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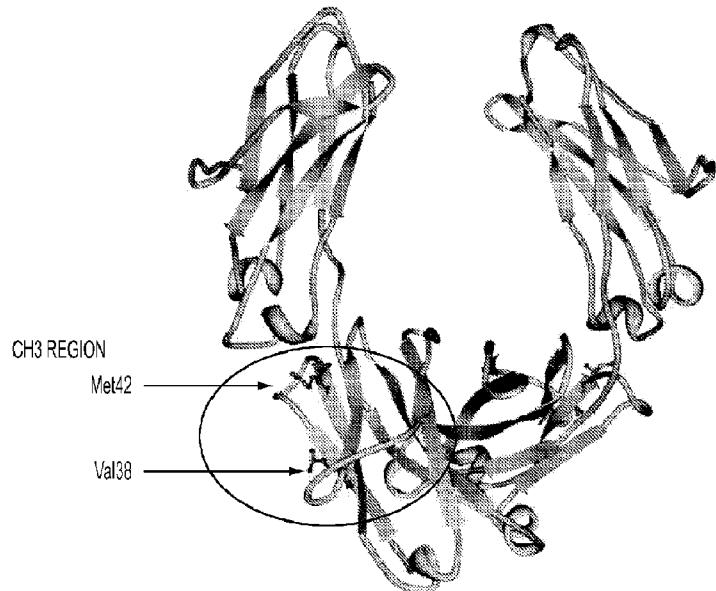
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(54) Title: IMMUNOGLOBULIN FC POLYPEPTIDES



(57) Abrégé/Abstract:

Methods and compositions involving polypeptides having an aglycosylated antibody Fc domain. In certain embodiments, polypeptides have an aglycosylated Fc domain that contains one or more substitutions compared to a native Fc domain. Additionally, some embodiments involve an Fc domain that binds some Fc receptors but not others. For example, polypeptides are provided with an aglycosylated Fc domain that selectively binds FcγRI at a level within 2-fold of a glycosylated Fc domain, but that is significantly reduced for binding to other Fc receptors. Furthermore, methods and compositions are provided for promoting antibody-dependent cell-mediated toxicity (ADCC) using a polypeptide having a modified aglycosylated Fc domain and a second non-Fc binding domain, which can be an antigen binding region of an antibody or a non-antigen binding region. Some embodiments concern antibodies with such polypeptides, which may have the same or different non-Fc binding domain.

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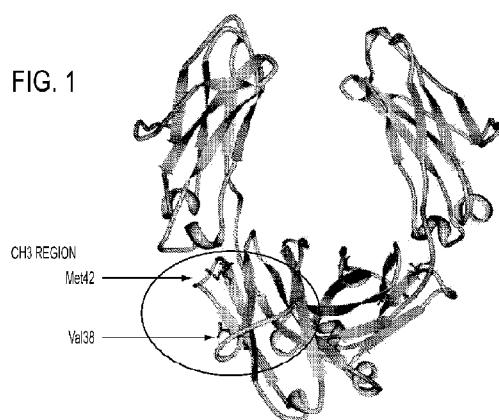
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FIG. 1



(57) **Abstract:** Methods and compositions involving polypeptides having an aglycosylated antibody Fc domain. In certain embodiments, polypeptides have an aglycosylated Fc domain that contains one or more substitutions compared to a native Fc domain. Additionally, some embodiments involve an Fc domain that binds some Fc receptors but not others. For example, polypeptides are provided with an aglycosylated Fc domain that selectively binds Fc γ RI at a level within 2-fold of a glycosylated Fc domain, but that is significantly reduced for binding to other Fc receptors. Furthermore, methods and compositions are provided for promoting antibody-dependent cell-mediated toxicity (ADCC) using a polypeptide having a modified aglycosylated Fc domain and a second non-Fc binding domain, which can be an antigen binding region of an antibody or a non-antigen binding region. Some embodiments concern antibodies with such polypeptides, which may have the same or different non-Fc binding domain.

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DESCRIPTION**IMMUNOGLOBULIN FC POLYPEPTIDES****BACKGROUND OF THE INVENTION**

[0001] This application claims priority to U.S. Application No. 61/221,999 filed on June 30, 2009.

1. Field of the Invention

[0002] The present invention relates generally to the field of protein engineering. More particularly, it concerns improved methods and compositions for the screening of combinatorial antibody Fc libraries expressed in bacteria.

2. Description of Related Art

[0003] Currently recombinant therapeutic antibodies have sales of well over \$10 bn/yr and with a forecast of annual growth rate of 20.9%, they are projected to increase to \$25 bn/yr by 2010. Monoclonal antibodies (mAbs) comprise the majority of recombinant proteins currently in the clinic, with more than 150 products in studies sponsored by companies located worldwide (Pavlou and Belsey, 2005). In terms of therapeutic focus, the mAb market is heavily focused on oncology and arthritis, immune and inflammatory disorders, and products within these therapeutic areas are set to continue to be the key growth drivers over the forecast period. As a group, genetically engineered mAbs generally have higher probability of FDA approval success than small-molecule drugs. At least 50 biotechnology companies and all the major pharmaceutical companies have active antibody discovery programs in place.

