding), and blocking of ligand-mediated cell signaling in cell growth differentiation, and/or proliferation pathways (see, e.g., Ludwig et al, Oncogene (2003) 22: 9097- 9106). Apoptosis, or programmed cell death, can be triggered by several different mechanisms. For example, the activation of signaling pathways through cell membrane-bound "death receptors", e.g., members of the tumor necrosis factor receptor (TNFR) superfamily, can lead to induction of direct cell death. Likewise, dimerization or cross-linking of surface antigen, e.g., CD20, can also induce direct cell death (see, e.g., Ludwig et al, Oncogene (2003) 22: 9097-9106). The orientation of the variable domains of e.g. IgG type antibodies seem to play a crucial role regarding antibody mediated induction of direct cell death.

The interface between the VH and CH1 domains comprises conserved amino acids (see e.g., Lesk and Chothia, Nature (1988) 335(8):188-190). The area of contact can be described as a "molecular ball-and-socket joint". This joint determines the "elbow motion" and also the so called "elbow angle" of the VH and VL regions with respect to the CH1 and CL regions, and prevents a rigid contact from forming between the V and C regions (Lesk and Chothia, Nature (1988) 335(8): 188-190)). The “socket” of this ball-and-socket joint is formed by amino acid

residues in the VH framework region whereas the "ball" is formed by amino acid residues in the CH1 domain. Differences in the amino acids at these positions can dictate the elbow angle that is formed between the V and C regions, and therefore the orientation of the VH-VL dimer (see Lesk and Chothia, Nature (1988) 335(8): 188-190). Conformational alterations of the elbow angle have been identified to be responsible for significant changes in binding behavior of the antibody/antigen interaction without changing overall affinity.

SUMMARY

Recognizing the tremendous therapeutic potential of modified antibodies that have reduced ability to induce effector functions and altered ability to induce direct cell death, the present inventors developed such antibodies, as well as a method for producing such antibodies. *Inter alia*, this method involves producing recombinant, chimeric antibodies or chimeric fragments thereof.

There remains a need for monoclonal antibodies with improved therapeutic potential for human therapy. Modulating induction of effector function and enhancing signaling pathways with monoclonal antibodies is very challenging, but modulating antigens associated with cell signaling, including, but not limited to, the induction of direct cell death while controlling effector function, is much needed for improved cancer therapy. Unexpectedly, the present inventors found that the point mutation Asn297Asp leads to reduced or abolished effector function but residual ADCP function. The inventors further found that the mutation Pro329Gly also leads to reduced or abolished effector function but residual ADCP function and the Asn297Asp and Pro329Gly mutations unexpectedly display a comparable and unique pattern of activation of effector functions. Surprisingly, this mutation can be combined with mutations in the elbow hinge part of the antibodies as disclosed herein, leading to altered induction of direct cell death. In combination, the modifications as disclosed herein allow modulation of induction of direct cell death and selective ablation of effector functions as compared to non-modified parent antibodies.

Accordingly, one aspect of the invention is an antibody comprising a variant heavy chain region comprising at least one amino acid substitution relative to the parent non-substituted heavy chain region, leading to strongly reduced or abolished ADCC and CDC function, residual ADCP function, reduced or abolished binding to Fc receptors, reduced or abolished binding to Clq and reduced or abolished toxicities. Another aspect of the invention is an antibody comprising a variant heavy chain region comprising at least one amino acid

substitution relative to the parent non-substituted heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residue Asn297, and wherein said substitution is Asn297Asp. Another aspect of the invention is an antibody comprising a variant heavy chain region comprising at least one amino acid substitution relative to the parent non-substituted heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residue Pro329, and wherein said substitution is Pro329Gly. A further aspect of the invention is an antibody as described herein, wherein induction of effector function is reduced compared to effector function induced by an antibody comprising the parent non-substituted heavy chain region.

In a specific aspect of the invention said parent non-substituted antibody is an anti-CD20 antibody. In another specific aspect of the invention the parent non-substituted antibody is a type I anti-CD20 antibody. In yet another specific aspect of the invention the parent non-substituted antibody is a type II anti-CD20 antibody. In a preferred aspect of the invention the parent non-substituted antibody is obinutuzumab. In another preferred aspect of the invention the parent non-substituted antibody is rituximab.

Another aspect of the invention is an antibody as described herein, wherein FcγRIII binding by the antibody comprising the variant heavy chain region is abolished compared to binding to FcγRIII by the parent non-substituted antibody comprising asparagine at position 297. Yet another aspect of the invention is an antibody as described herein, wherein ADCC function induced by the antibody comprising the variant heavy chain region is abolished or strongly reduced compared to ADCC function induced by the parent non-substituted antibody comprising asparagine at position 297. Yet another aspect of the invention is an antibody as described herein, wherein FcγRI binding by the antibody comprising the variant heavy chain region is reduced compared to binding to FcγRI by the parent non-substituted antibody comprising asparagine at position 297. Yet another aspect of the invention is an antibody as described herein, wherein induction of ADCP function induced by the antibody comprising the variant heavy chain region is reduced compared to ADCP function induced by the parent non-substituted antibody comprising asparagine at position 297, wherein said antibody comprising the variant heavy chain retains residual ADCP function. Yet another aspect of the invention is an antibody as described herein, wherein induction of CDC function induced by the antibody comprising the variant heavy chain region is strongly reduced compared to CDC function induced by the parent non-substituted antibody comprising asparagine at position 297. Another aspect of the invention is an antibody as described herein wherein the parent non-substituted

antibody comprises the amino acid residue Pro329, wherein FcγRIII binding by the antibody comprising the variant heavy chain region is abolished compared to binding to FcγRIII by the parent non-substituted antibody comprising proline at position 329. Yet another aspect of the invention is an antibody as described herein, wherein ADCC function induced by the antibody comprising the variant heavy chain region is abolished or strongly reduced compared to ADCC function induced by the parent non-substituted antibody comprising proline at position 329. Yet another aspect of the invention is an antibody as described herein, wherein FcγRI binding by the antibody comprising the variant heavy chain region is reduced compared to binding to FcγRI by the parent non-substituted antibody comprising proline at position 329. Yet another aspect of the invention is an antibody as described herein, wherein induction of ADCP function induced by the antibody comprising the variant heavy chain region is reduced compared to ADCP function induced by the parent non-substituted antibody comprising proline at position 329, wherein said antibody comprising the variant heavy chain retains residual ADCP function. Yet another aspect of the invention is an antibody as described herein, wherein induction of CDC function induced by the antibody comprising the variant heavy chain region is strongly reduced compared to CDC function induced by the parent non-substituted antibody comprising proline at position 329.

Yet another aspect of the invention is an antibody as described herein, wherein the variant heavy chain region comprises a further amino acid substitution relative to the parent non-substituted heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residue Pro151, and wherein said further substitution is at said amino acid residue Pro151, wherein direct cell death induced by the antibody comprising the variant heavy chain region is altered compared to direct cell death induced by the antibody comprising proline at position 151. Another aspect of the invention is the antibody as described herein, wherein direct cell death induced by the antibody comprising the variant heavy chain region is increased compared to direct cell death induced by the antibody comprising proline at position 151. Another aspect of the invention is the antibody as described herein, wherein direct cell death induced by the antibody comprising the variant heavy chain region is decreased compared to direct cell death induced by the antibody comprising proline at position 151.

Another aspect of the invention is the antibody as described herein, wherein Pro151 is substituted with an amino acid selected from the group consisting of alanine and phenylalanine. A specific aspect to the invention is the antibody described herein, wherein

Pro151 is substituted with phenylalanine. Another specific aspect to the invention is the antibody described herein, wherein Pro151 is substituted with alanine.

Yet another aspect of the invention is an antibody as described herein, wherein the variant heavy chain region comprises a further amino acid substitution relative to the parent non-substituted heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residue Val11, and wherein said further substitution is at said amino acid residue Val11, wherein direct cell death induced by the antibody comprising the variant heavy chain region is altered compared to direct cell death induced by the antibody comprising valine at position 11. Another aspect of the invention is the antibody as described herein, wherein direct cell death induced by the antibody comprising the variant heavy chain region is increased compared to direct cell death induced by the antibody comprising valine at position 11. Another aspect of the invention is the antibody as described herein, wherein direct cell death induced by the antibody comprising the variant heavy chain region is decreased compared to direct cell death induced by the antibody comprising valine at position 11.

Another aspect of the invention is the antibody as described herein, wherein Val11 is substituted with an amino acid selected from the group consisting of alanine, glycine, phenylalanine, threonine and tryptophan. A specific aspect of the invention is the antibody as described herein, wherein Val11 is substituted with an amino acid selected from the group consisting of phenylalanine, threonine and tryptophan. Another specific aspect of the invention is the antibody as described herein, wherein Val11 is substituted with an amino acid selected from the group consisting of alanine and glycine.

Yet another aspect of the invention is an anti-CD20 antibody comprising a variant heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residues Asn297 and Pro151, said variant heavy chain region comprising an amino acid substitution relative to the parent non-substituted heavy chain region, wherein said substitution is Asn297Asp, wherein induction of effector function is reduced compared to effector function induced by an antibody comprising the parent non-substituted heavy chain region, said variant heavy chain region comprising at least one further amino acid substitution at position Pro151 in the heavy chain, wherein direct cell death induced by the antibody comprising the variant heavy chain region is altered compared to direct cell death induced by the antibody comprising proline at position 151. Yet another aspect of the invention is an anti-CD20 antibody comprising a variant heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residues Pro329 and Pro151, said

variant heavy chain region comprising an amino acid substitution relative to the parent non-substituted heavy chain region, wherein said substitution is Pro329Gly, wherein induction of effector function is reduced compared to effector function induced by an antibody comprising the parent non-substituted heavy chain region, said variant heavy chain region comprising at least one further amino acid substitution at position Pro151 in the heavy chain, wherein direct cell death induced by the antibody comprising the variant heavy chain region is altered compared to direct cell death induced by the antibody comprising proline at position 151.

Yet another aspect of the invention is an anti-CD20 antibody comprising a variant heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residues Asn297 and Val11, said variant heavy chain region comprising an amino acid substitution relative to the parent non-substituted heavy chain region, wherein said substitution is Asn297Asp, wherein induction of effector function is reduced compared to effector function induced by an antibody comprising the parent non-substituted heavy chain region, said variant heavy chain region comprising at least one further amino acid substitution at position Val11 in the heavy chain, wherein direct cell death induced by the antibody comprising the variant heavy chain region is altered compared to direct cell death induced by the antibody comprising valine at position 11. Yet another aspect of the invention is an anti-CD20 antibody comprising a variant heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residues Pro329 and Val11, said variant heavy chain region comprising an amino acid substitution relative to the parent non-substituted heavy chain region, wherein said substitution is Pro329Gly, wherein induction of effector function is reduced compared to effector function induced by an antibody comprising the parent non-substituted heavy chain region, said variant heavy chain region comprising at least one further amino acid substitution at position Val11 in the heavy chain, wherein direct cell death induced by the antibody comprising the variant heavy chain region is altered compared to direct cell death induced by the antibody comprising valine at position 11.

Another aspect of the invention is the antibody as described herein, wherein the antibody specifically binds to CD20. In a specific aspect of the invention, the antibody binds to CD20 with a dissociation constant (Kd) on cells of 10 nM or less as determined by scatchard analysis.

Another aspect of the invention is a polynucleotide encoding a variant heavy chain region of an antibody as described herein. Another aspect of the invention is a polynucleotide encoding a light chain region of an antibody as described herein. Yet another aspect of the invention is a

vector comprising at least one of the polynucleotides as described herein. Another aspect of the invention is a polycistronic vector comprising the polynucleotides as described herein. Another aspect of the invention is a host cell comprising the vector or a polynucleotide as described herein.

Another aspect of the invention is a method for the production of an antibody as described herein comprising (i) culturing the host cell as described herein under conditions permitting the expression of said polynucleotide; and (ii) recovering said antibody from the culture medium.

Another aspect of the invention is a pharmaceutical composition comprising an antibody as described herein and a pharmaceutically acceptable carrier. Yet another aspect of the invention is an antibody as described herein for use as a medicament. Yet another aspect of the invention is an antibody as described herein for use in treating a disease selected from the group consisting of proliferative disorder and autoimmune disease.

Another aspect of the invention is an antibody as described herein for use in treating a proliferative disorder as described herein, characterized in that said proliferative disorder is a CD20 expressing cancer. Yet another aspect of the invention is an antibody as described herein for use in treating a CD20 expressing cancer, characterized in that said cancer is selected from the group consisting of lymphoma and lymphocytic leukemia. Yet another aspect of the invention is an antibody as described herein for use in treating an autoimmune disease as described herein, characterized in that said autoimmune disease is selected from the group consisting of rheumatoid arthritis, lupus, multiple sclerosis, Sjögren's syndrome and transplant rejection.

Another aspect of the invention is a method for treating a disease selected from the group consisting of proliferative disorder and autoimmune disease comprising administering to an individual an effective amount of the antibody as described herein. Yet another aspect of the invention is a method of treating an individual having cancer comprising administering to the individual an effective amount of the antibody as described herein, wherein said proliferative disorder is a CD20 expressing cancer. Yet another aspect of the invention is a method of treating an individual having cancer comprising administering to the individual an effective amount of the antibody as described herein, wherein said cancer is selected from the group consisting of lymphoma and lymphocytic leukemia. Yet another aspect of the invention is a method of treating an individual having an autoimmune disease comprising administering to

the individual an effective amount of the antibody as described herein, wherein said autoimmune disease is selected from the group consisting of rheumatoid arthritis, lupus, multiple sclerosis, Sjögren's syndrome and transplant rejection.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1

Induction of cell death by CD20 antibody (obinutuzumab, rituximab) Fc variants as measured by Annexin V binding and PI staining was determined using CD20-expressing mantle cell lymphoma (Z-138). Fc variants (wildtype, glycoenineered (GE), P329G L234A L235A, N297D) were tested using antibody concentrations from 16 ng/ml to 10 µg/ml for 21 hours.

Figure 2

Induction of ADCC by CD20 antibody (obinutuzumab, rituximab) Fc variants as measured by tumor cell lysis after 4 hours of incubation with the antibody Fc variants (wildtype, glycoenineered (GE), P329G L234A L235A and N297D)

A) Lysis of SU-DHL-4 cells as induced by peripheral blood mononuclear cells (PBMCs) in the presence of antibody Fc variants measured by FACS analysis

B) Lysis of Z-138 cells as induced by PBMCs in the presence of antibody Fc variants measured by FACS analysis

C) Degranulation of natural killer cell (NK) after coculture with SU-DHL-4 cells in the presence of antibody Fc variants as assessed by binding of anti-CD107a and flow cytometry

D) Degranulation of natural killer cells (NK) after coculture with Z-138 cells in the presence of antibody Fc variants as assessed by binding of anti-CD107a and flow cytometry

Figure 3

CDC-dependent lysis of CD20 expressing tumor cells induced by CD20 (obinutuzumab, rituximab) Fc variants was measured after incubation with the antibody Fc variants (wildtype, glycoenineered (GE), P329G L234A L235A and N297D) in the presence of rabbit complement.

A) Tumor cell lysis after 2 hours of incubation of SU-DHL-4 cells with rabbit complement in the presence of antibody Fc variants

B) Tumor cell lysis after 2 hours of incubation of Z-138 cells with rabbit complement in the presence of antibody Fc variants

C) Tumor cell lysis after 22 hours of incubation of SU-DHL-4 cells with rabbit complement in the presence of antibody Fc variants

D) Tumor cell lysis after 22 hours of incubation of Z-138 cells with rabbit complement in the presence of antibody Fc variants

Figure 4

B cell depletion in whole blood from healthy donors induced by obinutuzumab and rituximab Fc variants (wildtype, glycoenineered (GE), P329G L234A L235A and N297D) was measured by counting CD20/CD19 positive cells. Human whole blood was incubated with the antibody variants for 20 hours.

A) B cell depletion as measured by FACS analysis in whole blood from Donor 1

B) B cell depletion as measured by FACS analysis in whole blood from Donor 2

Figure 5

ADCP induced by obinutuzumab and rituximab Fc variants (wildtype, glycoenineered (GE) and N297D) in the presence of human M1 or M2c effector cells was measured by FACS analysis after co-incubation with target cells.

A) ADCP after 4 hours of incubation of SU-DHL-4 cells with human M1 effector cells in the presence of antibody Fc variants

B) ADCP after 4 hours of incubation of Z-138 cells with human M1 effector cells in the presence of antibody Fc variants

C) ADCP after 4 hours of incubation of SU-DHL-4 cells with human M2c effector cells in the presence of antibody Fc variants

D) ADCP after 4 hours of incubation of Z-138 cells with human M2c effector cells in the presence of antibody Fc variants

Figure 6

Tumor volume development and tumor growth inhibition (TGI) values after induction of SC SU-DHL-4 tumors in SCID beige mice until day 49 were measured. Monotherapy treatment using obinutuzumab and rituximab Fc variants (wildtype, glycoenineered (GE), P329G L234A L235A, N297D (aglyco)) started at day 21 after tumor cell inoculation and continued administered as once weakly dose for 4 weeks.

Figure 7

FcγRI binding as measured on a Biacore T100 system (GE Healthcare) with immobilized anti-His capturing antibody.

A) FcγRI binding as compared between Herceptin, P-Selectin and five different concentrations of the obinutuzumab variant N297D (aglyco)

B) FcγRI binding as compared between Herceptin, P-Selectin and obinutuzumab N297D

C) FcγRI binding and steady state affinity of the obinutuzumab variant N297D (aglyco)

D) FcγRI binding as compared between different human IgG variants

Figure 8

FcγRIII binding as measured on a Biacore T100 system (GE Healthcare) with immobilized anti-His capturing antibody.

A) FcγRIII binding as compared between Herceptin, P-Selectin and five different concentrations of the obinutuzumab (GA101) variant N297D (aglyco)

B) FcγRIII binding as compared for five different concentrations of the obinutuzumab (GA101) variant N297D (aglyco)

C) FcγRIII binding as compared between different human IgG variants

Figure 9

FcγRII binding as measured on a Biacore T100 system (GE Healthcare) with immobilized anti-His capturing antibody.

A) FcγRII binding as compared between different human IgG variants

B) FcγRII binding as compared between different human IgG variants

Figure 10

Induction of direct cell death by CD20 antibody (obinutuzumab, GA101) variants as measured by Annexin V binding and PI staining was determined using CD20-expressing mantle cell lymphoma (Z-138).

A) GA101 variants (GA101-wildtype, GA101-V11A, GA101-V11G, GA101-V11T, GA101-V11F, GA101-V11W, GA101-Ser114del, GA101-Ser114ins, GA101-P151A, GA101-LC 108, GA101-P151F, GA101-hinge ins, GA101-hinge del, GA101-P329F) were tested at an antibody concentration of 10 μg/ml;

B) GA101 variants (GA101-wildtype, GA101-V11A, GA101-V11G, GA101-V11T, GA101-V11F, GA101-V11W, GA101-Ser114del, GA101-Ser114ins, GA101-P151A, GA101-LC 108, GA101-P151F, GA101-hinge ins, GA101-hinge del, GA101-P329F) were tested at an antibody concentration of 0.1 µg/ml;

C) GA101 Fab variants (GA101-wildtype, GA101-V11F and GA101-P151F) and combined Fab/Fc variants (GA101-P329G L234A L235A, GA101-V11F P329G L234A L235A, GA101-P151F P329G L234A L235A and GA101-hinge del P329G L234A L235A) were compared for induction of direct cell death;

D) Fab variants (V11F, P151F and hinge del) and combined Fab variants (V11F P151F, P151F hinge del and V11F P151F hinge del) both on a GA101 P329G L234A L235A backbone were compared for induction of direct cell death.

Figure 11

B cell depletion induced by obinutuzumab variants (GA101-wildtype, GA101-P329G L234A L235A, GA101-V11F P329G L234A L235A and GA101-P151F P329G L234A L235A) was measured by counting CD20/CD19 positive cells. Human whole blood was incubated with antibody variants at different concentrations and for one or two days, respectively.

Figure 12

Concentration-time profile for GA101-V11F P329G L234A L235A, GA101-P151F P329G L234A L235A and GA101-P329G L234A L235A in SCID beige mice.

Figure 13 A and B

Melting temperature profile for GA101-N297D (aglyco), GA101-P329G, GA201-N297D (aglyco) and GA201-P329G as measured by Tryptophan fluorescence.

DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION

I. DEFINITIONS

Terms are used herein as generally used in the art, unless otherwise defined as follows and herein.

The term "heavy chain", "heavy chain domain" and "heavy chain region" are used interchangeably herein and refer to a polypeptide chain which essentially consists of the heavy chain of an immunoglobulin heavy chain or fragments thereof retaining the same functionality compared to the immunoglobulin heavy chain. In the present specification and claims, the numbering of the residues in an immunoglobulin heavy chain is that of Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991), expressly incorporated herein by reference in its entirety. Kabat et al. defined a numbering system for variable domain sequences that is applicable to any antibody. One of ordinary skill in the art can unambiguously assign this system of amino acid residue numbering to any heavy chain region sequence, without reliance on any experimental data beyond the sequence itself. As used herein, "Kabat numbering" refers to the numbering system set forth by Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991) for variable domain sequences. Unless stated otherwise herein, references to residue number 11 of a heavy chain region means residue numbering by the Kabat numbering system (Kabat numbering). The "EU numbering" system or "EU index as in Kabat" can be used when referring to a residue in an immunoglobulin heavy chain constant region. The EU index as in Kabat refers to the residue numbering of the human IgG1 EU antibody. The numbering of residues can be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a "standard" Kabat numbered sequence. Unless stated otherwise herein, references to residue numbers 151 and 297 means residue numbering by the EU numbering system (EU numbering) set forth by Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991). Examples of two anti-CD20 antibodies numbered according to Kabat (Kabat numbering or EU numbering), in particular positions 11 (Kabat numbering), 151 (EU numbering) and 297 (EU numbering) are included herein (SEQ ID NO: 01, SEQ ID NO: 02). Unless stated otherwise herein, references to residue number 329 means residue numbering by the EU numbering system.

An "*acceptor human framework*" for the purposes herein is a framework comprising the amino acid sequence of a light chain variable domain (VL) framework or a heavy chain variable

domain (VH) framework derived from a human immunoglobulin framework or a human consensus framework, as defined below. An acceptor human framework "derived from" a human immunoglobulin framework or a human consensus framework may comprise the same amino acid sequence thereof, or it may contain amino acid sequence changes. In some embodiments, the number of amino acid changes are 10 or less, 9 or less, 8 or less, 7 or less, 6 or less, 5 or less, 4 or less, 3 or less, or 2 or less. In some embodiments, the VL acceptor human framework is identical in sequence to the VL human immunoglobulin framework sequence or human consensus framework sequence.

"*Affinity*" refers to the strength of the sum total of noncovalent interactions between a single binding site of a molecule (e.g., an antibody) and its binding partner (e.g., an antigen). Unless indicated otherwise, as used herein, "binding affinity" refers to intrinsic binding affinity which reflects a 1:1 interaction between members of a binding pair (e.g., antibody and antigen). The affinity of a molecule X for its partner Y can generally be represented by the dissociation constant (Kd). Affinity can be measured by common methods known in the art, including those disclosed herein. Specific illustrative embodiments for measuring binding affinity are disclosed herein.

An "*affinity matured*" antibody refers to an antibody with one or more alterations in one or more hypervariable regions (HVRs), compared to a parent antibody which does not possess such alterations, such alterations resulting in an improvement in the affinity of the antibody for its antigen.

As used herein, the term "*agonist activity*" is intended to refer to activity of an agent (e.g., an antigen binding molecule) when it interacts with (for example, binds to) a molecule associated with a cell surface and initiates or induces a reaction.

As used herein, the term "*altered cell signaling activity*" is intended to refer to an increase or decrease in the ability of an antibody to induce or inhibit cell signaling activity of a target antigen.

As used herein, the term "*altered cross-linking of one or more target antigens*" is intended to refer to an increase or decrease in the ability of an antibody to bring into closer proximity to each other, and/or into closer proximity with other membrane-associated molecules, and/or into a more favorable conformation for interaction target antigens that are capable of forming

complexes (e.g., through cross-linking of proteins, or oligomerization of membrane-associated receptors) to initiate cell signaling activity.

As used herein, the term "*altered induction of direct cell death*" is intended to refer to an increase or decrease in the ability of an antibody to induce direct cell death.

As used herein, the term "*altered induction of effector function*" or "*altered effector function*" is intended to refer to an increase or decrease in the ability of an antibody to induce effector function.

As used herein, "*amino acid substitution*" is intended to refer to replacing one or more amino acids in a reference sequence (e.g., a parent molecule, such as an antibody). In one embodiment, amino acid substitution can be achieved by, for example, a point mutation in the sequence of a nucleic acid encoding a polypeptide as compared to a parent non-substituted sequence. In another embodiment, substitution of an amino acid residue can be achieved by replacing the entire framework region of the parent polypeptide with, for example, an Fc region sequence that comprises the desired amino acid at the position to be substituted in reference to the parent.

As used herein, the term "*antagonist activity*" is intended to refer to activity of an agent (e.g., an antigen binding molecule) when it interacts with (for example, binds to) a molecule on a cell and prevents initiation or induction of a reaction or discontinues an ongoing reaction.

As used herein, the term "*antibody*" is intended to include whole antibody molecules, including monoclonal, polyclonal and multispecific (e.g., bispecific) antibodies, as well as antibody fragments retaining binding specificity, and fusion proteins that include a region equivalent to the heavy chain region of an immunoglobulin and that retain binding specificity. Also encompassed are "*antibody fragments*" that retain binding specificity including, but not limited to, VH fragments, VL fragments, Fab fragments, $F(ab')_2$ fragments, scFv fragments, Fv fragments, minibodies, diabodies, triabodies, and tetrabodies (see, e.g., Hudson and Souriau, Nature Med. 9: 129-134 (2003), hereby incorporated by reference in its entirety). Also encompassed are humanized, primatized and chimeric antibodies. As used herein, "*whole antibody*" refers to an immunoglobulin molecule comprising two "*heavy chains*" and two "*light chains*", each of which comprises a variable and constant region. As used herein, the term "*modified antibody*" is intended to refer to an antibody comprising at least one amino acid residue substitution in the heavy chain variable region and/or CH1 region and/or at least

one amino acid residue substitution in the light chain variable region and/or CL region and/or at least one amino acid substitution in the Fc region.

An antibody "*which binds*" an antigen of interest, e.g. a tumor-associated polypeptide antigen target, is one that binds the antigen with sufficient affinity such that the antibody is useful as a therapeutic agent in targeting a cell or tissue expressing the antigen, and does not significantly cross-react with other proteins. In such embodiments, the extent of binding of the antibody to a "non-target" protein will be less than about 10% of the binding of the antibody to its particular target protein as determined by fluorescence activated cell sorting (FACS) analysis or radioimmunoprecipitation (RIA). With regard to the binding of an antibody to a target molecule, the term "specific binding" or "specifically binds to" or is "specific for" a particular polypeptide or an epitope on a particular polypeptide target means binding that is measurably different from a non-specific interaction. Specific binding can be measured, for example, by determining binding of a molecule compared to binding of a control molecule, which generally is a molecule of similar structure that does not have binding activity. For example, specific binding can be determined by competition with a control molecule that is similar to the target, for example, an excess of non-labeled target. In this case, specific binding is indicated if the binding of the labeled target to a probe is competitively inhibited by excess unlabeled target. The term "specific binding" or "specifically binds to" or is "specific for" a particular polypeptide or an epitope on a particular polypeptide target as used herein can be exhibited, for example, by a molecule having a Kd for the target of about 10^{-4} M or less, alternatively about 10^{-5} M or less, alternatively about 10^{-6} M or less, alternatively about 10^{-7} M or less, alternatively about 10^{-8} M or less, alternatively about 10^{-9} M or less, alternatively about 10^{-10} M or less, alternatively about 10^{-11} M or less, alternatively about 10^{-12} M or less, or less. In one embodiment, the term "specific binding" refers to binding where a molecule binds to a particular polypeptide or epitope on a particular polypeptide without substantially binding to any other polypeptide or polypeptide epitope.

The terms "*anti-CD20 antibody*" and "*an antibody that specifically binds to CD20*" refer to an antibody that is capable of binding CD20 with sufficient affinity such that the antibody is useful as a diagnostic and/or therapeutic agent in targeting CD20. In one embodiment, the extent of binding of an anti-CD20 antibody to an unrelated, non-CD20 protein is less than about 10% of the binding of the antibody to CD20 as measured by a radioimmunoassay (RIA). In certain embodiments, an antibody that binds to CD20 has a dissociation constant (Kd) of $\leq$ 1μM, $\leq$ 100 nM, $\leq$ 10 nM, $\leq$ 1 nM, $\leq$ 0.1 nM, $\leq$ 0.01 nM, or $\leq$ 0.001 nM (e.g. 10^{-8} M or less, e.g. from 10^{-8} M to 10^{-13} M, e.g., from 10^{-9} M to 10^{-13} M) as measured by scatchard analysis.

In certain embodiments, an anti-CD20 antibody binds to an epitope of CD20 that is conserved among CD20 from different species. Depending on binding properties and biological activities of anti-CD20 antibodies to the CD20 antigen, two types of anti-CD20 antibodies (type I and type II anti-CD20 antibodies) can be distinguished according to Cragg, M.S., et al., Blood 103 (2004) 2738-2743; and Cragg, M.S., et al., Blood 101 (2003) 1045-1052, see Table 1.

Table 1: Properties of Type I and Type II anti-CD20 Antibodies

Type I anti-CD20 antibodies	**Type II anti-CD20 antibodies**
Type I CD20 epitope	Type II CD20 epitope
Localize CD20 to lipid rafts	Do not localize CD20 to lipid rafts
Increased CDC (if IgG1 isotype)	Decreased CDC (if IgG1 isotype)
ADCC activity (if IgG1 isotype)	ADCC activity (if IgG1 isotype)
Full binding capacity	Reduced binding capacity
Homotypic aggregation	Stronger homotypic aggregation

One essential property of type I and type II anti-CD20 antibodies is their mode of binding. Thus type I and type II anti-CD20 antibody can be classified by the ratio of the binding capacities to CD20 on Raji cells (ATCC-No. CCL-86) of said anti-CD20 antibody compared to rituximab.

An antibody which "*induces direct cell death*" or "*induces apoptosis*" or "*induces apoptosis-like direct cell death*" is one which induces programmed cell death as determined by binding of annexin V, cell shrinkage, dilation of endoplasmic reticulum, cell fragmentation, and/or formation of membrane vesicles (called apoptotic bodies). The cell is usually one which expresses a CD20 polypeptide. Preferably the cell is a tumor cell. More preferably the cell is a hematopoietic cell, such as a malignant B cell or a B cell involved in autoimmunity. Various methods are available for evaluating the cellular events associated with direct cell death. For example, phosphatidyl serine (PS) translocation can be measured by annexin binding.

An antibody which "*induces cell death*" is one which causes a viable cell to become nonviable. The cell is one which expresses a CD20 polypeptide and is of a cell type which specifically expresses or overexpresses a CD20 polypeptide. The cells may be cancerous or normal cells of the particular cell type. The cell may be a normal B cell involved in

autoimmunity. The cell may be a cancer cell. Preferably the cell is a malignant B cell. Cell death *in vitro* may be determined in the absence of complement and immune effector cells to distinguish cell death induced by effector function. Thus, the assay for cell death may be performed using heat inactivated serum (i.e., in the absence of complement) and in the absence of immune effector cells. To determine whether the antibody is able to induce cell death, loss of membrane integrity as evaluated by uptake of propidium iodide (PI), trypan blue (see Moore et al. Cytotechnology 17:1-11 (1995)) or 7-AAD can be assessed relative to untreated cells.

By "*antibody having altered antibody-dependent cell-mediated cytotoxicity*" ("*ADCC*") is meant an antibody, as that term is defined herein, having altered ADCC as determined by any suitable method known to those of ordinary skill in the art. One accepted *in vitro* ADCC assay is as follows:

1) the assay uses target cells that are known to express the target antigen recognized by the antigen-binding region of the antibody;

2) the assay uses human peripheral blood mononuclear cells (PBMCs), isolated from blood of a randomly chosen healthy donor, as effector cells;

3) the assay is carried out according to the following protocol:

i) the PBMCs are isolated using standard density centrifugation procedures and are suspended at 5 x 10^6 cells/ml in RPMI cell culture medium;

ii) the target cells are grown by standard tissue culture methods, harvested from the exponential growth phase with a viability higher than 90%, washed in RPMI cell culture medium, labeled with 100 micro-Curies of ^{51}Cr, washed twice with cell culture medium, and resuspended in cell culture medium at a density of 10^5 cells/ml;

iii) 100 microliters of the final target cell suspension above are transferred to each well of a 96-well microtiter plate;

iv) the antibody is serially-diluted from 4000 ng/ml to 0.04 ng/ml in cell culture medium and 50 microliters of the resulting antibody solutions are added to the target cells in the 96-well microtiter plate, testing in triplicate various antibody concentrations covering the whole concentration range above;

v) for the maximum release (MR) controls, 3 additional wells in the plate containing the labeled target cells, receive 50 microliters of a 2% (V/V) aqueous solution of non-ionic detergent (Nonidet, Sigma, St. Louis), instead of the antibody solution (point iv above);

vi) for the spontaneous release (SR) controls, 3 additional wells in the plate containing the labeled target cells, receive 50 microliters of RPMI cell culture medium instead of the antibody solution (point iv above);

vii) the 96-well microtiter plate is then centrifuged at 50 x g for 1 minute and incubated for 1 hour at 4°C;

viii) 50 microliters of the PBMC suspension (point i above) are added to each well to yield an effector:target cell ratio of 25:1 and the plates are placed in an incubator under 5% CO_2 atmosphere at 37°C for 4 hours;

ix) the cell-free supernatant from each well is harvested and the experimentally released radioactivity (ER) is quantified using a gamma counter;

x) the percentage of specific lysis is calculated for each antibody concentration according to the formula (ER-MR)/(MR-SR) x 100, where ER is the average radioactivity quantified (see point ix above) for that antibody concentration, MR is the average radioactivity quantified (see point ix above) for the MR controls (see point v above), and SR is the average radioactivity quantified (see point ix above) for the SR controls (see point vi above);

4) "*increased ADCC*" is defined as either an increase in the maximum percentage of specific lysis observed within the antibody concentration range tested herein, and/or a reduction in the concentration of antibody required to achieve one half of the maximum percentage of specific lysis observed within the antibody concentration range tested herein. The increase in ADCC is relative to the ADCC, measured with the above assay, mediated by the same antibody, produced by the same type of host cells, using the same standard production, purification, formulation and storage methods, which are known to those skilled in the art, but that has not been modified by amino acid substitution as disclosed herein or glycoengineering. Thus, "de*creased ADCC*" is defined as either a decrease in the maximum percentage of specific lysis observed within the antibody concentration range tested herein, and/or an increase in the concentration of antibody required to achieve one half of the maximum percentage of specific lysis observed within the antibody concentration range tested herein. The decrease in ADCC is relative to the ADCC, measured with the above assay, mediated by the same antibody, produced by the same type of host cells, using the same standard production, purification, formulation and storage methods, which are known to those skilled in the art, but that has not been modified by amino acid substitution as disclosed herein or glycoengineering.

As used herein, the term "*apoptosis*" is intended to refer to programmed cell death, which is characterized by certain cellular events such as nuclear fragmentation and/or formation of apoptotic bodies by condensation of cytoplasm, plasma membranes and/or organelles.

As used herein, the term "*binding*" or "*specifically binding*" refers to the binding of the antibody to an epitope of the tumor antigen in an *in vitro* assay. The *in vitro* assay can be a plasmon resonance assay (BIAcore, GE-Healthcare Uppsala, Sweden) with purified wild-type antigen. For anti-CD20 antibodies the *in vitro* assay is preferably a scatchard analysis. The affinity of the binding is defined by the term KD. Binding or specifically binding means a binding affinity (KD) of 10^{-8} M or less, preferably 10^{-8} M to 10^{-13} M, more preferably 10^{-9} M to 10^{-13} M.

The term "*CD20*," as used herein, refers to any native CD20 from any vertebrate source, including mammals such as primates (e.g. humans) and rodents (e.g., mice and rats), unless otherwise indicated. The term encompasses "full-length" unprocessed CD20 as well as any form of CD20 that results from processing in the cell. The term also encompasses naturally occurring variants of CD20, e.g., splice variants or allelic variants. CD20 (also known as B-lymphocyte antigen CD20, B-lymphocyte surface antigen B1, Leu-16, Bp35, BM5, and LF5; the sequence is characterized by the SwissProt database entry P11836) is a hydrophobic transmembrane protein with a molecular weight of approximately 35 kD located on pre-B and mature B lymphocytes (Valentine, M.A. et al., J. Biol. Chem. 264 (1989) 11282-11287; Tedder, T.F., et al., Proc. Natl. Acad. Sci. U.S.A. 85 (1988) 208-212; Stamenkovic, I., et al., J. Exp. Med. 167 (1988) 1975-1980; Einfeld, D.A., et al., EMBO J. 7 (1988) 711-717; Tedder, T.F., et al., J. Immunol. 142 (1989) 2560-2568). The corresponding human gene is Membrane-spanning 4-domains, subfamily A, member 1, also known as MS4A1. This gene encodes a member of the membrane-spanning 4A gene family. Members of this nascent protein family are characterized by common structural features and similar intron/exon splice boundaries and display unique expression patterns among hematopoietic cells and nonlymphoid tissues. This gene encodes the B-lymphocyte surface molecule which plays a role in the development and differentiation of B-cells into plasma cells. This family member is localized to 11q12, among a cluster of family members. Alternative splicing of this gene results in two transcript variants which encode the same protein. The terms "CD20" and "CD20 antigen" are used interchangeably herein, and include any variants, isoforms and species homologs of human CD20 which are naturally expressed by cells or are expressed on cells transfected with the CD20 gene. Binding of an antibody of the invention to the CD20 antigen mediate the killing of cells expressing CD20 by signaling through CD20, by inactivating CD20 or by cross-linking CD20. Preferably, the cell is a tumor cell. The killing of the cells expressing CD20 may occur by one or more of the following mechanisms: Direct cell death/apoptosis induction, ADCC,

CDC and ADCP. Synonyms of CD20, as recognized in the art, include B-lymphocyte antigen CD20, B-lymphocyte surface antigen B1, Leu-16, Bp35, BM5, and LF5.

The term "*CD20 expressing cancer*" as used herein refers preferably to lymphomas (preferably B-Cell Non-Hodgkin's lymphomas (NHL)) and lymphocytic leukemias. Such lymphomas and lymphocytic leukemias include follicular lymphomas, Small Non-Cleaved Cell Lymphomas/ Burkitt's lymphoma (including endemic Burkitt's lymphoma, sporadic Burkitt's lymphoma and Non-Burkitt's lymphoma), marginal zone lymphomas (including extranodal marginal zone B cell lymphoma (Mucosa-associated lymphatic tissue lymphomas, MALT), nodal marginal zone B cell lymphoma and splenic marginal zone lymphoma), Mantle cell lymphoma (MCL), Large Cell Lymphoma (including diffuse large B-cell lymphoma (DLBCL), Diffuse Mixed Cell Lymphoma, Immunoblastic Lymphoma, Primary Mediastinal B-Cell Lymphoma, Angiocentric Lymphoma-Pulmonary B-Cell Lymphoma), hairy cell leukemia, lymphocytic lymphoma, Waldenstrom's macroglobulinemia, acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL)/ small lymphocytic lymphoma (SLL), B-cell prolymphocytic leukemia, plasma cell neoplasms, plasma cell myeloma, multiple myeloma, plasmacytoma, Hodgkin's disease. More preferably, the term CD20 expressing cancer refers to Non-Hodgkin's lymphomas (NHL), follicular lymphomas, diffuse large B-cell lymphoma (DLBCL) and chronic lymphocytic leukemia (CLL).

The term "*complement-dependent cytotoxicity (CDC)*" refers to lysis of human tumor target cells by the antibody according to the invention in the presence of complement. CDC is measured preferably by the treatment of a preparation of CD20 expressing cells with an anti-CD20 antibody according to the invention in the presence of complement. CDC is found if the antibody induces at a concentration of 100 nM the lysis (cell death) of 20% or more of the tumor cells after 4 hours. The assay is performed preferably with ^{51}Cr or Eu labeled tumor cells and measurement of released ^{51}Cr or Eu. Controls include the incubation of the tumor target cells with complement but without the antibody.

Typically, type I and type II anti-CD20 antibodies of the IgG1 isotype show characteristic CDC properties. Type I anti-CD20 antibodies have an increased CDC (if IgG1 isotype) and type II anti-CD20 antibodies have a decreased CDC (if IgG1 isotype) compared to each other. Preferably both type I and type II anti-CD20 antibodies are IgG1 isotype antibodies.

As used herein, "*cell signaling mechanism*" or "*cell signaling activity*" is intended to refer to the entire signaling (i.e., signal transduction) pathway that leads to a particular cellular event or biological function, as well as any signaling steps along the pathway.

As used herein, the term "*CH1 region*" is intended to refer to the domain of the heavy chain of an immunoglobulin that is just C-terminal to the variable region and N-terminal to the hinge region. In an immunoglobulin of the IgG type, for example, CH1 is normally defined by Kabat positions 114 to 223 (Kabat numbering).

