ACTIVE PROTEASE-RESISTANT ANTIBODY Fc MUTANTS

BACKGROUND

Prior Application

This application claims priority to U.S. application Serial No. 61/426,619, filed December 23, 2010 and U.S. application Serial No. 61/540,882 filed September 29, 2011, which is entirely incorporated herein by reference.

Field of the Invention

The invention relates to human antibody IgG constant regions, especially the Fc regions, mutated so as to alter proteolytic cleavage sites conferring resistance to endogenous and pathogen-derived proteases and further altered to specifically bind Fcγ receptors and activate mitogenic responses by immune cells by Fc receptor mediated cross-linking or activation of the complement cascade. The novel sequences may be incorporated into therapeutic antibody compositions wherein proteolytic resistance and cytotoxic effector functionality is desirable.

Discussion of the Field

The IgG isotype of human antibodies consists of subtypes IgG1, IgG2, IgG3, and IgG4, each containing two antigen binding arms (Fabs) connected to a single Fc domain by the hinge region. IgG1, the predominant subclass represented in therapeutic monoclonal antibodies, are considered stable molecules with long half-life in circulation of 17.6 to 56.2 days (Salfeld, Nat Biotechnol 25(12):1369-72, 2007). However, IgG1 is susceptible to proteolysis in the hinge region by a number of physiologically-relevant proteases associated with invasive cancer (e.g. matrix metalloproteinases) and pathological microorganisms. Cleavage above the disulfide bonds (core hinge) between the heavy chains, liberates the monovalent Fab and bilateral cleavage below the disulfide bonds liberates a bivalent structure, the $F(ab')_2$ fragment. Several metalloproteinases and two bacterial enzymes, glutamyl endopeptidase V8 of *Staphylococcus aureus* (GluV8) and Immunoglobulin degrading enzyme of *Streptococcus pyogenes* (IdeS), act on IgG1 in the lower hinge (below the disulfide bonds (Fig. 1) and ultimately produce a $F(ab')_2$ and an Fc fragment (Ryan et al., Mol Immunol 45(7):1837-46 2008) .

Where the efficacy of therapeutic monoclonal antibodies (mAbs) directed against cell surface antigens is related to elimination of the target cell, the antibody "effector functions"

imparted by the Fc domain are demonstrably involved and are important in the overall therapeutic effect of the antibody. (Bibeau et al., J Clin Oncol 27:1122-1129 2009; Cartron et al., Blood 99:754-758 2002; and Musolino et al., J Clin Oncol 26:1789-1796 2008). The Fc domain of the antibody interacting with Fc gamma receptors (FcγR) expressed on immune cells, as well as Fc domain interactions with complement are believed to contribute to the action of several monoclonal antibodies (mAbs) directed against cell surface antigens. These interactions can lead to the elimination of the mAb targeted cell by antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC).

Recently, it has been shown that a single proteolytic cleavage in one of the heavy chain polypeptides of an IgG1, is not disruptive to the association of the chains and therefore maintains longevity in the circulation antigen binding capacity; but causes a loss of the IgG1's ability to bind FcγRs and drive Fc-mediated effector functions (Brezski et al., Proc Natl Acad Sci USA 106:17864-17869 2009). Both single and multiple cleavages of therapeutic monoclonal antibodies may lead to species that bind target but have lost some or all efficacy. Thus, engineering protease-resistant, but effector function-enhanced Fc-platforms could provide a significant advantage for improving the efficacy of anti-cancer and anti-infectious disease therapeutics among other uses wherein the destruction of target cells or tissues is desired.

SUMMARY OF THE INVENTION

The present invention provides compositions of modified, immunoglobulin constant domains useful in engineering of antibody or antibody-like therapeutics. Immunoglobulins of the IgG class used as therapeutics, comprising an Fc region and targeting cell surface ligands, are particular candidates for modification using the present compositions. The modified immunoglobulins of the invention are resistant to proteolytic enzymes in the milieu targeted by the antigen binding domain of the IgG, for example, the intratumoral milieu comprising a cancer cell or tumor vasculature associated antigen, yet retain important effector functions associated with the Fc region of the antibody.

In accordance with the invention, a means is provided of generating protease-resistant IgG1 monoclonal antibodies by incorporating mutations in the IgG1 Fc region that confer protease-resistance. In a further aspect, functional activities can be restored to IgGs containing such mutations by introduction of additional amino acid changes at distal positions in the Fc region.

The composition of the invention is a protease-resistant IgG1 antibody comprising altered amino acid residues in the lower hinge of IgG1, which residues have involvement in Fc engagement of FcγR as well as having been identified as sites of physiologically-relevant proteolytic cleavage. In one aspect, the residues of human IgG1 subclass are replaced with the corresponding lower hinge residues of human IgG2. In one embodiment, the sequence of E233-L234-L235-G236 of human IgG1 is replaced with IgG2 P233-V234-A235 with G236 deleted (EU numbering (Edelman et al., Proc Natl Acad Sci USA 63:78-85 1969)). In another embodiment, the IgG1 residues E233-L234-L235-G236-G237 are replaced with P233-V234-A235 with G236 deleted, and G237 is substituted with an amino acid selected from A, D, P, Q or S. In still another embodiment, all of the residues in the IgG1 constant domain corresponding to residues 214-236, KVEPKSCDKT HTCPPCPAPEL LG (SEQ ID NO: 3), are replaced with a sequence of the corresponding position in human IgG2 or TVERKCCVEC PPCPAPPVA (SEQ ID NO: 4).

In a second aspect, the ability of the protease-resistant IgG1 mutant of the invention to engage FcγR and/or complement can be restored by incorporating further mutations. Thus, without such further substitutions, the protease-resistant IgG1 mutant comprising a protease-resistant hinge region comprising P233-V234-A235 with G236 deleted, or the mutant wherein all of the IgG1 constant region sequences of residues 214-236 are replaced with the corresponding sequences from IgG2, (SEQ ID NO: 4), has reduced ability to engage FcγR. To overcome the loss of effector function related to FcR and C1q engagement, additional mutations are incorporated in the non-hinge constant region so that the modified IgG1 molecule retains measurable or even enhanced ability to engage FcγR as compared with the wild-type human IgG1. Thus, in one embodiment of the invention, the protease resistant IgG1 comprises those molecules comprising an Fc domain having the sequence of a human IgG1 in the hinge and CH2 regions, from about EU residues 214 to about residue 330, where at least residues 233-237 are substituted with PVA/ (G236 deletion) and, additionally, specific substitutions selected from S239D/I332E; K326A/E333A; H268F/S324T/I332E; F243L/R292P/Y300L; S239D/H268F/S324T/I332E; S267E/H268F/S324T/I332E; E333A/K334A; G237A/S239D/I332E; and G237S/S239D/I332E; S298A/E333A/K334A; S239D/K326A/E333A and S267E/I332E. In a specific embodiment, the IgG molecule derived from the wild-type IgG1 constant domain sequences comprises SEQ ID NO: 6. In another embodiment, the protease-resistant IgG is derived from the wild-type IgG2 constant domains with substitutions at of S239D and I332E. Thus, the inclusion of these additional mutations allowed Fc functions while maintaining protease resistance, and, in some cases, improved the

Fc-mediated effector function over IgG1 wild type in both in vitro FcγR-binding assays as well as in vitro cellular assays such as antibody-dependent cellular cytotoxicity (ADCC). A protease-resistant monoclonal antibody with effector function incorporating an Fc domain of the present invention is useful for anti-cancer and anti-microbial therapeutics where IgG cleavage due to elevated local protease presence at the sites of tumor growth or infection.

In another aspect, the compositions as described herein find utility as components of therapeutic molecules administered to mammalian subjects in need thereof. In one method of using the composition, an antibody comprising wild-type human IgG1 sequences and a ligand binding domain used in treating subjects in need thereof, is modified to incorporate the protease-resistant hinge sequences P233-V234-A235 with G236 deleted, and, where desired, effector function restoring modifications selected from S239D/I332E; K326A/E333A; H268F/S324T/I332E; F243L/R292P/Y300L; K326A/I332E/E333A, S239D/K326A/E333A, and S267E/I332E S239D/H268F/S324T/I332E; S267E/H268F/S324T/I332E; E333A/K334A; G237A/S239D/I332E; and G237S/S239D/I332E. In one embodiment, the IgG1 Fc mutant composition is used in an indication where activation of FcγRs associated with immune and effector functions such as i) antibody dependent cytotoxicity (ADCC), ii) complement dependent cytotoxicity (CDC), iii) antibody dependent cellular phagocytosis (ADCP) iv) FcR-mediated cellular activation (e.g. cytokine release through FcR cross-linking), and v) and FcR-mediated platelet activation/depletion is essential to the efficacy of the composition. In one aspect, the IgG1 Fc mutations are incorporated into therapeutic antibodies or Fc-fusions of multivalent binders targeting ligands on cells of proliferative disorders, such as tumor cells; tumor vasculature cells; fibroblasts; activated B-cell or T-cells, or to pathogenic host or non-host derived cells, especially bacterial cells.

In another embodiment, the IgG1 Fc mutant comprises a pharmaceutical composition. In another embodiment the IgG1 Fc mutant comprises a portion of a pharmaceutically active molecule. The pharmaceutical compositions comprising the IgG1 Fc mutant or active IgG1 Fc mutant-comprising molecules are useful for the treatment of diseases characterized by unwanted or uncontrolled proliferation or migration of cells. In one aspect, the protease-resistant constant domain compositions of the invention, combined with a ligand binding domain, are administered to a subject in need thereof using a route of administration to a body compartment in which one or more proteases capable of degrading IgG1 subclass molecules is found using systemic delivery methods or local delivery methods.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 is a depiction of a human IgG1 antibody accompanied by the amino acid sequence found in the hinge region, a region critical for interaction with FcγRs and complement, and mapped protease cleavage points.

Figure 2 shows an alignment of the amino acid sequences of portions of the constant regions of wild type human IgG1(SEQ ID NO: 1) and IgG2 (SEQ ID NO: 2) aligned with new constructs 2h S239D/I332E (SEQ ID NO: 8) and 2h E333A/K334A (SEQ ID NO: 9) showing the corresponding EU numbering for each residue; where the hinge region of both IgG1and IgG2 both comprise the cysteine residues at EU residue 226 and 229 as do the new constructs.

Figures 3A-B show digestion analyses of antibodies comprising different IgG isotypes (A) and new constructs thereof as described in Fig. 2 (B) with the protease IdeS at different time points.

Figure 4A-D shows digestion of IgG constructs with IdeS (A) GluV8 (B), MMP-3 (C), and MMP-12 (D) after a 24 hour incubation (n=2).

Figure 5A-G show an FcγR-binding results from ALPHASCREEN® analysis for groups of protease-resistant mAb constructs: FcγRI (A), FcγRIIa (B and C), FcγRIIb (D and E), and FcγRIIIa (F and G) where the reduction from % Maximal Signal represents the ability of an unlabeled construct to compete with biotinylated IgG1 from binding (n=2).

Figure 6A-C are graphs from separate ADCP assays performed with protease-resistant mAb constructs and wildtype IgG_1 and IgG_2 where the % Phagocytosis on the Y-axis is relative to the total number of sampled cells.

Figure 7A-C are graphs from separate ADCC assays performed with protease-resistant mAb constructs and wildtype IgG_1 and IgG_2 where the % Lysis on the Y-axis is relative to 100% lysis of the same number of cells by detergent (n=2).

Figure 8 is a graph from a CDC assay performed with protease-resistant mAb constructs and wildtype IgG_1 and IgG_2 where the % Lysis on the Y-axis is relative to 100% lysis of the same number of cells by detergent (n=2).