[0004] The original method for isolation and production of mAbs was first reported at 1975 by Milstein and Kohler (Kohler and Milstein, 1975), and it involved the fusion of mouse lymphocyte and myeloma cells, yielding mouse hybridomas. Therapeutic murine mAbs entered clinical study in the early 1980s; however, problems with lack of efficacy and rapid clearance due to patients' production of human anti-mouse antibodies (HAMA) became apparent. These issues, as well as the time and cost consuming related to the technology became driving forces for the evolution of mAb production technology. Polymerase Chain Reaction (PCR) facilitated the cloning of monoclonal antibodies genes directly from lymphocytes of

immunized animals and the expression of combinatorial library of fragments antibodies in bacteria (Orlandi *et al.*, 1989). Later libraries were created entirely by *in vitro* cloning techniques using naïve genes with rearranged complementarity determining region 3 (CDR3) (Griffiths and Duncan, 1998; Hoogenboom *et al.*, 1998). As a result, the isolation of antibody fragments with the desired specificity was no longer dependent on the immunogenicity of the corresponding antigen. Moreover, the range of antigen specificities in synthetic combinatorial libraries was greater than that found in a panel of hybridomas generated from an immunized mouse. These advantages have facilitated the development of antibody fragments to a number of unique antigens including small molecular compounds (haptens) (Hoogenboom and Winter, 1992), molecular complexes (Chames *et al.*, 2000), unstable compounds (Kjaer *et al.*, 1998) and cell surface proteins (Desai *et al.*, 1998).

[0005] In microbial cells, display screening may be carried out by flow cytometry. In particular, Anchored Periplasmic Expression (APEx) is based on anchoring the antibody fragment on the periplasmic face of the inner membrane of *E.coli* followed by disruption of the outer membrane, incubation with fluorescently labeled target and sorting of the spheroplasts (U.S. Patent 7,094,571). APEx was used for the affinity maturation of antibody fragments (Harvey *et al.*, 2004; Harvey *et al.*, 2006). In one study over 200-fold affinity improvement was obtained after only two rounds of screening.

[0006] One important mechanism underlying the potency of antibody therapeutics is the ability of antibody to recruit immune cells to a target antigen (or cell). Thus, the Fc region of an antibody is crucial for recruitment of immunological cells and antibody dependent cytotoxicity (ADCC). In particular, the nature of the ADCC response elicited by antibodies depends on the interaction of the Fc region with receptors (FcRs) located on the surface of many cell types. Humans contain five different classes of Fc receptors. In addition haplotypes, or genetic variants of different FcRs belonging to a particular class are known. The binding of an antibody to FcRs determines its ability to recruit other immunological cells and the type of cell recruited. Hence, the ability to engineer antibodies that can recruit only certain kinds of cells can be critically important for therapy.

[0007] However, to the inventors' knowledge, previous attempts to engineer Fc domains have been performed using mammalian-expressed IgG molecules. Mammalian antibodies are glycosylated. The carbohydrate chain is attached to the Fc region and alters the conformation of the protein and enables the antibody to bind to FcRs. In contrast, aglycosylated antibodies produced in bacteria cannot bind to FcRs and therefore are unable to elicit ADCC. It is desirable to engineer aglycosylated antibodies that are capable of eliciting ADCC and thus benefit from the lower production costs that are derived from bacterial expression.

[0008] Second, and most importantly, mammalian antibodies with engineered Fc regions display increased binding to a particular FcR of interest but in addition they are still capable of binding to other FcRs with normal affinity. Thus, while such antibodies are more selective than the molecules naturally produced by the immune system they can nonetheless still mediate undesirable immunological responses.

[0009] Nonetheless, all high throughput antibody screening technologies available to-date rely on microbial expression of antibody fragments. The use of antibody fragments rather than intact or full length IgGs, in the construction and screening of libraries has been dictated by limitations related to the expression of the much larger IgGs in microorganisms. IgG libraries have never before been expressed or screened using microorganisms such as bacteria or yeasts. As a result the isolation of antigen binding proteins has been carried out exclusively using antibody fragments that are smaller and much easier to produce. Once isolated, such antibody fragments have to then be fused to vectors that express full length immunoglobulins which in turn are expressed preferentially in mammalian cells such as CHO cells.