In the case where there are two or more definitions of a term which is used and/or accepted within the art, the definition of the term as used herein is intended to include all such meanings unless explicitly stated to the contrary. A specific example is the use of the term "*complementarity determining region*" ("*CDR*") to describe the non-contiguous antigen combining sites found within the variable region of both heavy and light chain polypeptides. This particular region has been described by Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991) and by Chothia et al., J MoI Biol. 196:901-917 (1987), each of which is hereby incorporated by reference in its entirety, where the definitions include overlapping or subsets of amino acid residues when compared against each other. Nevertheless, application of either definition to refer to a CDR of an antibody or variants thereof is intended to be within the scope of the term as defined and used herein. The exact residue numbers which encompass a particular CDR will vary depending on the sequence and size of the CDR. Those skilled in the art can routinely determine which residues comprise a particular CDR given the variable region amino acid sequence of the antibody.

"*Conservative*" amino acid substitutions are those made by replacing one amino acid with another amino acid having similar structural and/or chemical properties, i.e., conservative amino acid replacements, and may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature, and/or the bulk sizes of the residues involved. For example, nonpolar (hydrophobic) amino acids include glycine, alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid. "*Substitutions*", "*insertions*" or "*deletions*" are preferably in the range of about 1 to 20 amino acids, more preferably 1 to 10 amino acids, more preferably 1 to 4 amino acids, most preferably 1 amino

acid. The variation allowed may be experimentally determined by systematically making insertions, deletions, or substitutions of amino acids in a polypeptide molecule using recombinant DNA techniques and assaying the resulting recombinant variants for activity.

"*Cytokine release syndrome*", which is an "*infusion reaction*", is a common immediate complication occurring with the use of antibody infusions such as e.g., the CD20-antibody rituximab. The pathogenesis is characterized in that the antibodies bind to T cell receptors, activating said T cells. The cytokines released by the activated T cells produce a type of systemic inflammatory response similar to that found in severe infection characterised by hypotension, pyrexia and rigors. Deaths due to cytokine release syndrome have been reported, and it can cause life-threatening pulmonary edema if the patient is fluid overloaded.

The term "*cytotoxic agent*" as used herein refers to a substance that inhibits or prevents a cellular function and/or causes cell death or destruction. Cytotoxic agents include, but are not limited to, radioactive isotopes (including but not limited to, ^{211}Astatine, ^{131}Iodine, ^{125}Iodine, ^{90}Yttrium, ^{186}Rhenium, ^{188}Rhenium, ^{153}Samarium, ^{212}Bismuth, ^{32}Phosphorus, ^{212}Lead and radioactive isotopes of Lutetium); chemotherapeutic agents or drugs (including but not limited to, methotrexate, adriamicin, vinca alkaloids (vincristine, vinblastine, etoposide), doxorubicin, melphalan, mitomycin C, chlorambucil, daunorubicin or other intercalating agents); growth inhibitory agents; enzymes and fragments thereof such as nucleolytic enzymes; antibiotics; toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof; and the various antitumor or anticancer agents disclosed below.

An "*effective amount*" of an agent, e.g., a pharmaceutical formulation, refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic or prophylactic result.

As used herein, the term "*effector function*" or "*Fc-mediated effector function*" refers to those biological activities attributable to the Fc region (a native sequence Fc region or amino acid sequence variant Fc region) of an antibody, and vary with the antibody isotype. Examples of antibody effector functions include, but are not limited to: C1q binding and complement dependent cytotoxicity (CDC), Fc receptor binding affinity, antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and cytokine secretion. As used herein, the term "*reduced*" in conjunction with effector function refers to a measurable reduction of effector function induced by an antibody modified according to the

invention compared to effector function induced by the corresponding parent non-substituted antibody. Effector function can be measured as disclosed herein and with reference to the examples as disclosed herein. As used herein, the terms "*strongly reduced*", "*abolished*" and "*residual*" in conjunction with effector function are considered to refer to a reduction of the named effector function induced by an antibody modified according to the invention compared to effector function induced by the the corresponding parent non-substituted antibody. "Strongly reduced" means a reduction to 50% or less, "abolished" means a reduction to 10% or less and "residual" means more than 10% compared to the respective effector function induced by the corresponding parent non-substituted antibody. Accordingly, antibodies of the present invention comprising an Fc variant of the invention comprise at least one or more of the following properties: reduced or abolished ADCC, reduced or abolished CDC, reduced or abolished ADCP, reduced or abolished binding to Fc receptors, reduced or abolished binding to Clq and reduced or abolished infusion reaction (cytokine release syndrome). In one preferred embodiment, provided is an antibody wherein ADCP function induced by the antibody comprising the variant heavy chain region is reduced compared to ADCP function induced by the parent non-substituted antibody, wherein more than 10% of ADCP function induced by the antibody comprising the variant heavy chain region is retained compared to ADCP function induced by the parent non-substituted antibody.

As used herein, the terms "*engineer, engineered, engineering, glycoengineer, glycoengineered, glycoengineering*", "*glycosylation engineering*" and "*GE*" are considered to include any manipulation of the glycosylation pattern of a naturally occurring or recombinant polypeptide, such as an antibody, or fragment thereof. Glycosylation engineering includes metabolic engineering of the glycosylation machinery of a cell, including genetic manipulations of the oligosaccharide synthesis pathways to achieve altered glycosylation of glycoproteins expressed in cells. In one embodiment, the glycosylation engineering is an alteration in glycosyltransferase activity. In a particular embodiment, the engineering results in altered glucosaminyltransferase activity and/or fucosyltransferase activity.

The term "*expression of the CD20 antigen*" is intended to indicate a significant level of expression of the CD20 antigen in a cell, preferably on the cell surface of a T- or B- cell, more preferably a B-cell, from a tumor or cancer, respectively, preferably a non-solid tumor. Patients having a "CD20 expressing cancer" can be determined by standard assays known in the art. "Expression of the CD20" antigen is also preferable intended to indicate a significant level of expression of the CD20 antigen in a cell, preferably on the cell surface of a T- or B-cell, more preferably a B-cell, in an autoimmune disease. CD20 antigen expression is

measured e.g., using immunohistochemical (IHC) detection, FACS or via PCR-based detection of the corresponding mRNA.

As used herein, the term "*Fc region*" is intended to refer to a C-terminal region of an IgG heavy chain. Although the boundaries of the Fc region of an IgG heavy chain might vary slightly, the human IgG heavy chain Fc region is usually defined to stretch from the amino acid residue at position Cys226 to the carboxyl-terminus.

As used herein, the term "*Fc-mediated cellular cytotoxicity*" includes "*antibody-dependent cell-mediated cytotoxicity*" (ADCC) and cellular cytotoxicity mediated by a soluble Fc-fusion protein containing a human Fc-region. It is an immune mechanism leading to the lysis of "antibody-targeted cells" by "human immune effector cells", wherein the human immune effector cells are a population of leukocytes that display Fc receptors on their surface through which they bind to the Fc-region of antibodies or of Fc-fusion proteins and perform effector functions. Such a population may include, but is not limited to, peripheral blood mononuclear cells (PBMC) and/or natural killer (NK) cells. The antibody-targeted cells are cells bound by the antibodies or Fc-fusion proteins. The antibodies or Fc fusion-proteins bind to target cells via the protein part N-terminal to the Fc region.

As used herein, the terms "*fusion*" and "*chimeric*", when used in reference to polypeptides such as antibodies refer to polypeptides comprising amino acid sequences derived from two or more heterologous polypeptides, such as portions of antibodies from different species. For chimeric antibodies, for example, the non-antigen binding components may be derived from a wide variety of species, including primates such as chimpanzees and humans. The constant region of the chimeric antibody is most preferably substantially identical to the constant region of a natural human antibody; the variable region of the chimeric antibody is most preferably derived from a non-human (i.e., donor) antigen binding molecule that specifically binds an antigen of interest. The chimeric antibody may comprise the entire donor variable region; alternatively, the chimeric antibody may comprise a humanized or primatized antibody. Humanized antibodies are a particularly preferred form of fusion or chimeric antibody. Other forms of "chimeric antibodies" encompassed by the present invention are those in which the class or subclass has been modified or changed from that of the original antibody. Such "chimeric" antibodies are also referred to as "class-switched antibodies". Methods for producing chimeric antibodies involve conventional recombinant DNA and gene transfection techniques now well known in the art. See, e.g., Morrison, S.L., et al., Proc. Natl. Acad Sci. USA 81 (1984) 6851-6855; US 5,202,238 and US 5,204,244.

As used herein, the term *"heavy chain variable region"* is intended to refer to the N-terminal domain of an immunoglobulin heavy chain. In one example, the heavy chain variable region is defined by Kabat positions 1 to 113 (with possible insertions at particular residues as designated by Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)). According to one embodiment of the present invention, a modified antibody can comprise a functional fragment of a heavy chain variable region.

As used herein, the term *"heavy chain constant region"* is intended to refer to the C terminal domain of an immunoglobulin heavy chain. There are five naturally-occurring classes of heavy chain constant regions: IgA, IgG, IgE, IgD, and IgM. In one example, the heavy chain constant region comprises a CH1 domain, a CH2 domain, and a CH3 domain.

A "*human consensus framework*" is a framework which represents the most commonly occurring amino acid residues in a selection of human immunoglobulin VL or VH framework sequences. Generally, the selection of human immunoglobulin VL or VH sequences is from a subgroup of variable domain sequences. Generally, the subgroup of sequences is a subgroup as in Kabat et al., Sequences of Proteins of Immunological Interest, Fifth Edition, NIH Publication 91-3242, Bethesda MD (1991), vols. 1-3. In one embodiment, for the VL, the subgroup is subgroup kappa I as in Kabat et al., supra. In one embodiment, for the VH, the subgroup is subgroup III as in Kabat et al., supra.

A "*human antibody*" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human and/or has been made using any of the techniques for making human antibodies as disclosed herein. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues. Human antibodies can be produced using various techniques known in the art, including phage-display libraries. Hoogenboom and Winter, J. Mol. Biol., 227:381 (1991); Marks et al., J. Mol. Biol., 222:581 (1991). Also available for the preparation of human monoclonal antibodies are methods described in Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985); Boerner et al., J. Immunol., 147(1):86-95 (1991). Disclosed also in van Dijk and van de Winkel, Curr. Opin. Pharmacol., 5: 368-74 (2001). Human antibodies can be prepared by administering the antigen to a transgenic animal that has been modified to produce such antibodies in response to antigenic challenge, but whose endogenous loci have been disabled, e.g., immunized xenomice (disclosed, e.g. in, U.S. Pat. Nos. 6,075,181 and 6,150,584

regarding XENOMOUSETM technology). See also, for example, Li et al., Proc. Natl. Acad. Sci. USA, 103:3557-3562 (2006) regarding human antibodies generated via a human B-cell hybridoma technology.

As used herein, the term *"humanized"* is used to refer to an antigen binding molecule derived from a non-human antigen-binding molecule, for example, a murine antibody, that retains or substantially retains the antigen-binding properties of the parent molecule but which is less immunogenic in humans. This may be achieved by various methods including (a) grafting the entire non-human variable domains onto human constant regions to generate chimeric antibodies, (b) grafting only the non-human CDRs onto human framework and constant regions with or without retention of critical framework residues (e.g., those that are important for retaining good antigen binding affinity or antibody functions), or (c) transplanting the entire non-human variable domains, but "cloaking" them with a human-like section by replacement of surface residues. Such methods are disclosed in Jones et al., Morrison et al., Proc. Natl. Acad. Sd., 81:6851-6855 (1984); Morrison and Oi, Adv. Immunol, 44:65-92 (1988); Verhoeyen et al., Science, 239:1534-1536 (1988); Padlan, Molec. Immun., 28:489-498 (1991); Padlan, Molec. Immun., 31(3):169-217 (1994), each of which is hereby incorporated by reference in its entirety. There are generally three complementarity determining regions (CDRs) (CDR1, CDR2 and CDR3), in each of the heavy and light chain variable domains of an antibody, which are flanked by four framework subregions (i.e., FRl, FR2, FR3, and FR4) in each of the heavy and light chain variable domains of an antibody: FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4. A discussion of humanized antibodies can be found, *inter alia*, in U.S. Patent No. 6,632,927, and in published U.S. Application No. 2003/0175269, each of which is hereby incorporated by reference in its entirety. Similarly, as used herein, the term *"primatized"* is used to refer to an antigen-binding molecule derived from a non-primate antigen-binding molecule, for example, a murine antibody, that retains or substantially retains the antigen-binding properties of the parent molecule but which is less immunogenic in primates.

As used herein, a nucleic acid that *"hybridizes under stringent conditions"* to a nucleic acid sequence of the invention, refers to a polynucleotide that hybridizes in an overnight incubation at 42°C in a solution comprising 50% formamide, 5x SSC (750 mM NaCl, 75 mM sodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 μg/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65°C.

As used herein, the term "*host cell*" covers any kind of cellular system which can be engineered to generate the polypeptides and antigen-binding molecules of the present invention. Host cells include cultured cells, including but not limited to, mammalian cultured cells, such as CHO cells, BHK cells, HEK293-EBNA cells, NSO cells, SP2/0 cells, Y0 myeloma cells, P3X63 mouse myeloma cells, PER cells, PER.C6 cells or hybridoma cells, yeast cells, insect cells, and plant cells, to name only a few, but also cells comprised within a transgenic animal, transgenic plant or cultured plant or animal tissue. In one embodiment, the host cell is engineered to allow the production of an antigen binding molecule with modified glycoforms. In a preferred embodiment, the antigen binding molecule is an antibody, antibody fragment, or fusion protein.

An "*immunoconjugate*" is an antibody conjugated to one or more heterologous molecule(s), including but not limited to a cytotoxic agent.

An "*individual*" or "*subject*" is a mammal. Mammals include, but are not limited to, domesticated animals (e.g., cows, sheep, cats, dogs, and horses), primates (humans and non-human primates such as monkeys), rabbits, and rodents (e.g., mice and rats). In certain embodiments, the individual or subject is a human.

An "*isolated antibody*" is one which has been separated from a component of its natural environment. In some embodiments, an antibody is purified to greater than 95% or 99% purity as determined by, for example, electrophoretic (e.g., SDS-PAGE, isoelectric focusing (IEF), capillary electrophoresis) or chromatographic (e.g., ion exchange or reverse phase HPLC). A review of methods for assessment of antibody purity is disclosed, *inter alia*, in Flatman et al., J. Chromatogr. B 848:79-87 (2007).

An "*isolated nucleic acid*" refers to a nucleic acid molecule that has been separated from a component of its natural environment. An isolated nucleic acid includes a nucleic acid molecule contained in cells that ordinarily contain the nucleic acid molecule, but the nucleic acid molecule is present extrachromosomally or at a chromosomal location that is different from its natural chromosomal location.

"*Isolated nucleic acid encoding an anti-CD20 antibody*" refers to one or more nucleic acid molecules encoding antibody heavy and light chains (or fragments thereof), including such nucleic acid molecule(s) in a single vector or separate vectors, and such nucleic acid molecule(s) present at one or more locations in a host cell.

The term "*monoclonal antibody*" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical and/or bind the same epitope, except for possible variant antibodies, e.g., containing naturally occurring mutations or arising during production of a monoclonal antibody preparation, such variants generally being present in minor amounts. In contrast to polyclonal antibody preparations, which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody of a monoclonal antibody preparation is directed against a single determinant on an antigen. Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by a variety of techniques, including but not limited to the hybridoma method, recombinant DNA methods, phage-display methods, and methods utilizing transgenic animals containing all or part of the human immunoglobulin loci, such methods and other exemplary methods for making monoclonal antibodies being disclosed herein.

A "*naked antibody*" refers to an antibody that is not conjugated to a heterologous moiety (e.g., a cytotoxic moiety) or radiolabel. The naked antibody may be present in a pharmaceutical formulation.

"*Native antibodies*" refer to naturally occurring immunoglobulin molecules with varying structures. For example, native IgG antibodies are heterotetrameric glycoproteins of about 150'000 daltons, composed of two identical "*light chains*" and two identical "*heavy chains*" that are disulfide-bonded. From N- to C-terminus, each heavy chain has a variable region (VH), also called a variable heavy domain or a heavy chain variable domain, followed by three constant domains (CH1, CH2, and CH3). Similarly, from N- to C-terminus, each light chain has a variable region (VL), also called a variable light domain or a light chain variable domain, followed by a constant light (CL) domain. The light chain of an antibody may be assigned to one of two types, called kappa (κ) and lambda (λ), based on the amino acid sequence of its constant domain.

The term "*nucleic acid molecule*", as used herein, is intended to include DNA molecules and RNA molecules. A nucleic acid molecule may be single-stranded or double-stranded, but preferably is double-stranded DNA.

The term "*package insert*" is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, combination therapy, contraindications and/or warnings concerning the use of such therapeutic products.

As used herein, the term "*parent antibody*", or "*parent non-modified antibody*", or "parent non-substituted antibody" refers to an antibody having a particular amino acid sequence encoded by a polynucleotide sequence. The sequence of the parent molecule (i.e., the "*parent sequence*") serves as a reference sequence for making amino acid residue substitutions that alter the ability of the resulting molecule to induce effector function and/or to induce signaling activity and/or cross-linking of antigen. Likewise, the activity of a parent molecule (e.g., the "parent non-substituted antibody) serves as the reference when determining whether a substitution has an effect on effector function and/or cell signaling activity and/or cross-linking of antigen, and, where relevant, the extent of that effect.

A "*pharmaceutically acceptable carrier*" refers to an ingredient in a pharmaceutical formulation, other than an active ingredient, which is nontoxic to a subject. A pharmaceutically acceptable carrier includes, but is not limited to, a buffer, excipient, stabilizer, or preservative.

The terms "*polypeptide*" and "*protein*" are used interchangeably to refer to a polymer of amino acid residues, comprising natural or non-natural amino acid residues, and are not limited to a minimum length. Thus, peptides, oligopeptides, dimers, multimers, and the like are included within the definition. Both full-length proteins and fragments thereof are encompassed by the definition. The terms also include post-translational modifications of the polypeptide, including, for example, glycosylation, sialylation, acetylation, and phosphorylation.

Furthermore, a "*polypeptide*" herein also refers to a modified protein such as single or multiple amino acid residue deletions, additions, and substitutions to the native sequence, as long as the protein maintains a desired activity. For example, a serine residue may be substituted to eliminate a single reactive cysteine or to remove disulfide bonding or a conservative amino acid substitution may be made to eliminate a cleavage site. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts which produce the proteins or errors due to polymerase chain reaction (PCR) amplification.

The "ratio of the binding capacities to CD20 on Raji cells (ATCC-No. CCL-86) of an anti-CD20 antibodies compared to rituximab" is determined by direct immunofluorescence measurement (the mean fluorescent intensities (MFI) is measured) using said anti-CD20 antibody conjugated with Cy5 and rituximab conjugated with Cy5 in a FACSArray (Becton Dickinson) with Raji cells (ATCC-No. CCL-86), and calculated as follows:

Ratio of the binding capacities to CD20 on Raji cells (ATCC-No. CCL-86) =

$$\frac{\text{MFI(Cy5-anti-CD20 antibody)}}{\text{MFI(Cy5-rituximab)}} \times \frac{\text{Cy5-labeling ratio(Cy5-rituximab)}}{\text{Cy5-labeling ratio(Cy5-anti-CD20 antibody)}}$$

MFI is the mean fluorescent intensity. The "Cy5-labeling ratio" as used herein means number of Cy5-label molecules per molecule antibody.

The term "*recombinant human antibody*", as used herein, is intended to include all human antibodies that are prepared, expressed, created or isolated by recombinant means, such as antibodies isolated from a host cell such as a NS0 or CHO cell or from an animal (e.g. a mouse) that is transgenic for human immunoglobulin genes or antibodies expressed using a recombinant expression vector transfected into a host cell. Such recombinant human antibodies have variable and constant regions derived from human germline immunoglobulin sequences in a rearranged form. The recombinant human antibodies according to the invention have been subjected to *in vivo* somatic hypermutation. Thus, the amino acid sequences of the recombinant antibodies are sequences that, while derived from and related to human germline sequences, may not naturally exist within the human antibody germline repertoire *in vivo*.

As used herein, "*treatment*" (and grammatical variations thereof such as "*treat*" or "*treating*") refers to clinical intervention in an attempt to alter the natural course of the individual being treated, and can be performed either for prophylaxis or during the course of clinical pathology. Desirable effects of treatment include, but are not limited to, preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, preventing metastasis, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis. In some embodiments, antibodies of the invention are used to delay development of a disease or to slow the progression of a disease.

The "*variable region*" or "*variable domain*" of an antibody refers to the amino-terminal domains of the heavy or light chain of the antibody. The variable domain of the heavy chain may be referred to as "VH". The variable domain of the light chain may be referred to as "VL". These domains are generally the most variable parts of an antibody and contain the antigen-binding sites.

By "*variant protein*", "*protein variant*" or "*variant*" as used herein is meant a protein that differs from that of a parent protein by virtue of at least one amino acid modification. Variant refers to the protein itself, a composition comprising the polypeptide, or the amino sequence that encodes it. Preferably, the protein variant has at least one amino acid modification compared to the parent protein, e.g. from about one to about seventy amino acid modifications, and preferably from about one to about five amino acid modifications compared to the parent. The protein variant sequence as disclosed herein will preferably possess at least about 80% homology with a parent protein sequence, and most preferably at least about 90% homology, more preferably at least about 95% homology. Variant protein can refer to the variant protein itself, compositions comprising the protein variant, or the DNA sequence that encodes it. Accordingly, by *"antibody variant"*, *"variant antibody"* or *"variant heavy chain region"* as used herein is meant an antibody, or part of an antibody, that differs from a parent antibody by virtue of at least one amino acid modification including but not limited to amino acid substitution, deletion or insertion. By "*IgG variant*" or "*variant IgG*" as used herein is meant an antibody that differs from a parent IgG by virtue of at least one amino acid modification, and "*immunoglobulin variant*" or "*variant immunoglobulin*" as used herein is meant an immunoglobulin sequence that differs from that of a parent immunoglobulin sequence by virtue of at least one amino acid modification.

In the context of the present invention, the term "variant" is used interchangeably with the term "mutated". Accordingly, by *"antibody mutant"*, *"mutated antibody"* or *"mutaded heavy chain region"* as used herein is meant an antibody, or part of an antibody, that differs from a parent antibody by virtue of at least one amino acid modification including but not limited to amino acid substitution, deletion or insertion. "*IgG mutant*" or "mutated *IgG*" as used herein is meant an antibody that differs from a parent IgG by virtue of at least one amino acid modification, and "*immunoglobulin mutant*" or "mutant *immunoglobulin*" as used herein is meant an immunoglobulin sequence that differs from that of a parent immunoglobulin sequence by virtue of at least one amino acid modification.

The term "*variable*" in relation with the term "variable domain" refers to the fact that certain segments of the variable domains differ extensively in sequence among antibodies. The V domain mediates antigen binding and defines specificity of a particular antibody for its particular antigen. However, the variability is not evenly distributed across the 110-amino acid span of the variable domains. Instead, the V regions consist of relatively invariant stretches called framework regions (FRs) of 15-30 amino acids separated by shorter regions of extreme variability called "hypervariable regions" that are each 9-12 amino acids long. The variable domains of native heavy and light chains each comprise four FRs, largely adopting a β-sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the β-sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cell-mediated cytotoxicity (ADCC).

The term "*vector*", as used herein, refers to a nucleic acid molecule capable of propagating another nucleic acid to which it is linked. The term includes the vector as a self-replicating nucleic acid structure as well as the vector incorporated into the genome of a host cell into which it has been introduced. Certain vectors are capable of directing the expression of nucleic acids to which they are operatively linked. Such vectors are referred to herein as "*expression vectors*". Recombinant variants encoding same or similar polypeptides may be synthesized or selected by making use of the "redundancy" in the genetic code. Various codon substitutions, such as the silent changes which produce various restriction sites, may be introduced to optimize cloning into a plasmid or viral vector or expression in a particular prokaryotic or eukaryotic system. Mutations in the polynucleotide sequence may be reflected in the polypeptide or domains of other peptides added to the polypeptide to modify the properties of any part of the polypeptide, to change characteristics such as ligand-binding affinities, interchain affinities, or degradation/turnover rate.

The term "*wildtype polypeptide*" and "*wildtype (human) Fc region*" as used herein refers to a polypeptide and Fc region, respectively, comprising an amino acid sequence which lacks one or more of the Fc region modifications disclosed herein, because they have not been introduced, and serve for example as controls. The wildtype polypeptide may comprise a

native sequence Fc region or an Fc region with pre-existing amino acid sequence modifications (such as additions, deletions and/or substitutions).

By a nucleic acid or polynucleotide having a nucleotide sequence at least, for example, 95% "identical" to a reference nucleotide sequence of the present invention, it is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the polynucleotide sequence may include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence.

As a practical matter, whether any particular nucleic acid molecule or polypeptide is at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to a nucleotide sequence or polypeptide sequence of the present invention can be determined conventionally using known computer programs. A preferred method for determining the best overall match between a query sequence (a sequence of the present invention) and a subject sequence, also referred to as a global sequence alignment, can be determined using the FASTDB computer program based on the algorithm of Brutlag et al, Comp. App. Biosci. 6:237-245 (1990). In a sequence alignment the query and subject sequences are both DNA sequences. An RNA sequence can be compared by converting U's to T's. The result of said global sequence alignment is in percent identity. Preferred parameters used in a FASTDB alignment of DNA sequences to calculate percent identity are: Matrix=Unitary, k-tuple=4, Mismatch Penalty=l, Joining Penalty=30, Randomization Group Length=0, Cutoff Score=l, Gap Penalty=5, Gap Size Penalty 0.05, Window Size=500 or the length of the subject nucleotide sequence, whichever is shorter.

If the subject sequence is shorter than the query sequence because of 5' or 3' deletions, not because of internal deletions, a manual correction must be made to the results. This is because the FASTDB program does not account for 5' and 3' truncations of the subject sequence when calculating percent identity. For subject sequences truncated at the 5' or 3' ends, relative to the query sequence, the percent identity is corrected by calculating the number of bases of the query sequence that are 5' and 3' of the subject sequence, which are not matched/aligned, as a percent of the total bases of the query sequence. Whether a nucleotide is matched/aligned is determined by results of the FASTDB sequence alignment. This percentage is then subtracted

from the percent identity, calculated by the FASTDB program as disclosed herein using the specified parameters, to arrive at a final percent identity score. This corrected score is what is used for the purposes of the present invention. Only bases outside the 5' and 3' bases of the subject sequence, as displayed by the FASTDB alignment, which are not matched/aligned with the query sequence, are calculated for the purposes of manually adjusting the percent identity score.

For example, a 90 base subject sequence is aligned to a 100 base query sequence to determine percent identity. The deletions occur at the 5' end of the subject sequence and therefore, the FASTDB alignment does not show a matched/alignment of the first 10 bases at the 5' end. The 10 unpaired bases represent 10% of the sequence (number of bases at the 5' and 3' ends not matched/total number of bases in the query sequence) so 10% is subtracted from the percent identity score calculated by the FASTDB program. If the remaining 90 bases were perfectly matched the final percent identity would be 90%. In another example, a 90 base subject sequence is compared with a 100 base query sequence. This time the deletions are internal deletions so that there are no bases on the 5' or 3' end of the subject sequence which are not matched/aligned with the query. In this case the percent identity calculated by FASTDB is not manually corrected. Once again, only bases on the 5' and 3' end of the subject sequence which are not matched/aligned with the query sequence are manually corrected for. No other manual corrections are to be made for the purposes of the present invention.

By a polypeptide having an amino acid sequence at least, for example, 95% "identical" to a query amino acid sequence of the present invention, it is intended that the amino acid sequence of the subject polypeptide is identical to the query sequence except that the subject polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the query amino acid sequence. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a query amino acid sequence, up to 5% of the amino acid residues in the subject sequence may be inserted, deleted, or substituted with another amino acid. These alterations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

As a practical matter, whether any particular polypeptide is at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to a reference polypeptide can be determined conventionally using known computer programs. A preferred method for determining the best overall match

between a query sequence (a sequence of the present invention) and a subject sequence, also referred to as a global sequence alignment, can be determined using the FASTDB computer program based on the algorithm of Brutlag et al, Comp. App. Biosci. 6:237-245 (1990). In a sequence alignment the query and subject sequences are either both nucleotide sequences or both amino acid sequences. The result of said global sequence alignment is in percent identity. Preferred parameters used in a FASTDB amino acid alignment are: Matrix=PAM 0, k-tuple=2, Mismatch Penalty=l, Joining Penalty=20, Randomization Group Length=O, Cutoff Score=l, Gap Penalty=5, Gap Size Penalty=0.05, Window Size=500 or the length of the subject amino acid sequence, whichever is shorter.

If the subject sequence is shorter than the query sequence due to N- or C-terminal deletions, not because of internal deletions, a manual correction must be made to the results. This is because the FASTDB program does not account for N- and C-terminal truncations of the subject sequence when calculating global percent identity. For subject sequences truncated at the N- and C-termini, relative to the query sequence, the percent identity is corrected by calculating the number of residues of the query sequence that are N- and C-terminal of the subject sequence, which are not matched/aligned with a corresponding subject residue, as a percent of the total bases of the query sequence. Whether a residue is matched/aligned is determined by results of the FASTDB sequence alignment. This percentage is then subtracted from the percent identity, calculated by the FASTDB program as disclosed herein using the specified parameters, to arrive at a final percent identity score. This final percent identity score is what is used for the purposes of the present invention. Only residues to the N- and C-termini of the subject sequence, which are not matched/aligned with the query sequence, are considered for the purposes of manually adjusting the percent identity score. That is, only query residue positions outside the farthest N- and C-terminal residues of the subject sequence.

For example, a 90 amino acid residue subject sequence is aligned with a 100 residue query sequence to determine percent identity. The deletion occurs at the N-terminus of the subject sequence and therefore, the FASTDB alignment does not show a matching/alignment of the first 10 residues at the N-terminus. The 10 unpaired residues represent 10% of the sequence (number of residues at the N- and C- termini not matched/total number of residues in the query sequence) so 10% is subtracted from the percent identity score calculated by the FASTDB program. If the remaining 90 residues were perfectly matched the final percent identity would be 90%. In another example, a 90 residue subject sequence is compared with a 100 residue query sequence. This time the deletions are internal deletions so there are no residues at the N- or C-termini of the subject sequence which are not matched/aligned with the query. In this

case the percent identity calculated by FASTDB is not manually corrected. Once again, only residue positions outside the N- and C-terminal ends of the subject sequence, as displayed in the FASTDB alignment, which are not matched/aligned with the query sequence are manually corrected for. No other manual corrections are to be made for the purposes of the present invention.

II. EMBODIMENTS ACCORDING TO THE INVENTION

Modified Antibodies According to the Invention

There remains a need for monoclonal antibodies with improved therapeutic potential for human therapy. Enforcing the induction of direct cell death while reducing effector function, is much needed for improved cancer therapy. Unexpectedly, the present inventors found that the substitutions Asn297Asp or Pro329Gly in the Fc region as disclosed herein strongly reduced CDC and ADCC function but retained residual ADCP function. Further surprisingly, this mutation can be combined with mutations in the elbow hinge region according to the present invention, leading to altered (increased or reduced) direct cell death induction. In combination, the modifications according to the present invention allow a selective and independent modulation of induction of effector function and/or induction of direct cell death as compared to non-substituted parent antibodies.

The present inventors surprisingly found that substitution of the asparagine residue at Kabat position 297 or of the proline residue at Kabat position 329 leads to reduced effector function. More specifically, substitution of the asparagine residue at position 297 to aspartic acid or of the proline residue at Kabat position 329 to glycine resulted with strongly reduced or abolished ADCC and CDC effector functions but residual ADCP function. Accordingly, in one embodiment, an antibody is provided, comprising a variant heavy chain region comprising at least one amino acid substitution relative to the parent non-substituted heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residue Asn297, and wherein said substitution is Asn297Asp. In a further embodiment, an antibody is provided, comprising a variant heavy chain region comprising at least one amino acid substitution relative to the parent non-substituted heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residue Pro329, and wherein said substitution is Pro329Gly.

In a further aspect, an antibody is provided, wherein induction of effector function is reduced compared to effector function induced by an antibody comprising the parent non-substituted heavy chain region. In one aspect, the present invention is related to antibodies with amino acid modifications in the Fc region leading to strongly reduced or abolished ADCC and/or CDC function, residual ADCP function, reduced or abolished binding to Fc receptors, reduced or abolished binding to Clq and reduced or abolished toxicities.

In another embodiment said antibodies comprising a Fc variant exhibit a reduced affinity to a human Fc receptor (FcγR) and/or a human complement receptor as compared to the antibody comprising the wildtype human Fc region. In a further embodiment the affinity to at least one of the FcγRI, FcγRII, FcγRIII is reduced, in a still further embodiment the affinity to the FcγRI and FcγRIII is reduced, and in a still further embodiment the affinity to the FcγRII and FcγRIII is reduced and in a still further embodiment the affinity to the FcγRI, FcγRII and FcγRIII is reduced, in still a further aspect of the invention the affinity to the FcγRI receptor, FcγRIII receptor and Clq is reduced, and in still a further aspect of the invention the affinity to the FcγRI, FcγRII, FcγRIII and Clq receptor is reduced.

The parent non-modified antibody can be any of any class (for example, but not limited to IgG, IgM, and IgE). In certain embodiments, antibodies of the invention are members of the IgG class of antibodies. In a specific embodiment, antibodies of the invention are of the IgG1, IgG2 or IgG4 subclass. In still another aspect of the invention the antibody comprising a heavy chain constant region variant and an Fc variant comprises a human IgGl or IgG4 Fc region. In still a further aspect of the invention the variants are IgG1 or IgG4 antibodies. In a specific embodiment, antibodies of the invention are of the IgG1 subclass, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residue Asn297, wherein the residues are numbered according to the EU index as in Kabat. In a further specific embodiment, antibodies of the invention are of the IgG1 subclass, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residue Pro329, wherein the residues are numbered according to the EU index as in Kabat. In another specific embodiment, antibodies of the invention are of the IgG1 subclass, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residue Asn297, wherein the variant heavy chain region comprises the amino acid substitution Asn297Asp of the Fc region. In another specific embodiment, antibodies of the invention are of the IgG1 subclass, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residue Pro329, wherein the variant heavy chain region comprises the amino acid

substitution Pro329Gly of the Fc region. In one embodiment, antibodies of the invention comprising the amino acid substitution Pro329Gly have a higher melting temperature compared to a variant of the parent non-substituted antibody comprising an amino acid substitution at amino acid residue Asn297 as measured by Tryptophan fluorescence. In one embodiment, an antibody of the invention comprising the amino acid substitution Pro329Gly has a higher melting temperature compared to a variant of the parent non-substituted antibody comprising the Asn297Asp substitution as measured by Tryptophan fluorescence.

Parent non-substituted antibodies according to the invention include, but are not limited to, monoclonal antibodies. In one aspect antibodies of the invention are so-called chimaeric antibodies, humanized antibodies or fully human antibodies. In a further aspect antibodies of the invention are full length antibodies or antibody fragments having the same biological activity including amino acid sequence variants and/or glycosylation variants of such antibodies or fragments. Parent non-substituted antibodies according to the invention include, but are not limited to, 3F8 (anti-GD2), Abagovomab (anti CA-125), Abciximab (anti CD41 (integrin alpha-IIb)), Adalimumab (anti-TNF-α), Adecatumumab (anti- EpCAM, CD326), Afelimomab (anti-TNF-α); Afutuzumab (anti-CD20), Alacizumab pegol (anti-VEGFR2), ALD518 (anti-IL-6), Alemtuzumab (Campath, MabCampath, anti- CD52), Altumomab (anti-CEA), Anatumomab (anti-TAG-72), Anrukinzumab (IMA-638, anti-IL-13), Apolizumab (anti-HLA-DR), Arcitumomab (anti-CEA), Aselizumab (anti-L-selectin, CD62L), Atlizumab (tocilizumab, Actemra, RoActemra, anti-IL-6 receptor), Atorolimumab (anti-Rhesus factor), Bapineuzumab (anti-beta amyloid), Basiliximab (Simulect, antiCD25 (α chain of IL-2 receptor)), Bavituximab (anti-phosphatidylserine), Bectumomab (LymphoScan, anti-CD22), Belimumab (Benlysta, LymphoStat-B, anti-BAFF), Benralizumab (anti-CD125), Bertilimumab (anti-CCLl 1 (eotaxin-1)), Besilesomab (Scintimun, anti-CEA-related antigen), Bevacizumab (Avastin, anti-VEGF-A), Biciromab (FibriScint, anti-fibrin II beta chain), Bivatuzumab (anti-CD44 v6), Blinatumomab (BiTE, anti-CD19), Brentuximab (cAC10, anti-CD30 TNFRSF8), Briakinumab (anti-IL-12, IL23), Canakinumab (Ilaris, anti-IL-1), Cantuzumab (C242, anti-CanAg), Capromab, Catumaxomab (Removab, anti-EpCAM, anti-CD3), CC49 (anti-TAG-72), Cedelizumab (anti-CD4), Certolizumab pegol (Cimzia anti-TNF-a), Cetuximab (Erbitux, IMC- C225, anti-EGFR), Citatuzumab bogatox (anti-EpCAM), Cixutumumab (anti-IGF-1), Clenoliximab (anti-CD4), Clivatuzumab (anti-MUC 1), Conatumumab (anti-TRAIL-R2), CR6261 (anti-Influenza A hemagglutinin), Dacetuzumab (anti-CD40), Daclizumab (Zenapax, anti-CD25 (a chain of IL-2 receptor)), Daratumumab (anti-CD38, cyclic ADP ribose hydrolase), Denosumab (Prolia, anti-RANKL), Detumomab (anti-B-lymphoma cell), Dorlimomab, Dorlixizumab, Ecromeximab (anti-GD3

ganglioside), Eculizumab (Soliris, anti-C5), Edobacomab (anti-endotoxin), Edrecolomab (Panorex, MAbl7-1A, anti-EpCAM), Efalizumab (Raptiva, anti-LFA-1 (CD 11a)), Efungumab (Mycograb, anti-Hsp90), Elotuzumab (anti-SLAMF7), Elsilimomab (anti-IL-6), Enlimomab pegol (anti-ICAM-1 (CD54)), Epitumomab (anti-episialin), Epratuzumab (anti-CD22), Erlizumab (anti-ITGB2 (CD 18)), Ertumaxomab (Rexomun, anti-HER2/neu, CD3), Etaracizumab (Abegrin, anti-integrin $\alpha_v\beta_3$), Exbivirumab (anti-hepatitis B surface antigen), Fanolesomab (NeutroSpec, anti-CD 15), Faralimomab (anti-interferon receptor), Farletuzumab (anti-folate receptor 1), Felvizumab (anti-respiratory syncytial virus), Fezakinumab (anti-IL-22), Figitumumab (anti-IGF-1 receptor), Fontolizumab (anti-IFN-γ), Foravirumab (anti-rabies virus glycoprotein), Fresolimumab (anti-TGF-β), Galiximab (anti-CD80), Gantenerumab (anti- beta amyloid), Gavilimomab (anti-CD147 (basigin)), Gemtuzumab (anti-CD33), Girentuximab (anti-carbonic anhydrase 9), Glembatumumab (CR011, anti-GPNMB), Golimumab (Simponi, anti-TNF-a), Gomiliximab (anti-CD23 (IgE receptor)), Ibalizumab (anti-CD4), Ibritumomab (anti-CD20), Igovomab (Indimacis-125, anti-CA-125), Imciromab (Myoscint, anti-cardiac myosin), Infliximab (Remicade, anti-TNF-a), Intetumumab (anti-CD51), Inolimomab (anti-CD25 (a chain of IL-2 receptor)), Inotuzumab (anti-CD22), Ipilimumab (anti-CD 152), Iratumumab (anti-CD30 (TNFRSF8)), Keliximab (anti-CD4), Labetuzumab (CEA-Cide, anti-CEA), Lebrikizumab (anti-IL-13). Lemalesomab (anti-NCA-90 (granulocyte antigen)), Lerdelimumab (anti-TGF beta 2), Lexatumumab (anti-TRAIL-R2), Libivirumab (anti-hepatitis B surface antigen), Lintuzumab (anti-CD33)), Lucatumumab (anti-CD40), Lumiliximab (anti-CD23 (IgE receptor), Mapatumumab (anti-TRAIL-R1), Maslimomab (anti-T-cell receptor), Matuzumab (anti-EGFR), Mepolizumab (Bosatria, anti-IL-5), Metelimumab (anti-TGF beta 1), Milatuzumab (anti-CD74), Minretumomab (anti-TAG-72), Mitumomab (BEC-2, anti-GD3 ganglioside), Morolimumab (anti-Rhesus factor), Motavizumab (Numax, anti-respiratory syncytial virus), Muromonab-CD3 (Orthoclone OKT3, anti-CD3), Nacolomab (anti-C242), Naptumomab (anti-5T4), Natalizumab (Tysabri, anti-integrin α_4), Nebacumab (anti-endotoxin), Necitumumab (anti-EGFR), Nerelimomab (anti-TNF-a), Nimotuzumab (Theracim, Theraloc, anti-EGFR), Nofetumomab, Obinutuzumab (anti-CD20), Ocrelizumab (anti-CD20), Odulimomab (Afolimomab, anti-LFA-1 (CD11a)), Ofatumumab (Arzerra, anti-CD20), Olaratumab (anti-PDGF-Rα), Omalizumab (Xolair, anti-IgE Fc region), Oportuzumab (anti-EpCAM), Oregovomab (OvaRex, anti-CA-125), Otelixizumab (anti-CD3), Pagibaximab (anti-lipoteichoic acid), Palivizumab (Synagis, Abbosynagis, anti-respiratory syncytial virus), Panitumumab (Vectibix, ABX-EGF, anti- EGFR), Panobacumab (anti-Pseudomonas aeruginosa), Pascolizumab (anti-IL-4), Pemtumomab (Theragyn, anti-MUCl), Pertuzumab (Omnitarg, 2C4, anti-IIER2/neu), Pexelizumab (anti-C5), Pintumomab (anti-adenocarcinoma

antigen), Priliximab (anti-CD4), Pritumumab (anti- vimentin), PRO 140 (anti-CCR5), Racotumomab (1E10, anti-(N-glycolylneuraminic acid (NeuGc, NGNA)-gangliosides GM3)), Rafivirumab (anti-rabies virus glycoprotein), Ramucirumab (anti-VEGFR2), Ranibizumab (Lucentis, anti-VEGF-A), Raxibacumab (anti-anthrax toxin, protective antigen), Regavirumab (anti-cytomegalovirus glycoprotein B), Reslizumab (anti-IL-5), Rilotumumab (anti-HGF), Rituximab (MabThera, Rituxan, anti- CD20), Robatumumab (anti-IGF-1 receptor), Rontalizumab (anti-IFN-a), Rovelizumab (LeukArrest, anti-CD11, CD 18), Ruplizumab (Antova, anti-CD 154 (CD40L)), Satumomab (anti-TAG-72), Sevirumab (anti-cytomegalovirus), Sibrotuzumab (anti-FAP), Sifalimumab (anti-IFN-a), Siltuximab (anti-IL-6), Siplizumab (anti-CD2), (Smart) MI95 (anti-CD33), Solanezumab (anti-beta amyloid), Sonepcizumab (anti-spingosine-1-phosphate), Sontuzumab (anti-episialin), Stamulumab (anti-myostatin), Sulesomab (LeukoScan, (anti-NCA-90 (granulocyte antigen), Tacatuzumab (anti-alpha-fetoprotein), Tadocizumab (anti-integrin $\alpha_{IIb}\beta_3$), Talizumab (anti-IgE), Tanezumab (anti-NGF), Taplitumomab (anti-CD19), Tefibazumab (Aurexis, (anti-clumping factor A)), Telimomab, Tenatumomab (anti-tenascin C), Teneliximab (anti-CD40), Teplizumab (anti-CD3), TGN1412 (anti-CD28), Ticilimumab (Tremelimumab, (anti-CTLA-4)), Tigatuzumab (anti-TRAIL-R2), TNX-650 (anti-IL-13), Tocilizumab (Atlizumab, Actemra, RoActemra, (anti-IL-6 receptor)), Toralizumab (anti-CD 154 (CD40L)), Tositumomab (anti-CD20), Trastuzumab (Herceptin, (anti-HER2/neu)), Tremelimumab (anti-CTLA-4), Tucotuzumab celmoleukin (anti-EpCAM), Tuvirumab (anti-hepatitis B virus), Urtoxazumab (anti-Escherichia coli), Ustekinumab (Stelara, anti-IL-12, IL-23), Vapaliximab (anti-AOC3 (VAP-1)), Vedolizumab (anti-integrin $\alpha_4\beta_7$), Veltuzumab (anti-CD20), Vepalimomab (anti-AOC3 (VAP-1), Visilizumab (Nuvion, anti-CD3), Vitaxin (anti-vascular integrin avb3), Volociximab (anti-integrin $\alpha_5\beta_1$), Votumumab (HumaSPECT, anti-tumor antigen CTAA16.88), Zalutumumab (HuMax-EGFr, (anti-EGFR)), Zanolimumab (HuMax-CD4, anti-CD4), Ziralimumab (anti-CD 147 (basigin)), Zolimomab (anti-CD5), Etanercept (Enbrel®), Alefacept (Amevive®), Abatacept (Orencia®), Rilonacept (Arcalyst), 14F7 (anti-IRP-2 (Iron Regulatory Protein 2)), 14G2a (anti-GD2 ganglioside, from Nat. Cancer Inst, for melanoma and solid tumors), J591 (anti-PSMA, Weill Cornell Medical School for prostate cancers), 225.28S (anti-HMW-MAA (High molecular weight-melanoma-antigen), Sorin Radiofarrnaci S.R.L. (Milan, Italy) for melanoma), COL-1 (anti-CEACAM3, CGM1 , from Nat. Cancer Inst. USA for colorectal and gastric cancers), CYT-356 (Oncoltad®, for prostate cancers), HNK20 (OraVax Inc. for respiratory syncytial virus), ImmuRAIT (from Immunomedics for NHL), Lym-1 (anti-HLA-DR10, Peregrine Pharm. for Cancers), MAK-195F (anti-TNF (tumor necrosis factor; TNFA, TNF-alpha; TNFSF2), from Abbott / Knoll for Sepsis toxic shock), MEDI-500 (T10B9, anti-CD3, TRαβ (T cell receptor

alpha/beta), complex, from Medlmmune Inc for Graft-versus-host disease), RING SCAN (anti-TAG 72 (tumour associated glycoprotein 72), from Neoprobe Corp. for Breast, Colon and Rectal cancers), Avicidin (anti-EPCAM (epithelial cell adhesion molecule), anti-TACSTD1 (Tumor-associated calcium signal transducer 1), anti- GA733-2 (gastrointestinal tumor-associated protein 2), anti-EGP-2 (epithelial glycoprotein 2); anti-KSA (KS 1/4 antigen; M4S; tumor antigen 17-1A; CD326, from NeoRx Corp. for Colon, Ovarian, Prostate cancers and NHL) ; LymphoCide (Immunomedics, NJ), Smart ID10 (Protein Design Labs), Oncolym (Techniclone Inc, CA), Allomune (BioTransplant, CA), anti-VEGF (Genentech, CA); CEAcide (Immunomedics, NJ), IMC-1C11 (ImClone, NJ) and Cetuximab (ImClone, NJ) .