BRIEF DESCRIPTION OF THE SEQUENCE LISTING

SEQ ID NO:	Description
1	IgG1– Fc; Human Ig gamma class, subclass , hinge, CH2 and CH3 domains
2	IgG2 – Fc; Human Ig gamma class, subclass 2 hinge, CH2 and CH3 domains
3	IgG1 hinge region, EU 214-236
4	IgG2 hinge region (2 hc), EU 214-235
5	IgG hybrid hinge region (2h), IgG2 EU 233-235
6	2hc (EU 214-447)
7	2h (EU 214-447)
8	2h S239D/I332E
9	2h E333A/K334A
10	2h F243L/R292P/Y300L
11	2h H268F/S324T/I332E
12	2h S239D/H268F/S324T/I332E
13	2h S267E/H268F/S324T/I332E
14	2h K326A/E333A
15	2h G237X/S239D/I332E where X is A, D, P, Q or S
16	2hc S239D/I332E
17	IgG2 S239D/I332E
18	2h K326A/ I332E/ E333A
19	2h S239D/ K326A /E333A
20	2h S267E/ I332E

DETAILED DESCRIPTION OF THE INVENTION

Abbreviations

ADCC = antibody-dependent cellular cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; CDC = complement-dependent cytotoxicity; FDCR = Fc-dependent cytokine release; FcγR or FcgammaR = Fc gamma receptor; GluV8 = glutamyl endopeptidase V8 of *Staphylococcus aureus*; IdeS = Immunoglobulin degrading enzyme of *Streptococcus pyogenes* IgG = immunoglobulin G; ITAM = immunoreceptor tyrosine-based activating motif ; ITIM = immunoreceptor tyrosine-based inhibitory motif; Mab = monoclonal antibody; MMP = matrix metalloproteinase; the term protease is equivalent to proteinase and are used interchangeably; PR = protease resistant.

Definitions & Explanation of Terminology

"Antibody-dependent cellular cytotoxicity" "Antibody-dependent cell-mediated cytotoxicity" or ADCC" refers to a form of cytotoxicity in which secreted Ig bound onto Fc receptors (FcRs) present on certain cytotoxic cells (e.g., Natural Killer (NK) cells, neutrophils, and macrophages) enables these cytotoxic effector cells to bind specifically to an antigen-bearing target cell and subsequently kill the target cell with cytotoxins. Ligand specific high-affinity IgG antibodies directed to the surface of target cells stimulate the cytotoxic cells and are absolutely required for such killing. Lysis of the target cell is extracellular, requires direct cell-to-cell contact, and does not involve complement.

The ability of any particular antibody to mediate lysis of the target cell by ADCC can be assayed. To assess ADCC activity, an antibody of interest is added to target cells displaying the target ligand in combination with immune effector cells, which may be activated by the antigen antibody complexes resulting in cytolysis of the target cell. Cytolysis is generally detected by the release of label (e.g. radioactive substrates, fluorescent dyes or natural intracellular proteins) from the lysed cells. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Specific examples of in vitro ADCC assays are described in Wisecarver et al, 1985, 19:211; Bruggemann et al, 1987, J Exp Med 166:1351; Wilkinson et al, 2001, J Immunol Methods 258:183; Patel et al, 1995 J Immunol Methods 184:29 (each of which is incorporated by reference). Alternatively, or additionally, ADCC activity of the antibody of interest may be assessed in vivo, e.g., in a animal model such as that disclosed in Clynes et al, 1998, PNAS USA 95:652, the contents of which is incorporated by reference in its entirety. Where the effector cell acts largely through

phagocytosis, the process can be described as Antibody Dependent Cellular Phagocytosis (ADCP).

"Complement-directed cytotoxicity" or CDC refers to the form of cytotoxicity in which the complement cascade is activated by the complement component C1q binding to antibody Fc.

The term "effector functions" include those interactions of Fc with Fc gamma receptors (FcγR) expressed on immune cells, as well as Fc domain interactions with complement leading to elimination of the antigen-expressing cell by lytic processes or phagocytosis by effector cells and complement components.

The terms "Fc," "Fc-containing protein" or "Fc-containing molecule" as used herein refer to a monomeric, dimeric or heterodimeric protein having at least an immunoglobulin CH2 and CH3 domain. The CH2 and CH3 domains can form at least a part of the dimeric region of the protein/molecule (e.g., antibody).

The term "antibody" as used herein is a specific form of an Fc-containing protein comprising at least one ligand binding domain which contains, or retains substantial homology to, at least one of a heavy or light chain antibody variable domain of at least one species of animal antibody.

Wild type human IgG subclass constant sequences are cataloged in the UniProt database available on-line as P01857 (IgG1), P01859 (IgG2), P01860 (IgG3), and P01861 (IgG4). As used herein, "wild type human IgG1 Fc region" refers to a human IgG Fc region that comprises the amino acid sequence of SEQ ID NO: 1 or a fragment thereof, which is from residue K214 to residue K447 of the human IgG heavy chain, according to the EU numbering of Kabat. Amino acids in the constant region are numbered by alignment with the human IgG1 antibody, EU (see Cunningham et al., 1970Biochemistry., 9: 3161-70). That is, the heavy and light chains of an antibody are aligned with the heavy and light chains of EU to maximize amino acid sequence identity and each amino acid in the antibody is assigned the same number as the corresponding amino acid in EU. The EU numbering system is conventionally used in the art (see generally, Kabat et al, Sequences of Protein of Immunological Interest, NIH Publication No. 91-3242, US Department of Health and Human Services (1991)). According to the convention, the alignment between the wildtype IgG2 constant region and that of EU results in an empty amino acid at positions 221-223 and 236 (Fig. 2, SEQ ID NO: 2).

The constant domain sequences of the mammalian IgG heavy chain are designated in sequence as CH1-hinge-CH2-CH3. The "hinge", "hinge region" or "hinge domain" of an IgG

is generally defined as including Glu216 and terminating at Pro230 of human IgG1 according to the Kabat system but functionally, the flexible portion of the chain may be considered to include additional residues termed the upper and lower hinge regions, such as from Glu216 to Gly237 (Roux et al. J. Immunol. 1998, 161:4083) and the lower hinge has been referred to as residues 233 to 239 of the Fc region where FcgammaR binding was generally attributed. Hinge regions of other IgG isotypes may be aligned with the IgG1 sequence by placing the first and last cysteine residues forming inter-heavy chain S--S binds. Although boundaries may vary slightly, as numbered according to the Kabat system, the CH1 domain is adjacent to the VH domain and amino terminal to the hinge region of an immunoglobulin heavy chain molecule and includes the first (most amino terminal) constant region domain of an immunoglobulin heavy chain, e.g., from about EU positions 118-215. The Fc domain extends from amino acid 231 to amino acid 447; the CH2 domain is from about Ala231 to Lys340 or Gly341 and the CH3 from about Gly341 or Gln342 to Lys447. The residues of the IgG heavy chain constant region of the CH1 region terminate at Lys.

The term "protease resistant" refers to the ability of a molecule comprised of peptide bonds, to resist hydrolytic cleavage of one or more of its peptide bonds in the presence of a proteolytic enzyme. The resistance to proteolytic enzymes is a relative property and is compared to a molecule which is less able to withstand hydrolytic cleavage of one or more of its peptide bonds over a specified time period and under specified conditions, including the pH and or temperature at which the cleavage resistance is tested. One result of proteolytic cleavage indicative that cleavage has occurred is the generation of smaller fragments (lower molecular weight) as compared to the molecular weight of the intact, non-cleaved parent molecule.

The term "therapeutically effective amount" refers to an amount of an therapeutic agent as described herein comprising an Fc-domain which may be an antibody, antibody fragment, or derivative to treat a disease or disorder in a subject.

Overview

The present invention was motivated by an interest in identifying an Fc domain for use in the manufacture of therapeutic antibody, Fc-fusions, and like biopharmaceuticals with improved resistance to proteolysis *in situ* and with retained ability to cause cytokine release or damage or kill target antigen displaying cells and tissues surrounding targeted cells.

Proteases are divided into five major groups according to the structure of catalytic site and the amino acid (as one of the constituents) essential for its activity: serine proteinases, threonine proteinases, cysteine (thiol) proteinases, aspartic proteinases, and metalloproteinases.

Various extracellular proteases function throughout the body and in body compartments performing critical regulatory and metabolic processes. Acid-resistant proteases secreted into the stomach (such as pepsin) and serine proteases present in duodenum (trypsin and chymotrypsin) enable degradation of food protein within the gastrointestinal tract; proteases present in blood or serum (thrombin, plasmin, Hageman factor, etc.) play important role in blood-clotting, as well as lysis of the clots, and regulation of cells of the immune system. Proteases are present in or released from leukocytes (elastase, cathepsin G). Proteases determine the lifetime of other proteins thus playing an important metabolic role. Unlike hormones, interleukins or chemokines, no intracellular signaling or alteration in protein expression machinery is required, making proteolytic control one of the fastest regulatory switching mechanisms. Further, cooperative action of the proteases as in cascade reactions, results in a rapid and efficient amplification of an organism's response to a physiological signal.

Human IgG isotypes (the subclasses of mature gamma globulin class G antibodies; IgG1, IgG2, IgG3 and IgG4) exhibit differential capacity to recruit immune functions. For example, antibody-dependent cellular cytotoxicity (ADCC) is promoted by IgG1 and IgG3, antibody-dependent cellular phagocytosis (ADCP) is promoted by IgG1, IgG2, IgG3 and IgG4, and complement dependent cytotoxicity (CDC) is promoted by IgG1 and IgG3. Isotype-specific engagement of such immune functions is based on selectivity for Fc receptors on distinct immune cells and the ability to bind C1q thereby activating the assembly of a membrane attack complex (MAC). Among the various isotypes, relative affinity for Fcγ receptors, which include FcγRI, FcγRIIa/b/c, and FcγRIIIa/b; is high for IgG1 and IgG3. However, Fcγ affinity for IgG2 is considerably lower with the exception of FcγRIIa H131 polymorphism and IgG4 only has measurable affinity for FcγRI. Using comparative sequence analysis and co-crystal structures, the key contact residues for receptor binding have been mapped to the amino acid residues spanning the lower hinge and CH2 region ((Hezereh et al., J Virol 75:12161-12168 2001; Shields et al., J Biol Chem 276:6591-6604 2001).

Many previous studies concluded that substitutions in the residues of the lower hinge of IgG2 (EU positions 233-235) abrogates FcγR-mediated function and complement activation. In one report, the substitution of E233P-L234V-L235A with G236 deleted in the lower hinge of IgG1 was one of a panel of Fc constructs of human IgG1 shown to have lost Fc-mediated effector functions (Armour et al., 1999 Eur J Immunol 29:2613-2624). This information

suggests that replacing the residues in or near the hinge of IgG1 with the corresponding IgG2 residues resulted in a profound loss affinity for binding to FcγRs and loss of ability to affect complement fixation and cell killing.

Secondly, several of the same residue positions are where proteases cleave the IgG1 (Fig. 1) sequence and which are occupied by different amino acids in the more proteolytic resistant IgG2 sequence as shown in by alignment of human IgG1 and IgG2 (Fig. 2). The present inventors used this information as a starting point to design a protease-resistant but effector-function competent IgG-Fc.

The present invention is a demonstration for the first time of substitutions in multiple positions of the IgG1 constant regions (Fc) which unexpectedly provide a protease-resistant and functional (FcgR-engaging) Fc domain. As appreciated in the art, once the properties of a Fc-domain having a specific amino acid sequence are known, the information can be applied to the construction or modification of existing antibodies engineering or Fc-polypeptide fusions. The protease-resistance conferred by the compositions of the invention include, for example the proteases that cleave at residues of IgG1 hinge region that are substituted by alternate amino acids derived from the corresponding IgG2 residue, such as MMP-3, MMP-7, MMP-12, HNE, plasmin, cathepsin G, pepsin, IdeS, or glutamyl endopeptidase I from Staph aureus (Fig. 1) (Ryan et al. 2008 *supra*).