In a preferred embodiment, the parent non-substituted antibody is an anti-CD20 antibody. Such antibodies preferably are monoclonal antibodies. Also preferably, said antibodies are selected from the group consisting of chimeric antibodies, humanized antibodies or fully human antibodies. In a most preferred aspect, said antibodies are selected from the group consisting of full length anti-CD20 antibodies and anti-CD20 antibody fragments having the same biological activity including amino acid sequence variants and glycosylation variants of such antibodies or fragments. Humanized anti-CD20 parent non-substituted antibodies according to the invention are specified with the INN names rituximab (see e.g., US Patent No. 7,381,560 and EP2000149B1 of Anderson et. al., see e.g., figures 4 and 5), ocrelizumab (as disclosed in WO 2004/056312 and WO 2006/084264), ibritumomab (see WO 94/011026), tositumomab (WHO Drug Information, Vol. 12, No. 4, 1998, p.281), veltuzumab (WHO Drug Information, Vol. 22, No. 3, 2008, p.28) and obinutuzumab (recommended INN, WHO Drug Information, Vol. 26, No. 4, 2012, p. 453).

A parent non-substituted CD20 antibody according to the invention is rituximab (a type I anti-CD20 antibody) which is sold by Genentech Inc. and F. Hoffmann-La Roche Ltd under the trade name MABTHERA™ or RITUXAN™. Rituximab is a genetically engineered chimeric human gamma 1 murine constant domain containing monoclonal antibody directed against the human CD20 antigen. This chimeric antibody contains human gamma 1 constant domains and is identified by the name "C2B8" in US 5,736,137 (Anderson et. al.) issued on April 17, 1998, assigned to IDEC Pharmaceuticals Corporation. Rituximab is approved for the treatment of patients with relapsed or refracting low-grade or follicular, CD20 positive, B cell non-Hodgkin's lymphoma. *In vitro* mechanism of action studies have shown that rituximab exhibits human complement-dependent cytotoxicity (CDC) (Reff, M.E., et. al., Blood 83 (1994) 435-

445). Additionally, it exhibits significant activity in assays that measure antibody-dependent cell-mediated cytotoxicity (ADCC). Rituximab is not afucosylated.

In a preferred embodiment the parent non-substituted antibody according to the invention is a humanized B-Ly1 antibody. The term "*humanized B-Ly1 antibody*" refers to humanized B-Ly1 antibodies as disclosed in WO 2005/044859 and WO 2007/031875, which were obtained from the murine monoclonal anti-CD20 antibody B-Ly1 (variable region of the murine heavy chain (VH): SEQ ID NO: 3; variable region of the murine light chain (VL): SEQ ID NO: 4 (see Poppema, S. and Visser, L., Biotest Bulletin 3 (1987) 131-139)) by chimerization with a human constant domain from IgG1 and following humanization (see WO 2005/044859 and WO 2007/031875). These humanized B Ly1 antibodies are disclosed in detail in WO 2005/044859 and WO 2007/031875. In one embodiment, the humanized B-Ly1 antibody has variable region of the heavy chain (VH) selected from group of SEQ ID NO: 5 to SEQ ID NO: 21 (B-HH2 to B-HH9 and B-HL8 to B-HL17 of WO 2005/044859 and WO 2007/031875). In one specific embodiment, such variable domain is selected from the group consisting of SEQ ID NOs: 5, 6, 9, 11, 13, 15 and 17 (B-HH2, BHH-3, B-HH6, B-HH8, B-HL8, B-HL11 and B-HL13 of WO 2005/044859 and WO 2007/031875). In one specific embodiment, the humanized B-Ly1 antibody has a variable region of the light chain (VL) of SEQ ID NO: 22 (B-KV1 of WO 2005/044859 and WO 2007/031875). In one specific embodiment, the humanized B-Ly1 antibody has a variable region of the heavy chain (VH) of SEQ ID NO: 9 (B-HH6 of WO 2005/044859 and WO 2007/031875) and a variable region of the light chain (VL) of SEQ ID NO: 22 (B-KV1 of WO 2005/044859 and WO 2007/031875). Furthermore, in one embodiment, the humanized B-Ly1 antibody is an IgG1 antibody. According to one aspect of the invention such afucosylated humanized B-Ly1 antibodies are glycoengineered (GE) in the Fc region according to the procedures described in WO 2005/044859, WO 2004/065540, WO 2007/031875, Umana, P. et al., Nature Biotechnol. 17 (1999) 176-180 and WO 99/154342. In one embodiment, the parent non-substituted antibody according to the invention is the afucosylated glycoengineered (GE) humanized B-Ly1 B-HH6-B-KV1 GE. In a preferred embodiment, the parent non-substituted antibody according to the invention is obinutuzumab (recommended INN, WHO Drug Information, Vol. 26, No. 4, 2012, p. 453). As used herein, obinutuzumab is synonymous for GA101. This replaces all previous versions (e.g. Vol. 25, No. 1, 2011, p.75-76), and is formerly known as afutuzumab (recommended INN, WHO Drug Information, Vol. 23, No. 2, 2009, p. 176;Vol. 22, No. 2, 2008, p. 124).

In a preferred embodiment, the parent non-substituted antibody is a type I anti-CD20 antibody. One essential property of type I and type II anti-CD20 antibodies is their mode of binding. In particular, type I and type II anti-CD20 antibodies can be classified by the ratio of the binding capacities to CD20 on Raji cells (ATCC-No. CCL-86) of said anti-CD20 antibody compared to rituximab. The type I anti-CD20 antibodies have a ratio of the binding capacities to CD20 on Raji cells (ATCC No. CCL-86) of said anti-CD20 antibody compared to rituximab of 0.8 to 1.2, preferably of 0.9 to 1.1. Preferred type I parent non-substituted anti-CD20 antibodies include rituximab, in EP2000149B1 (Anderson et. al., see figures 4 and 5), 1F5 IgG2a (ECACC, hybridoma; Press et al., Blood 69/2:584-591 (1987)), HI47 IgG3 (ECACC, hybridoma), 2C6 IgG1 (as disclosed in WO 2005/103081), 2F2 IgG1 or ofatumumab (as disclosed and WO 2004/035607 and WO 2005/103081) and 2H7 IgG1 (as disclosed in WO 2004/056312) and WO 2006/084264 (including but not limited to the variants disclosed in tables 1 and 2). Preferably said type I parent non-substituted anti-CD20 antibody is a monoclonal antibody that binds to the same epitope as rituximab. In one embodiment, a type I anti-CD20 antibody is provided, comprising a variant heavy chain region comprising at least one amino acid substitution relative to the parent non-substituted heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residue Asn297, and wherein said substitution is Asn297Asp, wherein induction of effector function is reduced compared to effector function induced by an antibody comprising the parent non-substituted heavy chain region. In a preferred embodiment, the parent non-substituted antibody is rituximab.

In another preferred embodiment, the parent non-substituted antibody is a type II anti-CD20 antibody. The type II anti-CD20 antibodies have a ratio of the binding capacities to CD20 on Raji cells (ATCC No. CCL-86) of said anti-CD20 antibody compared to Rituximab of 0.3 to 0.6, preferably of 0.35 to 0.55, more preferably 0.4 to 0.5. Preferred type II parent non-substituted anti-CD20 antibodies comprise, obinutuzumab, tositumomab (B1 IgG2a), humanized B-Ly1 antibody IgG1 (a chimeric humanized IgG1 antibody as disclosed in WO 2005/044859), 11B8 IgG1 (as disclosed in WO 2004/035607), and AT80 IgG1. Preferably said type II parent non-substituted anti-CD20 antibody is a monoclonal antibody that binds to the same epitope as humanized B-Ly1 antibody (as disclosed in WO 2005/044859). In a further embodiment, a type II anti-CD20 antibody is provided, comprising a variant heavy chain region comprising at least one amino acid substitution relative to the parent non-substituted heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residue Asn297, and wherein said substitution is Asn297Asp, wherein induction of effector function is reduced compared to effector function

induced by an antibody comprising the parent non-substituted heavy chain region. In a preferred embodiment, the parent non-substituted antibody is obinutuzumab.

The wildtype polypeptide comprises an Fc region. Generally the Fc region of the wildtype polypeptide comprises a native or wildtype sequence Fc region, and preferably a human native sequence Fc region (human Fc region). However, the Fc region of the wildtype polypeptide may have one or more pre-existing amino acid sequence alterations or modifications from a native sequence Fc region. For example, the Clq or Fcγ binding activity of the Fc region may have been previously altered (other types of Fc region modifications are described in more detail herein). In a further embodiment the parent polypeptide Fc region is "conceptual" and, while it does not physically exist, the person skilled in the art selects a desired variant Fc region amino acid sequence and generates a polypeptide comprising that sequence or a DNA encoding the desired variant Fc region amino acid sequence. In the preferred embodiment of the invention, however, a nucleic acid encoding an Fc region of a wildtype polypeptide (e.g., a wildtype heavy chain region) is available and this nucleic acid sequence is altered to generate a variant nucleic acid sequence encoding the Fc region variant.

One embodiment of the invention encompasses polypeptides comprising an Fc region of an antibody, comprising the addition, substitution, or deletion of at least one amino acid residue to the Fc region resulting in reduced or abolished affinity for at least one Fc receptor. The Fc region interacts with a number of receptors or ligands including but not limited to Fc receptors (e.g., FcγRI, FcγRII, FcγRIII), the complement protein Clq, and other molecules such as proteins A and G. These interactions are essential for a variety of effector functions and downstream signaling events including, but not limited to, antibody dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP) and complement dependent cytotoxicity (CDC). Accordingly, in certain embodiments the variants of the invention have reduced or abolished affinity for an Fc receptor responsible for an effector function compared to a polypeptide having the same amino acid sequence as the polypeptide comprising a Fc variant of the invention but not comprising the addition, substitution, or deletion of at least one amino acid residue to the Fc region (also referred to herein as "parent non-substituted" polypeptide or antibody). In certain embodiments, antibodies comprising a Fc variant of the invention comprise at least one or more of the following properties: reduced or abolished effector (ADCC and/or CDC and/or ADCP) function, reduced or abolished binding to Fc receptors, reduced or abolished binding to Clq and reduced or abolished infusion reaction (cytokine release syndrome). More specifically, embodiments of the invention provide anti-CD20 (same as obinutuzumab or rituximab), anti-CD9 (same as TA), anti-Selectin (pSel), anti-

CD37, anti-HER2 and anti-EGFR antibodies with reduced affinity for Fc receptors (e.g. FcγRI, FcγRII, FcγRIII) and/or the complement protein Clq.

In one embodiment, antibodies of the invention comprise an Fc region comprising at least one amino acid substitution at position Asn297, wherein the numbering system of the heavy chain is that of the EU index as in Kabat. In a specific embodiment, antibodies of the invention comprise the amino acid substitution Asn297Asp in the heavy chain region, wherein induction of effector function is reduced compared to effector function induced by an antibody comprising the parent non-substituted antibody heavy chain region. In a further embodiment, antibodies of the invention comprise an Fc region comprising at least one amino acid substitution at position Pro329, wherein the numbering system of the heavy chain is that of the EU index as in Kabat. In a specific embodiment, antibodies of the invention comprise the amino acid substitution Pro329Gly in the heavy chain region, wherein induction of effector function is reduced compared to effector function induced by an antibody comprising the parent non-substituted antibody heavy chain region. In a further embodiment said antibodies comprise at least one or more of the following properties: reduced or abolished effector (ADCC and/or CDC and/or ADCP) function, reduced or abolished binding to Fc receptors, reduced or abolished binding to Clq and reduced or abolished infusion reaction (cytokine release syndrome).

Accordingly, in one embodiment, an antibody is provided, comprising at least one amino acid substitution relative to the parent non-substituted heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residue Asn297, and wherein said substitution is Asn297Asp, wherein induction of effector function is reduced compared to effector function induced by an antibody comprising the parent non-substituted heavy chain region, wherein FcγRIII binding by the antibody comprising the variant heavy chain region is abolished compared to binding to FcγRIII by the parent non-substituted antibody comprising asparagine at position 297. In a further embodiment, an antibody as described herein is provided, wherein ADCC function induced by the antibody comprising the variant heavy chain region is abolished or strongly reduced compared to ADCC function induced by the parent non-substituted antibody comprising asparagine at position 297. In a further embodiment, an antibody as described herein is provided, wherein FcγRI binding by the antibody comprising the variant heavy chain region is reduced compared to binding to FcγRI by the parent non-substituted antibody comprising asparagine at position 297. In yet a futher embodiment, an antibody as described herein is provided, wherein induction of ADCP

function induced by the antibody comprising the variant heavy chain region is reduced compared to ADCP function induced by the parent non-substituted antibody comprising asparagine at position 297, wherein the antibody comprising the variant heavy chain retains residual ADCP function. In yet a futher embodiment, an antibody as described herein is provided, wherein induction of CDC function induced by the antibody comprising the variant heavy chain region is abolished compared to CDC function induced by the parent non-substituted antibody comprising asparagine at position 297. In a further embodiment, an antibody is provided, comprising at least one amino acid substitution relative to the parent non-substituted heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residue Pro329, and wherein said substitution is Pro329Gly, wherein induction of effector function is reduced compared to effector function induced by an antibody comprising the parent non-substituted heavy chain region, wherein FcγRIII binding by the antibody comprising the variant heavy chain region is abolished compared to binding to FcγRIII by the parent non-substituted antibody comprising proline at position 329. In a further embodiment, an antibody as described herein is provided, wherein ADCC function induced by the antibody comprising the variant heavy chain region is abolished or strongly reduced compared to ADCC function induced by the parent non-substituted antibody comprising proline at position 329. In a further embodiment, an antibody as described herein is provided, wherein FcγRI binding by the antibody comprising the variant heavy chain region is reduced compared to binding to FcγRI by the parent non-substituted antibody comprising proline at position 329. In yet a futher embodiment, an antibody as described herein is provided, wherein induction of ADCP function induced by the antibody comprising the variant heavy chain region is reduced compared to ADCP function induced by the parent non-substituted antibody comprising proline at position 329, wherein the antibody comprising the variant heavy chain retains residual ADCP function. In yet a futher embodiment, an antibody as described herein is provided, wherein induction of CDC function induced by the antibody comprising the variant heavy chain region is abolished compared to CDC function induced by the parent non-substituted antibody comprising prolilne at position 329.

In still another embodiment, the heavy chain variants of the present invention exhibit a reduced affinity to a human Fc receptor (FcγR) and/or a human complement receptor as compared to the parent antibody comprising the wildtype Fc region. In another embodiment, said antibody comprising a variant heavy chain region exhibits a reduced affinity to a human Fc receptor (FcγR) and/or a human complement receptor as compared to the parent antibody

comprising the wildtype human Fc region. In a further embodiment the affinity to at least one of the FcγRI, FcγRII, FcγRIII is reduced, in a still further embodiment the affinity to the FcγRI and FcγRIII is reduced, and in a still further embodiment the affinity to the FcγRI, FcγRII and FcγRIII is reduced, in still a further aspect of the invention the affinity to the FcγRI receptor, FcγRIII receptor and Clq is reduced, and in still a further aspect of the invention the affinity to the FcγRI, FcγRII, FcγRIII and Clq receptor is reduced. In still a further embodiment the ADCC induced by said antibody comprising a heavy chain variant is reduced. In still a further aspect of the invention, the ADCC and CDC induced by the antibody comprising the wildtype Fc polypeptide is reduced or abolished. In a still further aspect the antibody comprising an Fc variant disclosed herein exhibit a decreased ADCC, CDC and ADCP compared to the parent antibody comprising the wildtype Fc polypeptide. In yet a further embodiment the present invention is directed to antibodies comprising the amino acid substitution Asn297Asp in the Fc region of the antibodies leading to reduced or abolished infusion reaction (cytokine release syndrome) compared to the parent non-substituted antibody. In yet a further embodiment the present invention is directed to antibodies comprising the amino acid substitution Pro329Gly in the Fc region of the antibodies leading to reduced or abolished infusion reaction (cytokine release syndrome) compared to the parent non-substituted antibody. In another specific embodiment, the parent non-substituded antibody is obinutuzumab. In another specific embodiment, the parent non-substituted antibody is rituximab.

In a further aspect, the present invention is directed to antibodies with modified Fc region resulting with reduced effector function as described herein further comprising a modified heavy chain CH1 and/or VH region, whereby the ability of these antibodies to induce cell signaling activity of a target antigen and/or mediate cross-linking of target antigen can be enhanced (i.e., induced or increased) or reduced (i.e., inhibited or decreased). In a further aspect, said antibodies comprise a modified Fc region, whereby the induction of antibody dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP) and complement dependent cytotoxicity (CDC) is reduced and a modified CH1 region wherein induction of direct cell death is altered. In a further aspect, said antibodies comprise a modified Fc region, whereby the induction of antibody dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP) and complement dependent cytotoxicity (CDC) is reduced and a modified VH region wherein induction of direct cell death is altered. In a further aspect said modification to the Fc region leads to reduced but not abolished induction of ADCP function. In yet a further aspect an antibody modified according to the invention retains residual ADCP function.

In one embodiment the present invention is directed to antibodies as described herein having a modification at position Asn297 in the Fc region of the antibodies resulting with reduced induction of antibody dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP) and complement dependent cytotoxicity (CDC), said antibodies further comprising a modification at position Pro151 in the CH1 region and/or a modification at Leu11 in the VH region, resulting with altered signaling behavior of the antibodies. In one embodiment the present invention is directed to antibodies as described herein having a modification at position Pro329 in the Fc region of the antibodies resulting with reduced induction of antibody dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP) and complement dependent cytotoxicity (CDC), said antibodies further comprising a modification at position Pro151 in the CH1 region and/or a modification at Leu11 in the VH region, resulting with altered signaling behavior of the antibodies. In certain embodiments, antibodies comprising a variant Fc and/or CH1 and/or VH of the invention comprise at least one or more of the following properties: increased or decreased induction of direct cell death, reduced or abolished effector (ADCC and/or CDC and/or ADCP) function, reduced or abolished binding to Fc receptors, reduced or abolished binding to Clq and reduced or abolished infusion reaction (cytokine release syndrome). More specifically, embodiments of the invention provide anti-CD20 antibodies with increased induction of direct cell death and reduced affinity for Fc receptors (e.g., FcγRI, FcγRII, FcγRIII).

The modified heavy chain CH1, VH and Fc regions of the antibodies of the present invention differ from the corresponding non-substituted parent polypeptide regions by at least one amino acid substitution. The "parent", "starting", "nonmodified" or "non-substituted" polypeptide preferably comprises at least a portion of an antibody heavy chain region, and can be prepared using techniques available in the art for generating polypeptides comprising an Fc region as well as a heavy chain CH1 and VH region or portions thereof.

Accordingly, in one embodiment, an antibody is provided, comprising a variant heavy chain region comprising at least one amino acid substitution relative to the parent non-substituted heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residue Asn297, and wherein said substitution is Asn297Asp, wherein induction of effector function is reduced compared to effector function induced by an antibody comprising the parent non-substituted heavy chain region, wherein the variant heavy chain region comprises a further amino acid substitution relative to the parent non-substituted heavy chain region, wherein the heavy chain region of the parent non-substituted antibody

comprises the amino acid residue Pro151, and wherein said further substitution is at said amino acid residue Pro151, wherein direct cell death induced by the antibody comprising the variant heavy chain region is altered compared to direct cell death induced by the antibody comprising proline at position 151. Accordingly, in one embodiment, an antibody is provided, comprising a variant heavy chain region comprising at least one amino acid substitution relative to the parent non-substituted heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residue Pro329, and wherein said substitution is Pro329Gly, wherein induction of effector function is reduced compared to effector function induced by an antibody comprising the parent non-substituted heavy chain region, wherein the variant heavy chain region comprises a further amino acid substitution relative to the parent non-substituted heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residue Pro151, and wherein said further substitution is at said amino acid residue Pro151, wherein direct cell death induced by the antibody comprising the variant heavy chain region is altered compared to direct cell death induced by the antibody comprising proline at position 151. In another embodiment, the antibody as described herein is provided, wherein direct cell death induced by the antibody comprising the variant heavy chain region is increased compared to direct cell death induced by the antibody comprising proline at position 151. In yet another embodiment, the antibody as described herein is provided, wherein direct cell death induced by the antibody comprising the variant heavy chain region is decreased compared to direct cell death induced by the antibody comprising proline at position 151. In yet another embodiment, the antibody as described herein is provided, wherein Pro151 is substituted with an amino acid selected from the group consisting of alanine and phenylalanine. In yet another embodiment, the antibody as described herein is provided, wherein Pro151 is substituted with phenylalanine, wherein direct cell death induced by the antibody comprising the variant heavy chain region is increased compared to direct cell death induced by the antibody comprising proline at position 151. In yet another embodiment, the antibody as described herein is provided, wherein Pro151 is substituted with alanine, wherein direct cell death induced by the antibody comprising the variant heavy chain region is decreased compared to direct cell death induced by the antibody comprising proline at position 151. In another specific embodiment, the parent non-substituted antibody is obinutuzumab and said variant heavy chain region comprises the amino acid substitutions Pro151Phe of the CH1 region and Asn297Asp of the Fc region relative to obinutuzumab. In another specific embodiment, the parent non-substituted antibody is obinutuzumab and said variant heavy chain region comprises the amino acid substitutions Pro151Phe of the CH1 region and Pro329Gly of the Fc region relative to obinutuzumab. In another specific embodiment, the parent non-substituted antibody is rituximab and said variant

heavy chain region comprises the amino acid substitutions Pro151Phe of the CH1 region and Asn297Asp of the Fc region relative to rituximab. In another specific embodiment, the parent non-substituted antibody is rituximab and said variant heavy chain region comprises the amino acid substitutions Pro151Phe of the CH1 region and Pro329Gly of the Fc region relative to rituximab.

In a further embodiment, an antibody is provided, comprising a variant heavy chain region comprising at least one amino acid substitution relative to the parent non-substituted heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residue Asn297, and wherein said substitution is Asn297Asp, wherein induction of effector function is reduced compared to effector function induced by an antibody comprising the parent non-substituted heavy chain region, wherein the variant heavy chain region comprises a further amino acid substitution relative to the parent non-substituted heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residue Val11, and wherein said further substitution is at said amino acid residue Val11, wherein direct cell death induced by the antibody comprising the variant heavy chain region is altered compared to direct cell death induced by the antibody comprising valine at position 11. In a further embodiment, an antibody is provided, comprising a variant heavy chain region comprising at least one amino acid substitution relative to the parent non-substituted heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residue Pro329, and wherein said substitution is Pro329Gly, wherein induction of effector function is reduced compared to effector function induced by an antibody comprising the parent non-substituted heavy chain region, wherein the variant heavy chain region comprises a further amino acid substitution relative to the parent non-substituted heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residue Val11, and wherein said further substitution is at said amino acid residue Val11, wherein direct cell death induced by the antibody comprising the variant heavy chain region is altered compared to direct cell death induced by the antibody comprising valine at position 11. In another embodiment, the antibody as described herein is provided, wherein direct cell death induced by the antibody comprising the variant heavy chain region is increased compared to direct cell death induced by the antibody comprising valine at position 11. In yet another embodiment, the antibody as described herein is provided, wherein direct cell death induced by the antibody comprising the variant heavy chain region is decreased compared to direct cell death induced by the antibody comprising valine at position 11. In yet another embodiment, the antibody as described herein is provided, wherein Val11 is substituted with an amino acid selected from the group

consisting of alanine, glycine, phenylalanine, threonine and tryptophan. In yet another embodiment, the antibody as described herein is provided, wherein Val11 is substituted with an amino acid selected from the group consisting of phenylalanine, threonine and tryptophan, wherein direct cell death induced by the antibody comprising the variant heavy chain region is increased compared to direct cell death induced by the antibody comprising valine at position 11. In yet another embodiment, the antibody as described herein is provided, wherein Val11 is substituted with an amino acid selected from the group consisting of alanine and glycine, wherein direct cell death induced by the antibody comprising the variant heavy chain region is decreased compared to direct cell death induced by the antibody comprising valine at position Val11. In another specific embodiment, the parent non-substituted antibody is obinutuzumab and said variant heavy chain region comprises the amino acid substitutions Val11Phe of the VH region and Asn297Asp of the Fc region relative to obinutuzumab. In another specific embodiment, the parent non-substituted antibody is rituximab and said variant heavy chain region comprises the amino acid substitutions Val11Phe of the VH region and Asn297Asp of the Fc region relative to rituximab. In another specific embodiment, the parent non-substituted antibody is obinutuzumab and said variant heavy chain region comprises the amino acid substitutions Val11Phe of the VH region and Pro329Gly of the Fc region relative to obinutuzumab. In another specific embodiment, the parent non-substituted antibody is rituximab and said variant heavy chain region comprises the amino acid substitutions Val11Phe of the VH region and Pro329Gly of the Fc region relative to rituximab.

Accordingly, the amino acid sequence of the parent polypeptide can be modified according to the invention to generate an antibody having a modification at position Asn297 and/or Pro329 in the Fc region, with reduced ability to induce effector function, as well as a modification at position Pro151 in the heavy chain CH1 and/or position Leu11 in the heavy chain VH region, which results with altered ability to induce cell signaling activity of a target antigen when the modified antibody is complexed with (e.g., bound to) the target antigen. The cell signaling activity can be agonist activity or antagonist activity. According to one aspect of the invention, agonist activity is induced by a modified antigen binding molecule when it binds to a cell membrane-associated receptor and initiates a cell signaling pathway. In a specific embodiment, the cell signaling pathway is an apoptosis pathway. In another embodiment, the cell signaling pathway is a cell differentiation pathway. According to another aspect of the invention, antagonist activity by a modified antigen binding molecule occurs, for example, when the antibody binds to a cell membrane-associated receptor and prevents the induction of a cell signaling pathway or disrupts an ongoing signal. Antagonist activity can be achieved, for example, by blocking the binding and subsequent signal transduction of an endogenous ligand

and/or by preventing the cross-linking or oligomerization of receptors or other molecules that would be necessary for induction of a cell signaling pathway. In one embodiment, the cell signaling pathway that is inhibited or disrupted is a cell growth pathway. In another embodiment, the cell signaling pathway that is inhibited or disrupted is a cell division pathway. In another embodiment the cell signaling pathway that is inhibited or disrupted is a cell survival pathway. Likewise, the amino acid sequence of the parent polypeptide can also be modified to generate an antibody according to the present invention having a modification at position Asn297 and/or Pro329 in the Fc region, with reduced ability to induce effector function, as well as a modification at position Pro151 in the heavy chain CH1 and/or position Leu11 in the heavy chain VH region, with altered ability to mediate cross-linking of one or more target antigens when the modified antibody is complexed with (e.g., bound to) the target antigen(s). In one embodiment, the bound target antigens (e.g., cell surface receptor molecules) are brought into closer proximity to each other and/or a more favorable conformation for interaction than they would be by the corresponding non-substituted parent antibody, thereby increasing cross-linking and oligomerization between the bound antigens. In another embodiment, the bound target antigens (e.g., cell surface receptor molecules) are kept farther apart from each other, and/or in a less favorable conformation for interaction than they would be by the corresponding non-substituted parent antibody, thereby reducing or preventing cross-linking and oligomerization between the bound antigens. In a particular embodiment, the increased cross-linking or oligomerization results in increased direct cell death. In another embodiment, the increased cross-linking or oligomerization results in increased cell differentiation. In another embodiment, the reduction in cross-linking or oligomerization results in decreased cell growth, decreased cell division, or decreased cell survival.

In certain specific embodiments the present invention is directed to modified antibodies that have decreased ability to induce effector function and increased ability to induce apoptosis compared to the corresponding non-modified parent antibody. For example, a parent antibody that has little or no ability to induce apoptosis but strong ability to induce effector function can be modified according to the present invention to generate a modified antibody that does have the ability to induce apoptosis or that has an increased ability to induce apoptosis and that does no have the ability to induce strong effector function. Likewise a parent antibody that induces strong effector function can be modified to generate a modified antibody that induces weak effector function while retaining or increasing the potential to induce apoptosis. The present invention is also directed to modified antibodies that have increased ability to induce growth arrest or cell differentiation and reduced or abolished induction of effector function or cytokine

release activity as compared to the corresponding non-modified parent antibody. For example, a parent antibody that has little or no ability to induce growth arrest or cell differentiation can be modified according to the present invention to generate a modified antibody that does have the ability to induce growth arrest or differentiation or that has an increased ability to induce growth arrest or differentiation while reducing adverse events like e.g., infusion reaction (cytokine release syndrome).

A further aspect of the present invention is the provision of modified anti-CD20 antibodies. In a preferred embodiment, the antibody as disclosed herein specifically binds to CD20. In certain embodiments, the modified anti-CD20 antibody as disclosed herein has a dissociation constant (Kd) of $\leq 1\mu M$, ≤ 100 nM, ≤ 10 nM, ≤ 1 nM, ≤ 0.1 nM, ≤ 0.01 nM, or ≤ 0.001 nM, from 10^{-8} M to 10^{-13} M or from 10^{-9} M to 10^{-13} M. In a preferred embodiment, the antibody as disclosed herein binds to CD20 with a dissociation constant (Kd) on cells of 10 nM or less as determined by scatchard analysis. In one embodiment, the present invention is directed to a modified anti-CD20 antibody comprising at least two amino acid substitutions in the heavy chain region compared to a type I parent anti-CD20 antibody, wherein the substitutions result in decreased induction of effector function and increased induction of direct cell death by the modified anti-CD20 antibody. In another embodiment, the present invention is directed to modified type II anti-CD20 antibodies having decreased induction of effector function without loss of substantial ability to induce direct cell death as a result of amino acid substitutions as disclosed herein. In one embodiment, the type II anti-CD20 antibodies comprise a substitution in two or more amino acids in the heavy chain compared to a parent molecule. In another embodiment, the present invention is directed to a modified anti-CD20 antibody, comprising a variant heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residues Pro151 and Asn297, said variant heavy chain region comprising the amino acid substitutions Asn297Asp and Pro151Phe relative to the parent non-substituted heavy chain region, wherein effector function and direct cell death induced by the antibody comprising the variant heavy chain region is altered compared to effector function and direct cell death induced by the antibody comprising the parent non-substituted heavy chain region. In another embodiment, the present invention is directed to a modified anti-CD20 antibody, comprising a variant heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residues Pro151 and Pro329, said variant heavy chain region comprising the amino acid substitutions Pro329Gly and Pro151Phe relative to the parent non-substituted heavy chain region, wherein effector function and direct cell death induced by the antibody comprising the variant heavy chain region is altered compared to effector function and direct cell death induced by the

antibody comprising the parent non-substituted heavy chain region. In another embodiment, direct cell death induced by the antibody comprising the variant heavy chain region according to the invention is increased compared to direct cell death induced by the antibody comprising the parent non-substituted heavy chain region. In another embodiment, direct cell death induced by the antibody comprising the variant heavy chain region according to the invention is decreased compared to direct cell death induced by the antibody comprising the parent non-substituted heavy chain region. In another embodiment, effector functions induced by the antibody comprising the variant heavy chain region according to the invention are reduced or abolished compared to effector functions induced by the antibody comprising the parent non-substituted heavy chain region. In a preferred embodiment, direct cell death induced by the antibody comprising the variant heavy chain region according to the invention is increased compared to direct cell death induced by the antibody comprising the parent non-substituted heavy chain region and effector function induced by the antibody comprising the variant heavy chain region according to the invention is reduced or abolished compared to direct cell death induced by the antibody comprising the parent non-substituted heavy chain region. In a further preferred embodiment the antibody according to the invention, comprising said amino acid substitution Asn297Asp or Pro329Gly, retains residual ADCP function.

In a further embodiment of the present invention, a modified anti-CD20 antibody is provided, comprising a variant CH1 and/or VH region as disclosed herein and a variant Fc region as disclosed herein compared to the respective parent non-substituted antibody. In one embodiment an anti-CD20 antibody is provided, comprising a variant heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residues Pro151 and Asn297, said variant heavy chain region comprising the amino acid substitutions Pro151Phe and Asn297Asp relative to the parent non-substituted heavy chain region, wherein induction of direct cell death is increased, and wherein FcγRIII binding is abolished, wherein induction of ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, and wherein induction of ADCP function is reduced but residual ADCP function is preserved, compared to direct cell death and effector function induced by an antibody comprising the parent non-substituted antibody heavy chain region. In one embodiment an anti-CD20 antibody is provided, comprising a variant heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residues Pro151 and Pro329, said variant heavy chain region comprising the amino acid substitutions Pro151Phe and Pro329Gly relative to the parent non-substituted heavy chain region, wherein induction of direct cell death is increased, and wherein FcγRIII binding is

abolished, wherein induction of ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, and wherein induction of ADCP function is reduced but residual ADCP function is preserved, compared to direct cell death and effector function induced by an antibody comprising the parent non-substituted antibody heavy chain region. In a further embodiment an anti-CD20 antibody is provided, comprising a variant heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residues Val11 and Asn297, said variant heavy chain region comprising the amino acid substitutions Val11Phe and Asn297Asp relative to the parent non-substituted heavy chain region, wherein induction of direct cell death is increased, and wherein FcγRIII binding is abolished, wherein induction of ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, and wherein induction of ADCP function is reduced but residual ADCP function is preserved, compared to direct cell death and effector function induced by an antibody comprising the parent non-substituted antibody heavy chain region. In a further embodiment an anti-CD20 antibody is provided, comprising a variant heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residues Val11 and Pro329, said variant heavy chain region comprising the amino acid substitutions Val11Phe and Pro329Gly relative to the parent non-substituted heavy chain region, wherein induction of direct cell death is increased, and wherein FcγRIII binding is abolished, wherein induction of ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, and wherein induction of ADCP function is reduced but residual ADCP function is preserved, compared to direct cell death and effector function induced by an antibody comprising the parent non-substituted antibody heavy chain region. In a further aspect said antibodies exhibit a reduced affinity to the human FcγRIII and/or FcγRII and/or FcγRI compared to antibodies with wildtype Fc region. In a further aspect, said modifications in the Fc region lead to reduced or abolished effector (ADCC and/or CDC and/or ADCP) function, reduced or abolished binding to Fc receptors, reduced or abolished binding to Clq and reduced or abolished infusion reaction (cytokine release syndrome).

In a more specific embodiment, the present invention provides anti-CD20 antibodies with increased induction of direct cell death and reduced affinity to a human Fc receptor (FcγR) and/or a human complement receptor as compared to the polypeptide comprising the wildtype human heavy chain region. In a further embodiment the affinity to at least one of the FcγRI, FcγRII, FcγRIII is reduced, in a still further embodiment the affinity to the FcγRI and FcγRIII is reduced, and in a still further embodiment the affinity to the FcγRI, FcγRII and FcγRIII is reduced, in still a further aspect of the invention the affinity to the FcγRI receptor, FcγRIII

receptor and Clq is reduced, and in still a further aspect of the invention the affinity to the FcγRI, FcγRII, FcγRIII and Clq receptor is reduced.