The multi-substituted IgG1 mutants were selected on the bases of their relative affinities for human FcRs (FcγRI, FcγRIIa, FcγRIIb, FcγRIIIa assessed by AlphaScreen® competition assays). These mutants were further tested in the appropriate cellular systems for their ability to induce ADCC by PBMCs and ADCP by in vitro differentiated macrophages. In the experimental data provided herein, the IgG1with the specified introduced mutations (Table 2) were engineered protease-resistant mAbs derived from IgG1 with an Fc enhancing mutation.

Alternative compositions to IgG1 hinge region residues 214-237 in the EU numbering system (SEQ ID NO: 3) were made using the wildtype (wt) human IgG1 (SEQ ID NO: 1) conferring protease-resistance include either replacement of the complete IgG2 sequence (SEQ ID NO: 4) hinge or the chimeric hinge (SEQ ID NO: 5) with only residues 233-235 altered as shown in Table 1.

Table 1.

Fc Designation	IgG Scaffold (EU 214-447)	Hinge/Proximal CH2 (EU 214-236)
IgG1 wt (UniProt P01857, 96-329)	hIgG1	KVEPKSCDKTHTCPPCPAPELLG (SEQ ID NO:3)
IgG2 wt (UniProt P01859, 97-326)	hIgG2	TVERKCCVECPPCPAPPVA (SEQ ID NO:4)
2 hc	hIgG1 with complete IgG2 hinge region	SEQ ID NO:4
2h	hIgG1	KVEPKSCDKTHTCPPCPAPPVA (SEQ ID NO:5)

Compensating mutations in effector function enhancing regions can be selected from previously described substitutions as shown in Table 2 below.

Table 2.

Mutations (EU numbered positions)	Reference
S239D/I332E	1
E333A/K334A	5
F243L/R292P/Y300L	3
H268F/S324T/I332E	4
S239D/H268F/S324T/I332E	4
S267E/H268F/S324T/I332E	4
K326A/E333A	2
K326A/I332E/E333A	1, 2
S239D/K326A /E333A	1, 2
S267E/I332E	1, 4
G237X/S239D/I332E where X is A, D, P, Q, or S	1
S298A/E333A/K334A	5

Increased Fc function constructs, previously cited by:

1. Lazar, Proc Natl Acad Sci USA 103:4005-4010 (2006)
2. Idusogie, J Immunol 166:2571-2575 (2001)
3. Stavenhagen, Cancer Res 67(18):8882-90 (2007)
4. Moore, et al. MAbs 2(2):181-189 (2010)
5. Shields et al., J Biol Chem 276:6591-6604 (2001)

Using various measures of Fc-function based on in vitro assays, several protease-resistant Fc sequences were identified, that when incorporated into a complete IgG structure (H2L2), provide resistance to one or more of proteases acting at lower hinge residues (EU232-237) while having the ability to bind FcγR or promote cytolysis. The changes in Fc-related activities of selected constructs comprising a change at the hinge as well as in the CH2 region and categorized by receptor affinity and *in vitro* bioactivity is shown in Table 3.

Table 3.

Isotype / Construct	FcγRI	FcγRIIa	FcγRIIb	FcγRIIIa	ADCC	ADCP	CDC
IgG1	+++++	++++	++++	+++	+++++	+++++	+++++
IgG2	-	++	-	+/-	-	++	-
IgG1 2h (PVA)	-	-	-	+/-	+	++	-
IgG1 2h E333A/K334A	-	+	-	+/-	-	++	n.d.
IgG1 2h S239D/I332E	+++	+++++	++++	++++	+++++	++++++	-
IgG1 2h S239D/H268F/S324T/I332E	n.d.	++++	+++++	++++	++++++	++++++	-
IgG1 2h S267E/H268F/S324T/I332E	n.d.	+++++	++++++	+++	+/-	+	+++++
IgG1 2h K326A/E333A	n.d.	-	-	+/-	+/-	+++	+++++

Method of Making the Altered Fc-Containing Molecules

The sites for substitution were chosen based on the desire to produce a composition having the structural features of a native antibody Fc, maintain stability, retain FcR binding and the capacity to stimulate the complement cascade, cell lysis, cell phagocytosis or cytokine

release. Proteins, particularly long polypeptide chain multimers, with altered or mutated amino acids are conveniently created by modification of a nucleic acid within an expression vector encoding the parental sequence in order to change the corresponding genetic codon for the desired amino acid. The genetic code and such methods are well known in the art. Where a chimeric sequence is created, such as the Fc of the invention comprising portions of IgG1 and portions of IgG2, larger segments of the respective encoding nucleic acids may be spliced together or segments replaced by standard cloning techniques.

Proteolysis Testing

In order to determine whether one Fc-containing composition or antibody is more proteolytic resistant than another, or than the wild-type composition, the rate or extent to which a proteolytic enzyme degrades the different isolated Fc-containing compositions or antibodies is assessed. After a time period, the degradation is measured for the different compositions using a method capable of determining either scission of the chain directly, such as the formation of a unique cleavage site structure, or a measurement of newly formed fragments. Alternatively, where cleavage results in loss of activity, a functional assay can be performed, including a binding assay.

Proteolytic cleavage of an IgG1 can occur at any of the four polypeptide chains of the dimeric heterodimeric structure. Cleavage of IgG results in the generation of well characterized fragments such as Fab, $(Fab')_2$, and Fc fragments of approximate but unique molecular weights. The separation of such fragments generated during a proteolysis experiment can be accomplished using a size exclusion chromatography (SEC), by gel electrophoresis, by MALDI-TOF-MS (matrix-assisted laser/desorption ionization time- of-flight mass spectrometry) analyses after deglycosylation using PNGase F (peptide N-glycosidase F) as previously described (WO2007024743A2, WO2009045894A1).

Therefore, what is meant by reference to an Fc-containing composition resistant to proteolytic cleavage is that the composition is less likely to be degraded, lose activity, lose affinity for an Fc-binding partner such as an FcR upon exposure to a proteolytic enzyme than a comparator molecule, such as a wild-type human IgG1.

Biological Characterization of the Mutants

Fc-containing proteins can be compared for functionality by several well-known in vitro assays. In particular, affinity for members of the FcγRI, FcγRII, and FcγRIII family of Fcγ receptors is of interest. These measurements can be made using recombinant soluble forms

of the receptors or cell-associated forms of the receptors. In addition, affinity for FcRn, the receptor responsible for the prolonged circulating half-life of IgGs, can be measured, for example using a ligand bound bead format such as "ALPHASCREEN" with recombinant soluble FcRn. AlphaScreen®, used in high throughput screening, is a homogenous assay technology which allows detection of molecular events such as binding. Coated "Donor" and "Acceptor" beads are the basis of the AlphaScreen® assay technology. AlphaScreen®, a bead based assay, works through the interaction of the beads coming close to each other, resulting in a cascade of chemical reactions that act to produce a greatly amplified signal. Direct or indirect, e.g. competitive binding, measurements can be applied for assessing relative affinities and avidities among proteins.

There has been a natural evolution of human IgG isotypes (e.g. IgG1, IgG2, IgG3 and IgG4), each exhibiting a different spectrum of capacities to recruit immune functions, such as antibody-dependent cellular cytotoxicity (ADCC, e.g. IgG1 and IgG3), antibody-dependent cellular phagocytosis (ADCP, e.g. IgG1, IgG2, IgG3 and IgG4), and complement dependent cytotoxicity (CDC, e.g. IgG1, IgG3). The isotype-specific engagement of these functions is based on differential selectivity for Fc receptors which reside in distinct immune cells and the ability to bind C1q and activate the assembly of a membrane attack complex (MAC) resulting in CDC and CDP (complement dependent phagocytosis) through specific receptors binding complement components on effector macrophages. The hierarchy of ability to bind the initial component, C1q, of the complement cascade, of human isotypes is IgG1>IgG3>IgG2>IgG4 although complement activation by IgG2 and IgG4 in microbial infection is well-documented.

Cell-based functional assays, such as ADCC assays and CDC assays, provide insights into the likely functional consequences of particular construct structures. Antibody-dependent cell-mediated cytotoxicity (ADCC) is a cell-mediated reaction in which nonspecific cytotoxic cells that express Fc receptors (FcRs) (e.g., Natural Killer (NK) cells, neutrophils, and macrophages) recognize bound antibody on a target cell and subsequently cause lysis of the target cell. In one embodiment, the ADCC assay is configured to have NK cells as the primary effector cell, reflecting the functional effects on the FcγRIIIa which is the only activating Fcγ-type receptor known to be expressed by these cells.

Phagocytosis assays may also be used to compare immune effector functions of different mutants, as can assays that measure cellular responses, such as superoxide or inflammatory mediator release. In vivo models can be used, for example, in the case of using mutants of anti-CD3 antibodies to measure T cell activation in mice, an activity that is dependent on Fc domains engaging specific ligands, such as Fcγ receptors. Antibody directed

activation of macrophages mediates antibody-dependent cellular phagocytosis (ADCP), causing opsonized target cells to be engulfed and digested by macrophages. *In vitro*, differentiated macrophages expressing high levels of FcRs can be differentiated into the M1 phenotype using INFγ or GM-CSF to expressed elevated levels of all FcRs (FcγRI, FcγRIIa, FcγRIIIa) relative to monocytes.

Production of Antibody Mutants

The compositions of the invention are complex proteins which are most conveniently produced by engineered host cells. As described herein, the host cell chosen for expression of the recombinant Fc-containing protein or monoclonal antibody is an important contributor to the final composition, including, without limitation, the variation in composition of the oligosaccharide moieties decorating the protein in the immunoglobulin CH2 domain. Thus, one aspect of the invention involves the selection of appropriate host cells comprising polynucleotide sequences encoding the Fc-comprising constructs of the invention for use as or development of a production cell expressing the desired therapeutic protein.

Further, the host cell may be of mammalian origin or may be selected from COS-1, COS-7, HEK293, BHK21, CHO, BSC-1, Hep G2, 653, SP2/0, 293, HeLa, myeloma, lymphoma, yeast, insect or plant cells, or any derivative, immortalized or transformed cell thereof.

Alternatively, the host cell may be selected from a species or organism incapable of glycosylating polypeptides, e.g. a prokaryotic cell or organism, such as and of the natural or engineered *E. coli spp, Klebsiella spp.,* or *Pseudomonas spp., engineered plant or insect cells.*

Glycosylation at the naturally occurring glycosylation site within the IgG heavy chain (N297, EU numbering) also contributes to the Fc-binding affinity for FcγR. As the constant regions vary with isotype, each isotype possesses a distinct array of N-linked carbohydrate structures, which variably affect protein assembly, secretion or functional activity (Wright, A., and Morrison, S. L., Trends Biotech. 15:26-32 (1997)). The structure of the attached N-linked carbohydrate varies considerably, depending on the degree of processing, and can include high-mannose, multiply-branched as well as biantennary complex oligosaccharides and sialic acid (N-acetyl neuraminic acid or NANA), fucose, galactose and GlcNAc (N-acetyl glucosamine) residues as terminal sugars. The impact on effector functions of the host cell and oligosaccharide content of the antibodies has been recognized (Lifely, M. R., et al., 1995 Glycobiology 5:813-822; Jefferis, R., et al., 1998 Immunol Rev. 163:59-76; Wright, A. and Morrison, S. L., *supra*; Presta L. 2003. Curr Opin Struct Biol. 13(4):519-25). Furthermore, regarding a sugar chain in an antibody, it is reported that addition or modification of fucose at

the proximal N-acetylglucosamine at the reducing end in the N-glycoside-linked sugar chain of an antibody changes the ADCC activity of the antibody significantly (WO00/61739).

Further, the relative contributions of galactosylation of the biantennary oligosaccharides, the presence of bisecting GlcNac, and fucosylation indicate that non-fucosylated Mabs display a greater capacity to enhance ADCC as measured *in vitro* and *in vivo* than other modifications to the N-linked biantennary oligosaccharide structures (Shields, et al. 2002. J Biol Chem. 277:26733-40; Niwa, et al. 2004. Cancer Res. 64:2127-2133).

Expression or manufacture of a protease-resistant mAb of the invention using a host cell capable of, engineered to (as by knock-out or knock down of specific enzymes Shinkawa, et al. 2003 J. Biol. Chem., 278: 3466-3473; EP1176195A1) or induced, e.g. by environmental or nutritional manipulation, to produce a mAb having low fucose content is within the scope of the present invention.

Antibodies, Fc and Fc-fusion proteins

An antibody binding domain or fragments thereof can be produced using methods known to those skilled in the art and combined with the information provided herein can include sequences or be derived from any mammal, such as, but not limited to, a human, a mouse, a rabbit, a rat, a rodent, a primate, a goat, or any combination thereof. Such antibodies may provide the basic structures and components of binding domains for useful in producing the antibody constructs of the present invention. In one aspect, the antibodies binding domains are obtained from hybridomas prepared by immunizing a mouse or other animal with the target peptides, cells or tissues extracts. The antibodies can be obtained using any of the hybridoma techniques well known in the art, see, e.g., Harlow and Lane, antibodies, a Laboratory Manual, Cold Spring Harbor, NY (1989) entirely incorporated herein by reference or by selection of an antibody producing lymphocyte and cloning the nucleic acid sequences coding for the binding domains using techniques known in the art.

The present invention is directed to the constant region of a human IgG. Therefore, any antibody or fusion protein comprising a human Fc-domain wherein it is desirable that the final composition display both proteolytic-resistance (as described herein) and the ability to bind FcgR and promote ADCC, ADCP, and/or CDC in an *in vitro* assay is encompassed by the present invention. A targeting moiety including but not limited to an antibody Fv or single variable domain may be fused to the Fc-composition as desired. An"Fv" consists of a dimer of one heavy- and one light-chain variable region domain in tight, non-covalent association. From the folding of these two domains emanate six hypervariable loops (3 loops each from the H and

L chain) that contribute the amino acid residues for antigen binding and confer antigen binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although often at a lower affinity than the entire binding site. "Single-chain Fv" also abbreviated as "sFv" or "scFv" are antibody fragments that comprise the VH and VL antibody domains connected into a single polypeptide chain. The scFv polypeptide generally comprises a polypeptide linker between the VH and VL. The targeting moiety may also be selected from a paratope of an antibody (binding residues not limited to CDRs or variable domain structures); an enzyme; a hormone; a receptor; a cytokine; an immune cell surface antigen; and an adhesion molecule when the construct is produced entirely by recombinant methods. The targeting moiety may also be of non-proteinaceous nature such as a carbohydrate, a lipid, a lipopolysaccharide, an organic molecule, or a metal or metal complex. Generally, when present, the targeting molecule will be connected to the Fc- by a linker which may be a polypeptide or nonpolypeptide.

Fc-comprising proteins or Fc fragments described herein can be derived in several ways well known in the art. The antibodies binding domains or Fc-fusion proteins or components and domains thereof may also be obtained from selecting from libraries of such domains or components, e.g., a phage library. A phage library can be created by inserting a library of random oligonucleotides or a library of polynucleotides containing sequences of interest, such as from the B-cells of an immunized animal or human (Smith, G.P. 1985. Science 228: 1315-1317). Antibody phage libraries contain heavy (H) and light (L) chain variable region pairs in one phage allowing the expression of single-chain Fv fragments or Fab fragments (Hoogenboom, et al. 2000, Immunol. Today 21(8) 371-8). The diversity of a phagemid library can be manipulated to increase and/or alter the immunospecificities of the monoclonal antibodies of the library to produce and subsequently identify additional, desirable, human monoclonal antibodies. For example, the heavy (H) chain and light (L) chain immunoglobulin molecule encoding genes can be randomly mixed (shuffled) to create new HL pairs in an assembled immunoglobulin molecule. Additionally, either or both the H and L chain encoding genes can be mutagenized in a complementarity determining region (CDR) of the variable region of the immunoglobulin polypeptide, and subsequently screened for desirable affinity and neutralization capabilities. Antibody or Fc libraries also can be created synthetically by selecting one or more human framework sequences and introducing collections of CDR cassettes derived from human antibody repertoires or through designed variation (Kretzschmar and von Ruden 2000, Current Opinion in Biotechnology, 13:598-602). The

positions of diversity are not limited to CDRs, but can also include the framework segments of the variable regions or may include other than antibody variable regions, such as peptides. In one aspect, a phage library capable of displaying dimeric Fc structures linked to the phage coat protein pIX as described in applicants co-pending application (U.S. Serial No. 61/261767) may be used to select novel Fc-comprising structures according to the present invention.

Libraries of target binding components, which may include target binding components other than antibody variable regions, may include ribosome display libraries, yeast display libraries, and bacterial display libraries. Ribosome display is a method of translating mRNAs into their cognate proteins while keeping the protein attached to the RNA. The nucleic acid coding sequence is recovered by RT-PCR (Mattheakis, L.C. et al. 1994 Proc. Natl. Acad. Sci. USA 91, 9022). Yeast display is based on the construction of fusion proteins of the membrane-associated alpha-agglutinin yeast adhesion receptor, aga1 and aga2, a part of the mating type system (Broder, et al. 1997 Nature Biotechnology, 15:553-7). Bacterial display is based on fusion of the target to exported bacterial proteins that associate with the cell membrane or cell wall (Chen and Georgiou 2002. Biotechnol Bioeng, 79:496-503).

The invention also provides for nucleic acids encoding the compositions of the invention as isolated polynucleotides or as portions of expression vectors including vectors compatible with prokaryotic, eukaryotic or filamentous phage expression, secretion and/or display of the compositions or directed mutagens thereof.

Use of the Fc-Containing Molecules

The compositions (antibody, Fc-fusions, Fc fragments) generated by any of the above described methods may be used to diagnose, treat, detect, or modulate human disease or specific pathologies in cells, tissues, organs, fluid, or, generally, a host. As taught herein, modification of the Fc portion of an antibody, Fc-fusion protein, or Fc fragment to reduce or ablate proteolytic degradation while retaining measurable Fc gamma receptor binding or specified effector functions can be combined with a binding domain, such as the paratope of an antibody or a ligand binding domain, which retains the original targeting specificity and biological activity. Examplary binding domains are a paratope of an antibody, one or more antibody CDRs, or one or more antibody variable domains; an enzyme; a hormone; a receptor; an extracellular domain of a membrane receptor; a cytokine; an immune cell surface antigen; and an adhesion molecule. The resulting constructs provide for antibodies and Fc-constructs with a superior spectrum of activities, biophysical properties, stability and ability to persist in the body of a host.

The applicants discovery of Fc sequences with unique combinations of resistance to physiologically-relevant proteases and ability or nonability to engage one or more Fcgamma receptors and/or the ability to affect cell lysis by activation of effector cells or complement provide for the ability to purpose the binding molecule for maximal effectiveness in a specified indication. For example, the ability to target aberrant host cells such as those involved in neoplasia or other unwanted proliferation such as in inappropriate angiogenesis, inappropriate fibrosis would be best suited by a molecule comprising a eukaryotic protease-resistant Fc of the present invention capable of ADCC and ADCP. In contrast, bacterial cells are readily destroyed by complement-mediated mechanisms. Therefore, bacterial infections may be treated by a suitable Fc-construct of the invention which is resistant to bacterial proteases and has the ability to invoke CDC.

Applicants have identified methods of selecting an Fc having the appropriate combination of properties and provided working examples of purpose specific modified Fc-comprising molecules. Molecules which eukaryotic protease-resistant and capable of one or more of ADCC, ADCP, and CDC include an Fc domain having the sequence of a human IgG1 in the hinge and CH2 regions, from about EU residues 214 to about residue 330 where at least residues 233-237 are substituted with PVA/ (G236 deletion) and further comprising one or more substitutions in the CH2 domain whereby the molecule is capable of one or more of ADCC, ADCP, and CDC include the constructs **5**, **7**, **8**, **9**, **10**, **11**, **12**, **14**, **15**, **16**, and **17**. In a particular embodiment, such molecules include substitutions selected from I332E in combination with other substitutions such as S239D/I332E (**5, 14**), S239D/H268F/I332E (not made), H268F/S324T/I332E (**8**), S239D/H268F/S324T/I332E (**9**), S267E/H268F/S324T/I332E (**10**), G237X/S239D/I332E where X is A or S (**12**); , K326A/I332E/E333A (**15**), and S267E/I332E (**17**).

Molecules which are resistant to a prokaryotic protease and capable of CDC include those molecules comprising an Fc domain having the sequence of a human IgG1 in the hinge and CH2 regions, from about EU residues 214 to about residue 330 where at least residues 233-237 are substituted with PVA/ (G236 deletion) and further comprising one or more substitutions in the CH2 domain selected from K326A/E333A (**11**), S267E/H268F/S324T/I332E (**10**), K326A/I332E/E333A (**15**), S239D/ K326A/E333A (**16**), and S267E/I332E (**17**).

Molecules which are eukaryotic protease-resistant but do not promote target cell lysis may also be advantageously used to treat a disease or conditions wherein target cell modulation and not target cell destruction is the objective such as by using an antibody or other Fc-

construct as taught herein that is protease-resistant but has decreased ADCC, ADCP, or CDC as compared to wildtype IgG1. Molecules comprising a non-natural, non-wild type, Fc domain which are protease-resistant include those molecules comprising an Fc domain having the sequence of a human IgG1 in the hinge and CH2 regions, from about EU residues 214 to about residue 330 where at least residues 233-237 are substituted with PVA/ (G236 deletion).

Thus, based on the teachings and examples herein, the presently enabled Fc-comprising constructs demonstrating enhanced resistance to a protease occurring in the mammalian subject and, optionally, having the ability to target and antigen on a cell provide improved therapeutic molecules relative to the therapeutic molecule which is not protease resistant.

Administration

As proteolytic enzymes are localized according to their rate of formation or accumulation, for example pepsin in the digestive tract, or matrix metalloproteinases (MMPs) in regions of tissue remodeling or malignant growth, the compositions of the invention are particularly suited for uses in which a body compartment is known to contain proteases or abnormally high protease content.

The invention provides for stable formulations of a protease-resistant IgG composition such as an antibody, which is preferably an aqueous phosphate buffered saline or mixed salt solution, as well as preserved solutions and formulations as well as multi-use preserved formulations suitable for pharmaceutical or veterinary use, comprising at least one protease-resistant antibody in a pharmaceutically acceptable formulation. Suitable vehicles and their formulation, inclusive of other human proteins, e.g., human serum albumin, are described, for example, in e.g. Remington: The Science and Practice of Pharmacy, 21st Edition, Troy, D.B. ed., Lipincott Williams and Wilkins, Philadelphia, PA 2006, Part 5, Pharmaceutical Manufacturing pp 691-1092, See especially pp. 958-989.

A protease-resistant IgG composition with effector function in stable or preserved formulations as described herein or known in the art, can be administered to a patient in accordance with the present invention via a variety of delivery methods including intravenous (I.V.); intramusclular (I.M.); subcutaneously (S.C.); transdermal; pulmonary; transmucosal; using a formulation in an implant, osmotic pump, cartridge, micropump; or other means appreciated by the skilled artisan, as well-known in the art.

For site specific administration to a body compartment or cavity, the administration may be intrarticular, intrabronchial, intraabdominal, intracapsular, intracartilaginous, intracavitary, intracelial, intracelebellar, intracerebroventricular, intracolic, intracervical,

intragastric, intrahepatic, intramyocardial, intraosteal, intrapelvic, intrapericardiac, intraperitoneal, intrapleural, intraprostatic, intrapulmonary, intrarectal, intrarenal, intraretinal, intraspinal, intrasynovial, intrathoracic, intrauterine, intravesical, intralesional, vaginal, rectal, buccal, sublingual, intranasal, or by transdermal means.