In another aspect of the present invention, antibodies are provided, comprising at least one amino acid substitution at one of the amino acid residues selected from the group consisting of Val11, Pro151 and Asn297, wherein induction of effector function and/or binding to Fcγ receptors and induction of direct cell death by the antibody comprising the variant heavy chain region is altered. In another aspect of the present invention, antibodies are provided, comprising at least one amino acid substitution at one of the amino acid residues selected from the group consisting of Val11, Pro151, Asn297 and Pro329, wherein induction of effector function and/or binding to Fcγ receptors and induction of direct cell death by the antibody comprising the variant heavy chain region is altered. In one embodiment, binding to FcγRIII by the antibody comprising the variant heavy chain region is reduced. In preferred embodiments, binding to FcγRIII by the antibody comprising the variant heavy chain region is reduced to 90% or less, 80% or less, 70% or less, to 60% or less, to 50% or less, to 40% or less, to 30% or less, to 20% or less, to 10% or less of FcγRIII binding by the antibody comprising the parent non-substituted heavy chain region. In a more preferred embodiment, binding to FcγRIII by the antibody comprising the variant heavy chain region is reduced to 0% to 20% of FcγRIII binding by the antibody comprising the parent non-substituted heavy chain region. In a most preferred embodiment, binding to FcγRIII by the antibody comprising the variant heavy chain region is abolished compared to binding to FcγRIII by the antibody comprising the parent non-substituted heavy chain region. In another embodiment, induction of ADCC function by the antibody comprising the variant heavy chain region is reduced. In preferred embodiments, induction of ADCC function by the antibody comprising the variant heavy chain region is reduced to 90% or less, 80% or less, 70% or less, to 60% or less, to 50% or less, to 40% or less, to 30% or less, to 20% or less, to 10% or less of ADCC function induced by the antibody comprising the parent non-substituted heavy chain region. In a more preferred embodiment, induction of ADCC function by the antibody comprising the variant heavy chain region is reduced to 0% to 80%, and in yet a more preferred embodiment to 0% to 20% of ADCC function induced by the antibody comprising the parent non-substituted heavy chain region. In a most preferred embodiment, induction of ADCC function by the antibody comprising the variant heavy chain region is abolished compared to ADCC function induced by the antibody comprising the parent non-substituted heavy chain region. In a further embodiment, ADCC may be measured by quantification of LDH released into cell supernatants in the presence of effector cells. In yet another embodiment, binding to FcγRI by

the antibody comprising the variant heavy chain region is reduced. In another embodiment, binding to FcγRI by the antibody comprising the variant heavy chain region is abolished compared to binding to FcγRI by the antibody comprising the parent non-substituted heavy chain region. In preferred embodiments, binding to FcγRI by the antibody comprising the variant heavy chain region is reduced to 90% or less, 80% or less, 70% or less, to 60% or less, to 50% or less, to 40% or less, to 30% or less, to 20% or less, to 10% or less of FcγRI binding by the antibody comprising the parent non-substituted heavy chain region. In a more preferred embodiment, binding to FcγRI by the antibody comprising the variant heavy chain region is reduced to 10% to 90%, and in a most preferred embodiment to 20% to 60% of FcγRI binding by the antibody comprising the parent non-substituted heavy chain region. In another embodiment, induction of ADCP function by the antibody comprising the variant heavy chain region is reduced. In a further embodiment, induction of ADCP function by the antibody comprising the variant heavy chain region is abolished compared to ADCP function induced by the antibody comprising the parent non-substituted heavy chain region. In preferred embodiments, induction of ADCP function by the antibody comprising the variant heavy chain region is reduced to 90% or less, 80% or less, 70% or less, to 60% or less, to 50% or less, to 40% or less, to 30% or less, to 20% or less, to 10% or less of ADCP function induced by the antibody comprising the parent non-substituted heavy chain region. In a more preferred embodiment, induction of ADCP function by the antibody comprising the variant heavy chain region is reduced to 10% to 90%, and in yet a more preferred embodiment to 20% to 60% of ADCP function induced by the antibody comprising the parent non-substituted heavy chain region. In a most preferred embodiment, antibodies of the present invention preserve residual ADCP function. In a further embodiment, ADCP may be measured by quantification of CFSE-PKH26-double positive macrophages after co-incubation with target cells. In another embodiment, induction of CDC function by the antibody comprising the variant heavy chain region is reduced. In preferred embodiments, induction of CDC function by the antibody comprising the variant heavy chain region is reduced to 90% or less, 80% or less, 70% or less, to 60% or less, to 50% or less, to 40% or less, to 30% or less, to 20% or less, to 10% or less of CDC function induced by the antibody comprising the parent non-substituted heavy chain region. In a more preferred embodiment, induction of CDC function by the antibody comprising the variant heavy chain region is reduced to 0% to 80%, and in yet a more preferred embodiment to 0% to 20% of CDC function induced by the antibody comprising the parent non-substituted heavy chain region. In a most preferred embodiment, induction of CDC function by the antibody comprising the variant heavy chain region is abolished compared to CDC function induced by the antibody comprising the parent non-substituted heavy chain

region. In a further embodiment, CDC may be measured by quantification of LDH released into cell supernatants in the presence of complement. In another embodiment induction of infusion reaction (cytokine release syndrome) by the antibody comprising the variant heavy chain region is reduced. In preferred embodiments, induction of infusion reaction by the antibody comprising the variant heavy chain region is reduced to 90% or less, 80% or less, 70% or less, to 60% or less, to 50% or less, to 40% or less, to 30% or less, to 20% or less, to 10% or less of infusion reaction induced by the antibody comprising the parent non-substituted heavy chain region. In a most preferred embodiment, induction of infusion reaction by the antibody comprising the variant heavy chain region is abolished.

Accordingly, in a further embodiment, direct cell death induced by the antibody comprising the variant heavy chain region is increased compared to direct cell death induced by the antibody comprising the parent non-substituted heavy chain region. In another aspect of the present invention, direct cell death induced by the antibody comprising the variant heavy chain region is increased to at least 110% of the direct cell death induced by the antibody comprising the parent non-substituted heavy chain region. In a preferred embodiment, direct cell death induced by the antibody comprising the variant heavy chain region is increased to at least 120% of the direct cell death induced by the antibody comprising the parent non-substituted heavy chain region. In more preferred embodiments, direct cell death induced by the antibody comprising the variant heavy chain region is increased to at least 130%, to at least 140%, to at least 150%, to at least 160%, to at least 170%, to at least 180%, to at least 190% to at least 200% of the direct cell death induced by the antibody comprising the parent non-substituted heavy chain region. In a most preferred embodiment, direct cell death induced by the antibody comprising the variant heavy chain region is increased to 120% to 200% of the direct cell death induced by the antibody comprising the parent non-substituted heavy chain region. In yet a further aspect of the present invention, direct cell death induced by the antibody comprising the variant heavy chain region is decreased compared to direct cell death induced by the antibody comprising the parent non-substituted heavy chain region. In still a further embodiment, direct cell death induced by the antibody comprising the variant heavy chain region is decreased to 90% or less of the direct cell death induced by the antibody comprising the parent non-substituted heavy chain region. In a preferred embodiment, direct cell death induced by the antibody comprising the variant heavy chain region is decreased to 80% or less of the direct cell death induced by the antibody comprising the parent non-substituted heavy chain region. In more preferred embodiments, direct cell death induced by the antibody comprising the variant heavy chain region is decreased to 70% or less, to 60% or less, to 50% or less, to 40% or less, to 30% or less, to 20% or less, to 10% or less of the direct cell death

induced by the antibody comprising the parent non-substituted heavy chain region. In another more preferred embodiment, direct cell death induced by the antibody comprising the variant heavy chain region is decreased to 0% to 70%, and in yet a more preferred embodiment to 0% to 20% of the direct cell death induced by the antibody comprising the parent non-substituted heavy chain region. In still another embodiment, direct cell death induced by the antibody comprising the variant heavy chain region is abolished. In a further embodiment induction of direct cell death may be measured by Annexin V binding and PI staining.

While, it is preferred to alter binding to a FcγR, Fc region variants with altered binding affinity for the neonatal receptor (FcRn) are also contemplated herein. Fc region variants with improved affinity for FcRn are anticipated to have longer serum half-lives, and such molecules will have useful applications in methods of treating mammals where long half-life of the administered polypeptide is desired, e.g., to treat a chronic disease or disorder. Fc region variants with decreased FcRn binding affinity, on the contrary, are expected to have shorter half-lives, and such molecules may, for example, be administered to a mammal where a shortened circulation time may be advantageous, e.g. for *in vivo* diagnostic imaging or for polypeptides which have toxic side effects when left circulating in the blood stream for extended periods, etc. Fc region variants with decreased FcRn binding affinity are anticipated to be less likely to cross the placenta, and thus may be utilized in the treatment of diseases or disorders in pregnant women. Fc region variants with altered binding affinity for FcRn include those comprising an Fc region amino acid modification at any one or more of amino acid positions 238, 252, 253, 254, 255, 256, 265, 272, 286, 288, 303, 305, 307, 309, 311, 312, 317, 340, 356, 360, 362, 376, 378, 380, 382, 386, 388, 400, 413, 415, 424, 433, 434, 435, 436, 439 or 447. Those which display reduced binding to FcRn will generally comprise an Fc region amino acid modification at any one or more of amino acid positions 252, 253, 254, 255, 288, 309, 386, 388, 400, 415, 433, 435, 436, 439 or 447; and those with increased binding to FcRn will usually comprise an Fc region amino acid modification at any one or more of amino acid positions 238, 256, 265, 272, 286, 303, 305, 307, 311, 312, 317, 340, 356, 360, 362, 376, 378, 380, 382, 413, 424 or 434.

In a specific embodiment, an anti-CD20 antibody is provided, comprising a variant heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residue Asn297, said variant heavy chain region comprising the amino acid substitution Asn297Asp relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is reduced, compared to effector

function induced by the parent non-substituted antibody comprising asparagine at position 297. In a further specific embodiment, an anti-CD20 antibody is provided, comprising a variant heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residue Pro329, said variant heavy chain region comprising the amino acid substitution Pro329Gly relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is reduced, compared to effector function induced by the parent non-substituted antibody comprising proline at position 329.

In a specific embodiment, an anti-CD20 antibody is provided, comprising a variant heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residue Asn297, said variant heavy chain region comprising the amino acid substitution Asn297Asp relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, and wherein induction of ADCP function is reduced but residual ADCP function is preserved, compared to effector function induced by the parent non-substituted antibody comprising asparagine at position 297. In a further specific embodiment, an anti-CD20 antibody is provided, comprising a variant heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residue Pro329, said variant heavy chain region comprising the amino acid substitution Pro329Gly relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, and wherein induction of ADCP function is reduced but residual ADCP function is preserved, compared to effector function induced by the parent non-substituted antibody comprising proline at position 329.

In a specific embodiment, an anti-CD20 antibody is provided, comprising a variant heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residue Asn297, said variant heavy chain region comprising the amino acid substitution Asn297Asp relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is reduced but residual ADCP function is preserved, and wherein infusion reaction (cytokine release syndrome) is reduced compared to effector function induced by the parent non-substituted antibody comprising

asparagine at position 297. In a further specific embodiment, an anti-CD20 antibody is provided, comprising a variant heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residue Pro329, said variant heavy chain region comprising the amino acid substitution Pro329Gly relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is reduced but residual ADCP function is preserved, and wherein infusion reaction (cytokine release syndrome) is reduced compared to effector function induced by the parent non-substituted antibody comprising proline at position 329.

In a specific embodiment, an anti-CD20 antibody is provided, comprising a variant heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residues Pro151 and Asn297, said variant heavy chain region comprising the amino acid substitutions Pro151Phe and Asn297Asp relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is reduced, compared to effector function induced by the parent non-substituted antibody comprising asparagine at position 297, and wherein induction of direct cell death is increased compared to direct cell death induced by the parent non-substituted antibody comprising proline at position 151. In another specific embodiment, an anti-CD20 antibody is provided, comprising a variant heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residues Pro151 and Pro329, said variant heavy chain region comprising the amino acid substitutions Pro151Phe and Pro329Gly relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is reduced, compared to effector function induced by the parent non-substituted antibody comprising proline at position 329, and wherein induction of direct cell death is increased compared to direct cell death induced by the parent non-substituted antibody comprising proline at position 151.

In a specific embodiment, an anti-CD20 antibody is provided, comprising a variant heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residues Pro151 and Asn297, said variant heavy chain region comprising the amino acid substitutions Pro151Phe and Asn297Asp relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or

strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is reduced but residual ADCP function is preserved, and wherein infusion reaction (cytokine release syndrome) is reduced compared to effector function induced by the parent non-substituted antibody comprising asparagine at position 297, and wherein induction of direct cell death is increased compared to direct cell death induced by the parent non-substituted antibody comprising proline at position 151. In another specific embodiment, an anti-CD20 antibody is provided, comprising a variant heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residues Pro151 and Pro329, said variant heavy chain region comprising the amino acid substitutions Pro151Phe and Pro329Gly relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is reduced but residual ADCP function is preserved, and wherein infusion reaction (cytokine release syndrome) is reduced compared to effector function induced by the parent non-substituted antibody comprising proline at position 329, and wherein induction of direct cell death is increased compared to direct cell death induced by the parent non-substituted antibody comprising proline at position 151.

In a specific embodiment, an anti-CD20 antibody is provided, comprising a variant heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residues Pro151 and Asn297, said variant heavy chain region comprising the amino acid substitutions Pro151Ala and Asn297Asp relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is reduced, compared to effector function induced by the parent non-substituted antibody comprising asparagine at position 297, and wherein induction of direct cell death is decreased compared to direct cell death induced by the parent non-substituted antibody comprising proline at position 151. In a specific embodiment, an anti-CD20 antibody is provided, comprising a variant heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residues Pro151 and Pro329, said variant heavy chain region comprising the amino acid substitutions Pro151Ala and Pro297Gly relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is reduced, compared to effector function induced by the parent non-substituted antibody comprising proline at position 329, and wherein induction of direct cell

death is decreased compared to direct cell death induced by the parent non-substituted antibody comprising proline at position 151.

In a specific embodiment, an anti-CD20 antibody is provided, comprising a variant heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residues Pro151 and Asn297, said variant heavy chain region comprising the amino acid substitutions Pro151Ala and Asn297Asp relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is reduced, and wherein infusion reaction (cytokine release syndrome) is reduced compared to effector function induced by the parent non-substituted antibody comprising asparagine at position 297, and wherein induction of direct cell death is decreased compared to direct cell death induced by the parent non-substituted antibody comprising proline at position 151.

In a specific embodiment, an anti-CD20 antibody is provided, comprising a variant heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residues Val11 and Asn297, said variant heavy chain region comprising the amino acid substitutions Val11Phe and Asn297Asp relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is reduced, compared to effector function induced by the parent non-substituted antibody comprising asparagine at position 297, and wherein induction of direct cell death is increased compared to direct cell death induced by the parent non-substituted antibody comprising valine at position 11. In a specific embodiment, an anti-CD20 antibody is provided, comprising a variant heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residues Val11 and Pro329, said variant heavy chain region comprising the amino acid substitutions Val11Phe and Pro329Gly relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is reduced, compared to effector function induced by the parent non-substituted antibody comprising proline at position 329, and wherein induction of direct cell death is increased compared to direct cell death induced by the parent non-substituted antibody comprising valine at position 11.

In a specific embodiment, an anti-CD20 antibody is provided, comprising a variant heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residues Val11 and Asn297, said variant heavy chain region comprising the amino acid substitutions Val11Ala and Asn297Asp relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is reduced, compared to effector function induced by the parent non-substituted antibody comprising asparagine at position 297, and wherein induction of direct cell death is decreased compared to direct cell death induced by the parent non-substituted antibody comprising valine at position 11.

In a specific embodiment, an anti-CD20 antibody is provided, comprising a variant heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residues Val11 and Asn297, said variant heavy chain region comprising the amino acid substitutions Val11Phe and Asn297Asp relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is reduced but residual ADCP function is preserved, and wherein infusion reaction (cytokine release syndrome) is reduced compared to effector function induced by the parent non-substituted antibody comprising asparagine at position 297, and wherein induction of direct cell death is increased compared to direct cell death induced by the parent non-substituted antibody comprising valine at position 11.

In a specific embodiment, an antibody is provided, comprising a variant heavy chain region, wherein the parent non-substituted antibody is obinutuzumab comprising the amino acid residue Asn297 in the heavy chain region, said variant heavy chain region comprising the amino acid substitution Asn297Asp relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is reduced, compared to effector function induced by obinutuzumab. In a specific embodiment, an antibody is provided, comprising a variant heavy chain region, wherein the parent non-substituted antibody is obinutuzumab comprising the amino acid residue Pro329 in the heavy chain region, said variant heavy chain region comprising the amino acid substitution Pro329GlyAsp relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced,

wherein induction of ADCP function is reduced, compared to effector function induced by obinutuzumab.

In a specific embodiment, an antibody is provided, comprising a variant heavy chain region, wherein the parent non-substituted antibody is obinutuzumab comprising the amino acid residue Asn297 in the heavy chain region, wherein the residues are numbered according to the EU index as in Kabat, said variant heavy chain region comprising the amino acid substitution Asn297Asp relative to the parent non-substituted heavy chain region, wherein effector (ADCC and/or CDC and/or ADCP) function induced by the antibody comprising the variant heavy chain region is reduced or abolished compared to effector function induced by obinutuzumab, wherein infusion reaction (cytokine release syndrome) induced by the antibody comprising the variant heavy chain region is reduced or abolished compared to infusion reaction induced by obinutuzumab.

In a specific embodiment, an antibody is provided, comprising a variant heavy chain region, wherein the parent non-substituted antibody is obinutuzumab comprising the amino acid residue Asn297 in the heavy chain region, said variant heavy chain region comprising the amino acid substitution Asn297Asp relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is reduced but residual ADCP function is preserved, compared to effector function induced by obinutuzumab.

In a specific embodiment, an antibody is provided, comprising a mutated heavy chain, wherein the parent non-mutated antibody is obinutuzumab comprising the amino acid residue Asn297 (EU numbering) in the heavy chain, said mutated heavy chain comprising the amino acid substitution Asn297Asp relative to the parent non-mutated heavy chain, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is reduced but residual ADCP function is preserved, compared to effector function induced by obinutuzumab.

In a specific embodiment, an antibody is provided, comprising a mutated heavy chain, wherein the parent non-mutated antibody is obinutuzumab comprising the amino acid residue Pro329 (EU numbering) in the heavy chain, said mutated heavy chain comprising the amino acid substitution Pro329Gly relative to the parent non-mutated heavy chain, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding

is reduced, wherein induction of ADCP function is reduced but residual ADCP function is preserved, compared to effector function induced by obinutuzumab.

In a specific embodiment, an antibody is provided, comprising a variant heavy chain region, wherein the parent non-substituted antibody is obinutuzumab comprising the amino acid residues Pro151 and Asn297 in the heavy chain region, said variant heavy chain region comprising the amino acid substitutions Pro151Phe and Asn297Asp relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is reduced but residual ADCP function is preserved, compared to effector function induced by obinutuzumab, and wherein induction of direct cell death is increased compared to direct cell death induced by obinutuzumab. In a specific embodiment, an antibody is provided, comprising a variant heavy chain region, wherein the parent non-substituted antibody is obinutuzumab comprising the amino acid residues Pro151 and Pro329 in the heavy chain region, said variant heavy chain region comprising the amino acid substitutions Pro151Phe and Pro329Gly relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is reduced but residual ADCP function is preserved, compared to effector function induced by obinutuzumab, and wherein induction of direct cell death is increased compared to direct cell death induced by obinutuzumab.

In a specific embodiment, an antibody is provided, comprising a variant heavy chain region, wherein the parent non-substituted antibody is obinutuzumab comprising the amino acid residues Pro151 and Asn297 in the heavy chain region, wherein the residues are numbered according to the EU index as in Kabat, said variant heavy chain region comprising the amino acid substitutions Pro151Phe and Asn297Asp relative to the parent non-substituted heavy chain region, wherein effector (ADCC and/or CDC and/or ADCP) function induced by the antibody comprising the variant heavy chain region is reduced or abolished compared to effector function induced by obinutuzumab, and wherein direct cell death induced by the antibody comprising the variant heavy chain region is increased compared to direct cell death induced by obinutuzumab.

In a specific embodiment, an antibody is provided, comprising a variant heavy chain region, wherein the parent non-substituted antibody is obinutuzumab comprising the amino acid residues Pro151 and Asn297 in the heavy chain region, said variant heavy chain region

comprising the amino acid substitutions Pro151Ala and Asn297Asp relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is reduced, compared to effector function induced by obinutuzumab, and wherein induction of direct cell death is decreased compared to direct cell death induced by obinutuzumab.

In a specific embodiment, an antibody is provided, comprising a variant heavy chain region, wherein the parent non-substituted antibody is obinutuzumab comprising the amino acid residues Pro151 and Asn297 in the heavy chain region, said variant heavy chain region comprising the amino acid substitutions Pro151Ala and Asn297Asp relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is reduced but residual ADCP function is preserved, compared to effector function induced by obinutuzumab, and wherein induction of direct cell death is decreased compared to direct cell death induced by obinutuzumab. In a specific embodiment, an antibody is provided, comprising a variant heavy chain region, wherein the parent non-substituted antibody is obinutuzumab comprising the amino acid residues Pro151 and Pro329 in the heavy chain region, said variant heavy chain region comprising the amino acid substitutions Pro151Ala and Pro329Gly relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is reduced but residual ADCP function is preserved, compared to effector function induced by obinutuzumab, and wherein induction of direct cell death is decreased compared to direct cell death induced by obinutuzumab.

In a specific embodiment, an antibody is provided, comprising a variant heavy chain region, wherein the parent non-substituted antibody is obinutuzumab comprising the amino acid residues Val11 and Asn297 in the heavy chain region, said variant heavy chain region comprising the amino acid substitutions Val11Phe and Asn297Asp relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is reduced, compared to effector function induced by obinutuzumab, and wherein induction of direct cell death is increased compared to direct cell death induced by obinutuzumab.

In a specific embodiment, an antibody is provided, comprising a variant heavy chain region, wherein the parent non-substituted antibody is obinutuzumab comprising the amino acid residues Val11 and Asn297 in the heavy chain region, said variant heavy chain region comprising the amino acid substitutions Val11Ala and Asn297Asp relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is reduced, compared to effector function induced by obinutuzumab, and wherein induction of direct cell death is decreased compared to direct cell death induced by obinutuzumab.

In a specific embodiment, an antibody is provided, comprising a variant heavy chain region, wherein the parent non-substituted antibody is obinutuzumab comprising the amino acid residues Val11 and Asn297 in the heavy chain region, said variant heavy chain region comprising the amino acid substitutions Val11Phe and Asn297Asp relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is reduced but residual ADCP function is preserved, compared to effector function induced by obinutuzumab, and wherein induction of direct cell death is increased compared to direct cell death induced by obinutuzumab. In a specific embodiment, an antibody is provided, comprising a variant heavy chain region, wherein the parent non-substituted antibody is obinutuzumab comprising the amino acid residues Val11 and Pro329 in the heavy chain region, said variant heavy chain region comprising the amino acid substitutions Val11Phe and Pro329Gly relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is reduced but residual ADCP function is preserved, compared to effector function induced by obinutuzumab, and wherein induction of direct cell death is increased compared to direct cell death induced by obinutuzumab.

In a specific embodiment, an antibody is provided, comprising a variant heavy chain region, wherein the parent non-substituted antibody is rituximab comprising the amino acid residue Asn297 in the heavy chain region, said variant heavy chain region comprising the amino acid substitution Asn297Asp relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is reduced, compared to effector function induced by rituximab.

In a specific embodiment, an antibody is provided, comprising a variant heavy chain region, wherein the parent non-substituted antibody is rituximab comprising the amino acid residue Asn297 in the heavy chain region, said variant heavy chain region comprising the amino acid substitution Asn297Asp relative to the parent non-substituted heavy chain region, wherein effector (ADCC and/or CDC and/or ADCP) function induced by the antibody comprising the variant heavy chain region is reduced or abolished compared to effector function induced by rituximab, wherein infusion reaction (cytokine release syndrome) induced by the antibody comprising the variant heavy chain region is reduced or abolished compared to infusion reaction induced by rituximab.

In a specific embodiment, an antibody is provided, comprising a variant heavy chain region, wherein the parent non-substituted antibody is rituximab comprising the amino acid residue Asn297 in the heavy chain region, said variant heavy chain region comprising the amino acid substitution Asn297Asp relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is reduced but residual ADCP function is preserved, compared to effector function induced by rituximab.

In a specific embodiment, an antibody is provided, comprising a mutated heavy chain, wherein the parent non-mutated antibody is rituximab comprising the amino acid residue Asn297 (EU numbering) in the heavy chain, said mutated heavy chain comprising the amino acid substitution Asn297Asp relative to the parent non-mutated heavy chain, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is reduced but residual ADCP function is preserved, compared to effector function induced by rituximab.

In a specific embodiment, an antibody is provided, comprising a mutated heavy chain, wherein the parent non-mutated antibody is rituximab comprising the amino acid residue Pro329 (EU numbering) in the heavy chain, said mutated heavy chain comprising the amino acid substitution Pro329Gly relative to the parent non-mutated heavy chain, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is reduced but residual ADCP function is preserved, compared to effector function induced by rituximab.

In a specific embodiment, an antibody is provided, comprising a variant heavy chain region, wherein the parent non-substituted antibody is rituximab comprising the amino acid residues Pro151 and Asn297 in the heavy chain region, said variant heavy chain region comprising the amino acid substitutions Pro151Phe and Asn297Asp relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is reduced but residual ADCP function is preserved, compared to effector function induced by rituximab, and wherein induction of direct cell death is increased compared to direct cell death induced by rituximab.

In a specific embodiment, an antibody is provided, comprising a variant heavy chain region, wherein the parent non-substituted antibody is rituximab comprising the amino acid residues Pro151 and Asn297 in the heavy chain region, said variant heavy chain region comprising the amino acid substitutions Pro151Phe and Asn297Asp relative to the parent non-substituted heavy chain region, wherein effector (ADCC and/or CDC and/or ADCP) function induced by the antibody comprising the variant heavy chain region is reduced or abolished compared to effector function induced by rituximab, and wherein direct cell death induced by the antibody comprising the variant heavy chain region is increased compared to direct cell death induced by rituximab.

In a specific embodiment, an antibody is provided, comprising a variant heavy chain region, wherein the parent non-substituted antibody is rituximab comprising the amino acid residues Pro151 and Asn297 in the heavy chain region, said variant heavy chain region comprising the amino acid substitutions Pro151Ala and Asn297Asp relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is reduced, compared to effector function induced by rituximab, and wherein induction of direct cell death is decreased compared to direct cell death induced by rituximab.

In a specific embodiment, an antibody is provided, comprising a variant heavy chain region, wherein the parent non-substituted antibody is rituximab comprising the amino acid residues Pro151 and Asn297 in the heavy chain region, said variant heavy chain region comprising the amino acid substitutions Pro151Ala and Asn297Asp relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is

reduced but residual ADCP function is preserved, compared to effector function induced by rituximab, and wherein induction of direct cell death is decreased compared to direct cell death induced by rituximab.

In a specific embodiment, an antibody is provided, comprising a variant heavy chain region, wherein the parent non-substituted antibody is rituximab comprising the amino acid residues Val11 and Asn297 in the heavy chain region, said variant heavy chain region comprising the amino acid substitutions Val11Phe and Asn297Asp relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is reduced, compared to effector function induced by rituximab, and wherein induction of direct cell death is increased compared to direct cell death induced by rituximab.

In a specific embodiment, an antibody is provided, comprising a variant heavy chain region, wherein the parent non-substituted antibody is rituximab comprising the amino acid residues Val11 and Asn297 in the heavy chain region, said variant heavy chain region comprising the amino acid substitutions Val11Ala and Asn297Asp relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is reduced, compared to effector function induced by rituximab, and wherein induction of direct cell death is decreased compared to direct cell death induced by rituximab.

In a specific embodiment, an antibody is provided, comprising a variant heavy chain region, wherein the parent non-substituted antibody is rituximab comprising the amino acid residues Val11 and Asn297 in the heavy chain region, said variant heavy chain region comprising the amino acid substitutions Val11Phe and Asn297Asp relative to the parent non-substituted heavy chain region, wherein FcγRIII binding is abolished, wherein ADCC is abolished or strongly reduced, wherein FcγRI binding is reduced, wherein induction of ADCP function is reduced but residual ADCP function is preserved, compared to effector function induced by rituximab, and wherein induction of direct cell death is increased compared to direct cell death induced by rituximab.

Antibodies according to the present invention comprising amino acid modifications (substitutions, additions, deletions) can be prepared by methods known in the art. These methods include, but are not limited to, preparation by site-directed (or oligonucleotide-mediated) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared

nucleic acid encoding the polypeptide. Site-directed mutagenesis is a preferred method for preparing substitution variants. This technique is well known in the art (see e.g., Carter et a Nucleic Acids Res. 13: 4431-4443 (1985) and Kunkel et. al, Proc. Natl. Acad. ScL USA 82: 488 (1987), each of which is hereby incorporated by reference in its entirety). Briefly, in carrying out site directed mutagenesis of DNA, the starting DNA is altered by first hybridizing an oligonucleotide encoding the desired mutation to a single strand of such starting DNA. After hybridization, a DNA polymerase is used to synthesize an entire second strand, using the hybridized oligonucleotide as a primer, and using the single strand of the starting DNA as a template. Thus, the oligonucleotide encoding the desired mutation is incorporated in the resulting double-stranded DNA.

PCR mutagenesis is also suitable for making amino acid sequence variants of the non-modified starting polypeptide (see, e.g., Vallette et. al, Nuc. Acids Res. 17: 723-733 (1989), hereby incorporated by reference in its entirety). Briefly, when small amounts of template DNA are used as starting material in a PCR, primers that differ slightly in sequence from the corresponding region in a template DNA can be used to generate relatively large quantities of a specific DNA fragment that differs from the template sequence only at the positions where the primers differ from the template.

Another method according to the invention for preparing the inventive antibody variants, cassette mutagenesis, is based on the technique described by Wells et al, Gene 34: 315-323 (1985), hereby incorporated by reference in its entirety. The starting material is the plasmid (or other vector) comprising the starting polypeptide DNA to be modified. The codon(s) in the starting DNA to be mutated are identified. There must be a unique restriction endonuclease site on each side of the identified mutation site(s). If no such restriction sites exist, they can be generated using the herein-disclosed oligonucleotide-mediated mutagenesis method to introduce them at appropriate locations in the starting polypeptide DNA. The plasmid DNA is cut at these sites to linearize it. A double-stranded oligonucleotide encoding the sequence of the DNA between the restriction sites but containing the desired mutation(s) is synthesized using standard procedures, wherein the two strands of the oligonucleotide are synthesized separately and then hybridized together using standard techniques. This double-stranded oligonucleotide is referred to as the cassette. This cassette is designed to have 5' and 3' ends that are compatible with the ends of the linearized plasmid, such that it can be directly ligated to the plasmid. This plasmid now contains the mutated DNA sequence.

Alternatively, or additionally, the desired amino acid sequence encoding an antibody variant can be determined, and a nucleic acid sequence encoding such an amino acid sequence variant can be generated synthetically.

By introducing the appropriate amino acid sequence modifications in a parent Fc region, one can generate a variant Fc region which (a) mediates one or more effector functions in the presence of human effector cells less effectively and/or (b) binds an Fcγ receptor (FcγR) with smaller affinity than the parent polypeptide. Such modified Fc regions will comprise at least one amino acid modification in the Fc region. Likewise, variant CH1 and VH regions mediating altered induction of direct cell death more or less effectively compared to the parent non-substituted polypeptide can be generated by introducing the appropriate amino acid sequence modifications. Combined Fc and CH1 and/or VH region variants can be generated by introducing substitutions either individually into parent region polypeptides and combining modified regions to a combined Fc and CH1 and/or VH variant or by introducing all amino acid substitutions at the same time.

In preferred embodiments, the parent polypeptide Fc region is a human Fc region, including but not limited to a native human Fc region human IgG1 (A and non-A allotypes), IgG2, IgG3, IgG4, and all allotypes known or discovered from any species Fc region. Such regions have sequences such as those disclosed in U.S. Provisional Patent Application No. 60/678,776, which is hereby incorporated by reference in its entirety.

In certain embodiments, in order to generate an antibody comprising one or more amino acid substitutions in the heavy chain CH1 and VH regions further comprising a modified Fc region with altered effector function (including but not limited to ADCC, CDC and ADCP), the parent polypeptide preferably has pre-existing effector funtion (e.g., the parent polypeptide comprises a human IgG1 or human IgG3 Fc region). In some embodiments, a modified Fc region with altered effector function mediates effector function (including but not limited to ADCC, CDC and ADCP) substantially less effectively than an antibody with a native sequence IgGl or IgG3 Fc region.

The polypeptides of the invention having modified heavy chain regions can be subjected to one or more further modifications, depending on the desired or intended use of the polypeptide. Such modifications involve, for example but are not limited to, further alteration of the amino acid sequence (substitution, insertion and/or deletion of amino acid residues), fusion to heterologous polypeptide(s) and/or covalent modifications. Such further

modifications can be made prior to, simultaneously with, or following, the amino acid modification(s) disclosed herein which result in an alteration of signaling activity and/or of Fc receptor binding and/or effector function.

In another aspect of the invention, an antibody provided herein has a dissociation constant (Kd) of $\leq$ 1 µM, $\leq$ 100 nM, $\leq$ 10 nM, $\leq$ 1 nM, $\leq$ 0.1 nM, $\leq$ 0.01 nM, or $\leq$0.001 nM. Preferably, the dissociation constant is 10^{-8} M or less, from 10^{-8} M to 10^{-13} M, from 10^{-9} M to 10^{-13} M.

In one aspect of the invention, the dissociation constant Kd is measured by scatchard analysis using Europium labeled antibodies and analyzing the bound/free ratio at different antibody concentrations. $2x10^5$ SU-DHL4 cells are seeded into V-bottom plates (NUNC) in culture medium containing 20% FCS (50 µl). Then, 50 µl Europium-labeled antibodies are added in different concentrations and incubated at 25°C for 1h. Thereafter, 150 µl complete medium is added and the plate is centrifuged. The cells are washed 2x by replacing the whole medium, transferred into a new plate and washed again 2x. Finally, the medium is removed after centrifugation and the pellet is resuspended in 200 µl enhancer solution, transferred into a black 96 well plate and put onto a shaker for 10 min. By this step, the coupled Europium is released into the supernatant, the fluorescence is enhanced and then analyzed on a BMG PheraStar machine (ex337/em615). The molarity of the antibodies bound is calculated by usage of relative fluorescence units (RFU) from a standard (Europium-antibody) titration curve. The amount of free antibody is calculated by subtraction of the bound antibody from the signal measured in the total antibody wells. The bound versus free antibody ratio is plotted against the number of bound antibody molecules and the slope of the curve (S) is determined. The affinity of the antibody is determined using the following formula: Kd (M) = 1/ -S.

According to another embodiment, the dissociation constant Kd is measured by a radiolabeled antigen or Fc receptor binding assay (RIA) performed with the Fab version of an antibody of interest and its antigen as disclosed by the following assay. Solution binding affinity of Fabs for antigen is measured by equilibrating Fab with a minimal concentration of (^{125}I)- labeled antigen in the presence of a titration series of unlabeled antigen, then capturing bound antigen with an anti-Fab antibody-coated plate (see, e.g., Chen, et al., J. Mol. Biol. 293 (1999) 865-881). To establish conditions for the assay, MICROTITER® multi-well plates (Thermo Scientific) are coated overnight with 5 µg/ml of a capturing anti-Fab antibody (Cappel Labs) in 50 mM sodium carbonate (pH 9.6), and subsequently blocked with 2% (w/v) bovine serum albumin in PBS for two to five hours at room temperature (approximately 23°C). In a non-

adsorbent plate (Sigma-Aldrich P7366), 100 pM or 26 pM [^{125}I]-antigen are mixed with serial dilutions of a Fab of interest (e.g., consistent with assessment of the anti-VEGF antibody, Fab-12, in Presta, et al., Cancer Res. 57 (1997) 4593-4599). The Fab of interest is then incubated overnight; however, the incubation may continue for a longer period (e.g., about 65 hours) to ensure that equilibrium is reached. Thereafter, the mixtures are transferred to the capture plate for incubation at room temperature for one hour. The solution is then removed and the plate washed eight times with 0.1% polysorbate 20 (TWEEN-20®) in PBS. When the plates have dried, 150 µl/well of scintillant (MICROSCINT-20™; Packard) is added, and the plates are counted on a TOPCOUNT™ gamma counter (Packard) for ten minutes. Concentrations of each Fab that give less than or equal to 20% of maximal binding are chosen for use in competitive binding assays.

According to another embodiment, the dissociation constant Kd of the antibody is measured using surface plasmon resonance assays using a BIACORE®-2000 or a BIACORE®-3000 (BIAcore, Inc., Piscataway, NJ) at 25°C with immobilized antigen or Fc receptor CM5 chips at ~10 response units (RU). Briefly, carboxy methylated dextran biosensor chips (CM5, BIACORE, Inc.) are activated with N-ethyl-N'-(3-dimethylaminopropyl)- carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) according to the supplier's instructions. Antigen is diluted with 10 mM sodium acetate, pH 4.8, to 5 µg/ml (~0.2 µM) before injection at a flow rate of 5 µl/minute to achieve approximately 10 response units (RU) of coupled protein. Following the injection of antigen, 1 M ethanolamine is injected to block unreacted groups. For kinetics measurements, two-fold serial dilutions of Fab (0.78 nM to 500 nM) are injected in PBS with 0.05% polysorbate 20 (TWEEN-20™) surfactant (PBST) at 25°C at a flow rate of approximately 25 µl/min. Association rates (kon) and dissociation rates (koff) are calculated using a simple one-to-one Langmuir binding model (BIACORE® Evaluation Software version 3.2) by simultaneously fitting the association and dissociation sensograms. Kd is calculated as the ratio koff/kon. See, e.g., Chen, et al., J. Mol. Biol. 293 (1999) 865-881. If the on-rate exceeds 10^6 M^{-1} s^{-1} by the surface plasmon resonance assay herein, then the on-rate can be determined by using a fluorescent quenching technique that measures the increase or decrease in fluorescence emission intensity (excitation = 295 nm; emission = 340 nm, 16 nm band-pass) at 25°C of a 20 nM anti-antigen antibody (Fab form) in PBS, pH 7.2, in the presence of increasing concentrations of antigen as measured in a spectrometer, such as a stop-flow equipped spectrophometer (Aviv Instruments) or a 8000-series SLM- AMINCO™ spectrophotometer (ThermoSpectronic) with a stirred cuvette.

In a specific embodiment, the parent non-substituted heavy chain region is from obinutuzumab, as disclosed in SEQ ID NO: 1, and further disclosed herein. The amino acid positions 11, 151, and 297 according to Kabat are underlined.

Obinutuzumab heavy chain amino acid sequence (SEQ ID NO: 1)

QVQLVQSGAE**V**KKPGSSVKVSCKASGYAFSYSWINWVRQAPGQGLEWMGR

IFPGDGDTDYNGKFKGRVTITADKSTSTAYMELSSLRSEDTAVYYCARNV

FDGYWLVYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKD

YF**P**EPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTY

ICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPK

DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY**N**S

TYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQV

YTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVL

DSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG

In another specific embodiment, the parent non-substituted heavy chain region is from rituximab, as disclosed in SEQ ID NO: 2, and further disclosed herein. The amino acid positions 11, 151, and 297 according to Kabat are underlined.

Rituximab heavy chain amino acid sequence (SEQ ID NO: 2)

QVQLQQPGAE**L**VKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLEWIGA

IYPGNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYCARST

YYGGDWYFNVWGAGTTVTVSAASTKGPSVFPLAPSSKSTSGGTAALGCLV

KDYF**P**EPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQ

TYICNVNHKPSNTKVDKKAEPKSCDKTHTCPPCPAPELLGGPSVFLFPPK

PKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY

NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREP

QVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP

VLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Expression of Modified Antibodies

Methods which are well known to those skilled in the art can be used to construct expression vectors containing the coding sequence of a modified antibody having substantially the same binding specificity of a parent antibody along with appropriate transcriptional/translational control signals. These methods include *in vitro* recombinant DNA techniques, synthetic techniques and *in vivo* recombination/genetic recombination. See, for example, the techniques described in Maniatis et al, MOLECULAR CLONING A LABORATORY MANUAL, Cold Spring Harbor Laboratory, N. Y. (1989) and Ausubel et at, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Greene Publishing Associates and Wiley Interscience, N.Y (1989).

A variety of host-expression vector systems can be utilized to express the coding sequence of the antibodies of the present invention. Preferably, mammalian cells are used as host cell systems transfected with recombinant plasmid DNA or cosmid DNA expression vectors containing the coding sequence of the protein of interest and the coding sequence of the fusion polypeptide. Most preferably, CHO cells, HEK293-EBNA cells, BHK cells, NSO cells, SP2/0 cells, YO myeloma cells, P3X63 mouse myeloma cells, PER cells, PER.C6 cells or hybridoma cells, other mammalian cells, yeast cells, insect cells, or plant cells are used as host cell system. Some examples of expression systems and selection methods are disclosed in the following references, and references therein: Borth et at, Biotechnol. Bioen. 71(4):266-73 (2000-2001), in Werner et al, Arzneimittelforschung/Drug Res. 48(8):870-80 (1998), in Andersen and Krummen, Curr. Op. Biotechnol. 13:117-123 (2002), in Chadd and Chamow, Curr. Op. Biotechnol. 12:188-194 (2001), and in Giddings, Curr. Op. Biotechnol. 12: 450-454 (2001). In alternate embodiments, other eukaryotic host cell systems may be contemplated, including yeast cells transformed with recombinant yeast expression vectors containing the coding sequence of an antibody of the present invention, such as the expression systems taught in U.S. Pat. Appl. No. 60/344,169 and WO 03/056914 (methods for producing human-like glycoprotein in a non-human eukaryotic host cell) (each of which is hereby incorporated by reference in its entirety); insect cell systems infected with recombinant virus expression vectors (e.g., baculo virus) containing the coding sequence of a modified antibody having substantially the same binding specificity of a parent antibody; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing the coding sequence of the antibody of the invention, including, but not limited to, the expression systems taught in U.S. Pat. No. 6,815, 184 (methods for expression and

secretion of biologically active polypeptides from genetically engineered duckweed); WO 2004/057002 (production of glycosylated proteins in bryophyte plant cells by introduction of a glycosyl transferase gene) and WO 2004/024927 (methods of generating extracellular heterologous non-plant protein in moss protoplast); and U.S. Pat. Appl. Nos. 60/365,769, 60/368,047, and WO 2003/078614 (glycoprotein processing in transgenic plants comprising a functional mammalian GnTIII enzyme) (each of which is hereby incorporated by reference in its entirety); or animal cell systems infected with recombinant virus expression vectors (e.g., adenovirus, vaccinia virus) including cell lines engineered to contain multiple copies of the DNA encoding a modified antibody having substantially the same binding specificity of a parent antibody either stably amplified (CHO/dhfr) or unstably amplified in double-minute chromosomes (e.g., murine cell lines). In one embodiment, the vector comprising the polynucleotide(s) encoding the antibody of the invention is polycistronic. In a preferred embodiment, the antibody is a humanized antibody.

In one embodiment, the present invention is directed to an expression vector and/or a host cell which comprise one or more isolated polynucleotides of the present invention. According to one aspect of the invention, the light and heavy chains can be expressed separately, using immunoglobulin light chain and immunoglobulin heavy chains in separate plasmids, or on a single (including but not limited to, a polycistronic) vector. Accordingly, in one aspect of the invention, a polynucleotide encoding a variant heavy chain region of an antibody is provided. In one aspect of the invention, a polynucleotide encoding a light chain region of an antibody is provided. In one aspect of the invention a vector comprising at least one polynucleotide encoding a variant heavy chain and/or a light chain of an antibody is provided. In a further aspect said vector is polycystronic. One embodiment of the present invention is directed to host cells comprising said polynucleotides or vectors. The present invention is also directed to a method for producing an antibody of the present invention in a host cell comprising (i) culturing the host cell under conditions permitting the expression of said at least one polynucleotide; and (ii) recovering said antibody from the culture medium.

For the methods of this invention, stable expression is generally preferred to transient expression because it typically achieves more reproducible results and also is more amenable to large-scale production. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with the respective coding nucleic acids controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and

then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows selection of cells which have stably integrated the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines.

A number of selection systems may be used, including, but not limited to, the herpes simplex virus thymidine kinase (Wigler et al, Cell 11:223 (1977)), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, Proc. Natl. Acad. Sd. USA 48:2026 (1962)), and adenine phosphoribosyltransferase (Lowy et al, Cell 22:817 (1980)) genes, which can be employed in tk^-, $hgprt^-$ or $aprt^-$ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler et al, Natl Acad. Sd. USA 77:3567 (1989); O'Hare et al, Proc. Natl Acad. ScL USA 78: 1527 (1981)); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, Proc. Natl Acad. ScL USA 78:2072 (1981)); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin et al, J. MoI Biol 150:1 (1981)); and hygro, which confers resistance to hygromycin (Santerre et al, Gene 30:147 (1984) genes. Recently, additional selectable genes have been described, namely trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman & Mulligan, Proc. Natl Acad. ScL USA S5:8047 (1988)); the glutamine synthase system; and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DEMO (McConlogue, in: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed. (1987)).

Therapeutic Applications of Modified Antibodies According to the Methods of the Invention

In the broadest sense, the modified antibodies of the present invention can be used to target cells *in vivo* or *in vitro* that express a target antigen, in particular, where said target antigen is expressed on the cell surface. The cells expressing a target antigen can be targeted for diagnostic or therapeutic purposes. In one aspect, the modified antibodies of the present invention can be used to control effector function while altering cell signaling activity in cells expressing a target antigen. In another aspect, the modified antibodies of the present invention can be used to suppress effector function and/or alter the cross-linking and/or oligomerization of one or more target antigens. Target antigens for the modified antibodies of the present invention can be cell surface receptors including, but not limited to CD20, CD21, CD22, CD19, CD47, CD99, CD2, CD45, Herl (EGFR), Her2/neu, Her3, Her4, TRAIL receptors (e.g., TRAILR1, TRAILR2), TNFR, FGF receptors (e.g., FGFR1), IGF receptors, PDGF receptors,

VEGF receptors, and other cell-surface associated receptors. In a particular embodiment, the target antigen is CD20. The modified antibodies of the invention also act to arrest the cell cycle, cause direct cell death of the target cells, inhibit angiogenesis and/or cause differentiation of target cells.

In another aspect, the invention is directed to a method for treating a disease that is treatable by suppressing effector function and/or alter cell signaling activity of a target antigen comprising administering a therapeutically effective amount of a modified antibody of the present invention to a subject in need thereof. In a specific embodiment the modified antibody is humanized. Examples of diseases for which the modified antibodies can be administered include, but are not limited to, cell proliferation diseases or disorders, autoimmune diseases or disorders, and diseases or disorders related to bacterial or viral infection.

In one embodiment, the invention is directed to a method for treating a disease selected from the group consisting of proliferative disorder and autoimmune disease comprising administering to an individual an effective amount of the antibody according to the present invention. In a further aspect, said method comprises administering to a subject a pharmaceutically effective amount of a composition containing at least one of the modified antibodies of the invention (conjugated, including but not limited to an immunotoxin, or unconjugated). In one embodiment, said proliferative disorder include, but is not limited to, neoplasms, cancers, malignancies and/or tumors located in the abdomen, bone, breast, digestive system, liver, pancreas, peritoneum, endocrine glands (adrenal;, parathyroid, pituitary, testicles, ovary, thymus, thyroid), eye, head and neck, nervous system (central and peripheral), lymphatic system, pelvic, skin, soft tissue, spleen, thoracic region, and urogenital system. Particular neoplasms, cancers, malignancies, and/or tumors that can be treated with the antibodies of the invention include, but are not limited to, epidermal and squamous cell carcinomas, gliomas, pancreatic cancer, ovarian cancer, prostate cancer, breast cancer, bladder cancer, head and neck cancer, renal cell carcinomas, colon cancer, colorectal cancer, lung cancer, brain tumor, malignant melanoma, leukemia, lymphomas, T cell lymphomas, multiple myeloma, gastric cancer, cervical cancer, endometrial carcinoma, esophageal cancer, liver cancer, cutaneous cancer, urinary tract carcinoma, choriocarcinoma, pharyngeal cancer, laryngeal cancer, thecomatosis, androblastoma, endometrium hyperplasy, endometriosis, embryoma, fibrosarcoma, Kaposi's sarcoma, hemangioma, cavernous hemangioma, angioblastoma, retinoblastoma, astrocytoma, neurofibroma, oligodendroglioma, medulloblastoma, ganglioneuroblastoma, glioma, rhabdomyosarcoma, hamartoblastoma, osteogenic sarcoma, leiomyosarcoma, thyroid sarcoma, Ewing's sarcoma, and Wilms tumor. In

a preferred embodiment, said proliferative disorder is a CD20 expressing cancer. In another preferred embodiment, said cancer is selected from the group consisting of lymphoma and lymphocytic leukemia. Such lymphomas and lymphocytic leukemias include, but are not limited to, follicular lymphomas, Small Non-Cleaved Cell Lymphomas/ Burkitt's lymphoma (including endemic Burkitt's lymphoma, sporadic Burkitt's lymphoma and Non-Burkitt's lymphoma), marginal zone lymphomas (including extranodal marginal zone B cell lymphoma (Mucosa-associated lymphatic tissue lymphomas, MALT), nodal marginal zone B cell lymphoma and splenic marginal zone lymphoma), Mantle cell lymphoma (MCL), Large Cell Lymphoma (including diffuse large B-cell lymphoma (DLBCL), Diffuse Mixed Cell Lymphoma, Immunoblastic Lymphoma, Primary Mediastinal B-Cell Lymphoma, Angiocentric Lymphoma-Pulmonary B-Cell Lymphoma), hairy cell leukemia, lymphocytic lymphoma, Waldenstrom's macroglobulinemia, acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL)/ small lymphocytic lymphoma (SLL), B-cell prolymphocytic leukemia, plasma cell neoplasms, plasma cell myeloma, multiple myeloma, plasmacytoma, Hodgkin's disease. In a preferred embodiment, said CD20 expressing cancer is selected from the group consisting of Non-Hodgkin's lymphomas (NHL), follicular lymphomas, diffuse large B-cell lymphoma (DLBCL) and chronic lymphocytic leukemia (CLL).

In a further aspect, the invention is directed to an improved method for treating B-cell proliferative disorders including B-cell lymphoma, based on B-cell depletion comprising administering a therapeutically effective amount of an antibody of the present invention to a human subject in need thereof. In a preferred embodiment, the antibody is an anti-CD20 antibody with a binding specificity substantially the same as that of the murine B-Ly1 antibody. In another preferred embodiment the antibody is humanized. In another preferred embodiment the antibody comprises a variant heavy chain region comprising at least one amino acid substitution relative to the parent non-substituted heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residues Pro151 and Asn297, wherein said substitutions are Pro151Phe and Asn297Asp, wherein induction of effector function is reduced or abolished, and wherein direct cell death induced by the antibody comprising the variant heavy chain region is increased compared to effector function and direct cell death induced by the antibody comprising the parent non-substituted heavy chain region. In this aspect of the invention, the antibodies of the invention are used to deplete the blood of normal B-cells for an extended period.

The subject invention further provides methods for inhibiting the growth of human tumor cells, treating a tumor in a subject, and treating a proliferative type disease in a subject. These

methods comprise administering to the subject an effective amount of the composition of the invention.

Other cell proliferation disorders can also be treated with the modified antibodies of the present invention. As encompassed by the present invention, said cell proliferation disorders include, but are not limited to hypergammaglobulinemia, lymphoproliferative disorders, paraproteinemias, purpura, sarcoidosis, Sezary Syndrome, Waldenström's Macroglobulinemia, Gaucher's Disease, histiocytosis, and any other cell proliferation disease, besides neoplasia, located in an organ system listed herein.

In another embodiment, the invention is directed to a method for treating an autoimmune disease. In one embodiment, said autoimmune disease include, but is not limited to, immune-mediated thrombocytopenias, such as acute idiopathic thrombocytopenic purpurea and chronic idiopathic thrombocytopenic purpurea, dermatomyositis, Sydenham's chorea, lupus nephritis, rheumatic fever, polyglandular syndromes, Henoch-Schonlein purpura, poststreptococcal nephritis, erythema nodosum, Takayasu's arteritis, Addison's disease, erythema multiforme, polyarteritis nodosa, ankylosing spondylitis, Goodpasture's syndrome, thromboangitis ubiterans, primary biliary cirrhosis, Hashimoto's thyroiditis, thyrotoxicosis, chronic active hepatitis, polymyositis/dermatomyositis, polychondritis, pamphigus vulgaris, Wegener's granulomatosis, membranous nephropathy, amyotrophic lateral sclerosis, tabes dorsalis, polymyaglia, pernicious anemia, rapidly progressive glomerulonephritis and fibrosing alveolitis, inflammatory responses such as inflammatory skin diseases including psoriasis and dermatitis (e.g., atopic dermatitis), systemic scleroderma and sclerosis, responses associated with inflammatory bowel disease (such as Crohn's disease and ulcerative colitis), respiratory distress syndrome (including adult respiratory distress syndrome, ARDS), dermatitis, meningitis, encephalitis, uveitis, colitis, glomerulonephritis, allergic conditions such as eczema and asthma and other conditions involving infiltration of T cells and chronic inflammatory responses, atherosclerosis, leukocyte adhesion deficiency, rheumatoid arthritis, systemic lupus erythematosus (SLE), diabetes mellitus (e.g., Type 1 diabetes mellitus or insulin dependent diabetes mellitus), multiple sclerosis, Reynaud's syndrome, autoimmune thyroiditis, allergic encephalomyelitis, Sjögren's syndrome, juvenile onset diabetes, and immune responses associated with acute and delayed hypersensitivity mediated by cytokines and T-lymphocytes typically found in tuberculosis, sarcoidosis, polymyositis, granulomatosis and vasculitis, pernicious amenia (Addison's disease), diseases involving leukocyte diapedesis, central nervous system (CNS) inflammatory disorder, multiple organ injury syndrome, hemolytic anemia (including, but not limited to cryoglobinemia or Coombs positive anemia), myasthenia

gravis, antigen-antibody complex mediated diseases, anti-glomerular basement membrane disease, antiphospholipid syndrome, allergic neuritis, Graves' disease, Lambert-Eaton myasthenic syndrome, pemphigoid bullous, pemphigus, autoimmune polyendocrinopathies, Reiter's disease, stiff-man syndrome, Behcet disease, giant cell arteritis, immune complex nephritis, IgA nephropathy, IgM polyneuropathies, immune thrombocytopenic purpura (ITP) or autoimmune thrombocytopenia. In a preferred embodiment, said autoimmune disease is selected from the group consisting of rheumatoid arthritis, lupus, multiple sclerosis, Sjögren's syndrome and transplant rejection.

In a further aspect, the invention is directed to an improved method for treating an autoimmune disease as defined herein, based on B-cell depletion comprising administering a therapeutically effective amount of an antibody of the present invention to a human subject in need thereof. In a preferred embodiment, the antibody is a anti-CD20 antibody with a binding specificity substantially the same as that of the murine B-Ly1 antibody. In another preferred embodiment the antibody is humanized. In another preferred embodiment the antibody comprises a variant heavy chain region comprising at least one amino acid substitution relative to the parent non-substituted heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residues Pro151 and Asn297, wherein said substitutions are Pro151Phe and Asn297Asp, wherein induction of effector function is reduced or abolished, and wherein direct cell death induced by the antibody comprising the variant heavy chain region is increased compared to effector function and direct cell death induced by the antibody comprising the parent non-substituted heavy chain region. In this aspect of the invention, the antibodies of the invention are used to deplete the blood of normal B-cells for an extended period.

The modified antibodies of the present invention can be used alone or in combination with other treatments or therapeutic agents to treat disorders that are treatable by altering effector function and/or increasing or decreasing cell signaling activity and/or cross-linking of one or more target antigens. In one embodiment, modified antibodies of the present invention can be used alone to target and kill tumor cells *in vivo*. The modified antibodies can also be used in conjunction with an appropriate therapeutic agent to treat human carcinoma. For example, the modified antibodies can be used in combination with standard or conventional treatment methods such as chemotherapy, radiation therapy or can be conjugated or linked to a therapeutic drug, or toxin, as well as to a lymphokine or a tumor-inhibitory growth factor, for delivery of the therapeutic agent to the site of the carcinoma. In particular embodiments, the conjugates of the modified antibodies of this invention include (1) immunotoxins (conjugates

of the modified antibody and a cytotoxic moiety) and (2) labeled (e.g., radiolabeled, enzyme-labeled, or fluorochrome-labeled) modified antibodies in which the label provides a means for identifying immune complexes that include the labeled antibody. The cytotoxic moiety of the immunotoxin may be a cytotoxic drug or an enzymatically active toxin of bacterial or plant origin, or an enzymatically active fragment ("A chain") of such a toxin. Enzymatically active toxins and fragments thereof used are diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from Pseudomonas aeruginosa), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, Phytolacca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, and enomycin. In another embodiment, the modified antibodies are conjugated to small molecule anticancer drugs. Conjugates of the modified antibody and such cytotoxic moieties are made using a variety of bifunctional protein coupling agents. Examples of such reagents are SPDP, IT, bifunctional derivatives of imidoesters such a dimethyl adipimidate HCl, active esters such as disuccinimidyl suberate, aldehydes such as glutaraldehyde, bis-azido compounds such as bis (p-azidobenzoyl) hexanediamine, bis-diazonium derivatives such as bis-(p-diazoniumbenzoyl)-ethylenediamine, diisocyanates such as tolylene 2,6-diisocyanate, and bis-active fluorine compounds such as 1,5- difluoro-2,4-dinitrobenzene. The lysing portion of a toxin may be joined to the Fab fragment of the modified antibodies. Additional appropriate toxins are known in the art, as evidenced in e.g., published U.S. Patent Application No. 2002/0128448, incorporated herein by reference in its entirety.

In one embodiment, the antigen binding molecule of the present invention is conjugated to an additional moiety, such as a radiolabel or a toxin. Such conjugated modified antibodies can be produced by numerous methods that are well known in the art.

A variety of radionuclides are applicable to the present invention and those skilled in the art are credited with the ability to readily determine which radionuclide is most appropriate under a variety of circumstances. For example, ^{131}iodine is a well known radionuclide used for targeted immunotherapy. However, the clinical usefulness of ^{131}iodine can be limited by several factors including: eight-day physical half-life; dehalogenation of iodinated antibody both in the blood and at tumor sites; and emission characteristics (eg, large gamma component) which can be suboptimal for localized dose deposition in tumor. With the advent of superior chelating agents, the opportunity for attaching metal chelating groups to proteins has increased the opportunities to utilize other radionuclides such as ^{111}indium and ^{90}yttrium. ^{90}Yttrium provides several benefits for utilization in radioimmunotherapeutic applications: the

64 hour half-life of ^{90}yttrium is long enough to allow antibody accumulation by tumor and, unlike eg, ^{131}iodine, ^{90}yttrium is a pure beta emitter of high energy with no accompanying gamma irradiation in its decay, with a range in tissue of 100 to 1000 cell diameters. Furthermore, the minimal amount of penetrating radiation allows for outpatient administration of ^{90}yttrium-labeled antibodies. Additionally, internalization of labeled antibody is not required for cell killing, and the local emission of ionizing radiation should be lethal for adjacent tumor cells lacking the target antigen.

Effective single treatment dosages (i.e., therapeutically effective amounts) of ^{90}yttrium labeled modified antibodies of the present invention range from between about 5 and about 75 mCi, more preferably between about 10 and about 40 mCi. Effective single treatment non-marrow ablative dosages of ^{13}iodine labeled antibodies of the present invention range from between about 5 and about 70 mCi, more preferably between about 5 and about 40 mCi. Effective single treatment ablative dosages (i.e., may require autologous bone marrow transplantation) of ^{131}iodine labeled antibodies of the present invention range from between about 30 and about 600 mCi, more preferably between about 50 and less than about 500 mCi. In conjunction with a chimeric antibody according to the present invention, owing to the longer circulating half life vis-a-vis murine antibodies, an effective single treatment non-marrow ablative dosages of ^{131}iodine labeled chimeric antibodies range from between about 5 and about 40 mCi, more preferably less than about 30 mCi. Imaging criteria for the ^{111}indium label, are typically less than about 5 mCi.

With respect to radiolabeled antibodies of the present invention, therapy therewith can also occur using a single therapy treatment or using multiple treatments. Because of the radionuclide component, it is preferred that prior to treatment, peripheral stem cells ("PSC") or bone marrow ("BM") be "harvested" for patients experiencing potentially fatal bone marrow toxicity resulting from radiation. BM and/or PSC are harvested using standard techniques, and then purged and frozen for possible reinfusion. Additionally, it is most preferred that prior to treatment a diagnostic dosimetry study using a diagnostic labeled antibody (including but not limited to using ^{111}indium) be conducted on the patient, a purpose of which is to ensure that the therapeutically labeled antibody (eg, using ^{90}yttrium) will not become unnecessarily "concentrated" in any normal organ or tissue.

In one embodiment, a chimeric, modified antibody of the present invention, is conjugated to ricin A chain. Most advantageously, the ricin A chain is deglycosylated and produced through

recombinant means. An advantageous method of making the ricin immunotoxin is described in Vitetta et al., Science 238, 1098 (1987), hereby incorporated by reference in its entirety.

When used to kill human cancer cells *in vitro* for diagnostic purposes, the conjugates will typically be added to the cell culture medium at a concentration of at least about 10 nM. The formulation and mode of administration for *in vitro* use are not critical. Aqueous formulations that are compatible with the culture or perfusion medium will normally be used. Cytotoxicity may be read by conventional techniques to determine the presence or degree of cancer.

As discussed herein, a cytotoxic radiopharmaceutical for treating cancer may be made by conjugating a radioactive isotope (e.g., I, Y, Pr) to a chimeric, modified antibody of the present invention. The term "cytotoxic moiety" as used herein is intended to include such isotopes.

In another embodiment, liposomes are filled with a cytotoxic drug and the liposomes are coated with the antibodies of the present invention. Because many of the target molecules for the modified antibodies of the present invention are expressed on the cell surface (e.g., there are many CD20 molecules on the surface of the malignant B-cell), this method permits delivery of large amounts of drug to the correct cell type.

Techniques for conjugating such therapeutic agents to antibodies are well known (see, e.g., Arnon et al., "Monoclonal Antibodies for Immunotargeting of Drugs in Cancer Therapy", in Monoclonal Antibodies and Cancer Therapy, Reisfeld et al. (eds.), pp. 243-56 (Alan R. Liss, Inc. 1985); Hellstrom et at, "Antibodies For Drug Delivery", in Controlled Drug Delivery (2nd Ed.), Robinson etal. (eds.), pp.623-53 (Marcel Defcker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in Monoclonal Antibodies '84: Biological And Clinical Applications, Pinchera et al. (eds.), pp. 475-506 (1985); and Thorpe et al., "The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates", Immunol. Rev., 62:119-58 (1982) (each of which is hereby incorporated by reference in its entirety).

Still other therapeutic applications for the antibodies of the invention include conjugation or linkage, including but not limited to conjugation by recombinant DNA techniques, to an enzyme capable of converting a prodrug into a cytotoxic drug and the use of that antibody-enzyme conjugate in combination with the prodrug to convert the prodrug to a cytotoxic agent at the tumor site (see, e.g., Senter et al., "Anti-Tumor Effects of Antibody-alkaline Phosphatase", Proc. Natl. Acad. ScL USA 55:4842-46 (1988); "Enhancement of the *in vitro* and *in vivo* Antitumor Activites of Phosphorylated Mitocycin C and Etoposide Derivatives by

Monoclonal Antibody-Alkaline Phosphatase Conjugates", Cancer Research 49:5789-5792 (1989); and Senter, "Activation of Prodrugs by Antibody-Enzyme Conjugates: A New Approach to Cancer Therapy," FASEB J. 4:188-193 (1990)).

Still another therapeutic use for the antibodies of the invention involves use, either unconjugated, or as part of an antibody-drug or antibody-toxin conjugate, to remove tumor cells from the bone marrow of cancer patients. According to this approach, autologous bone marrow may be purged *ex vivo* by treatment with the antibody and the marrow infused back into the patient (see, e.g., Ramsay et al., "Bone Marrow Purging Using Monoclonal Antibodies", J. Clin. Immunol, 8(2):81-88 (1988)).

Furthermore, it is contemplated that the invention comprises a single-chain immunotoxin comprising antigen binding domains that allow substantially the same specificity of binding as a parent antibody (including but not limited to, polypeptides comprising the CDRs of the parent antibody) and further comprising a toxin polypeptide. The single-chain immunotoxins of the invention may be used to treat human carcinoma *in vivo*.

Similarly, a fusion protein comprising at least the antigen-binding region of an antibody of the invention joined to at least a functionally active portion of a second protein having anti-tumor acitivty, including but not limited to, a lymphokine or oncostatin, can be used to treat human carcinoma *in vivo*.

Accordingly, the present invention provides a method for selectively killing tumor cells expressing cell surface receptors including, but not limited to CD20, Her1 (EGFR), Her2/neu, Her3, Her4, TRAIL receptors (e.g., TRAILR1, TRAILR2), TNFR, FGF receptors (e.g., FGFR1), IGF receptors, PDGF receptors, VEGF receptors, and other cell-surface associated receptors. This method comprises reacting the modified antibody of the invention (conjugated, e.g., as an immunotoxin, or unconjugated) with said tumor cells. These tumor cells maybe from a human carcinoma.

In a further aspect, the invention relates to an antibody according to the present invention for use as a medicament. In one embodiment, the invention relates to an antibody according to the present invention for use in treating a disease selected from the group consisting of proliferative disorder and autoimmune disease. According to one aspect of the invention said proliferative disorder is selected from the group consisting of B-cell lymphoma, lung cancer, non- small cell lung (NSCL) cancer, bronchioalviolar cell lung cancer, bone cancer, pancreatic

cancer, skin cancer, cancer of the head or neck, cutaneous or intraocular melanoma, uterine cancer, ovarian cancer, rectal cancer, cancer of the anal region, stomach cancer, gastric cancer, colon cancer, breast cancer, uterine cancer, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, Hodgkin's Disease, cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the urethra, cancer of the penis, prostate cancer, cancer of the bladder, cancer of the kidney or ureter, renal cell carcinoma, carcinoma of the renal pelvis, mesothelioma, hepatocellular cancer, biliary cancer, chronic or acute leukemia, lymphocytic lymphomas, neoplasms of the central nervous system (CNS), spinal axis tumors, brain stem glioma, glioblastoma multiforme, astrocytomas, schwannomas, ependymomas, medulloblastomas, meningiomas, squamous cell carcinomas, pituitary adenomas, including refractory versions of any of the herein cancers, or a combination of one or more of the herein cancers or a precancerous condition or lesion herein disclosed. The precancerous condition or lesion includes, for example, the group consisting of oral leukoplakia, actinic keratosis (solar keratosis), precancerous polyps of the colon or rectum, gastric epithelial dysplasia, adenomatous dysplasia, hereditary nonpolyposis colon cancer syndrome (HNPCC), Barrett's esophagus, bladder dysplasia, and precancerous cervical conditions. Preferably, the cancer is selected from the group consisting of B-cell lymphoma, breast cancer, bladder cancer, head and neck cancer, skin cancer, pancreatic cancer, lung cancer, ovarian cancer, colon cancer, prostate cancer, kidney cancer, and brain cancer. In a preferred embodiment, said proliferative disorder is a CD20 expressing cancer. In another preferred embodiment, said cancer is selected from the group consisting of lymphoma and lymphocytic leukemia.

Accordingly, in one aspect of the invention said autoimmune disease is selected from the group consisting of immune-mediated thrombocytopenias, such as acute idiopathic thrombocytopenic purpurea and chronic idiopathic thrombocytopenic purpurea, dermatomyositis, Sydenham's chorea, lupus nephritis, rheumatic fever, polyglandular syndromes, Henoch-Schonlein purpura, poststreptococcal nephritis, erythema nodosum, Takayasu's arteritis, Addison's disease, erythema multiforme, polyarteritis nodosa, ankylosing spondylitis, Goodpasture's syndrome, thromboangitis ubiterans, primary biliary cirrhosis, Hashimoto's thyroiditis, thyrotoxicosis, chronic active hepatitis, polymyositis/dermatomyositis, polychondritis, pamphigus vulgaris, Wegener's granulomatosis, membranous nephropathy, amyotrophic lateral sclerosis, tabes dorsalis, polymyaglia, pernicious anemia, rapidly progressive glomerulonephritis and fibrosing alveolitis,

inflammatory responses such as inflammatory skin diseases including psoriasis and dermatitis (e.g., atopic dermatitis), systemic scleroderma and sclerosis, responses associated with inflammatory bowel disease (such as Crohn's disease and ulcerative colitis), respiratory distress syndrome (including adult respiratory distress syndrome, ARDS), dermatitis, meningitis, encephalitis, uveitis, colitis, glomerulonephritis, allergic conditions such as eczema and asthma and other conditions involving infiltration of T cells and chronic inflammatory responses, atherosclerosis, leukocyte adhesion deficiency, rheumatoid arthritis, systemic lupus erythematosus (SLE), diabetes mellitus (e.g., Type 1 diabetes mellitus or insulin dependent diabetes mellitus), multiple sclerosis, Reynaud's syndrome, autoimmune thyroiditis, allergic encephalomyelitis, Sjögren's syndrome, juvenile onset diabetes, and immune responses associated with acute and delayed hypersensitivity mediated by cytokines and T-lymphocytes typically found in tuberculosis, sarcoidosis, polymyositis, granulomatosis and vasculitis, pernicious amenia (Addison's disease), diseases involving leukocyte diapedesis, central nervous system (CNS) inflammatory disorder, multiple organ injury syndrome, hemolytic anemia (including, but not limited to cryoglobinemia or Coombs positive anemia), myasthenia gravis, antigen-antibody complex mediated diseases, anti-glomerular basement membrane disease, antiphospholipid syndrome, allergic neuritis, Graves' disease, Lambert-Eaton myasthenic syndrome, pemphigoid bullous, pemphigus, autoimmune polyendocrinopathies, Reiter's disease, stiff-man syndrome, Behcet disease, giant cell arteritis, immune complex nephritis, IgA nephropathy, IgM polyneuropathies, immune thrombocytopenic purpura (ITP) or autoimmune thrombocytopenia. In a preferred embodiment, said autoimmune disease is selected from the group consisting of rheumatoid arthritis, lupus, multiple sclerosis, Sjögren's syndrome and transplant rejection.

Yet another embodiment is the use of the antibody according to the present invention for the manufacture of a medicament for the treatment or prophylaxis of cancer or for the treatment or prophylaxis of a precancerous condition or lesion or for the treatment of an autoimmune disease. Cancer and precancerous condition or lesions are defined as herein. In one embodiment, said cancer is a CD20 expressing cancer. In a specific embodiment said cancer is a lymphoma or lymphocytic leukemia. In another specific embodiment said cancer is selected from the group consisting of follicular lymphomas, Small Non-Cleaved Cell Lymphomas/ Burkitt's lymphoma (including endemic Burkitt's lymphoma, sporadic Burkitt's lymphoma and Non-Burkitt's lymphoma), marginal zone lymphomas (including extranodal marginal zone B cell lymphoma (Mucosa-associated lymphatic tissue lymphomas, MALT), nodal marginal zone B cell lymphoma and splenic marginal zone lymphoma), Mantle cell lymphoma (MCL), Large Cell Lymphoma (including diffuse large B-cell lymphoma (DLBCL), Diffuse Mixed

Cell Lymphoma, Immunoblastic Lymphoma, Primary Mediastinal B-Cell Lymphoma, Angiocentric Lymphoma-Pulmonary B-Cell Lymphoma), hairy cell leukemia, lymphocytic lymphoma, Waldenstrom's macroglobulinemia, acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL)/ small lymphocytic lymphoma (SLL), B-cell prolymphocytic leukemia, plasma cell neoplasms, plasma cell myeloma, multiple myeloma, plasmacytoma, Hodgkin's disease. In a preferred embodiment, said cancer is selected from the group consisting of Non-Hodgkin's lymphomas (NHL), follicular lymphomas, diffuse large B-cell lymphoma (DLBCL) and chronic lymphocytic leukemia (CLL). In a preferred embodiment, said cancer is selected from the group consisting of lymphoma and lymphocytic leukemia. In another preferred embodiment, said autoimmune disease is selected from the group consisting of rheumatoid arthritis, lupus, multiple sclerosis, Sjögren's syndrome and transplant rejection.

The present invention encompasses pharmaceutical compositions, combinations, uses, and methods for treating human carcinomas and autoimmune diseases. The invention includes pharmaceutical compositions for use in the treatment of human carcinomas and autoimmune diseases comprising a pharmaceutically effective amount of an antibody of the present invention and a pharmaceutically acceptable carrier.

The antibody compositions of the invention can be administered using conventional modes of administration including, but not limited to, intravenous, intraperitoneal, oral, intralymphatic or administration directly into the tumor. Intravenous administration is preferred.

The present invention is further directed to pharmaceutical compositions comprising the modified antibodies of the present invention and a pharmaceutically acceptable carrier. In one aspect of the invention, therapeutic formulations containing the antibodies of the invention are prepared for storage by mixing an antibody having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Pharmaceutically acceptable carriers are generally nontoxic to recipients at the dosages and concentrations employed, and include, but are not limited to: buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride; benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic

polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as polyethylene glycol (PEG).

Lyophilized formulations adapted for subcutaneous administration are described in WO 97/04801. Such lyophilized formulations may be reconstituted with a suitable diluent to a high protein concentration and the reconstituted formulation may be administered subcutaneously to the individual to be treated herein.

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desirable to further provide a cytotoxic agent, chemotherapeutic agent, cytokine or immunosuppressive agent (e.g. one which acts on T cells, such as cyclosporin or an antibody that binds T cells, e.g., one which binds LFA-1). The effective amount of such other agents depends on the amount of antagonist present in the formulation, the type of disease or disorder or treatment, and other factors discussed herein. These are generally used in the same dosages and with administration routes as used hereinbefore or about from 1 to 99% of the heretofore employed dosages.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980).

Sustained-release preparations may be prepared. Suitable sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antagonist, which matrices are in the form of shaped articles, e.g., films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ-ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT(TM) (injectable

microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid.

The formulations to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

The compositions of the invention may be in a variety of dosage forms which include, but are not limited to, liquid solutions or suspension, tablets, pills, powders, suppositories, polymeric microcapsules or microvesicles, liposomes, and injectable or infusible solutions. The preferred form depends upon the mode of administration and the therapeutic application.

The compositions of the invention also preferably include conventional pharmaceutically acceptable carriers and adjuvants known in the art such as human serum albumin, ion exchangers, alumina, lecithin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, and salts or electrolytes such as protamine sulfate.

The most effective mode of administration and dosage regimen for the pharmaceutical compositions of this invention depends upon the severity and course of the disease, the patient's health and response to treatment and the judgment of the treating physician. Accordingly, the dosages of the compositions should be titrated to the individual patient. Nevertheless, an effective dose of the compositions of this invention will generally be in the range of from about 0.01 to about 2000 mg/kg.

The dosages of the present invention may, in some cases, be determined by the use of predictive biomarkers. Predictive biomarkers are molecular markers that are used to determine (i.e., observe and/or quantitate) a pattern of expression and/or activation of e.g., tumor related genes or proteins, or cellular components of a tumor-related signaling pathway. Elucidating the biological effects of targeted therapies in tumor tissue and correlating these effects with clinical response helps identify the predominant growth and survival pathways operative in tumors, thereby establishing a profile of likely responders and conversely providing a rationale for designing strategies to overcoming resistance to therapy.

Predictive biomarkers may be measured by cellular assays that are well known in the art including, but not limited to immunohistochemistry, flow cytometry, immunofluorescence, capture-and-detection assays, and reversed phase assays, and/or assays set forth in U.S. Pat.

Appl. Pub. No. 2004/0132097 A1, the entire contents of which is hereby incorporated by reference in its entirety.

Thus, in one aspect, the present invention provides for a method for treating a disorder that is related to altered or dysregulated cell signaling by a target antigen and/or altered ability to mediate cross-linking and/or oligomerization of one or more target antigens, wherein reducing effector function mediated by the antibody therapy is beneficial for the patient, comprising predicting a response to therapy with a modified antibody in a human subject in need of treatment by assaying a sample from the human subject prior to therapy with one or a plurality of reagents that detect expression and/or activation of predictive biomarkers for a disorder that is related to altered or dysregulated cell signaling by a target antigen and/or altered ability to mediate cross-linking and/or oligomerization of one or more target antigens (such as cancer); determining a pattern of expression and/or activation of one or more of the predictive biomarkers, wherein the pattern predicts the human subject's response to the modified antibody therapy; and administering to a human subject who is predicted to respond positively to modified antibody treatment a therapeutically effective amount of a composition comprising a modified antibody of the present invention. As used herein, a human subject who is predicted to respond positively to modified antibody treatment is one for whom the modified antibody will have a measurable effect on the disease or disorder that is related to altered or dysregulated cell signaling by a target antigen and/or altered ability to mediate cross-linking and/or oligomerization of one or more target antigens (e.g., tumor regression/shrinkage) and for whom the benefits of modified antibody therapy are not outweighed by adverse effects (e.g., toxicity). As used herein, a sample means any biological sample from an organism, particularly a human, comprising one or more cells, including single cells of any origin, tissue or biopsy samples which has been removed from organs such as breast, lung, gastrointestinal tract, skin, cervix, ovary, prostate, kidney, brain, head and neck, or any other organ or tissue of the body, and other body samples including, but not limited to, smears, sputum, secretions, cerebrospinal fluid, bile, blood, lymph fluid, urine and feces.

The composition comprising a modified antibody of the present invention will be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular disease or disorder being treated, the particular mammal being treated, the clinic condition of the individual patient, the cause of the disease or disorder, the site of delivery of the agent, the method of administration, the scheduling of administration, and other factors known to medical practitioners. The

therapeutically effective amount of the antagonist to be administered will be governed by such considerations.

In a preferred embodiment, the antibody modified according to the present invention is a humanized antibody. Suitable dosages for such an unconjugated antibody are, for example, in the range from about 20 mg/m^2 to about 1000 mg/m^2. In one embodiment, the dosage of the antibody modified according to the present invention is equal to the dosage presently recommended for the non-substituted parent antibody. In one embodiment, the dosage of the antibody modified according to the present invention differs from the dosage presently recommended for the non-substituted parent antibody. In one embodiment, the dosage of the antibody modified according to the present invention is lower compared to the dosage presently recommended for the non-substituted parent antibody. In one embodiment, the dosage of the antibody modified according to the present invention is higher compared to the dosage presently recommended for the non-substituted parent antibody. In one embodiment, antibodies modified according to the present invention are administer to the patient in one or more doses of substantially less than 375 mg/m^2 of the antibody, including but not limited to, where the dose is in the range from about 20 mg/m^2 to about 250 mg/m^2, or from about 50 mg/m^2 to about 200 mg/m^2. In another embodiment, the modified antibodies are used in a therapeutically effective amount from about 375 mg/m^2 to about 1000 mg/m^2.

According to the invention one or more initial dose(s) of the antibody followed by one or more subsequent dose(s) are administered, wherein the mg/m^2 dose of the antibody in the subsequent dose(s) exceeds the mg/m^2 dose of the antibody in the initial dose(s). For example, the initial dose may be in the range from about 20 mg/m^2 to about 250 mg/m^2 and the subsequent dose may be in the range from about 250 mg/m^2 to about 1000 mg/m^2.

As noted herein, however, these suggested amounts of modified antibody are subject to a great deal of therapeutic discretion. The key factor in selecting an appropriate dose and scheduling is the result obtained, as indicated herein. For example, relatively higher doses may be needed initially for the treatment of ongoing and acute diseases. To obtain the most efficacious results, depending on the disease or disorder, the antagonist is administered as close to the first sign, diagnosis, appearance, or occurrence of the disease or disorder as possible or during remissions of the disease or disorder.

The modified antibody of the present invention is administered by any suitable means, including parenteral, subcutaneous, intraperitoneal, intrapulinonary, and intranasal, and, if

desired for local immunosuppressive treatment, intralesional administration. Parenteral infusions include intramuscular, intravenous, intraarterial, intraperitoneal, or subcutaneous administration. In addition, the antagonist may suitably be administered by pulse infusion, e.g., with declining doses of the antagonist. Preferably the dosing is given by injections, most preferably intravenous or subcutaneous injections, depending in part on whether the administration is brief or chronic.

According to the invention other compounds, such as cytotoxic agents, chemotherapeutic agents, immunosuppressive agents and/or cytokines are administered with the antagonists herein. The combined administration includes coadministration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities.

It would be clear that the dose of the composition of the invention required to achieve cures may be further reduced with schedule optimization.

In accordance with the practice of the invention, the pharmaceutical carrier may be a lipid carrier. The lipid carrier may be a phospholipid. Further, the lipid carrier may be a fatty acid. Also, the lipid carrier may be a detergent. As used herein, a detergent is any substance that alters the surface tension of a liquid, generally lowering it.

In one example of the invention, the detergent may be a nonionic detergent. Examples of nonionic detergents include, but are not limited to, polysorbate 80 (also known as Tween 80 or polyoxyethylenesorbitan monooleate), Brij, and Triton (for example Triton WR-1339 and Triton A-20).

Alternatively, the detergent may be an ionic detergent.

Additionally, in accordance with the invention, the lipid carrier may be a liposome. As used in this application, a "liposome" is any membrane bound vesicle which contains any molecules of the invention or combinations thereof.

Exemplary Embodiments

Antibodies according to the invention comprise a variant heavy chain region comprising at least one amino acid substitution relative to the parent non-substituted heavy chain region,

leading to strongly reduced or abolished ADCC and CDC function, residual ADCP function, reduced or abolished binding to Fc receptors, reduced or abolished binding to Clq and reduced or abolished toxicities. Accordingly, provided are exemplary embodiments as follows.

1. An antibody comprising a variant heavy chain region comprising at least one amino acid substitution relative to the parent non-substituted heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residue Asn297 and/or Pro329, and wherein said substitution is Asn297Asp and/or Pro329Gly.

2. The antibody according to embodiment 1, wherein induction of effector function is reduced compared to effector function induced by an antibody comprising the parent non-substituted heavy chain region.

3. The antibody according to any one of claims 1 or 2, wherein the antibody is an IgG1 antibody.

4. The antibody according to any one of embodiments 1 to 3, wherein the parent non-substituted antibody is an anti-CD20 antibody.

5. The antibody according to any one of embodiments 1 to 4, wherein the parent non-substituted antibody is a type I anti-CD20 antibody.

6. The antibody according to any one of embodiments 1 to 4, wherein the parent non-substituted antibody is a type II anti-CD20 antibody.