The diseases or pathologies that may be amenable to treatment using a composition provided by the invention include, but are not limited to, diseases in which unwanted proliferation, activation or migration of cells is deleterious such as malignancy, hyper-active or unbalanced immune responses, fibrotic tissue formation, or infection as the compositions provide for the activation of cytotoxic or cytolytic mechanisms of the host immune system through FcγR-driven mechanisms. Such diseases include malignancies: leukemia, acute leukemia, acute lymphoblastic leukemia (ALL), B-cell, T-cell or FAB ALL, acute myeloid leukemia (AML), chronic myelocytic leukemia (CML), chronic lymphocytic leukemia (CLL), hairy cell leukemia, myelodyplastic syndrome (MDS), a lymphoproliferative disease, cutaneous T-cell lymphoma, Hodgkin's disease, Castleman's disease, glioma, glioblastoma, astracytoma, a malignant lymphoma, non-Hodgkin's lymphoma, Burkitt's lymphoma, multiple myeloma, Kaposi's sarcoma, colorectal carcinoma, pancreatic carcinoma, renal cell carcinoma, breast cancer, ductal carcinoma, lipoma, nasopharyngeal carcinoma, prostate cancer, testicular cancer, ovarian cancer, retinoblastoma, malignant histiocytosis, hypercalcemia of malignancy, plasmacytomas, chondrosarcomas, sarcomas, Merkel cell cancer, heptocellular carcinoma, hepatoma, basal cell cancer, adenocarcinomas, squamous cell carcinomas, sarcomas (such as Ewings, Kaposi's, childhood soft tissue, adult), melanomas, metastatic melanoma, hemangioma, metastatic disease, osteosarcoma, rhabdomyosarcoma, thymoma and thymic carcinoma, cancer related bone resorption, endometrial cancer, vaginal cancer, uterine cancer, Wilms tumors, cancer related bone pain, and the like.

Targeted molecules capable of binding antigens on malignant lymphocytes include B-cell antigens such as CD19, CD20, and CD22. Solid tumors derived from epidermal tissue often display and are stimulated by ligand binding to epidermal growth factor receptors known as the ErbB1, ErbB2, ErbB3 and other receptor capable of signaling or causing a proliferative response or an anti-apoptotic response leading to unchecked growth of the tumor. Other common antigens on solid tumors are tissue factor or RON.

The compositions, when combined with an appropriate target binding domain, are also useful in treating an infectious disease caused by bacterial (such as Streptococcus, Staphylococcus, and E. coli.), viral (such as influenza, AIDS, RSV, SARS, and West Nile

Virus), fungal (such as Aspergillosis, coccidiodomycosis, cryptococcosis, or Candidiasis), or protozoan infection (such as trypanosomiasis, toxoplasmosis, giardia, or malaria).

The compositions are useful in treating general immunological and autoimmune disorders including but not limited to the rheumatic diseases, psoriasis, and scleroderma.

The compositions are useful in treating disorders associated with inappropriate angiogenesis. Angiogenesis is the process of generating new capillary blood vessels, and it results from activated proliferation of endothelial cells. Neovascularization is tightly regulated, and occurs only during embryonic development, tissue remodeling, wound healing and periodic cycle of corpus luteum development (Folkman and Cotran, Relation of vascular proliferation to tumor growth, Int. Rev. Exp. Pathol.'16, 207-248(1976)). Endothelial cells normally proliferate much more slowly than other types of cells in the body. However, if the proliferation rate of these cells becomes unregulated, pathological angiogenesis can result. Pathological angiogenesis is involved in many diseases. For example, cardiovascular diseases such as angioma, angiofibroma, vascular deformity, atherosclerosis, synechia and edemic sclerosis; and opthalmological diseases such as neovascularization after cornea implantation, neovascular glaucoma, diabetic retinopathy, angiogenic corneal disease, macular degeneration, pterygium, retinal degeneration, retrolental fibroplasias, and granular conjunctivitis are related to angiogenesis. Chronic inflammatory diseases such as arthritis; dermatological diseases such as psoriasis, telangiectasis, pyogenic granuloma, seborrheic dermatitis, venous ulcers, acne, rosacea (acne rosacea or erythematosa), warts (verrucas), eczema, hemangiomas, lymphangiogenesis are also angiogenesis-dependent.

Diabetic retinopathy can take one of two forms, non-proliferative or proliferative. Proliferative retinopathy is characterized by abnormal new vessel formation (neovascularization), which grows on the vitreous surface or extends into the vitreous cavity. Macular degeneration, likewise takes two forms, dry and wet. In exudative macular degeneration (wet form), which is much less common, there is formation of a subretinal network of choroidal neovascularization often associated with intraretinal hemorrhage, subretinal fluid, pigment epithelial detachment, and hyperpigmentation. Neovascular glaucoma occurs in patients with diabetes or central retinal vein occlusion or inflammatory precipitates associated with uveitis pulling the iris up into the angle (Ch. 99. The Merck Manual 17th Ed. 1999).

Rheumatoid arthritis, an inflammatory disease, also results in inappropriate angiogenesis. The growth of vascular endothelial cells in the synovial cavity is activated by the

inflammatory cytokines, and results in cartilage destruction and replacement with pannus in the articulation (Koch AK, Polverini PJ and Leibovich SJ, Arth; 15 Rhenium, 29, 471-479(1986); Stupack DG, Storgard CM and Cheresh DA, Braz. J. Med. Biol. Res., 32, 578-581(1999); Koch AK, Arthritis Rheum, 41, 951 962(1998)).

The compositions are useful in treating psoriasis, which is caused by uncontrolled proliferation of skin cells. Fast growing cell requires sufficient blood supply, and abnormal angiogenesis is induced in psoriasis (Folkman J., J. Invest. Dermatol. 59, 40- 48 (1972)).

A number of factors are involved in processes and events leading to angiogenesis: cell adhesion molecules, integrins, vascular endothelial growth factor (VEGF), TNFalpha, bFGF, and cytokines including IL-6 and IL-12. For example, the closely related but distinct integrins alphaVbeta3 and aVb5 have been shown to mediate independent pathways in the angiogenic process. An antibody generated against alphaVbeta3 blocked basic fibroblast growth factor (bFGF) induced angiogenesis, whereas an antibody specific to aVb5 inhibited vascular endothelial growth factor (VEGF) induced angiogenesis (Eliceiri, et al., J. Clin. Invest.103: 1227-1230 (1999); Friedlander et al., Science 270: 1500-1502 (1995)). Therefore, the invention encompasses the use of targeting binding domains directed to these targets in the compositions of the invention for use in treating diseases where the inhibition of angiogenesis is indicated.

The applicants co-pending published International Patent Application WO2009023457A1, hereby incorporated by reference into the present application, discloses a strategy to restore effector function to cleaved IgGs using anti-hinge cleavage site epitope specific monoclonal antibodies. Incorporating a protease-resistant and effector function competent Fc in accordance with the present invention with anti-hinge domains to produce a bivalent antibody would produce a therapeutic mAb capable of both restoring Fc-effector function to cleaved IgGs while rendering the therapeutic resistant to silencing by proteolytic cleavage. Accordingly, the present invention encompasses the incorporation of a protease-resistant Fc constant region of the invention with an anti-hinge variable region mAb as described.

While having described the invention in general terms, the embodiments of the invention will be further disclosed in the following examples that should not be construed as limiting the scope of the claims.

EXAMPLE 1: CONSTRUCTION OF AND TESTING OF Fc MUTANTS

A series of constructs (shown in Figure 2 and combining the hinge region of Table 1 with activity restoring mutations in the CH2 region selected from Table 2) were generated using standard recombinant technology. The designation 2hc denotes that the IgG1 constant domain corresponding to the Kabat numbering of the EU antibody (EU numbering) from 214 to 236 (SEQ ID NO: 3) have been replaced with the corresponding IgG2 sequence (SEQ ID NO: 4). The designation 2h denotes that the residues of IgG1 E233-L234-L235-G236 are replaced by the corresponding residues of IgG2 P233-V234-A235 with G235 deleted.

Table 4.

Con-struct	Designation/Construct	IgG Isotype Scaffold	EU 214-447 Sequence	CD142	CD20
1	IgG1 wt	hIgG1	SEQ ID NO: 1	X	X
2	IgG2 wt	hIgG2	SEQ ID NO: 2	X	X
3	2 hc (all hinge residues from IgG2)	hIgG1	SEQ ID NO: 6	X	
4	2h	hIgG1	SEQ ID NO: 7	X	X
5	2h S239D/I332E	hIgG1	SEQ ID NO: 8	X	X
6	2h E333A/K334A	hIgG1	SEQ ID NO: 9	X	
7	2h F243L/R292P/Y300L	hIgG1	SEQ ID NO: 10	X	X
8	2h H268F/S324T/I332E	hIgG1	SEQ ID NO: 11	X	X
9	2h S239D/H268F/S324T/I332E	hIgG1	SEQ ID NO: 12	X	X
10	2h S267E/H268F/S324T/I332E	hIgG1	SEQ ID NO: 13	X	X
11	2h K326A/E333A	hIgG1	SEQ ID NO: 14	X	X
12	2h G237X/S239D/I332E where X is A, D, P, Q or S	hIgG1	SEQ ID NO: 15	X	
13	2hc S239D/I332E	hIgG1	SEQ ID NO: 16	X	
14	IgG2 S239D/I332E	hIgG2	SEQ ID NO: 17	X	

The variable regions of the one set of antibody constructs bind to CD142 (tissue factor) which allowed testing of Fc-dependent cell-killing of the antibodies in cellular assays using MDA-MB-231 (ATCC, HTB-26™) expressing tissue factor (Brezski et al., Proc Natl Acad Sci USA 106:17864-17869 2009). An additional panel was generated with variable regions that bind to CD20 which allowed testing of CDC activity using WIL2-S cells

displaying CD20 (ATCC, CRL-8885) (Brezski et al., Proc Natl Acad Sci USA 106:17864-17869 2009). All of the antibodies were expressed transiently in 293T cells using standard cloning methods and procedures. MAbs were purified using protein A columns to greater than 95% purity prior to experimental analysis.

Protease Digestion of wild type and mAb constructs

Protease digestions of purified IgGs were carried out at pH 7.5 in phosphate-buffered saline (PBS) or, for the MMPs (MMP-3, MMP-7, MMP-12 and MMP-13 were all obtained from Enzo Life Sciences), in Tris-buffered saline buffer at 37 °C. IdeS was obtained from Genovis, and GluV8 was obtained from Pierce. $CaCl_2$ was included in the MMP reactions at 5 mM for all MMPs tested. Antibody concentrations were 0.5 mg/mL and reactions were initiated by addition of enzyme to approximately 1-2% (w/w) ratio to IgG unless otherwise specified. IgG cleavage was assessed by analysis of the electrophorograms after Agilent Biosizing microcapillary electrophoresis (Agilent Technologies). All digests were performed in duplicate.

ALPHASCREEN® competition binding assays

Competition binding studies were carried out in half-well volume 96-well opaque plates (Corning) in assay buffer (PBS, 0.05% BSA, 0.01% Tween-20) at pH 7.4. All competition studies were carried out against biotinylated IgG1 (1 IgG: 2 biotin, using EZ Link™ NHS-LC-biotin, Pierce) at a fixed concentration and competing wild type and protease-resistant constructs in serial 3-fold dilutions. FcγR concentrations were 0.2 μg/ml in final concentration of the assays. Biotinylated IgG1 (0.2 μg/ml final) and wild type and protease-resistant antibodies (10 μl) were sequentially added to each row of a 96-well plate in duplicates. Thereafter designated FcγRs were added followed by the sequential addition of 10 μl each of 1/50 diluted nickel chelate (Ni)-acceptor beads and streptavidin (SA)-donor beads. The opaque plate was covered with an aluminum seal to maintain light-safe conditions while shaking for 30 minutes on an Orbital shaker. Thereafter the seal was removed and the fluorescence was read on an ENVISION™ plate reader (PerkinElmer) equipped with appropriate filter set of AlphaScreen® excitation/emission spectra. Raw data was transferred to GraphPad PRISM™ software and normalized for maximal signal and competition curves were plotted using non-linear regression curve-fitting software.