7. The antibody according to any one of embodiments 1 to 4 or 6, wherein said parent non-substituted antibody is obinutuzumab.

8. The antibody according to any one of embodiments 1 to 5, wherein said parent non-substituted antibody is rituximab.

9. The antibody according to any one of embodiments 1 to 8, wherein FcγRIII binding by the antibody comprising the variant heavy chain region is abolished compared to binding to FcγRIII by the parent non-substituted antibody comprising asparagine at position 297 or proline at position 329.

10. The antibody according to any one of embodiments 1 to 9, wherein ADCC function induced by the antibody comprising the variant heavy chain region is abolished or strongly reduced compared to ADCC function induced by the parent non-substituted antibody comprising asparagine at position 297 or proline at position 329.

11. The antibody according to any one of embodiments 1 to 10, wherein FcγRI binding by the antibody comprising the variant heavy chain region is reduced compared to binding to FcγRI by the parent non-substituted antibody comprising asparagine at position 297 or proline at position 329.

12. The antibody according to any one of embodiments 1 to 11, wherein ADCP function induced by the antibody comprising the variant heavy chain region is reduced compared to ADCP function induced by the parent non-substituted antibody comprising asparagine at position 297 or proline at position 329, wherein the antibody comprising the variant heavy chain retains residual ADCP function.

13. The antibody according to any one of embodiments 1 to 12, wherein the variant heavy chain region comprises a further amino acid substitution relative to the parent non-substituted heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residue Pro151, and wherein said further substitution is at said amino acid residue Pro151, wherein direct cell death induced by the antibody comprising the variant heavy chain region is altered compared to direct cell death induced by the antibody comprising proline at position 151.

14. The antibody according to embodiment 13, wherein direct cell death induced by the antibody comprising the variant heavy chain region is increased compared to direct cell death induced by the antibody comprising proline at position 151.

15. The antibody according to embodiment 13, wherein direct cell death induced by the antibody comprising the variant heavy chain region is decreased compared to direct cell death induced by the antibody comprising proline at position 151.

16. The antibody according to embodiment 13, wherein Pro151 is substituted with an amino acid selected from the group consisting of alanine and phenylalanine.

17. The antibody according to any one of embodiments 13 or 14, wherein Pro151 is substituted with phenylalanine.

18. The antibody according to any one of embodiments 13 or 15, wherein Pro151 is substituted with alanine.

19. The antibody according to any one of embodiments 1 to 18, wherein the variant heavy chain region comprises a further amino acid substitution relative to the parent non-substituted heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residue Val11, and wherein said further substitution is at said amino acid residue Val11, wherein direct cell death induced by the antibody comprising the variant heavy chain region is altered compared to direct cell death induced by the antibody comprising valine at position 11.

20. The antibody according to embodiment 19, wherein direct cell death induced by the antibody comprising the variant heavy chain region is increased compared to direct cell death induced by the antibody comprising valine at position 11.

21. The antibody according to embodiment 19, wherein direct cell death induced by the antibody comprising the variant heavy chain region is decreased compared to direct cell death induced by the antibody comprising valine at position 11.

22. The antibody according to embodiment 19, wherein Val11 is substituted with an amino acid selected from the group consisting of alanine, glycine, phenylalanine, threonine and tryptophan.

23. The antibody according to any one of embodiments 19 or 20, wherein Val11 is substituted with an amino acid selected from the group consisting of phenylalanine, threonine and tryptophan.

24. The antibody according to any one of embodiments 19 or 21, wherein Val11 is substituted with an amino acid selected from the group consisting of alanine and glycine.

25. The antibody according to any one of embodiments 1 to 24, wherein the antibody specifically binds to CD20.

26. The antibody according to any one of embodiments 1 to 25, wherein the antibody binds to CD20 with a dissociation constant (Kd) on cells of 10 nM or less as determined by scatchard analysis.

27. A polynucleotide encoding a variant heavy chain region of an antibody of any one of embodiments 1 to 26.

28. A polynucleotide encoding a light chain region of an antibody of any one of embodiments 1 to 26.

29. A vector comprising at least one of the polynucleotides according to the embodiments 27 and 28.

30. The vector of embodiment 29 which is polycistronic.

31. A host cell comprising one of the vectors according to embodiments 29 and 30 or at least one of the polynucleotides according to the embodiments 27 and 28.

32. A method for the production of an antibody according to any one of embodiments 1 to 26 comprising (i) culturing the host cell of embodiment 30 under conditions permitting the expression of said at least one polynucleotide; and (ii) recovering said antibody from the culture medium.

33. A pharmaceutical composition comprising an antibody according to any one of embodiments 1 to 26 and a pharmaceutically acceptable carrier.

34. An antibody according to any one of embodiments 1 to 26 for use as a medicament.

35. An antibody according to any one of embodiments 1 to 26 for use in treating a disease selected from the group consisting of proliferative disorder and autoimmune disease.

36. An antibody for use according to embodiment 35, characterized in that said proliferative disorder is a CD20 expressing cancer.

37. An antibody for use according to embodiment 36, characterized in that said cancer is selected from the group consisting of lymphoma and lymphocytic leukemia.

38. An antibody for use according to embodiment 35, characterized in that said autoimmune disease is selected from the group consisting of rheumatoid arthritis, lupus, multiple sclerosis, Sjögren's syndrome and transplant rejection.

39. A method for treating a disease selected from the group consisting of proliferative disorder and autoimmune disease comprising administering to an individual an effective amount of the antibody according to any one of embodiments 1 to 26.

40. The method according to embodiment 39, characterized in that said proliferative disorder is a CD20 expressing cancer.

41. The method according to embodiment 40, characterized in that said cancer is selected from the group consisting of lymphoma and lymphocytic leukemia.

42. The method according to embodiment 39, characterized in that said autoimmune disease is selected from the group consisting of rheumatoid arthritis, lupus, multiple sclerosis, Sjögren's syndrome and transplant rejection.

43. Use of the antibody according to any one of embodiments 1 to 26 for the manufacture of a medicament.

44. The use of embodiment 43, wherein the medicament is for treatment of a disease selected from the group consisting of proliferative disorder and autoimmune disease.

45. The use of embodiment 44, characterized in that said proliferative disorder is a CD20 expressing cancer.

46. The use of embodiment 45, characterized in that said cancer is selected from the group consisting of lymphoma and lymphocytic leukemia.

47. The use of embodiment 44, characterized in that said autoimmune disease is selected from the group consisting of rheumatoid arthritis, lupus, multiple sclerosis, Sjorgen's syndrome and transplant rejection.

The examples below explain the invention in more detail. The following preparations and examples are given to enable those skilled in the art to more clearly understand and to practice

the present invention. The present invention, however, is not limited in scope by the exemplified embodiments, which are intended as illustrations of single aspects of the invention only, and methods which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those disclosed herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

III. EXAMPLES

The following are examples of methods and compositions of the invention. It is understood that various other embodiments may be practiced, given the general description provided above.

Example 1

Antibodies

For the experiments described below antibodies against CD20 (obinutuzumab (GA101), recommended INN, WHO Drug Information, Vol. 26, No. 4, 2012, p. 453 and rituximab, US Patent No. 7,381,560 and EP2000149B1) were used. All variants disclosed herein, e.g., obinutuzumab P329G L234A L235A, obinutuzumab N297D, rituximab P329G L234A L235A and rituximab N297D were generated using PCR based mutagenesis. IgG molecules were expressed in the HEK-EBNA or HEK293 system, and purified using protein A and size exclusion chromatography.

Example 2

Cell death induction of tumor targets by obinutuzumab and rituximab Fc variants

The induction of cell death by obinutuzumab and rituximab variants was tested using CD20-expressing mantle cell lymphoma (Z-138). Briefly, cells were harvested, counted, checked for viability and re-suspended at 0.526 x 10^6 cells/ml in RPMI1640 + 10 % FCS + 1 % Glutamax. 190 µl of cell suspension (containing 0.1 x 10^6 cells) were incubated in round-bottom 96-well plate for 20 hours to 24 hours at 37°C and 5 % CO_2 in the cell incubator with different concentrations of the obinutuzumab or rituximab Fc variants (16 ng/ml - 10 µg/ml). The final volume was 200 µl per well. Afterwards, the cells were washed once with Annexin V Binding Buffer (10 mM HEPES/NAOH pH7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) before incubation for 30 min at 4°C in the dark with 100 µl/well Annexin V FLUOS (Roche #11828681001, pre-diluted in Annexin V Binding Buffer 1:75). The cells were washed by addition of 80 µl/well Annexin V Binding Buffer and immediately analyzed by FACS using a

FACS CantoII (Software FACS Diva) after addition of pre-diluted PI solution (Sigma Aldrich #P4864, 1:4000).

Figure 1 shows the induction of Phosphatidylserine surface expression on Z-138 as measured by Annexin V binding as well as PI staining in the presence of different obinutuzumab or rituximab Fc variants. All obinutuzumab variants induced significant cell death of Z-138 during 21 hours incubation whereas all rituximab variants hardly induced cell death under the chosen conditions. The induction of cell death was independent of the Fc part of the antibody. Only at high antibody concentrations obinutuzumab P329G L234A L235A and obinutuzumab N297D seemed slightly inferior to obinutuzumab WT and GE.

Example 3

ADCC induction by obinutuzumab and rituximab Fc variants

The lysis of CD20-expressing tumor cells and subsequent NK cell activation mediated by obinutuzumab and rituximab Fc variants was assessed on Z-138 cells (mantle cell lymphoma) and SU-DHL-4 (B-NH). Human PBMCs were used as effectors and tumor cell lysis was detected after 4 hours of incubation with the different anti-CD20 antibodies. Briefly, target cells were harvested, washed, and plated at density of 30 000 cells/well using round-bottom 96-well plates. Peripheral blood mononuclear cells (PBMCs) were prepared by Histopaque density centrifugation of fresh blood obtained from healthy human donors. Fresh blood was diluted with sterile PBS and layered over Histopaque gradient (Sigma, #10771). After centrifugation (450 x g, 30 min, room temperature, no brake), the plasma above the PBMC-containing interphase was discarded and PBMCs transferred in a new falcon tube subsequently filled with 50 ml of PBS. The mixture was centrifuged (350 x g, 10 min, room temperature), the supernatant discarded and the PBMC pellet washed twice with sterile PBS (centrifugation steps 300 x g, 10 min). The resulting PBMC population was counted automatically (ViCell) and re-suspended in AIM V at 12.5 or 15 x 10^6 cells/ml. For the ADCC, the antibodies were added at the indicated concentrations (range of 0.01 ng/ml – 1000 ng/ml in triplicates). Furthermore, anti-CD107a (PE anti-human CD107a, Biolegend #328608) was added directly into the assay. PBMCs were added to target cells at final E:T ratio of 21:1 or 25:1. Tumor cell lysis was assessed after 4 hours of incubation at 37°C and 5% CO_2 by quantification of LDH released into cell supernatants by apoptotic/necrotic cells (LDH detection kit, Roche Applied Science, #11 644 793 001). Maximal lysis of the target cells (= 100%) was achieved by incubation of target cells with 1% Triton X-100. Minimal lysis (= 0%) refers to target cells co-incubated with effector cells without antibodies. For the assessment of NK cell activation occurring upon ADCC, cells were centrifuged at 400 x g for 4 min and washed twice with

FACS Buffer (PBS containing 2 % FCS + 5 mM EDTA + 0.25 % sodium acide). Surface staining for CD3 (PECy7 anti-human CD3, Biolegend # 300420) and CD56 (APC anti-human CD56, Biolegend # 318310) was performed according to the suppliers' indications. Cells were washed twice with 150 µl/well FACS Buffer and fixed using 150 µl/well FACS Lysing Solution (BD # 349202). Samples were analyzed using a BD FACS CantoII.

Figure 2 A and B show that obinutuzumab and rituximab GE antibodies induced a strong and target-specific killing of CD20+ target cells. Obinutuzumab GE was superior to rituximab GE and both antibodies were superior to the WT Fc variants of obinutuzumab and rituximab. Also as WT Fc variants, obinutuzumab was superior to rituximab. Neither N297D nor P329G L234A L235A Fc variants of obinutuzumab or rituximab induced detectable tumor cell lysis using Z-138 or SU-DHL-4 as targets. Analysis of NK cell degranulation by flow cytometry confirmed the data and activity of the tested Fc variants (Figure 2 C, D). Obinutuzumab and rituximab GE antibodies induced strong degranulation as measured by CD107a expression on NK cells. Obinutuzumab GE was superior to rituximab GE and both antibodies were superior to the WT Fc variants of obinutuzumab and rituximab. Also as WT Fc variants, obinutuzumab was superior to rituximab. Neither N297D nor P329G L234A L235A Fc variants of obinutuzumab or rituximab induced detectable CD107a expression.

Example 4

CDC induction by obinutuzumab and rituximab Fc variants

The induction of CDC-dependent lysis of CD20-expressing tumor cells mediated by obinutuzumab and rituximab Fc variants was assessed on Z-138 cells and SU-DHL-4. Rabbit complement (Low-Tox Rabbit Complement, Cedarlane Laboratories Limited # CL3051) were used as complement and tumor cell lysis was detected after 2 hours or 24 hours of incubation with the different anti-CD20 antibodies. Briefly, target cells were harvested, washed, and plated at density of 50 000 cells/well using round-bottom 96-well plates. For the CDC, the antibodies were added at the indicated concentrations (0.01 µg/ml – 100 µg/ml in triplicates) in 50 µl/well and incubated with the cells for 10 min at RT. Meanwhile, the complement was dissolved in 1 ml Cedarlane Cytotoxicity Medium (Cedarlane Laboratories Limited # CL95100) and 2 ml AIM V per vial. 50 µl/well prepared complement was added to the cells. Tumor cell lysis was assessed after 2 hours of incubation at 37°C and 5% CO_2 by quantification of LDH released into cell supernatants by apoptotic/necrotic cells (LDH detection kit, Roche Applied Science, #11 644 793 001). After addition of 35 µl/well AlamarBlue (Biosource # DAL1100) the cells were incubated for further 22 hours at 37°C and 5% CO_2. Tumor cell viability was measured using a Wallac Victor3 1420 Multilabel Counter

(excitation 584 nm, emission 612 nM). Maximal lysis of the target cells (= 100 %) was achieved by incubation of target cells with 1% Triton X-100. Minimal lysis (= 0 %) refers to target cells co-incubated with CDC without antibodies.

Figure 3 A to D show that rituximab GE and WT antibodies induced a strong and target-specific killing of CD20+ target cells after 2 hours and 22 hours. Obinutuzumab GE and WT Fc variants also induced CDC but were significantly inferior to rituximab GE and WT. Neither N297D nor P329G LALA Fc variants of obinutuzumab or rituximab induced detectable tumor cell lysis using Z-138 or SU-DHL-4 as targets.

Example 5

B cell depletion in human whole blood mediated by obinutuzumab or rituximab Fc variants

Normal B cell depletion mediated by obinutuzumab or rituximab Fc variants was also assessed using fresh heparinized human blood from healthy volunteers. Briefly, fresh blood was collected in heparin-containing syringes. Blood aliquots (190 µL/well) were placed in 96-deep well plates, supplemented with obinutuzumab or rituximab IgG variants dilutions (10 µL/well ranging from 0.1 ng/ml up to 1000 ng/ml) and incubated for 20 hours to 24 hours up at 37°C and 5 % CO_2 in a humidified cell incubator. After incubation, blood was mixed by pipetting up and down before 35 µL/well blood aliquots were transferred in 96-round-bottom plates and incubated with fluorescent anti-CD45 (APC anti-human CD45, BD # 555485), anti-CD19 (PE Anti-human CD19, Biolegend # 302208) and anti-CD3 (PE/Cy7Anti-human CD3, Biolegend # 300420) in total 55 µL volume for flow cytometry. After 15 min incubation at room temperature (in the dark) 200 µL/well of FACS lysis solution (BD # 349202) was added to deplete erythrocytes and to fix cells prior to flow cytometry using a BD FACSCantoII.

Figure 4 A (Donor 1) and B (Donor 2) show that obinutuzumab and rituximab GE antibodies induced a strong killing of CD20+/CD19+ B cells in healthy human whole blood. Obinutuzumab GE was superior to rituximab GE and both antibodies were superior to the WT Fc variants of obinutuzumab and rituximab. Also as WT Fc variants, obinutuzumab was superior to rituximab. Obinutuzumab N297D and P329G L234A L235A Fc variants also induced some B cell depletion which is due to the Fc-independent direct cell death effect mediated by obinutuzumab. In contrast to that, rituximab N297D and P329G L234A L235A Fc variants did not induce detectable B cell depletion. As type I CD20 binder and as shown in example 2, rituximab induced only minor direct cell death induction compared to

obinutuzumab. Table 2 shows the corresponding EC50 values calculated with GraphPad Prism.

Table 2 EC50 (ng/ml) of B cell depletion

	EC50 (ng/ml)_donor 1	EC50 (ng/ml)_donor 2
obinutuzumab GE	0.4	2.8
obinutuzumab WT	6.4	34.8
obinutuzumab P329G L234A L235A	91.0	120.5
obinutuzumab N297D	126.8	166.2
rituximab GE	5.4	23.9
rituximab WT	14.1	40.8
rituximab P329G L234A L235A	n.d.	n.d.
rituximab N297D	n.d.	n.d.

Example 6

ADCP induction by obinutuzumab and rituximab Fc variants

The antibody-dependent phagocytosis (ADCP) of CD20-expressing tumor cells by monocyte-derived macrophages (M1 or M2c) mediated by obinutuzumab and rituximab Fc variants was assessed on Z-138 cells (mantle cell lymphoma) and SU-DHL-4 (B-NH). Pan monocytes were isolated from human PBMCs (PBMC isolation see example 3) and differentiated into macrophages using 30 ng/ml M-CSF for 7 days followed by 2 day incubation with 10 ng/ml IL-10 to generate M2c macrophages or 1 day incubation with 100 ng/ml IFNg + 100 ng/ml LPS to generate M1 macrophages. Differentiated M1 and M2c macrophages were labeled with PKH26 (Sigma Aldrich) according to the manufacturer's instructions and used as effectors and phagocytosis was detected after 4 h of incubation with different anti-CD20 antibodies. Briefly, target cells were harvested, washed, and labeled with 2 µM CFSE (Sigma Aldrich) prior to plating at a density of 30 000 cells/well using 96-well UpCell plates. For ADCP, the antibodies were added at 50 ng/ml or 1000 ng/ml in triplicates in the presence or absence of 10 mg/ml Redimune (Behring). PKH26-labeled M1 or M2c macrophages were added to target cells at final E:T ratio of 3:1. ADCP was assessed after 4 hours of incubation at 37°C and 5% CO_2 by analyzing the CFSE-PKH26-double positive cells in flow cytometry using a BD FACS CantoII.

Figure 5 A to D show the ADCP after 4 hours incubation of SU-DHL-4 or Z-138 with M1 or M2c macrophages in the presence of 10 mg/ml unspecific human IgGs (Redimune). M1 and

M2c macrophages performed comparable phagocytosis under the chosen conditions. After 4 hours, phagocytosis of SUDHL4 was significantly higher in the presence of 1000 ng/ml anti-CD20 abs compared to Z-138. Obinutuzumab WT and N297D induced significant and comparable phagocytosis using SU-DHL-4 and Z-138. Obinutuzumab GE induced slightly stronger phagocytosis mainly of CD20+ SU-DHL-4 target cells using M2c as effectors compared to obinutuzumab WT and N297D in the presence of 10 mg/ml Redimune.

Example 7

Antitumor activity of a type II anti-CD20 antibody obinutuzumab (GA101), a type I anti-CD20 antibody rituximab and the obinutuzumab variants GA101 P329G L234A L235A and GA101 aglyco (N297D)

Test agents

The antibodies were provided as stock solution from Roche Glycart AG, Schlieren, Switzerland, in histidine buffer. The antibody was diluted with 0.9 % NaCl solution prior to in-vivo application.

Cell lines and culture conditions

The human SU-DHL-4 lymphoma cell line was cultured in RPMI 1640 supplemented with 10% fetal bovine serum (PAA Laboratories, Austria) and 2 mM L-glutamine at 37°C in a water-saturated atmosphere at 5% CO_2. For in-vivo xenograft experiments the cells were co-injected with Matrigel.

Animals

Female SCID beige mice, age 5 to 6 weeks at arrival (purchased from Charles River, Sulzfeld, Germany), were maintained in the quarantine part of the animal facility for one week and afterwards under specific-pathogen-free condition with daily cycles of 12 hours light / 12 hours darkness according to guidelines (GV-Solas; Felasa; TierschG). The experimental study protocol was reviewed and approved by Roche and the local government (Regierung von Oberbayern; registration no. 55.2-1-54-2531.2-26-09). Diet food (KLIBA NAFAG 3807) and water (filtered) were provided ad libitum.

Induction of SC SU-DHL-4 tumors in SCID beige mice

Five millions ($5x10^6$) SU-DHL-4 tumor cells in 100 µl of PBS with matrigel (50:50, BD Biosciences, France) were subcutaneously (SC) injected into the right flank of female SCID beige mice.

Monitoring

Animals were monitored daily for clinical symptoms and detection of adverse effects. During the experiment the body weight of animals was checked two times a week and tumor volume was measured by caliper.

Treatment of animals

Animal treatment started at the day of randomization 21 days after tumor cell inoculation. Humanized type II anti-CD20 antibody obinutuzumab (GA101), rituximab, GA101 P329G L234A L235A and obinutuzumab N297D was administered as single agent i.p. q7d once weekly (day 21, 28, 35 and 42) for 4 weeks at a dosage of 30 mg/kg. The corresponding vehicle was administered on the same days.

Tumor growth inhibition (TGI) on day 49

Monotherapy treatment using GA101 P329G L234A L235A, rituximab or obinutuzumab N297D resulted in tumor growth inhibition of 62 %, 71% or 93%, respectively (based on medians). Obinutuzumab treatment showed tumor regression (TGI>100%) on day 49 after tumor cell inoculation.

Nonparametric Treatment-to-Control-Ratios (TCRnpar) on day 49

Nonparametric Treatment-to-Control-Ratios (TCRnpar) and the two-sided nonparametric confidence intervals (CI) was calculated based on baseline corrected data by ratio to assess statistical significance on day 49 after tumor cell inoculation. Each treatment was statistically significant compared to the control group.

Table 3: Summary of results according to Figure 6

Treatment schedule	**TGI (%)**	**np TCR [95% CI] compared to vehicle**		**Tumor free Animals on Day 49**
Vehicle	--	--	[--]	0
obinutuzumab (GA101) 30 mg/kg; q7dx4; IP	>100	0	[0-0]	9
GA101 P329G LALA 30 mg/kg; q7dx4; IP	62	0.43	[0.31 -0.63]	0
GA101 N297D 30 mg/kg; q7dx4; IP	93	0.14	[0 -0.35]	2
rituximab 30 mg/kg; q7dx4; IP	71	0.37	[0.29 -0.54]	0

Example 8

FcγRI SPR assay

The SPR interaction analysis of captured FcγRI and IgG1 Fc variants was performed on a Biacore T100 system (GE Healthcare) with high immobilized anti-His capturing antibody (GE Healthcare). Immobilization of the anti-His capturing antibody was performed on a CM5 chip using the standard amine coupling kit (GE Healthcare) at pH 4.5 10 mM sodium acetate and 10 µg/ml anti-His solution. The immobilization level of anti-His reached more than 10.000 RU. FcγRI was applied in a 100 nM solution, in the running buffer HBS-P+ and captured with a pulse of 60 sec at a flow rate of 30 µl per min. Subsequently GA101 N297D was applied in a serial dilution from 1000-62.5 nM in HBS-P+ and a flow rate of 30 µl per min for 60 sec. The dissociation phase was monitored for 180 sec. The surface was regenerated by a 60 sec washing step with a 10 mM Glycine pH 1.5 at a flow rate of 30 µl per min. The stabilization period took 60 sec. The Biacore T100 evaluation software was used for data analysis.

FcγRI SPR assay steady state evaluation

For the steady state affinity concentration determination the response values from the end of association were used to plot the response against the concentration. The Biacore T100 evaluation software was used to calculate the affinity of samples for immobilized FcyRI receptor from the plot.

The interaction measurement of GA101 N297D to FcyRI in comparison to a wild type IgG1 (Herceptin WT) molecule and a IgG4SPLE molecule (P-Selectin), demonstrated a clear binding order (Figure 7 A). The IgG1 WT displayed the highest interaction signal of up to 250 Response Units (RU), the GA101 N297D displayed a RU signal of 150, whereas the P-Selectin displayed only a very low binding signal of 25 Response Units, measured at 200 nM antibody concentration (Figure 7 B). It is known for IgG1 Wt antibodies to bind FcyRI with an affinity in the nanomolar range. To determine the GA101 N297D affinity a steady state affinity determination was performed. The affinity was measured as 234 nM (Figure 7 C), which is roughly 50 fold below the IgG1 Wt affinity, but significant stronger compared with the IgG4SPLE FcyRI binding reduction, where it is not possible to determine a certain affinity.

Example 9

FcγRIII SPR assay

The SPR interaction analysis of captured FcγRIIIA and IgG1 Fc variants was performed on a Biacore T100 system (GE Healthcare) with high immobilized anti-His capturing antibody (GE Healthcare). Immobilization of the anti-His capturing antibody was performed on a CM5 chip using the standard amine coupling kit (GE Healthcare) at pH 4.5 10 mM sodium acetate and 10 µg/ml anti-His solution. The immobilization level of anti-His reached more than 10.000 RU. FcγRIIIA was applied in a 200 nM solution, in the running buffer HBS-P+ and captured with a pulse of 60 sec at a flow rate of 30 µl per min. Subsequently GA101 N297D was applied in a serial dilution from 1000 to 62.5 nM in HBS-P+ and a flow rate of 30 µl per min for 60 sec. The dissociation phase was monitored for 180 sec. The surface was regenerated by a 60 sec washing step with a 10 mM Glycine pH 1.5 at a flow rate of 30 µl per min. The stabilization period took 60 sec. The Biacore T100 evaluation software was used for data analysis.

The time resolved binding determination of GA101 N297D displayed no measurable interaction with FcγRIIIA, in contrast to the Herceptin WT IgG1 molecule (Figure 8 A). Performing the analogous steady state affinity determination experiment no clear concentration dependent binding signal could be measured. The highest concentration of 1µM displayed only a RU value of 5 and no saturation of the interaction could be observed (Figure 8 B). Consequently, the saturable interaction was below the value that could be determined by SPR (high µM range) comparably to the affinity of the IgG4 SPLE control, which did also not display a relevant interaction signal (Figure 8 A).

Example 10

SPR FcγR capture setup (Figure 7D, 8C,9A, 9B)

The SPR interaction analysis of captured FcγRs (FcγRIa, FcγRIIa (R131) and FcγRIIa (H131), FcγRIIIa (V158)) and IgG1 Fc variants was performed on a Biacore T200 system (GE Healthcare) with high immobilized anti-His capturing antibody (GE Healthcare). Immobilization of the anti-His capturing antibody was performed on a CM5 chip using the standard amine coupling kit (GE Healthcare) at pH 4.5 10mM sodium acetate and 10 µg/ml anti-His solution. The immobilization level of anti-His reached >10 000 RU. FcγRs were prepared as solution of 100nM each, using running buffer HBS-P+ and were captured with a pulse of 60 s at a flow rate of 30 µl/min. Subsequently, IgG1 Fc his-tagged variants were applied at a concentration of 100nM in HBS-P+ and a flow rate of 30 µl/min for 60 s.

The dissociation phase was monitored for 180 s. The surface was regenerated by a 60 s washing step with a 10mM Glycine pH 1.5 at a flow rate of 30 µl/min. The stabilization period was set to 60 s. The Biacore T200 evaluation software was used for data analysis.

Example 11

Thermostability

The melting temperature, Tm, was assessed by recording the intrinsic Tryptophan fluorescence with an Optim1000 instrument (Avacta Analytical Inc.). Samples were prepared at approx. 1 mg/mL in 20 mM Histidine chloride, 140 mM NaCl, pH 6.0 and transferred to a 9 µL multi-cuvette array. The multi-cuvette array was heated from 30 °C to 90 °C at a constant rate of 0.1 °C/minute. The instrument continuously records fluorescence emission spectra after excitation with a 266 nm laser, providing a data point approximately every 0.6 °C. The melting temperature, Tm, is determined by plotting the fluorescence intensity against the temperature and Tm is defined as the inflection point in these curves.

Both N297D variants display an aggregation temperature 6°C lower compared to both respective P329G variants (Figure 13). Additionally, the melting temperature of the deglycosylated N297D variants is below the more stable P329G variants. The P329G stays in an active confirmation at higher temperatures and displays therby a better structural integrity.

WHAT IS CLAIMED IS:

1. An antibody comprising a variant heavy chain region comprising at least one amino acid substitution relative to the parent non-substituted heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residue Asn297 and/or Pro329, and wherein said substitution is Asn297Asp and/or Pro329Gly, wherein the residues are numbered according to the EU index as in Kabat, wherein ADCP function induced by the antibody comprising the variant heavy chain region is reduced compared to ADCP function induced by the parent non-substituted antibody, wherein the antibody comprising the variant heavy chain retains residual ADCP function.

2. The antibody according to claim 1, wherein induction of effector function is reduced compared to effector function induced by an antibody comprising the parent non-substituted heavy chain region.

3. The antibody according to any one of claims 1 or 2, wherein the antibody is an IgG1 antibody.

4. The antibody according to any one of claims 1 to 3, wherein the parent non-substituted antibody is obinutuzumab or rituximab.

5. The antibody according to any one of claims 1 to 4, wherein FcγRIII binding by the antibody comprising the variant heavy chain region is abolished compared to binding to FcγRIII by the parent non-substituted antibody.

6. The antibody according to any one of claims 1 to 5, wherein ADCC function induced by the antibody comprising the variant heavy chain region is abolished or strongly reduced compared to ADCC function induced by the parent non-substituted antibody.

7. The antibody according to any one of claims 1 to 6, wherein FcγRI binding by the antibody comprising the variant heavy chain region is reduced compared to binding to FcγRI by the parent non-substituted antibody.

8. The antibody according to any one of claims 1 to 7, wherein the variant heavy chain region comprises a further amino acid substitution relative to the parent non-substituted

heavy chain region, wherein the heavy chain region of the parent non-substituted antibody comprises the amino acid residue Pro151, and wherein said further substitution is at said amino acid residue Pro151, wherein direct cell death induced by the antibody comprising the variant heavy chain region is altered compared to direct cell death induced by the antibody comprising proline at position 151.

9. The antibody according to claim 8, wherein direct cell death induced by the antibody comprising the variant heavy chain region is increased compared to direct cell death induced by the antibody comprising proline at position 151.

10. The antibody according to any one of claim 8 or 9, wherein Pro151 is substituted with phenylalanine.

11. The antibody according to any one of claims 1 to 10, wherein the antibody specifically binds to CD20.

12. A pharmaceutical composition comprising an antibody according to any one of claims 1 to 11 and a pharmaceutically acceptable carrier.

13. An antibody according to any one of claims 1 to 11 for use as a medicament.

14. An antibody according to any one of claims 1 to 11 for use in treating a disease selected from the group consisting of proliferative disorder and autoimmune disease.

15. An antibody for use according to claim 14, characterized in that said proliferative disorder is a CD20 expressing cancer.

16. A method for treating a disease selected from the group consisting of proliferative disorder and autoimmune disease comprising administering to an individual an effective amount of the antibody according to any one of claims 1 to 11.

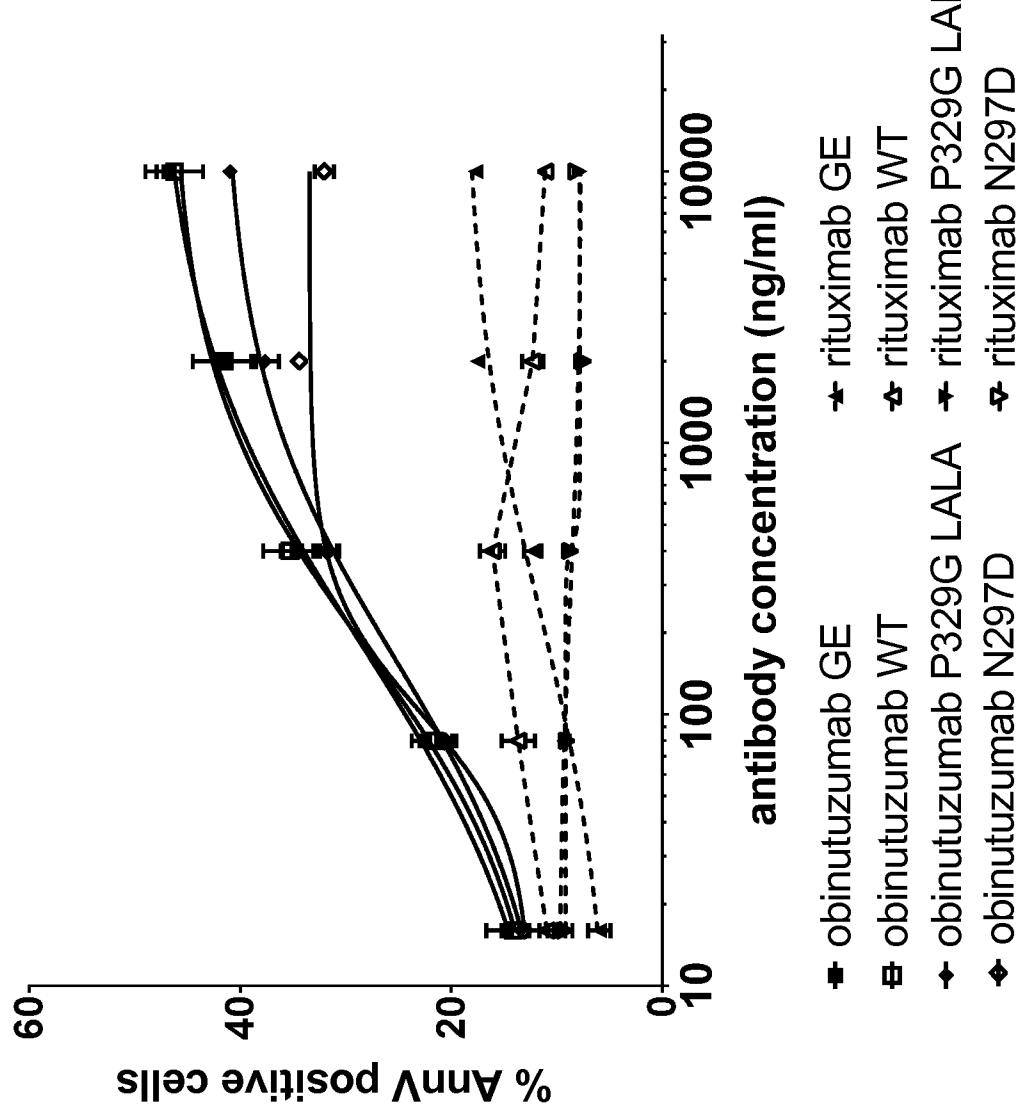
17. The method according to claim 16, characterized in that said proliferative disorder is a CD20 expressing cancer.

18. Use of the antibody according to any one of claims 1 to 11 for the manufacture of a medicament.

19. The use of claim 18, wherein the medicament is for treatment of a disease selected from the group consisting of proliferative disorder and autoimmune disease.

5

Figure 1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40
20
0
10
100
1000
10000
antibody concentration (ng/ml)
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rituximab GE
obinutuzumab WT
rituximab WT
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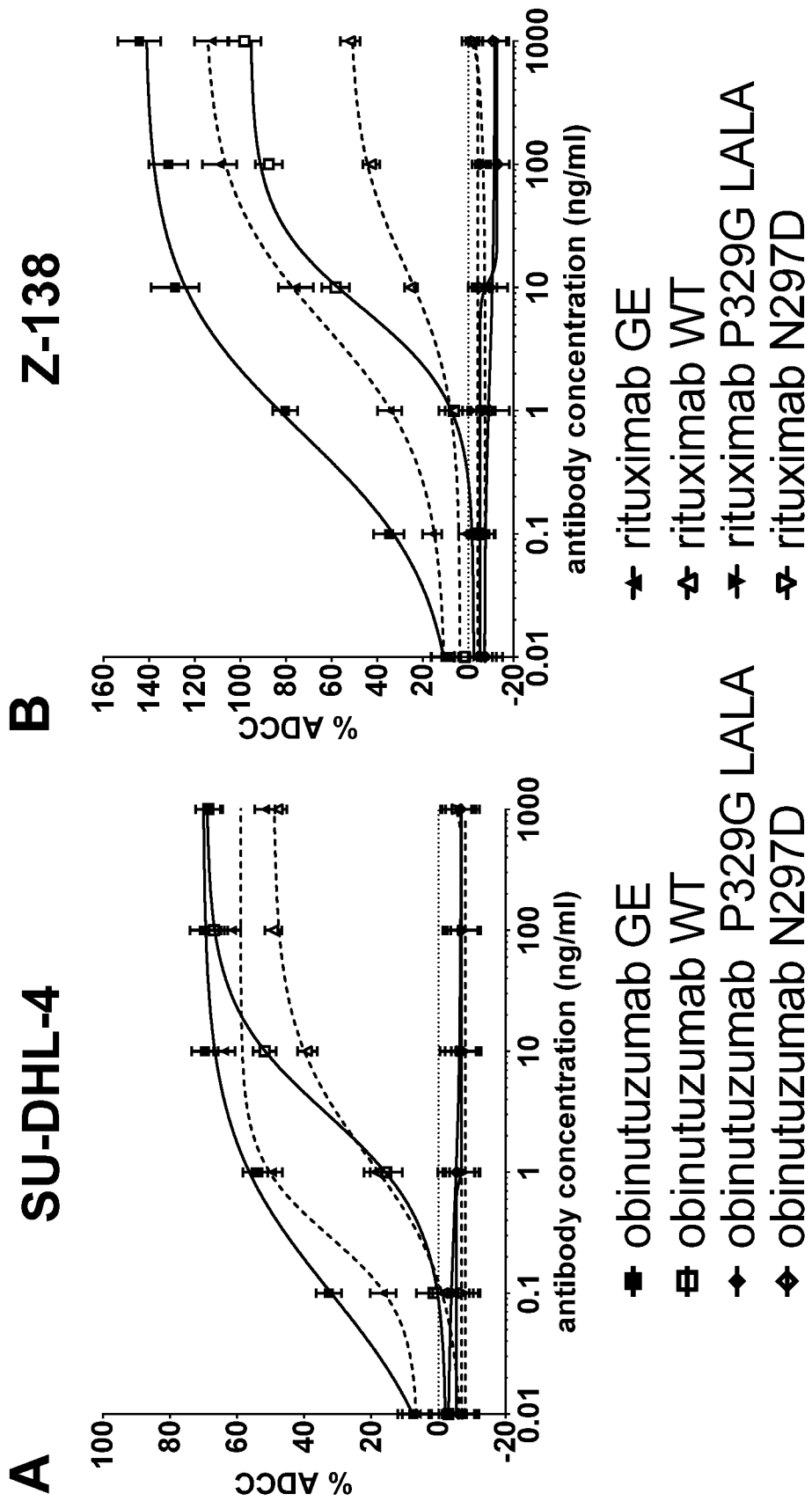
rituximab N297D

Figure 2 A,B


A
SU-DHL-4
% ADCC
antibody concentration (ng/ml)
B
Z-138
% ADCC
antibody concentration (ng/ml)
obinutuzumab GE
obinutuzumab WT
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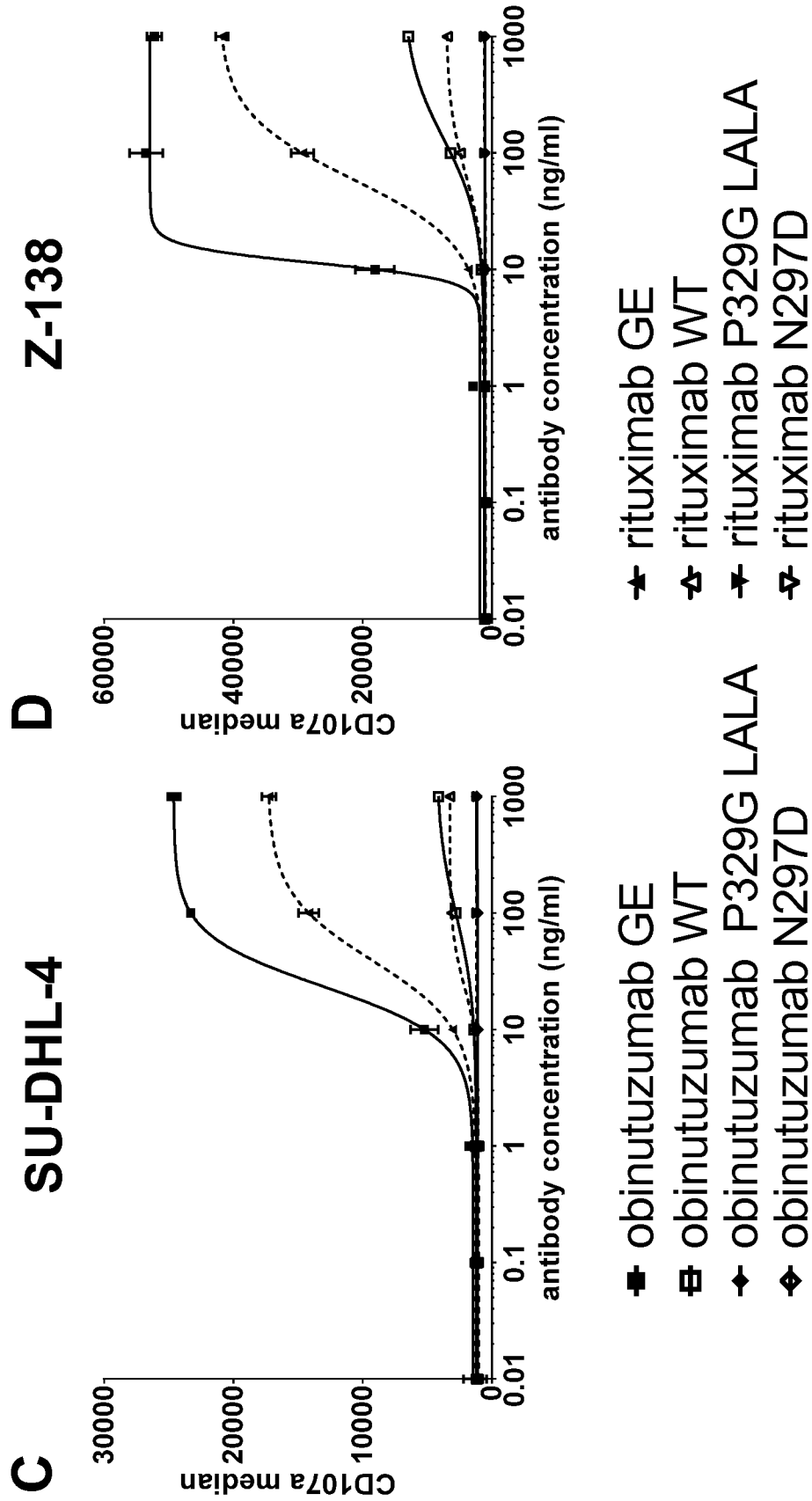
rituximab N297D

Figure 2 C,D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D
Z-138
CD107a median
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40000
20000
0
0.01
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10
100
1000
antibody concentration (ng/ml)
obinutuzumab GE
obinutuzumab WT
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rituximab WT
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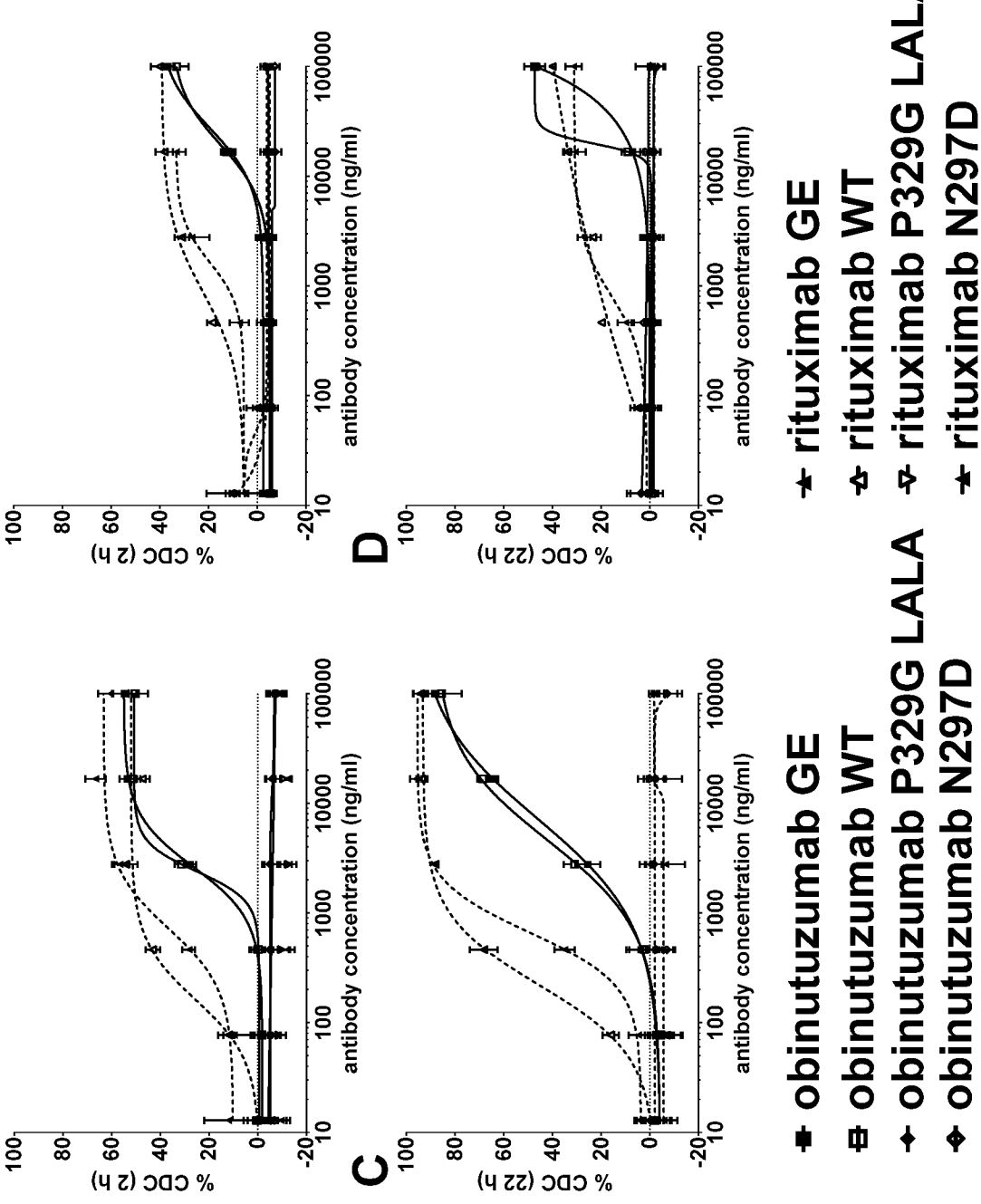
rituximab N297D

Figure 3 A-D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antibody concentration (ng/ml)
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antibody concentration (ng/ml)
C
% CDC (22 h)
antibody concentration (ng/ml)
D
% CDC (22 h)
antibody concentration (ng/ml)
obinutuzumab GE
obinutuzumab WT
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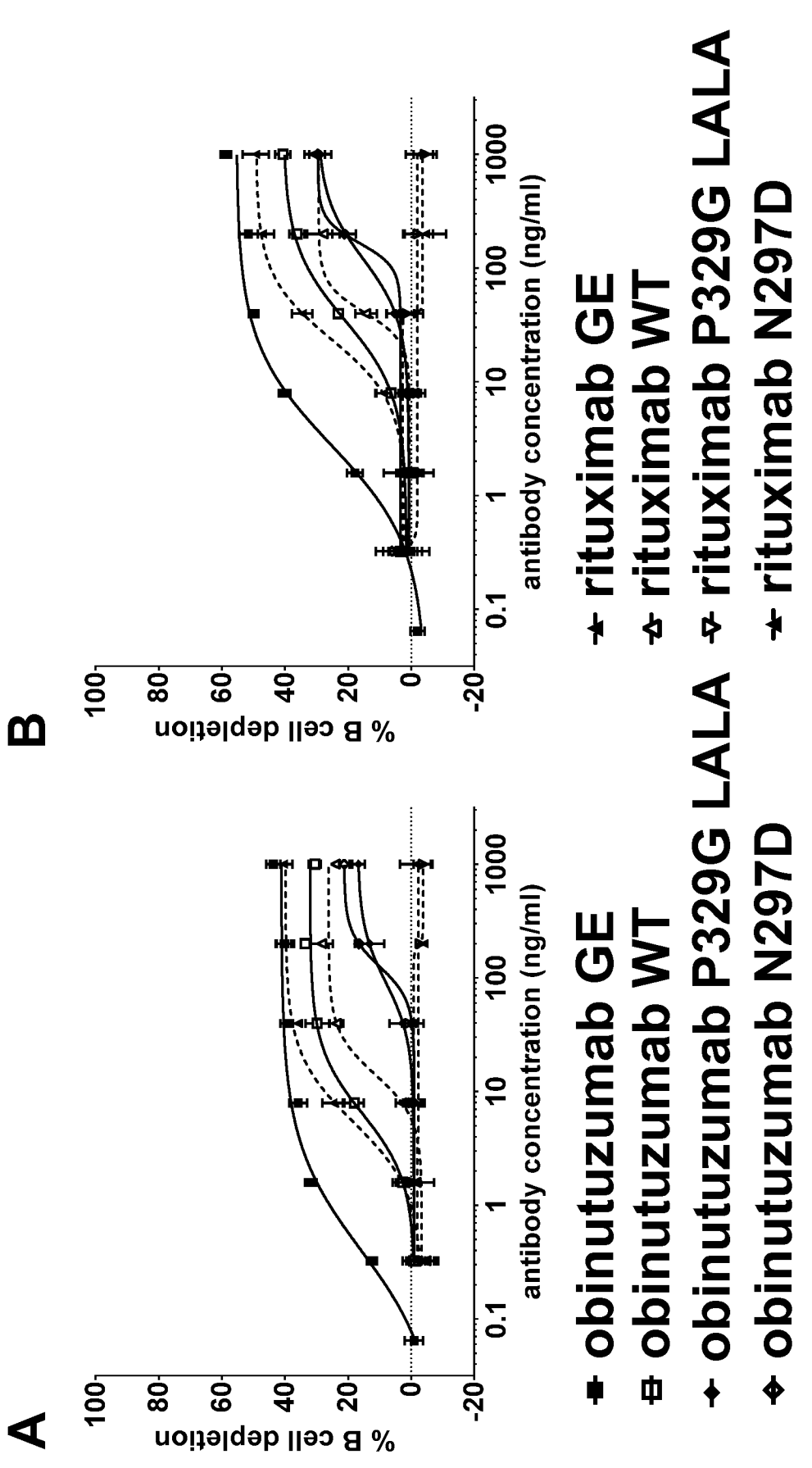
obinutuzumab N297D
rituximab GE
rituximab WT
rituximab P329G LALA
rituximab N297D

Figure 4 A,B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B
% B cell depletion
antibody concentration (ng/ml)
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60
40
20
0
-20
0.1
1
10
100
1000
obinutuzumab GE
obinutuzumab WT
obinutuzumab P329G LALA
obinutuzumab N297D
rituximab GE
rituximab WT
rituximab P329G LALA
rituximab N297D

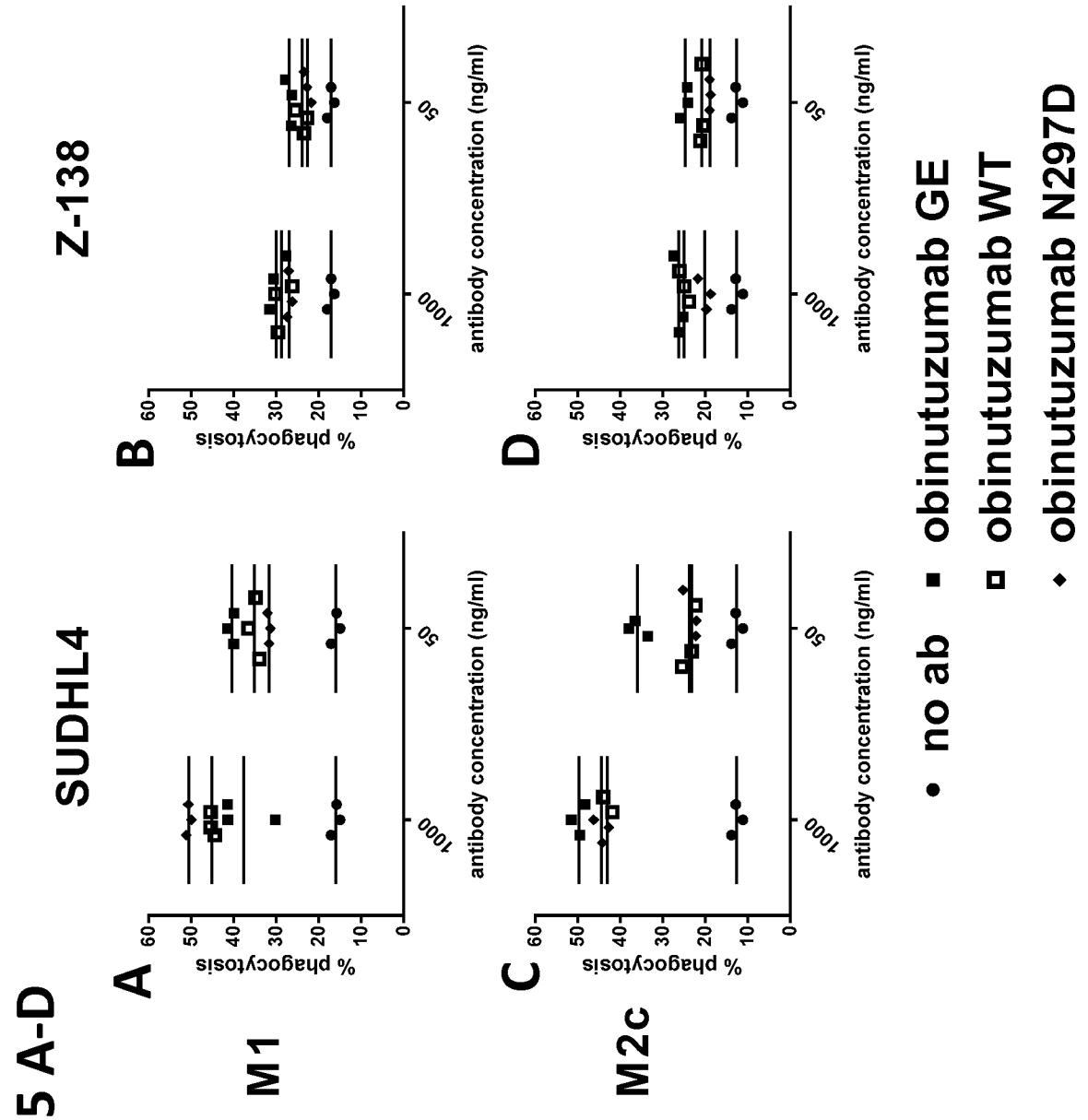
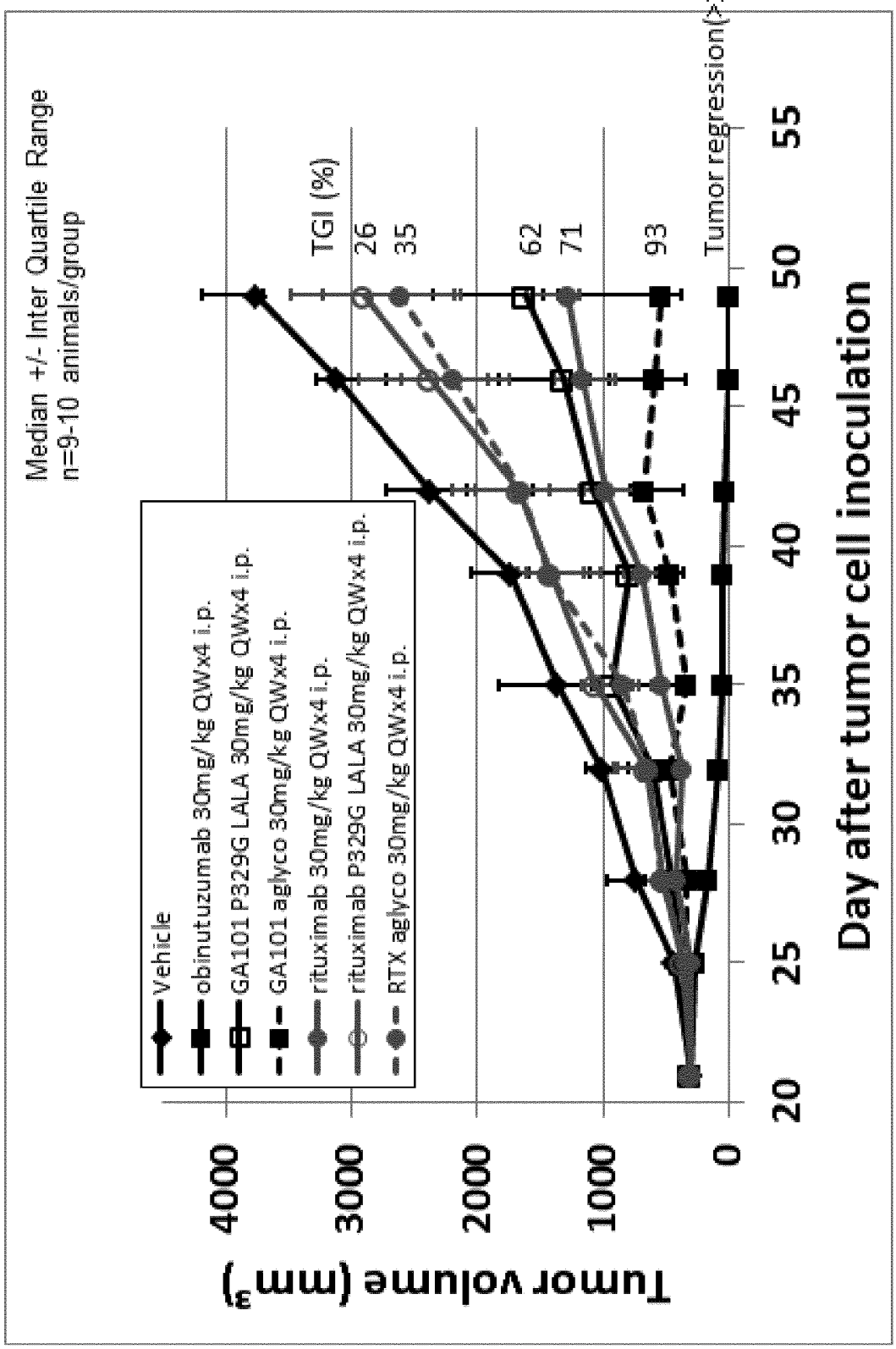


Figure 5 A-D
SUDHL4
Z-138
A
B
C
D
M1
M2c
% phagocytosis
antibody concentration (ng/ml)
0
10
20
30
40
50
60
50
1000
• no ab
▪ obinutuzumab GE
▫ obinutuzumab WT
◆ obinutuzumab N297D

Figure 6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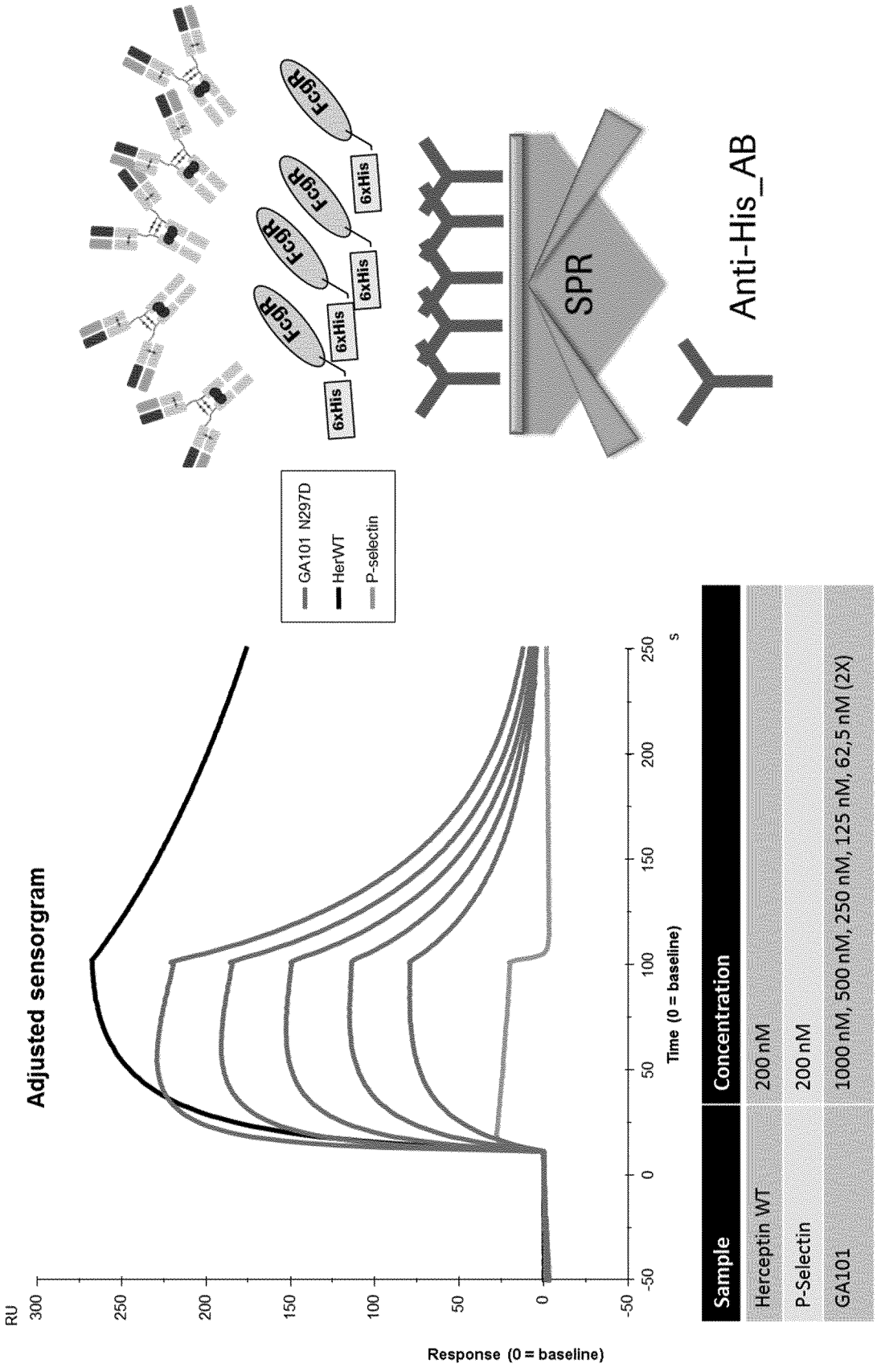
GA101 aglyco 30mg/kg QWx4 i.p.
rituximab 30mg/kg QWx4 i.p.
rituximab P329G LALA 30mg/kg QWx4 i.p.
RTX aglyco 30mg/kg QWx4 i.p.
TGI (%)
26
35
62
71
93
Tumor regression(>100)
Tumor volume (mm³)
4000
3000
2000
1000
0
20
25
30
35
40
45
50
55
Day after tumor cell inoculation

Figure 7 A



Sample	Concentration
Herceptin WT	200 nM
P-Selectin	200 nM
GA101	1000 nM, 500 nM, 250 nM, 125 nM, 62,5 nM (2X)

Figure 7 B

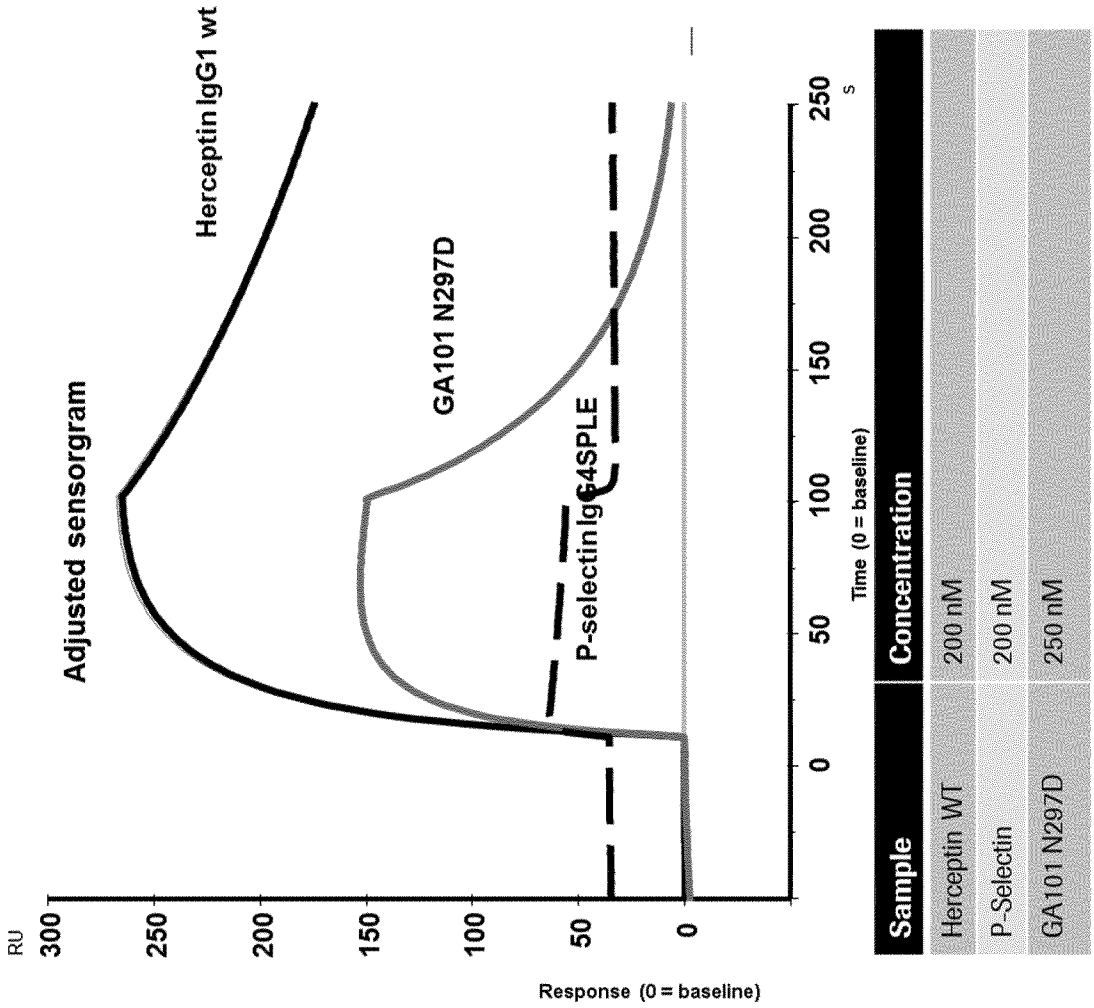

Adjusted sensorgram
RU
300
250
200
150
100
50
0
Herceptin IgG1 wt
GA101 N297D
P-selectin IgG4SPLE
0
50
100
150
200
250
s
Time (0 = baseline)
Response (0 = baseline)
Sample
Concentration
Herceptin WT
200 nM
P-Selectin
200 nM
GA101 N297D
250 nM

Figure 7 C

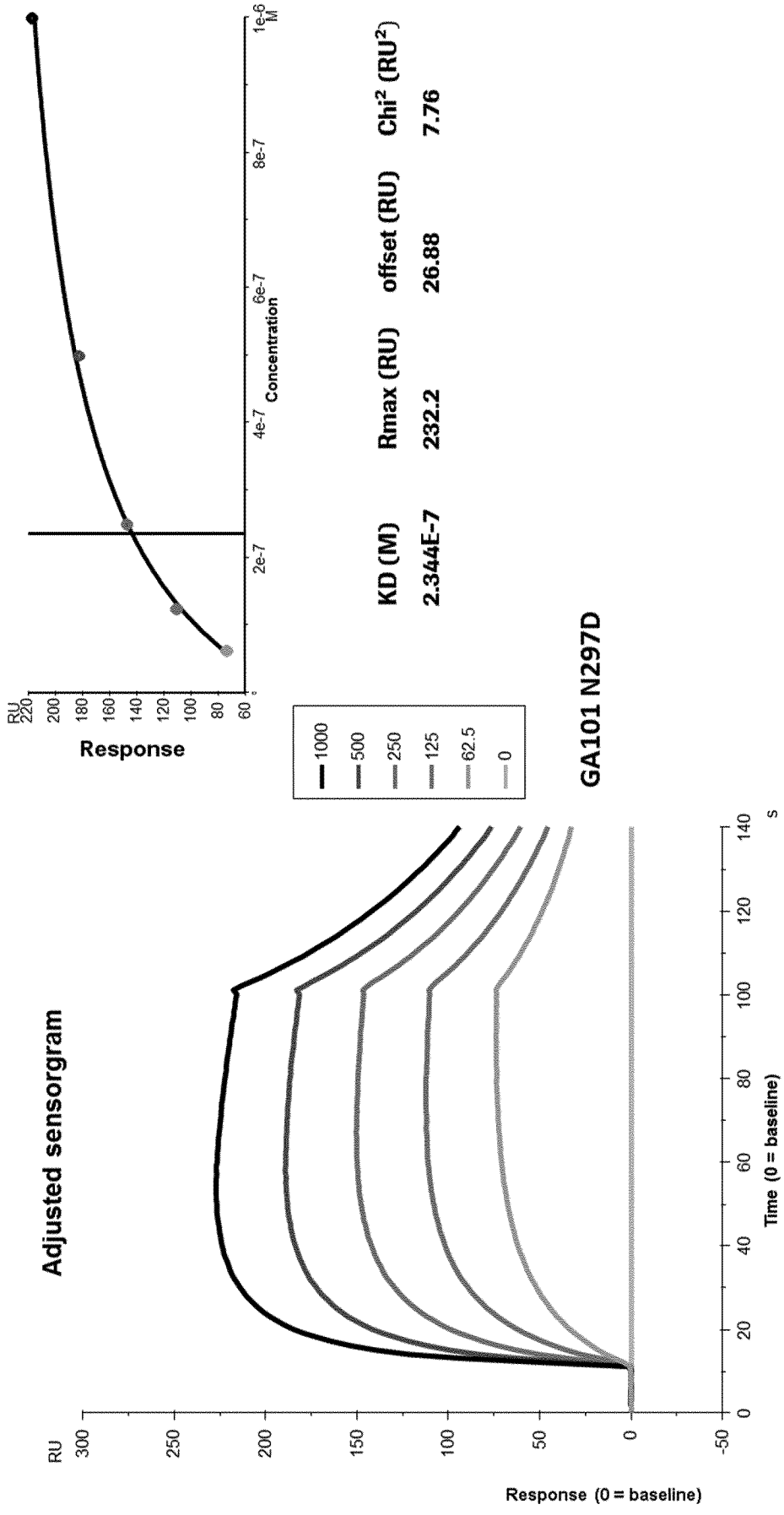

Steady state affinity
RU
Response
Concentration
KD (M)
Rmax (RU)
offset (RU)
Chi² (RU²)
2.344E-7
232.2
26.88
7.76
1000
500
250
125
62.5
0
GA101 N297D
Adjusted sensorgram
RU
Response (0 = baseline)
Time (0 = baseline)
s

Figure 7 D

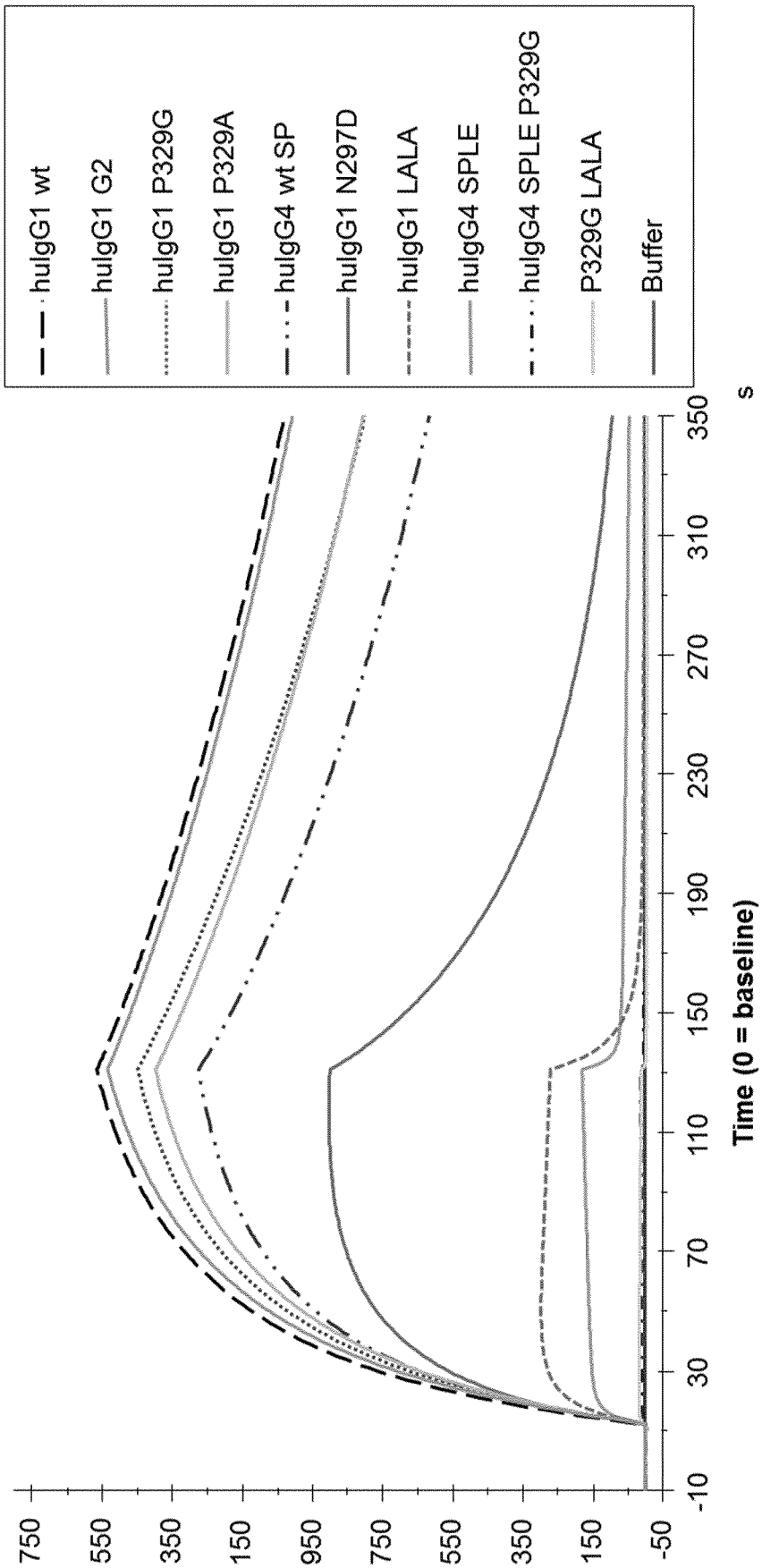
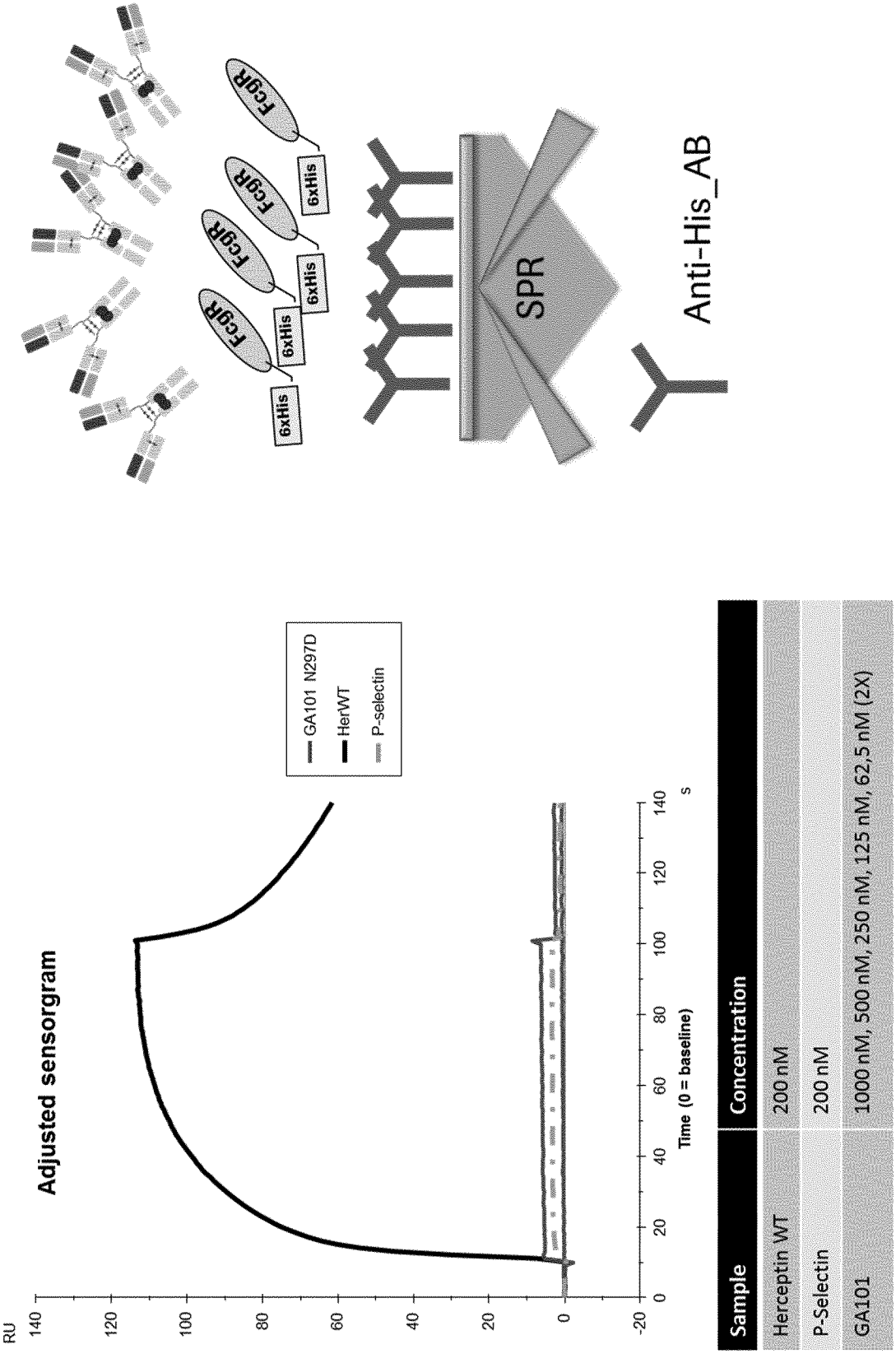

Adjusted sensorgram
RU
1750
1550
1350
1150
950
750
550
350
150
-50
-10
30
70
110
150
190
230
270
310
350
Time (0 = baseline)
s
hulgG1 wt
hulgG1 G2
hulgG1 P329G
hulgG1 P329A
hulgG4 wt SP
hulgG1 N297D
hulgG1 LALA
hulgG4 SPLE
hulgG4 SPLE P329G
P329G LALA
Buffer

Figure 8 A



Sample	Concentration
Herceptin WT	200 nM
P-Selectin	200 nM
GA101	1000 nM, 500 nM, 250 nM, 125 nM, 62,5 nM (2X)

Figure 8 B

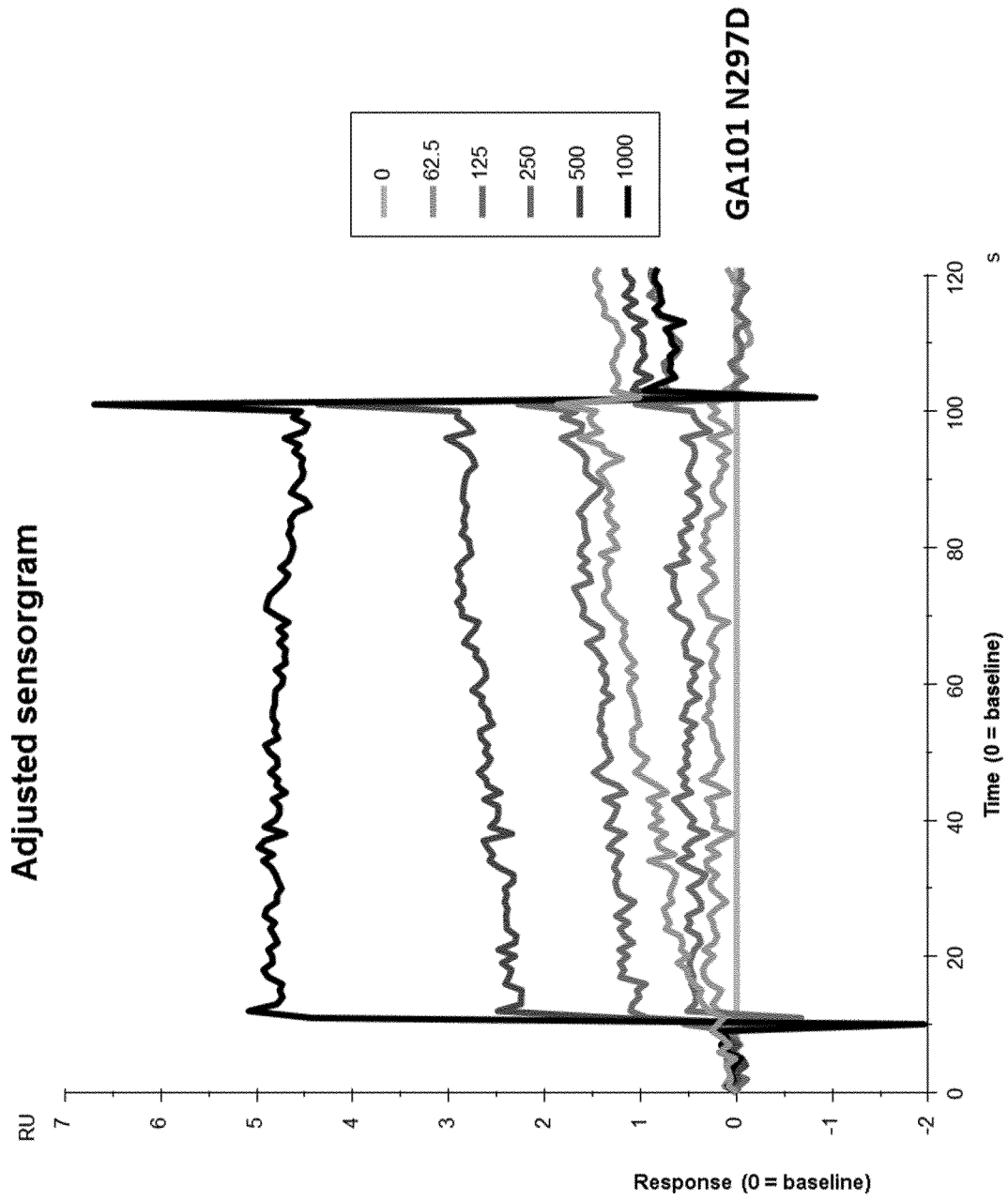

Adjusted sensorgram
GA101 N297D
0
62.5
125
250
500
1000
RU
7
6
5
4
3
2
1
0
-1
-2
0
20
40
60
80
100
120
s
Time (0 = baseline)
Response (0 = baseline)