Results

Of initial interest was to determine the susceptibility of mAb constructs to a number of physiologically-relevant proteases previously shown to cleave IgG1 in the lower hinge region;

MMP-3, MMP-7, MMP-12, MMP-13, GluV8 and IdeS. Constructs 1-5, 7, 9-11, 12 (G237A), 13-14 were tested as CD142 binding antibodies. The proteases cleaved IgG1 to a varying degree over 24 hours. Whereas MMP-3, MMP-12, IdeS elimnated all intact IgG1 (construct 1) within 24 hours, MMP-7 cleaved about 30%, MMP-13 about 40% and GluV8 about 60%. Construct 4 (2h) and those constructs with the 2h lower hinge modification were resistant to all of the MMPs to more or less the same degree. Construct 4 was resistant to GluV8 but not IdeS. Construct 2 were also more resistant to GluV8 digestion.

IdeS has been shown to cleave all human IgG isotypes (von Pawel-Rammingen et al., EMBO 21:1607-1615 2002). The data from a time course study (Figure 3A) indicate that IdeS rapidly converted IgG1 **(1)** and IgG2 **(2)** to the $F(ab')_2$ fragment (within 5 minutes of incubation) while the IgG1 sample 2h **(4)** had single-cleaved intermediate through 120 minutes. Of the constructs tested, 2h S239D/I332E **(5)** was the most proteolytic resistant construct to IdeS (Figure 3B), with intact IgG detected even after 24 hour incubation (the other antibody constructs had no detectable intact IgG by 5 minutes). The mutation of S239D in **(5)** which is near the IdeS cleavage point between G236 and G237, may contribute to the additional protease resistance as compared to IgG1 2h **(4)**.

A panel of 12 anti-CD142 antibody constructs (**1-5**, **7**, **9-13**, and **14**) was tested along with their wildtype IgG1 and IgG2 counterparts for susceptibility to proteolysis by IdeS, GluV8, MMP-3, and MMP-12, and. The data for intact IgG remaining after 24 hour incubation was calculated from the electrophorograms and shown in FIG. 4A-D.

The data demonstrated that IgG1 wt (**1**), IgG2 wt (**2**), 2hc (**3**), IgG1 2h (**4**), 2hc S239D/I332E (**13**), IgG2 S239D/I332E (**14**), and 2h K326A/E333A (**11**) were susceptible to proteolysis by IdeS after a 24 hour incubation. In contrast, the constructs 2h S239D/I332E (**5**), 2h G237A/S239D/I332E (**12**), 2h F243L/R292P/Y300L (**7**), 2h S239D/H268F/S324T/I332E (**9**), and 2h S267E/H268F/S324T/I332E (**10**) were resistant to proteolysis by IdeS. Surprisingly, constructs with more IgG2 hinge region substitutions (comprising SEQ ID NO: 4) were not resistant to IdeS proteolysis. The construct IgG2 S239D/I332E had less than 40% intact IgG remaining after 24 hours digestion with IdeS.

Digestion with the GluV8 protease from *Staph aureus* indicated that the constructs IgG2 S239D/I332E (**14**), 2h S239D/I332E (**5**), 2h G237A/S239D/I332E (**12**), and 2h F243L/R292P/Y300L (**7**) had a range of 40-60% intact IgG remaining after a 24 hour digestion, similar to the level of cleavage seen for IgG1 wt (**1**). The constructs IgG2 wt (**2**) , 2hc (**3**), 2hc S239D/I332E (**14**), IgG1 2h (**4**), IgG1 2h K326A/E333A (**11**), 2h S239D/H268F/S324T/I332E

(**9**), and 2h S267E/H268F/S324T/I332E (**10**) showed increased resistance to proteolysis by GluV8 (all having greater than 75% intact IgG remaining) relative to IgG1 wt.

MMP-3 and MMP-12 represent two types of cancer associated proteases. Less than 5% intact IgG1 wt was detected after digestion with both MMP-3 and MMP-12. In contrast, human IgG2 wt and all of the constructs tested displayed increased protease-resistance to both MMP-3 and MMP-12 as demonstrated by greater than 60% intact IgG remaining after 24 hours of digestion.

Fcγ Receptor Binding Results

The ability of some of the Fc- constructs to compete for binding to the Fcγ family of receptors with wt IgG1 was assessed. The ability of the constructs to reduce maxiumum signal produced by the biotinylated IgG1 are shown in Fig. 5A-G. The initial screens included Constructs **1-2, 4-6**. For the initial group of constructs tested, IgG2 (**2**), IgG1 2h (**4**), and 2h E333A/K334A (**6**) showed no detectable binding to the high affinity FcγRI, while a wild-type IgG1 showed robust binding. The 2h S239D/I332E (**5**) showed detectable but reduced binding to FcγRI compared to IgG1. The IgG1 2h (**4**) and 2h E333A/K334A (**6**) showed no detectable binding to FcγRIIa, while the 2h S239D/I332E (**5**) construct showed comparable binding to IgG1 wt (Fig. 5B). Three constructs: IgG2 (**2**), IgG1 2h (**4**), and 2h E333A/K334A (**6**) showed no detectable binding to FcγRIIb, while IgG1 (**1**) and 2h S239D/I332E (**5**) showed comparable binding (Fig. 5D). IgG2 (**2**), IgG1 2h (**4**), and 2h E333A/K334A (**6**) showed comparable, but reduced binding to FcγRIIIa compared to IgG1. The 2h S239D/I332E (**5**) construct displayed the highest level of binding to FcγRIIIa, even higher than IgG1 wt (Fig. 5F).

Additionally, the 2h S239D/H268F/S324T/I332E (**9**) construct displayed comparable binding to IgG1, whereas the 2h S267E/H268F/S324T/I332E (**10**) construct had increased binding to FcγRIIa compared to IgG1 wt (Figure 5C). Both of the constructs 2h S239D/H268F/S324T/I332E (**9**) and 2h S267E/H268F/S324T/I332E (**10**) displayed increased binding to FcγRIIb relative to IgG1 wt (Figure 5E). The construct 2h K326A/E333A (**11**) showed minimal detectable binding to both FcγRIIa (Figure 5C) and FcγRIIb (Figure 5E).

The construct 2h S267E/H268F/S324T/I332E (**10**) showed slightly decreased binding to FcγRIIIa compared to IgG1 wt, whereas the construct 2h S239D/H268F/S324T/I332E (**9**) had increased binding to FcγRIIIa (Figure 5G). The 2h K326A/E333A (**11**) displayed weak binding to FcγRIIIa compared to IgG1 wt (Figure 5G).

Summary

These results indicated that the proteolytic resistant constructs comprising 2h S239D/I332E (**5**), 2h S239D/H268F/S324T/I332E (**9**), and 2h S267E/H268F/S324T/I332E (**10**) were capable of binding to FcγRIIa, IIb, and IIIa to varying degrees, and all three of these constructs had increased binding relative to the 2h (**4**) mutation alone. The mutation of residues in the lower hinge of IgG1 combined with other CH2 mutations, which enhanced FcγRIIa binding affinity over IgG1 wt as with construct 2h S267E/H268F/S324T/I332E (**10**), enhanced affinity of 2h S239D/H268F/S324T/I332E (**9**) and 2h S267E/H268F/S324T/I332E (**10**) for FcγRIIb, enhanced FcγRIIIa binding affinity over IgG1 wt as with the constructs 2h S239D/I332E (**5**) and 2h S239D/H268F/S324T/I332E (**9**) were an unexpected results.

EXAMPLE 2. ANTIBODY-DEPENDENT CELLULAR PHAGOCYTOSIS (ADCP)

In order to test the ability of the protease-resistant mAbs to mediate Fc-dependent *in vitro* cell killing, ADCP assays were performed. In this assay, phagocytic cells are recruited to the target antigen displaying cell by antibody binding and target cell destruction is measured.

Procedure

PBMCs were isolated from normal human donors using Ficoll gradient centrifugation. CD14pos monocytes were purified from PBMCs by negative depletion using a CD14 Isolation kit that did not deplete CD16pos monocytes (Stem Cell Technologies). Monocytes were plated at $0.1x10^6$ cells/cm^2 in X-VIVO-10 medium (Lonza) containing 10% FBS and 20 ng/ml GM-CSF (R&D Systems) for 7 days. 100 ng/ml of IFNγ (R&D Systems) was added for the final 24 hours of differentiation. The target cells for the ADCP assay were GFP-expressing MDA-MB-231 cells. Isolated macrophages were incubated with GFP-expressing MDA-MB-231 at a ratio of 4:1 for 4 hours with or without wild type and protease-resistant mAb constructs in 96 well U-bottom plates. After incubation, cells were removed from the 96 well plates using Accutase (Sigma). Macrophages were identified with anti-CD11b and anti-CD14 antibodies (both from BD Biosciences) coupled to AlexaFluor 647 (Invitrogen), and then cells were acquired on a FACs Calibur (BD Biosciences). The data were analyzed using FloJo Software (Tree Star). The percent phagocytosis was determined by the following equation ((GFPpos, CD11bpos, CD14pos cells) / (GFPpos, CD11bpos, CD14pos cells plus GFPpos alone cells) × 100%.

Isolated monocytes were differentiated in vitro using GM-CSF and IFNγ as described. As was shown by others, the differentiated macrophages expressed all of the FcγRs (FcγRI, FcγRIIa, FcγRIIb, and FcγRIIIa) (data not shown).

Results

The data represented in Figure 6A indicates that IgG1 wt (**1**) and 2h S239D/I332E (**5**) acheived the highest levels of ADCP. The ADCP capacity of IgG2 wt (**2**), IgG1 2h (**4**), and 2h E333A/K334A (**6**) produced low but detectable ADCP capacity. These results indicate that the protease-resistant construct 2h S239D/I332E (**5**) was capable of phagocytosing tumor cells at a level comparable to IgG1 wt. In a separate experiment, an additional panel of CH2 constructs containing the IgG1 2h hinge region were tested for ADCP capacity (Figure 6B). In this group, the constructs 2h S239D/I332E (**5**), 2h F243L/R292P/Y300L (**7**), and 2h S239D/H268F/S324T/I332E (**9**) had similar ADCP as IgG1 wt (**1**), whereas the constructs 2h S267E/H268F/S324T/I332E (**10**), 2h H268F/S324T/I332E (**8**), and 2h G237A/S239D/I332E (**12**) had slightly decreased maximal phagocytosis relative to IgG1 wt. The constructs 2h K326A/E333A (**11**) had low but detectable ADCP, similar to IgG2 wt (**2**) and IgG1 2h (**4**). Finally, the constructs containing the complete hinge of IgG2 were tested for ADCP. The construct IgG2 S239D/I332E (**14**) displayed similar ADCP as IgG1 wt, whereas the constructs 2hc (**3**) and 2hc S239D/I332E (**13**) displayed low but detectable ADCP.

EXAMPLE 3. ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY (ADCC)

In this assay, mononuclear cells are recruited to the target antigen displaying cell and target cell destruction is measured.

Procedure

ADCC assays were performed as previously described (Scallon et al., Mol Immunol 44:1524-1534 2007). Briefly, PBMCs were purified from human blood by Ficoll gradients and used as effector cells for ADCC assays. MDA-MB-231 human breast carcinoma cells (ATCC HTB-26) were used as target cells with a ratio of 1 target cell to 50 effector cells. Target cells were pre-labeled with BATDA (PerkinElmer) for 20 minutes at 37°C, washed twice and resuspended in DMEM, 10% heat-inactivated FBS, 2mM L-glutamine (all from Invitrogen). Target (1×10^4 cells) and effector cells (0.5×10^6 cells) were combined and 100µl of cells were added to the wells of 96-well U-bottom plates. An additional 100µl was added with or without wild type and protease-resistant mAb constructs. All samples were performed in duplicate. The plates were centrifuged at 200g for 3 minutes, incubated at 37°C for 2 hours, and then centrifuged again at 200g for 3 minutes. A total of 20µl of supernatant was removed per well and cell lysis was measured by the addition of 200µl of the DELPHIA Europium-based reagent (PerkinElmer). Fluorescence was measured using an Envision 2101 Multilabel Reader (PerkinElmer). Data were normalized to maximal cytotoxicity with 0.67% Triton X-100

(Sigma Aldrich) and minimal control determined by spontaneous release of BATDA from target cells in the absence of any antibody. Data were fit to a sigmoidal dose-response model using GraphPad Prism v5.