Figure 8 C

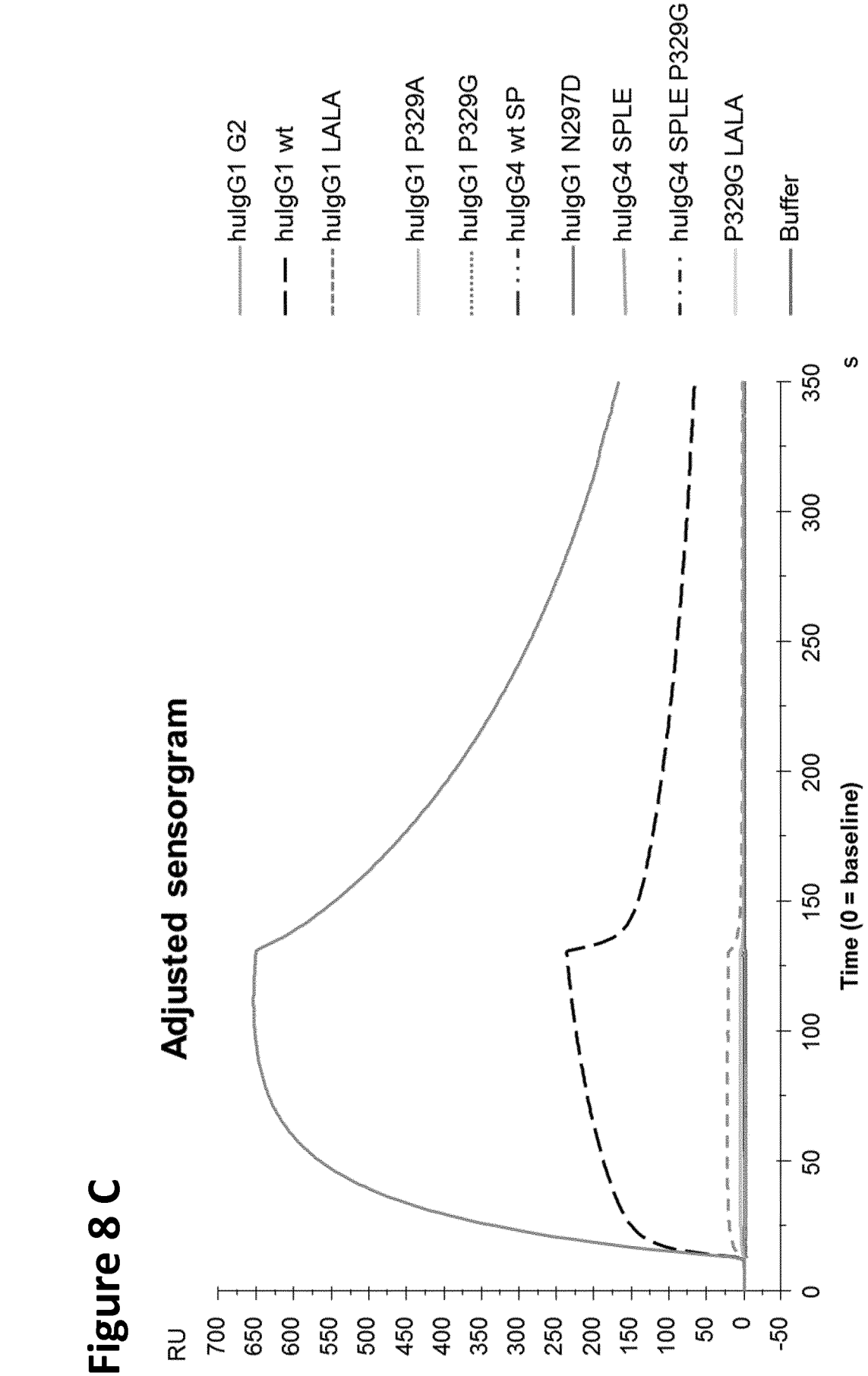

Adjusted sensorgram
RU
700
650
600
550
500
450
400
350
300
250
200
150
100
50
0
-50
0
50
100
150
200
250
300
350
s
Time (0 = baseline)
hulgG1 G2
hulgG1 wt
hulgG1 LALA
hulgG1 P329A
hulgG1 P329G
hulgG4 wt SP
hulgG1 N297D
hulgG4 SPLE
hulgG4 SPLE P329G
P329G LALA
Buffer

Figure 9 A

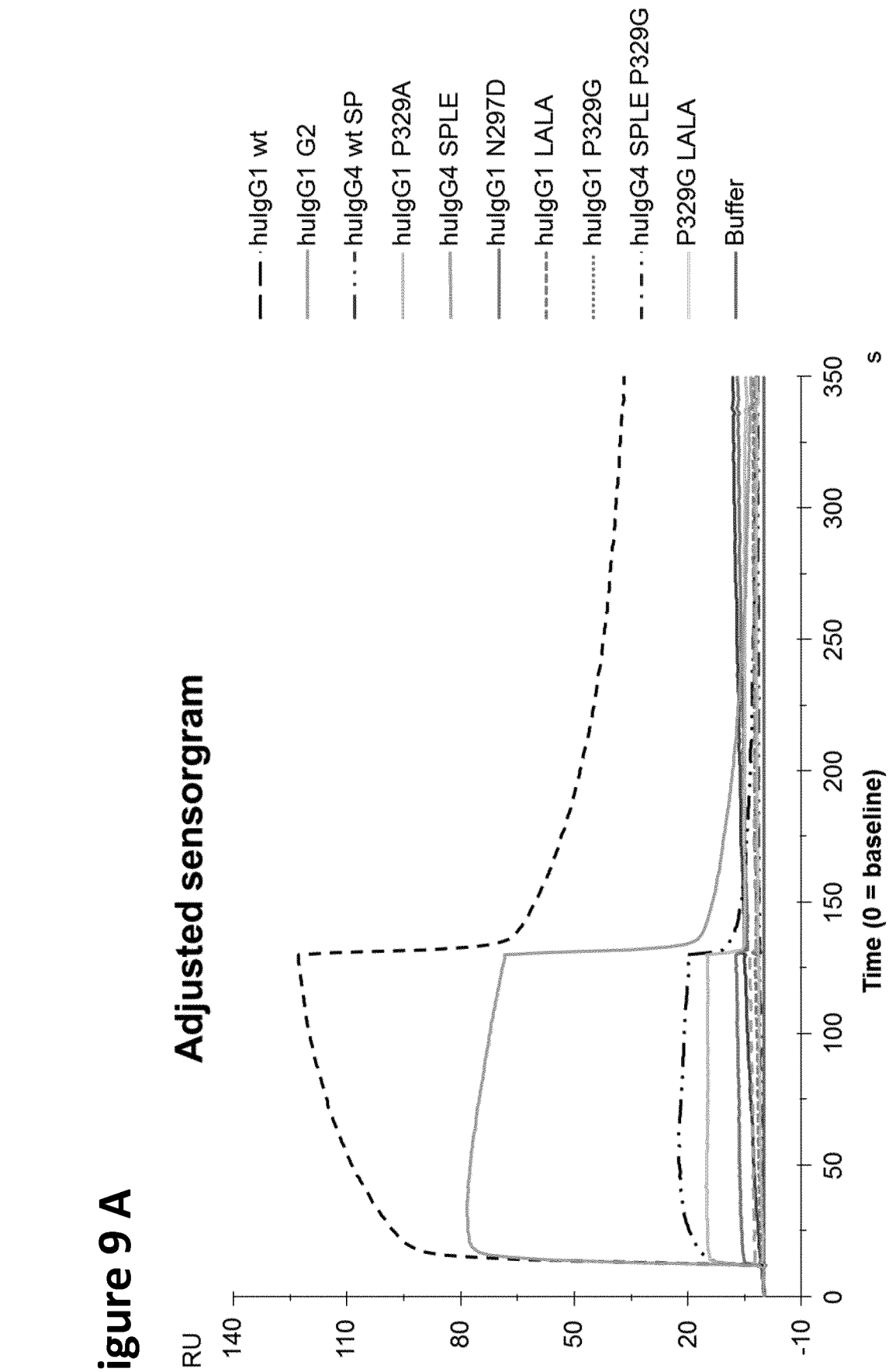

Adjusted sensorgram
RU
140
110
80
50
20
-10
0
50
100
150
200
250
300
350
s
Time (0 = baseline)
huIgG1 wt
huIgG1 G2
huIgG4 wt SP
huIgG1 P329A
huIgG4 SPLE
huIgG1 N297D
huIgG1 LALA
huIgG1 P329G
huIgG4 SPLE P329G
P329G LALA
Buffer

Figure 9 B

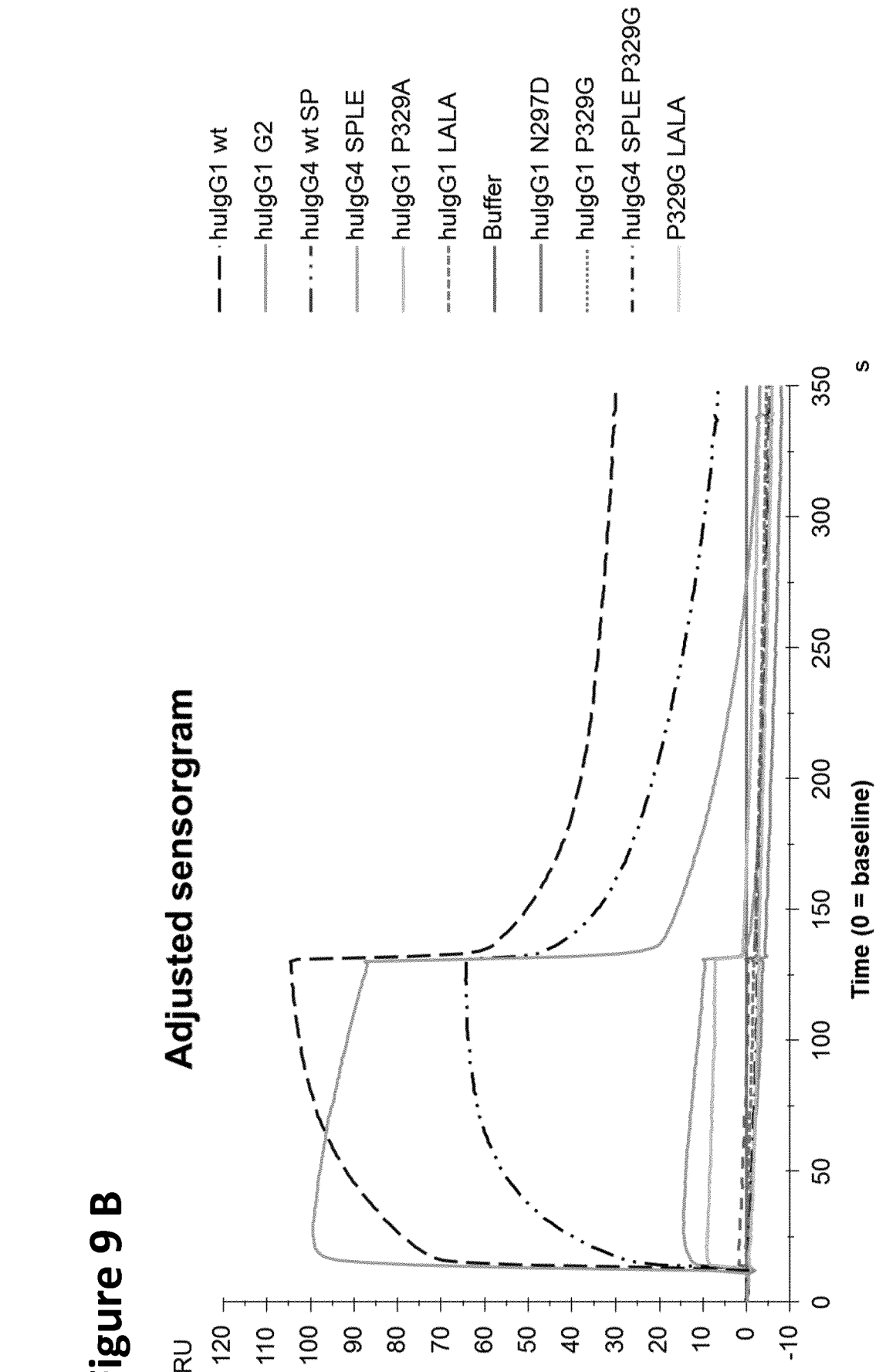

Adjusted sensorgram
RU
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
0
50
100
150
200
250
300
350
s
Time (0 = baseline)
huIgG1 wt
huIgG1 G2
huIgG4 wt SP
huIgG4 SPLE
huIgG1 P329A
huIgG1 LALA
Buffer
huIgG1 N297D
huIgG1 P329G
huIgG4 SPLE P329G
P329G LALA

Figure 10 A

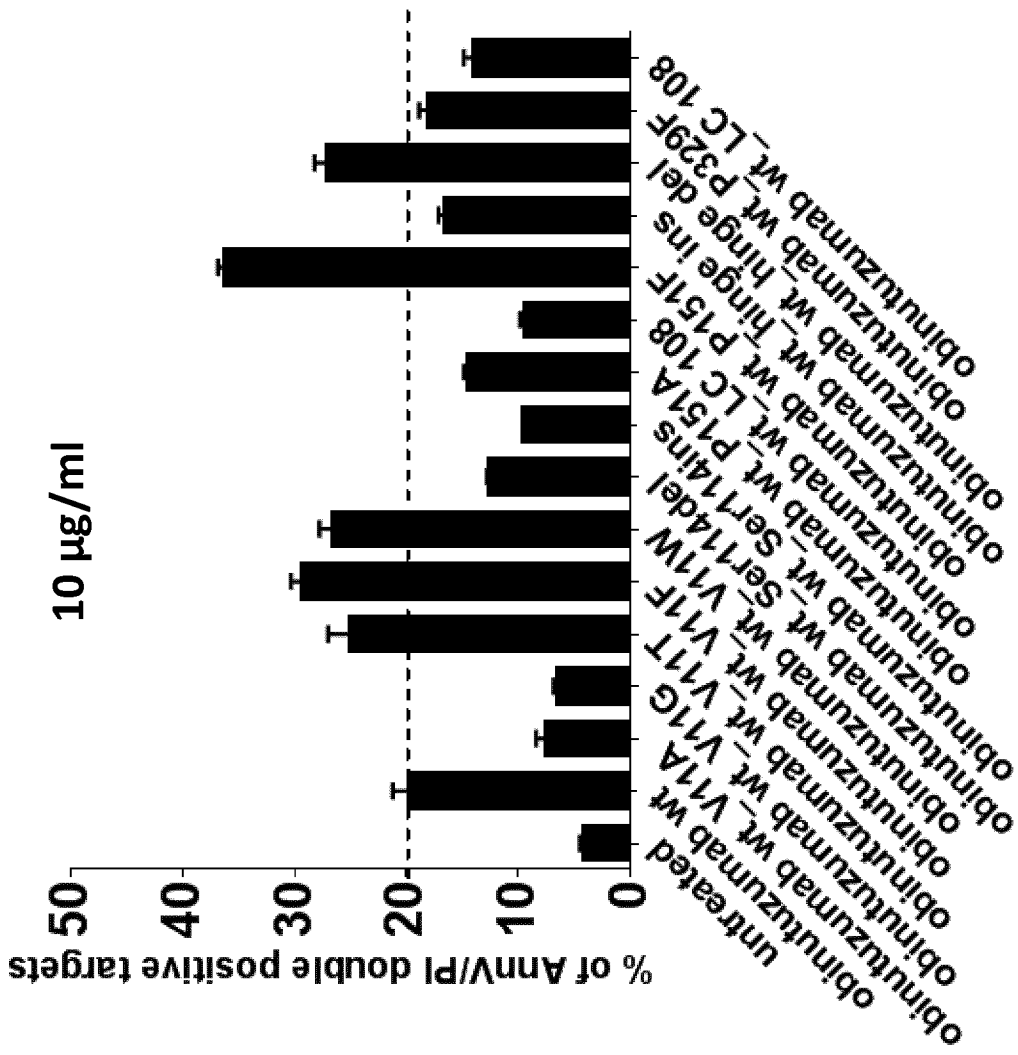

% of AnnV/PI double positive targets
0
10
20
30
40
50
10 µg/ml
untreated
obinutuzumab wt
obinutuzumab wt_V17A
obinutuzumab wt_V17G
obinutuzumab wt_V17T
obinutuzumab wt_V17F
obinutuzumab wt_V17W
obinutuzumab wt_Ser114del
obinutuzumab wt_P151ins
obinutuzumab wt_P151A
obinutuzumab wt_LC 108
obinutuzumab wt_hinge ins
obinutuzumab wt_hinge del
obinutuzumab wt_P329F
obinutuzumab wt_LC 108

Figure 10 B

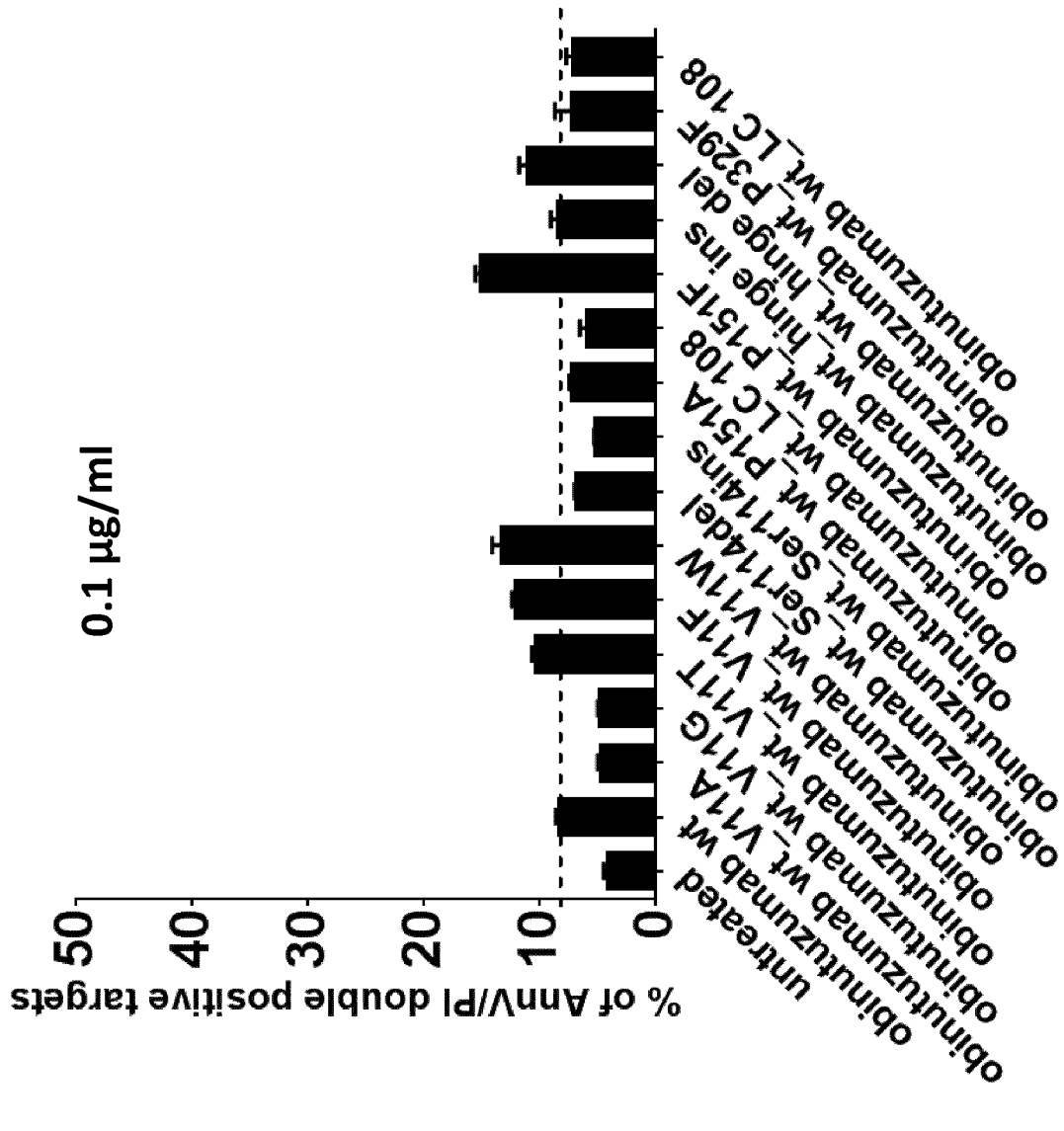

0.1 µg/ml
% of AnnV/PI double positive targets
0
10
20
30
40
50
untreated
obinutuzumab wt
obinutuzumab wt_V14A
obinutuzumab wt_V17G
obinutuzumab wt_V17T
obinutuzumab wt_V17F
obinutuzumab wt_V17W
obinutuzumab wt_Ser114del
obinutuzumab wt_Ser114ins
obinutuzumab wt_P151A
obinutuzumab wt_LC 108
obinutuzumab wt_P151F
obinutuzumab wt_hinge ins
obinutuzumab wt_hinge del
obinutuzumab wt_P329F
obinutuzumab wt_LC 108

Figure 10 C

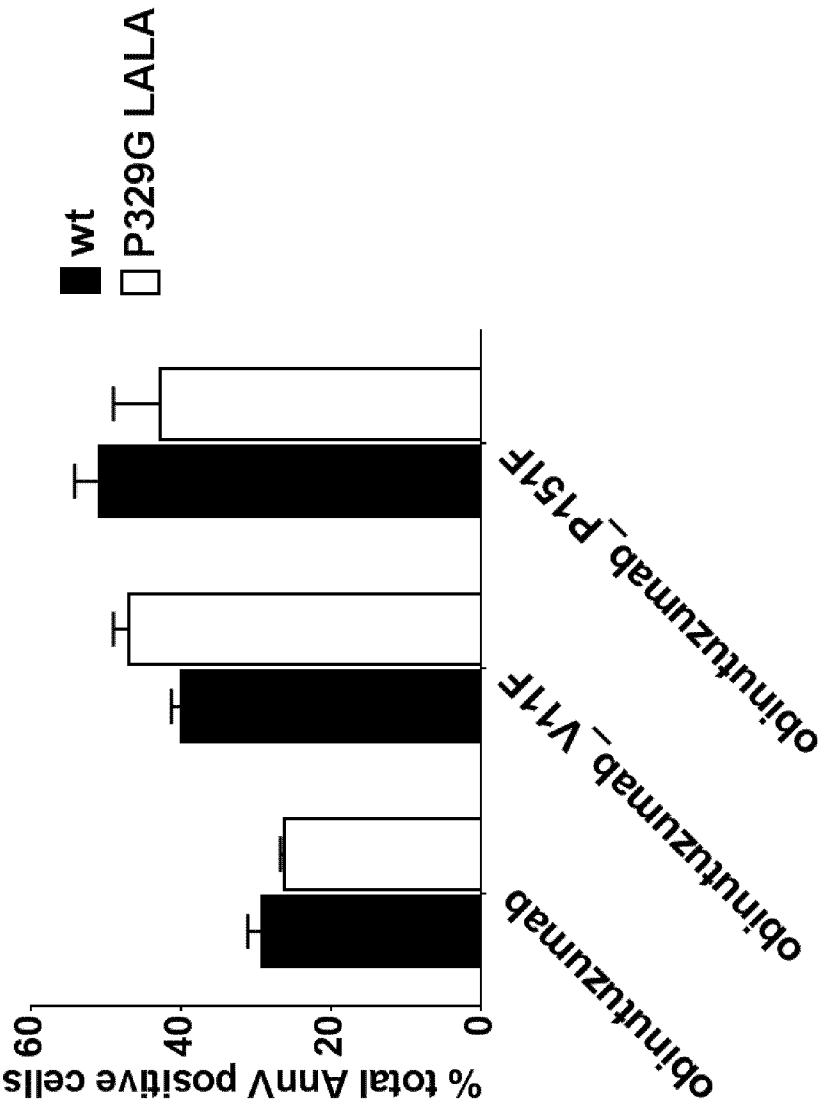

wt
P329G LALA
% total AnnV positive cells
0
20
40
60
obinutuzumab
obinutuzumab_V17F
obinutuzumab_P15TF

Figure 10 D

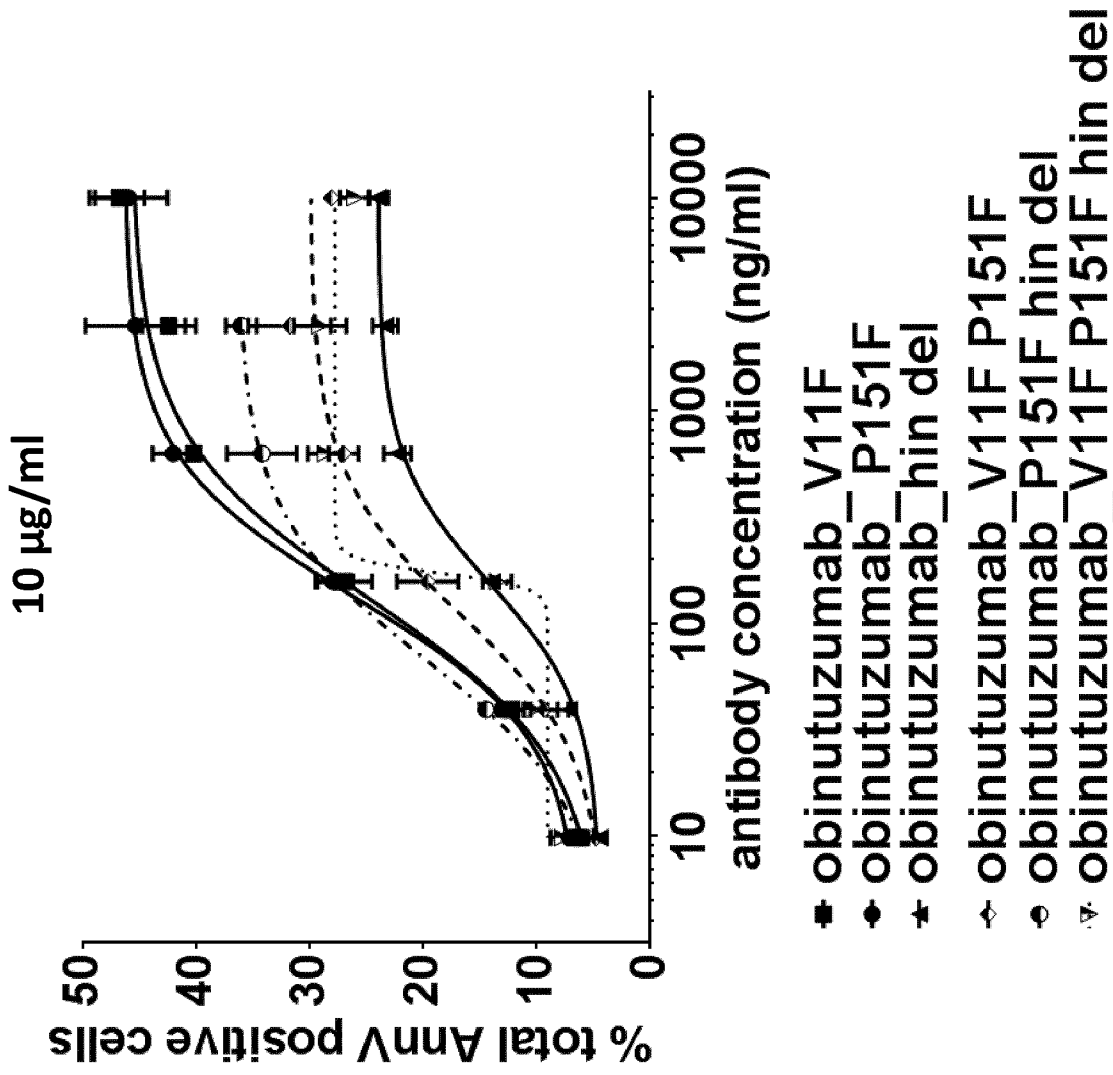

D
10 µg/ml
% total AnnV positive cells
0
10
20
30
40
50
10
100
1000
10000
antibody concentration (ng/ml)
obinutuzumab_V11F
obinutuzumab_P151F
obinutuzumab_hin del
obinutuzumab_V11F P151F
obinutuzumab_P151F hin del
obinutuzumab_V11F P151F hin del

Figure 11

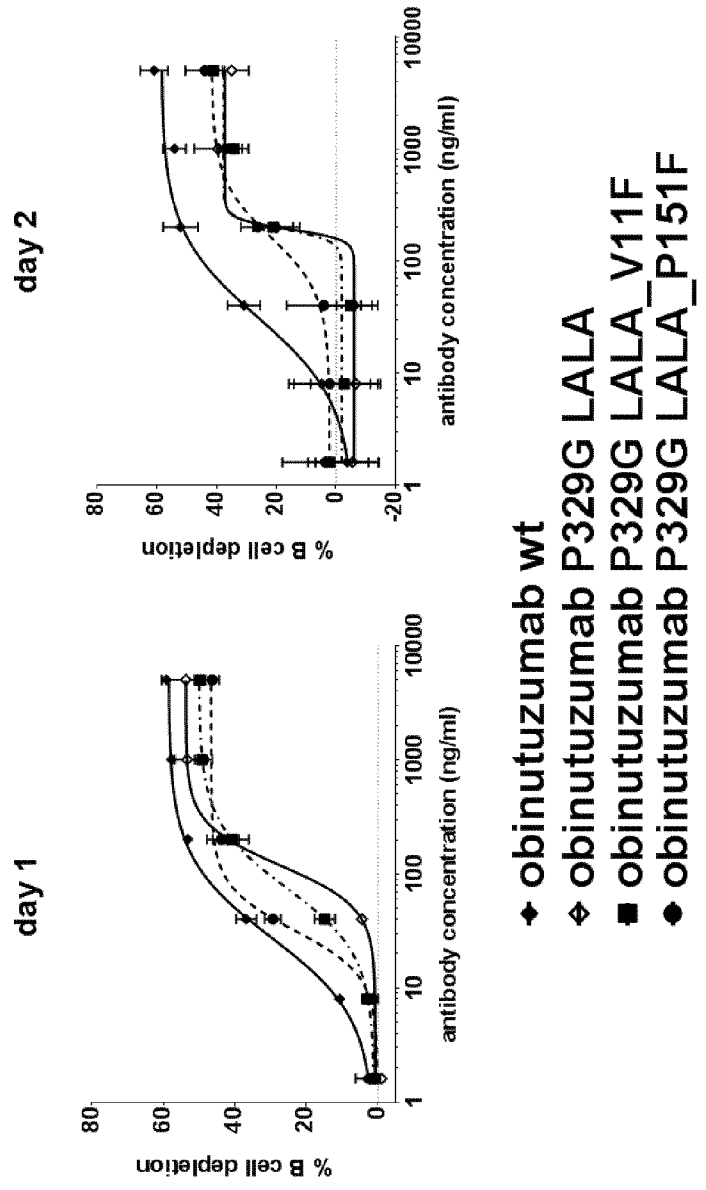

day 1
day 2
% B cell depletion
antibody concentration (ng/ml)
obinutuzumab wt
obinutuzumab P329G LALA
obinutuzumab P329G LALA_V11F
obinutuzumab P329G LALA_P151F

Figure 12

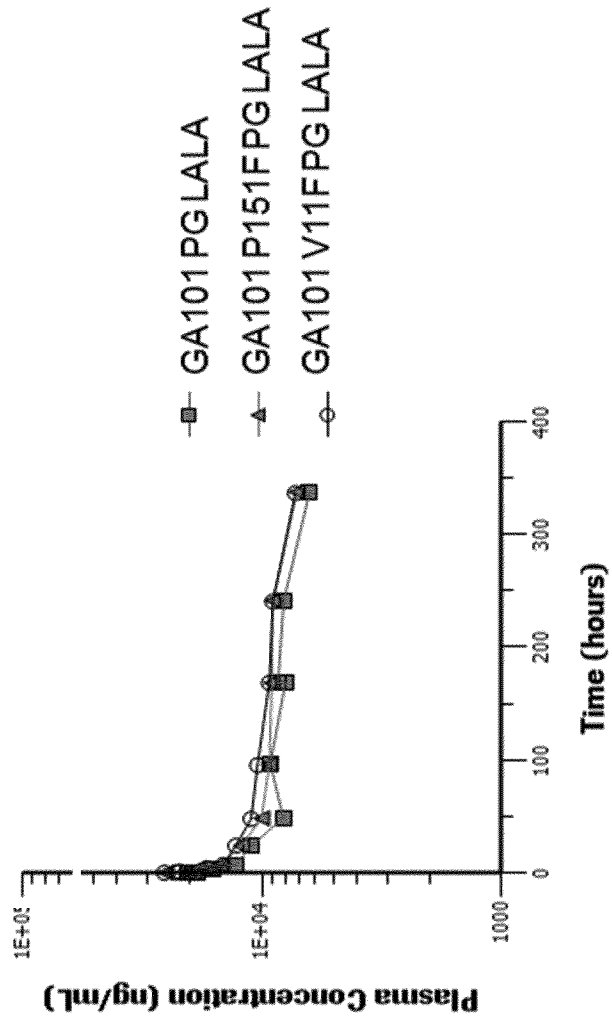

GA101 PG LALA
GA101 P151F PG LALA
GA101 V11F PG LALA
Plasma Concentration (ng/mL)
1E+01
1E+04
1000
0
100
200
300
400
Time (hours)

Figure 13 A

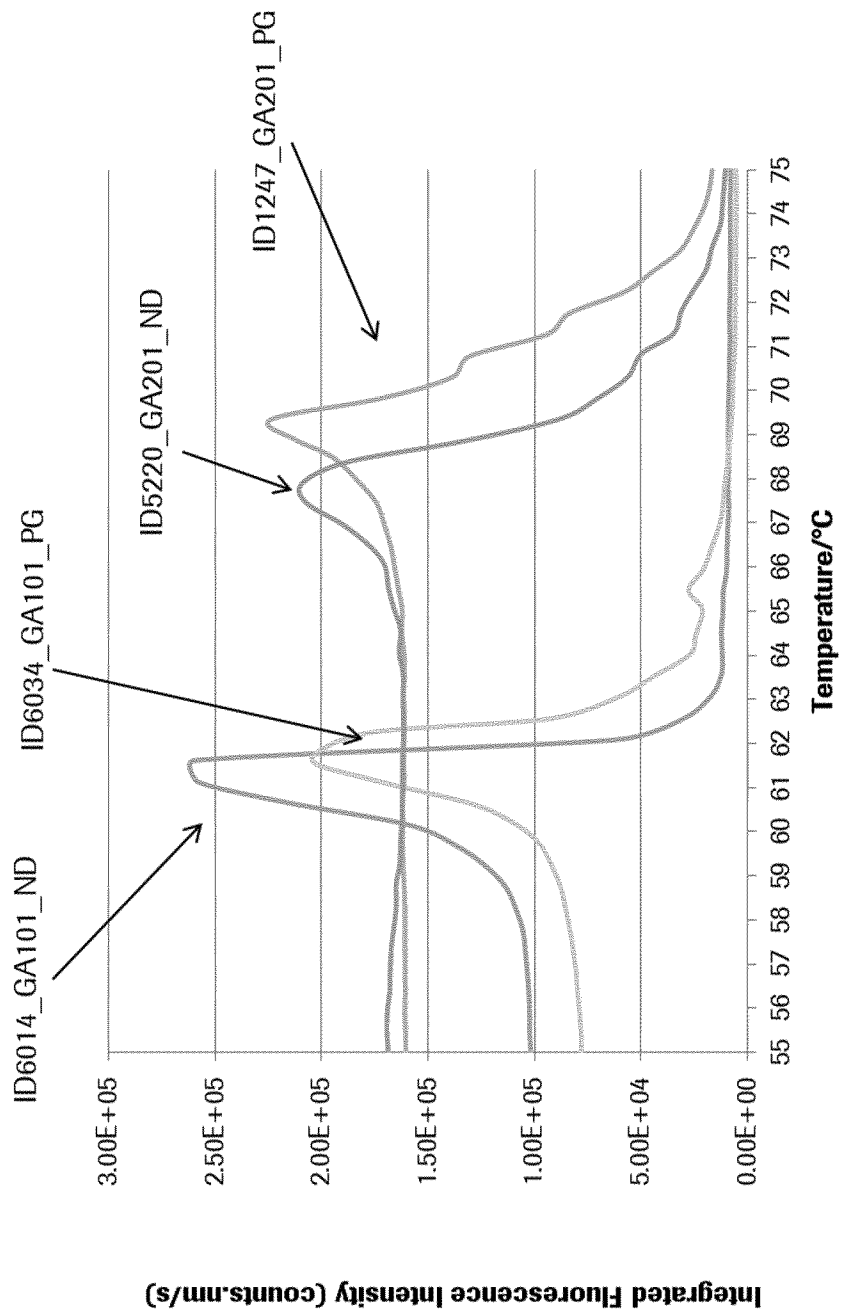
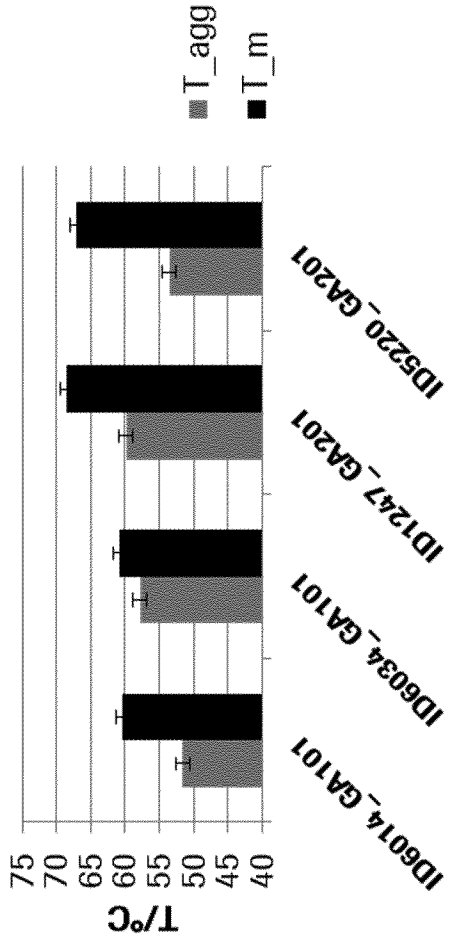

ID6014_GA101_ND
ID6034_GA101_PG
ID5220_GA201_ND
ID1247_GA201_PG
Integrated Fluorescence Intensity (counts.nm/s)
3.00E+05
2.50E+05
2.00E+05
1.50E+05
1.00E+05
5.00E+04
0.00E+00
55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75
Temperature/°C

Figure 13 B

Sample	T_agg/°C	T_m/°C
ID6014_GA101_ND	51.6	60.4
ID6034_GA101_PG	57.8	60.8
ID1247_GA201_PG	59.8	68.5
ID5220_GA201_ND	53.5	67.0



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/054542

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/28 A61K39/00
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 A61K

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, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2012/251531 A1 (BAEHNER MONIKA [DE] ET AL) 4 October 2012 (2012-10-04) claim all; figure all; example all	1-19
X	NZ 588 860 A (ROCHE GLYCART AG) 30 March 2012 (2012-03-30) claim all; figure all; example all	1-19

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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

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

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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 April 2017	22/05/2017

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

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2017/054542

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		CN 1902231 A	24-01-2007
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		CR 11848 A	04-04-2011
		CY 1110301 T1	14-01-2015
		DK 1692182 T3	31-05-2010
		DK 2077282 T3	30-01-2017
		DK 2380910 T3	19-10-2015
		EA 200600905 A1	29-12-2006
		EA 201100128 A1	30-06-2011
		EA 201300481 A1	30-01-2014
		EC SP066603 A	17-10-2006
		EP 1692182 A2	23-08-2006
		EP 2077282 A2	08-07-2009
		EP 2348051 A2	27-07-2011
		EP 2380910 A1	26-10-2011
		EP 2380911 A1	26-10-2011
		ES 2341009 T3	14-06-2010
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Information on patent family members

International application No
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		PT 2380910 E	26-11-2015
		RS 51334 B	28-02-2011
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		US 2016075794 A1	17-03-2016
		US 2016076009 A1	17-03-2016
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		ZA 200604547 B	25-07-2007