Results

The data were plotted so that the level of cell lysis is represented on the Y-axis as a function of antibody concentration. The data shown in Figure 7A indicate that 2h S239D/I332E (**5**) construct had the highest level of ADCC capacity which was approximately an 8-fold (as evidenced by the shift in apparent EC50) improvement over IgG1 wt in the depicted assay.

In another experiment, the ADCC capacity of an extended panel of constructs was compared. Figure 7B depicts the curves generated by the data. Three constructs (2h S239D/I332E (**5**), 2h F243L/R292P/Y300L (**7**), and 2h S239D/H268F/S324T/I332E (**9**) had increased ADCC capacity relative to IgG1 wt. The 2h G237A/S239D/I332E (**12**) and 2h H268F/S324T/I332E (**8**) constructs had slightly increased ADCC over IgG1 wt, whereas the constructs 2h K326A/E333A (**11**) and 2h S267E/H268F/S324T/I332E (**10**) had detectable, but decreased ADCC relative to IgG1 wt. Figure 7C depicts ADCC results from a panel of constructs that contained the complete hinge region of IgG2. The IgG2 S239D/I332E (**14**) construct had a lower EC50 than IgG1 wt, but also a lower maximal lysis. The constructs 2hc (**3**) and 2hc S239D/I332E (**13**) had detectable ADCC above IgG2 wt, but lower than IgG1 wt. Taken together, these results demonstrate that mutation of critical residues in the lower hinge can be compensated for to restore ADCC and FcγR-binding by a number of CH2 mutations. However, not all of the CH2 mutations made to the IgG1 2h (**3**) backbone tested were capable of restoring/enhancing ADCC relative to IgG1 wt.

These results were consistent with the FcγRIIIa binding assay (Figure 5F-G) showing enhanced affinity of 2h S239D/I332E (**5**) and 2h S239D/H268F/S324T/I332E (**9**), because FcγRIIIa-expressing NK cells are thought to be the relevant effector cell in ADCC.

EXAMPLE 4. COMPLEMENT-DEPENDENT CYTOTOXICITY (CDC)

In this assay, complement components are recruited to the target antigen displaying cell and target cell destruction is measured.

Procedure

CDC assays were performed as previously described (Brezski et al. J Immunol. 181(5):3183-3192 2008). WIL2-S cells were used as target cells for CDC assays. 50µl of cells

were added to the wells of 96-well plates for a final concentration of 8×10^4 cells per well in RPMI, 5% heat-inactivated FBS, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate (all from Invitrogen). An additional 50 µl was added to the wells with or without antibodies and the plates were incubated at room temperature for 2 hours. 50µl of 10% rabbit complement (Invitrogen) was added to the wells and the plates were incubated for 20 minutes at 37°C. All samples were performed in duplicate. The plates were centrifuged at 200g for 3 minutes and 50µl of supernatant was removed to separate plates and CDC was measured with LDH cytotoxicity detection kit (Roche). Absorbance was measured using a Spectra max Plus 384 (PerkinElmer). Data were normalized to maximal cytotoxicity with Triton X-100 (Sigma Aldrich) and minimal control containing only cells and complement alone. Data were fit to a sigmoidal dose-response model using GraphPad Prism v5.

Results

The data shown in Figure 8 indicate that the constructs 2h S267E/H268F/S324T/I332E (**10**) and 2h K326A/E333A (**11**) both achieved similar levels of cell lysis as IgG1 wt. The constructs IgG1 2h (**4**), 2h S239D/I332E (**5**), 2h F243L/R292P/Y300L (**7**), and 2h S239D/H268F/S324T/I332E (**9**) had minimal CDC capacity which was similar to that measured for IgG2 wt (**2**).

EXAMPLE 5: ADDITIONAL PROTEASE-RESISTANT CONTRUCTS

Only two CH2 mutations, in combination with the E233P/L234V/L235A with G236 deleted, were capable of CDC activity comparable to IgG1 wt, namely 2h K326A/K334A (**11**) and 2h S267E/H268F/S324T/I332E (**10**). However, 2h K326A/K334A (**11**) had minimal ADCC and ADCP activity, and 2h S267E/H268F/S324T/I332E (**10**) had reduced ADCC activity relative to IgG1 wt. It would be beneficial to engineer protease-resistant constructs that have all three activities (ADCC, ADCP, and CDC). The H268F/S324T mutations alone were previously not shown to increase affinity to FcγRs (Moore et al.), whereas the construct 2h H268F/S324T/I332E had increased ADCC relative to 2h. Therefore, the I332E mutation alone may restore ADCC to the 2h protease-resistant hinge construct. Therefore, constructs will be generated that combine both ADCC/ADCP restoration with CDC restoration to the 2h parent hinge including 2h K326A/I332E/E333A (**15**) (SEQ ID NO: 18), S239D/K326A/E333A (SEQ ID NO: 19) (**16**), and S267E/I332E (**17**) (SEQ ID NO: 20).

The three constructs were tested using the materials and methods described in Example 1. The three constructs displayed resistance to MMP-3 and MMP-12 compared to IgG1 wt.

As was previously demonstrated, IgG2 wt (**2**) was resistant to GluV8, whereas IgG1 wt (**1**) had less than 60% intact IgG left after a 24 hour digestion. The three constructs had increased resistance to GluV8 compared to IgG1 wt. However, constructs 2h K326A/I332E/E333A (**15**) and 2h S267E/I332E (**17**) had decreased resistance to GluV8 compared to IgG2 wt, while 2h S239D/K326A/E333A (**16**) had resistance comparable to IgG2 wt. These data suggest that mutations which introduce an additional Glu into the CH2 in combination with the lower hinge mutation creates a novel GluV8 cleavage site (e.g. 2h S239D/I332E (**5**), 2h K326A/I332E/E333A (**15**) and 2h S267E/I332E (**17**)), whereas mutations that do not incorporate a Glu into the CH2 display resistance to GluV8 similar to IgG2 wt (e.g. 2h K326A/E333A (**11**) and 2h S239D/K326A/E333A (**16**)).

Both IgG1 wt and IgG2 wt were susceptible to proteolysis by IdeS. The two constructs 2h K326A/I332E/E333A (**15**) and 2h S267E/I332E (**17**) had greater than 90% intact IgG remaining after a 24 hour incubation with IdeS, whereas the construct 2h S239D/K326A/E333A (**16**) had less than 20% intact IgG remaining. These results suggest that the addition of a Glu into the CH2 in combination with the lower hinge mutation increases the protease-resistance to IdeS, a property which the lower hinge mutation alone, 2h (**4**), does not impart.

The three constructs were tested for their ability to perform ADCP, ADCC, and CDC. The three constructs had increased ADCP capacity compared to both IgG2 wt and 2h (**4**), but decreased maximum ADCP compared to IgG1 wt. Two of the constructs, 2h K326A/I332E/E333A (**15**) and 2h S239D/K326A/E333A (**16**) had slightly increased ADCC relative to IgG1 wt. The construct 2h S267E/I332E (**17**) had decreased ADCC relative to IgG1 wt, but increased ADCC relative to IgG2 wt and 2h (**4**). All three constructs had increased CDC capacity relative to IgG2 wt and 2h (**4**); however, the CDC for all three was slightly decreased relative to IgG1 wt.

EXAMPLE 6: SUMMARY OF BENEFICIAL MUTATIONS

The following eleven Fc variants were shown to provide an antibody composition resistant to one or more proteases capable of cleaving IgG1 in the lower hinge while providing one or more effector functions exhibited by the wild-type human IgG1. The symbol 2h designates IgG1 with E233P/L234V/L235A – G236 deleted.

Table5.

Construct	Abbreviation (#)
IgG1 2h S239D/I332E	2h DE (**5**)
IgG1 2h F243L/R292P/Y300L	2h LPL(**7**)
IgG1 2h H268F/S324T/I332E	2h FTE (**8**)
IgG1 2h S239D/H268F/S324T/I332E	2h DFTE (**9**)
IgG1 2h S267E/H268F/S324T/I332E	2h EFTE (**10**)
IgG1 2h K326A/E333A	2h AA (**11**)
IgG1 2h G237A/S239D/I332E	2h XDE (**12**)
IgG2 S239D/I332E	IgG2 DE (**14**)
IgG1 2h K326A/ I332E/ E333A	2h AEA (**15**)
IgG1 2h S239D/ K326A /E333A	2h DAA (**16**)
IgG1 2h S267E/ I332E	2h EE (**17**)

Where sufficient data was available, EC50 values calculated when a cell killing was complete or near complete for the *in vitro* assays used as a proxy for various effector function is shown below. The data shown in Tables 6A and 6B were generated under identical conditions except that the donor PBMC source was different. Therefore, the fold change from the IgG1 wt (1) in each experiment is used to standardize the relative biological activity.

Table 6A.

Construct (#)	ADCC EC50 (ng/ml)	fold	ADCP EC50 (ng/ml)	fold	CDC EC50 (ng/ml)	fold
IgG1 wt (**1**)	4.8	1	27	1	96	1

Construct (#)	ADCC EC50 (ng/ml)	fold	ADCP EC50 (ng/ml)	fold	CDC EC50 (ng/ml)	fold
2h DE (**5**)	0.53	9	54	0.5	n/a	n/a
2h G237A/DE (**13**)	0.47	10	24	1.1	n.d.	n.d.
2h LPL (**7**)	0.31	15	30	0.9	n/a	n/a
2h AA (**11**)	21	0.2	n/a	n/a	157	0.6
2h DFTE (**9**)	0.70	7	34	0.8	n/a	n/a
2h EFTE (**10**)	n/a	n/a	44	0.6	77	1.2

Table 6B

Construct (#)	ADCC EC50 (ng/ml)	fold	ADCP EC50 (ng/ml)	fold	CDC EC50 (ng/ml)	fold
IgG1 wt	0.42	1	186	1	114	1
2h AEA (15)	0.17	2.5	44*	4.2	202	0.56
2h DAA (16)	0.14	3.4	42*	4.4	308	0.37
2h EE (17)	5.6	0.08	52*	3.6	592	0.19

n/a = not applicable (insufficient binding curve data to determine EC50), * submaximal lysis achieved

n.d. = no data

fold = EC50 IgG1 wt / EC50 construct

The effect on ADCC and ACDP of an additional modification at G237 in the lower hinge in addition to S239D/I332E and 233PVA/236, was examined using constructs listed in the Table 7 below. The G237A construct was tested and found to have resistance to MMPs,

IdeS, and GluV8. The other constructs were not evaluated in the digestion assays. These data indicate that Ala(A) and Ser (S) are tolerated at 237 but do not increase the cytolytic activity of the Fc above that displayed by the parent molecule, 2h DE (**5**).

Table 7.

Construct	ADCC	ADCP
IgG1 2h DE	++++++	+++++
IgG1 2h ADE	++++++	++++
IgG1 2h DDE	+	+
IgG1 2h PDE	++	+
IgG1 2h QDE	+	+
IgG1 2h SDE	+++++	++

A summary of the combined relative protease resistance (PR) to specific physiologically relevant proteases and the *in vitro* results for proxy assays indicating potential effector function (ADCC, ADCP, and CDC) are shown in Table 8 below where those constructs with combined protease resistance and one or more demonstrable effector activities are in white.

Table 8.

Isotype/ Construct	PR MMPs	PR IdeS	PR GluV8	ADCC	ADCP	CDC
IgG1 wt (1)	-	-	+	+++++	+++++	+++++
IgG2 wt (2)	+++++	-	+++++	-	++	-
IgG1 2hc (3)	+++++	-	+++++	+	++	n.d.
IgG1 2hc DE (13)	+++++	-	+++++	+	++	n.d.
IgG1 2h (4)	+++++	-	+++++	+	++	-
IgG1 2h DE (5)	+++++	++++	++	++++++	+++++	-
IgG1 2h LPL (7)	+++++	++++	++	++++++	+++++	-
IgG1 2h FTE (8)	+++++	++++	+++++	++++++	+++	-
IgG1 2h DFTE (9)	+++++	+++++	++++	++++++	+++++	-
IgG1 2h EFTE (10)	+++++	++++	++++	+	++++	+++++

Isotype/ Construct	PR MMPs	PR IdeS	PR GluV8	ADCC	ADCP	CDC
2h AA (11)	+++++	-	+++++	+++	++	+++++
2h ADE (12)	+++++	++++	++	++++++	++++	n.d.
IgG2 DE (14)	+++++	+++	++	++++++	++++	n.d.
2h AEA (**15**)	++++	+++++	+++	++++++	+++	++++
2h DAA (**16**)	++++	+	+++++	+++++	+++	++++
2h EE (**17**)	++++	+++++	+++	+	+++	++++

Summary of Results

The study of Fc constructs presented herein demonstrated that substitution of residues EU 233-236 with PVA/, the sites where proteases were shown to cleave the IgG1 molecule, produced an Fc that was resistant to MMP-3, MMP-12, and GluV8; proteases that cleave between residues 232 and 234 (Fig. 1). When combined with these substitutions, additional modifications produced resistance to the Staphylococcus protease IdeS whether the residue positions substituted include a modification at the presumed cleavage site (EU236-237) or at a more distal position.

Substitution of the residues EU 233-236 with PVA/ (2h, construct **4**) alone resulted in the loss of cytolytic functions measureable by the *in vitro* assays described for ADCC, ADCP, and CDC. With respect to the combination of IgG1 Fc modifications previously reported to enhance one or more effector functions (Table 2) with the lower hinge PVA/ substitution, unexpectedly restored one or more aspects of in vitro cytolytic activity. Thus, no single construct was both protease-resistant and had measurable or enhanced activity for all three effector functions as measured by in vitro cell killing or cell lysis assays for ADCC, ADCP, and CDC.

1. Eight constructs had protease-resistance and enhanced or comparable ADCC compared to IgG1 wt: **5**, **7**, **8**, **9**, **12**, **14**, **15**, and **16**. Six of these incorporate the I332E substitution: including IgG1 2h DE (**5**), IgG1 2h FTE (**8**), IgG1 2h DFTE (**9**), IgG1 2h ADE (**12**),IgG2 DE (**14**) and IgG1 2h AEA (**15**).

2. Three PR constructs had similar ADCP compared to IgG1 wt including IgG1 2h DE (**5**), IgG1 2h LPL (**7**), and IgG1 2h DFTE (**9**). Three constructs had slightly reduced ADCP compared to IgG1 including IgG1 2h FTE (**8**), IgG1 2h EFTE (**10**), IgG1 2h ADE (**12**), and IgG2 DE (**14**).

3. Five PR mutations restored CDC capacity, IgG1 2h AA (**11**), IgG 2h EFTE (**10**), 2h AEA (**15**), 2h DAA (**16**), and 2h EE (**17**). In addition, all five had detectable, but reduced ADCP compared to IgG1 wt. Two variants, 2h AEA (**15**), 2h DAA (**16**), also had enhance ADCC as compared to IgG1 wt (**1**).

   Two constructs (**8** and **9**) comprising H268F/S324T mutations did not have restored CDC when a protease-resistant hinge was present. The S267E mutation (EFTE (**10**)) restored CDC but decreased FcγRIIIa binding (also noted by Moore et al. *mAbs* 2010 2(2):181.) The S267E mutation increased affinity to FcγRIIb.

What is claimed is:

1. An isolated modified Fc-containing molecule or a fragment thereof comprising a wild type human IgG1 Fc region of SEQ ID NO: 1 comprising a hinge, a CH2 domain and a CH3 domain, wherein

a) the sequence of E233-L234-L235-G236 in the hinge is replaced with P233-V234-A235 with G236 deleted; and

b) the CH2 domain comprises at least one substitution selected from S239D/1332E, K326A/E333A, H268F/S324T/1332E, F243L/R292P/Y300L, S239D/H268F/S324T/1332E, S267E/H268F/S324T/1332E, K326A/1332E/E333A, S239D/K326A/E333A, S267E/I332E and G237X/S239D/1332E where X is A, D, P, Q or S; wherein amino acid residues are numbered according to EU numbering.

2. The isolated modified Fc-domain containing molecule of claim **1**, wherein the molecule is resistant to proteolytic degradation by a protease that cleaves the wild type human IgG1 molecule between or at residues 222-237 (EU numbering).

3. The isolated modified Fc-domain containing molecule of claim **1**, wherein the molecule is capable of promoting antibody-dependent cellular phagocytosis (ADCP) measured in the presence of CD14 positive and/or CD11b positive human monocyte-derived macrophages, is capable of promoting antibody-dependent cell-mediated cytotoxicity (ADCC) measured in the presence of blood mononuclear cells, and/or is capable of promoting complement-dependent cytotoxicity (CDC) measured by cell lysis in the presence of complement.

4. The isolated modified Fc-domain containing molecule of claim **3**, wherein the molecule induces IL-10 secretion by human monocyte-derived macrophages by about no more than three times more when compared to the IL-10 secretion by the human monocyte-derived macrophages in the absence of the isolated modified Fc-containing molecule.

5. The isolated modified Fc-domain containing molecule of claim **4**, wherein the molecule has an IC_{50} fold change ratio value of 0.04 or less measured in a competition assay with biotinylated human IgG1 to 0.2 μg/ml soluble human FcγRI, wherein the IC_{50} fold change ratio is a ratio of an IC_{50} value for a wild type human IgG1 to an IC_{50} value for the isolated modified Fc-domain containing protein of claim **1**.

6. The isolated modified Fc-domain containing molecule of claim **5**, wherein the molecule at a concentration of 0.5

μg/ml induces interferon-gamma (IFNγ) secretion by pheripheral blood mononuclear cells (PBMCs) by at least three times more when compared to the IFNγ secretion by the PBMCs in the absence of the isolated modified Fc-domain containing molecule.

7. The isolated modified Fc-domain containing molecule of claim **1**, wherein the protease that cleaves the wild type IgG1 molecule between or at residues 222-237 is matrix metalloprotease (MMP) 2 (MMP-2), MMP-3, MMP-7, MMP-9, MMP-12, MMP-13, immunoglobulin degrading enzyme from *Strep. Pyrongenes* (IdeS), glutamyl endopeptidase I from *Staph. aureus* (GluV8), human neutrophil elastase (HNE), plasmin, cathepsin G, or pepsin.

8. The isolated modified Fc-domain containing molecule of claim **7**, wherein the protease that cleaves the wild type IgG1 molecule between or at residues 222-237 is MMP-3, MMP-7, MMP-12, MMP-13, IdeS, or GluV8.

9. The isolated modified Fc-domain containing molecule of claim **1**, wherein the Fc-containing molecule comprises the polypeptide sequence of SEQ ID NOs: 8, 10-15 or 18-20.

10. The isolated modified Fc-domain containing molecule of claim **1**, wherein the Fc-containing molecule is an antibody or an Fc fusion protein.

11. An isolated antibody or fragment thereof comprising a modified Fc-containing molecule comprising a wild type human IgG1 Fc region of SEQ ID NO: 1 comprising a hinge, a CH2 domain and a CH3 domain, wherein

a) the sequence of E233-L234-L235-G236 in the hinge is replaced with P233-V234-A235 with G236 deleted; and

b) the CH2 domain comprises at least one substitution selected from S239D/I332E, K326A/E333A, H268F/S324T/I332E, F243L/R292P/Y300L, S239D/H268F/S324T/I332E, S267E/H268F/S324T/I332E, K326A/I332E/E333A, S239D/K326A/E333A, S267E/I332E and G237X/S239D/I332E where X is A, D, P, Q or S; wherein amino acid residues are numbered according to EU numbering.

12. The isolated antibody of claim **11**, wherein the antibody is resistant to proteolytic degradation by a protease that cleaves the wild type human IgG1 molecule between or at residues 222-237.

13. The isolated antibody of claim **11**, wherein the antibody is capable of promoting antibody-dependent cellular phagocytosis (ADCP) measured in the presence of CD14 positive and/or CD11b positive human monocyte-derived macroph-

ages, is capable of promoting antibody-dependent cell-mediated cytotoxicity (ADCC) measured in the presence of blood mononuclear cells, or is capable of promoting complement-dependent cytotoxicity (CDC) measured by cell lysis in the presence of complement.

14. The isolated antibody of claim **11**, wherein the antibody is capable of promoting antibody-dependent cellular phagocytosis (ADCP) measured in the presence of CD14 positive and/or CD11b positive human monocyte-derived macrophages, is capable of promoting antibody-dependent cell-mediated cytotoxicity (ADCC) measured in the presence of blood mononuclear cells, and is capable of promoting complement-dependent cytotoxicity (CDC) measured by cell lysis in the presence of complement.

15. The isolated antibody of claim **13** or **14**, wherein the antibody induces IL-10 secretion by human monocyte-derived macrophages by about no more than three times more when compared to the IL-10 secretion by the human monocyte-derived macrophages in the absence of the isolated antibody.

16. The isolated antibody of claim **14**, wherein the antibody has an IC_{50} fold change ratio value of 0.04 or less measured in a competition assay with biotinylated human IgG1 to 0.2 μg/ml soluble human FcγRI, wherein the IC_{50} fold change ratio is a ratio of an IC_{50} value for a wild type human IgG1 to an IC_{50} value for the isolated antibody of claim **11**.

17. The isolated antibody of claim **16**, wherein the antibody at a concentration of 0.5 μg/ml induces interferon-gamma (IFNγ) secretion by pheripheral blood mononuclear cells (PBMCs) by at least three times more when compared to the IFNγ secretion by the PBMCs in the absence of the isolated antibody.

18. The isolated antibody of claim **12**, wherein the protease that cleaves the wild type IgG1 molecule between or at residues 222-237 is matrix metalloprotease (MMP) 2 (MMP-2), MMP-3, MMP-7, MMP-9, MMP-12, MMP-13, immunoglobulin degrading enzyme from *Strep. Pyrongenes* (IdeS), or glutamyl endopeptidase I from *Staph. aureus* (GluV8), human neutrophil elastase (HNE), plasmin, cathepsin G, or pepsin.

19. The isolated antibody of claim **18**, wherein the protease that cleaves the wild type IgG1 molecule between or at residues 222-237 is MMP-3, MMP-7, MMP-12, MMP-13, IdeS, or GluV8.

20. The isolated antibody of claim **11**, wherein the antibody comprises the polypeptide sequence of SEQ ID NOs: 8, 10-15 or 18-20.

21. The isolated antibody of claim **11**, wherein the antibody binds to an antigen on a tumor cell, tumor matrix, or tumor vasculature.

22. The antibody of claim **21**, wherein the antibody binds CD20, ErbB1, ErbB2, ErbB3, VEGF, RON, or tissue factor.

23. A pharmaceutical composition comprising the isolated modified Fc-domain containing molecule of claim **1** or the isolated antibody of claim **11**.

24. The isolated antibody of claim **11**, wherein the CH2 domain comprises the substitution S239D/K326A/E333A having the polypeptide sequence of SEQ ID NO: 19.

25. The isolated antibody of **claim 24, wherein the antibody** binds CD20, ErbB1, ErbB2, ErbB3, VEGF, RON, or tissue factor.

26. The isolated antibody of **claim 25, wherein the antibody** binds CD20 or tissue factor.

27. The isolated antibody of **claim 26, wherein the antibody** binds tissue factor.