[0010] *E.coli* possesses a reducing cytoplasm that is unsuitable for the folding of proteins with disulfide bonds which accumulate in an unfolded or incorrectly folded state (Baneyx and Mujacic, 2004). In contrast to the cytoplasm, the periplasm of *E. coli* is maintained in an oxidized state that allows the formation of protein disulfide bonds. Notably, periplasmic expression has been employed successfully for the expression of antibody fragments such as Fvs, scFvs, Fabs or F(ab')2s (Kipriyanov and Little, 1999). These fragments can be made relatively quickly in large quantities with the retention of antigen binding activity. However, because antibody fragments lack the Fc domain, they do not bind the FcRn receptor and are cleared quickly; thus,

they are only occasionally suitable as therapeutic proteins (Knight *et al.*, 1995). Until recently, full-length antibodies could only be expressed in *E. coli* as insoluble aggregates and then refolded *in vitro* (Boss *et al.*, 1984; Cabilly *et al.*, 1984). Clearly this approach is not amenable to the high throughput screening of antibody libraries since with the current technology it is not possible to refold millions or tens of millions of antibodies individually. A further problem is that since *E. coli* expressed antibodies are not glycosylated, they fail to bind to complement factor 1q (C1q) or Fc and many other Fc receptors. However, aglycosylated Fc domains can bind to the neonatal Fc receptor efficiently (FcRn). Consequently bacterially expressed aglycosylated antibodies do exhibit serum persistence and pharmacokinetics similar to those of fully glycosylated IgGs produced in human cells. Nonetheless, since the aglycosylated antibodies fail to elicit complement activation and can not mediate the recruitment of immune cells such as macrophages, they have previously been ineffective for many therapeutic applications.

[0011] Moreover, some studies have reported that binding of some Fc receptors by Fc domains can have an activating effect while others have an inhibitory one (Boruchov *et al.* 2005; Kalergis *et al.*, 2002). Different Fc γ R effector functions include (antibody-dependent cell-mediated cytotoxicity (ADCC), cytokine release, phagocytosis, and maturation. Fc domains engineered to have selective effector functions could provide physiological benefits.

SUMMARY OF THE INVENTION

[0012] This disclosure provides compounds and methods involving aglycosylated antibody Fc domains that bind to Fc receptors.

[0013] In some embodiments, there are compositions involving a polypeptide that has an aglycosylated Fc domain from an antibody (“antibody Fc domain”). In additional embodiments, the aglycosylated Fc domain is a variant of a wild-type Fc domain such that the variation allows the Fc domain to specifically bind to one or more Fc receptors. In some embodiments, a polypeptide with an aglycosylated Fc domain variant is able to bind only a subset of Fc receptors that a polypeptide with glycosylated version of the wild-type Fc domain (“glycosylated wild-type Fc domain”) can bind. In specific embodiments, the polypeptide with an aglycosylated

Fc domain variant can specifically bind Fc γ RI; in some cases, it has the affinity or binding ability that is within 2-fold of a polypeptide having a glycosylated wild-type Fc domain. In other embodiments, additionally or alternatively, the polypeptide with an aglycosylated Fc domain variant has significantly reduced affinity or binding ability (50-fold or greater reduction) compared to a polypeptide having a glycosylated wild-type Fc domain. In certain embodiments, the polypeptide with an aglycosylated Fc domain variant has a significantly reduced affinity to or ability to bind Fc γ RIIb. It is contemplated that a polypeptide may have an affinity or binding ability for Fc γ RI that is comparable (within 2-fold), as well as significantly reduced affinity or binding ability for Fc γ RIIB, both as compared to a polypeptide having a glycosylated wild-type Fc domain.

[0014] As used herein, the term "affinity" refers to the equilibrium constant for the reversible binding of two agents and is expressed as Kd. Affinity of a binding domain to its target can be, for example, from about 100 nanomolar (nM) to about 0.1 nM, from about 100 nM to about 1 picomolar (pM), or from about 100 nM to about 1 femtomolar (fM); alternatively, it can be between 100 nM and 1 nM or between 0.1 nM and 10 nM. Moreover, it is contemplated that agents specifically bind when there is an affinity between the two agents that is in the affinity ranges discussed above.

[0015] An antibody Fc domain may be the Fc domain of an IgA, IgM, IgE, IgD or IgG antibody or a variant thereof. In certain embodiments, the domain is an IgG antibody Fc domain such as an IgG1, IgG2a, IgG2b, IgG3 or IgG4 antibody Fc domain. Furthermore, the antibody Fc domain may be defined as a human Fc domain, in which case it specifically binds one or more human Fc receptors. In certain aspects, the Fc domain may be an IgG1 Fc domain, such as the Fc domain of an anti-HER2 antibody, more specifically, the Fc domain of trastuzumab. It is contemplated that in some embodiments an entire polypeptide is aglycosylated or that in other embodiments only a portion of the polypeptide is aglycosylated, such as the Fc domain. It is also contemplated that a polypeptide may contain one or more regions from an antibody in addition to the Fc domain. A polypeptide may contain an antigen binding domain from an antibody. Moreover, multiple polypeptides may form an antibody or antibody-like protein.

[0016] In some embodiments, there is a polypeptide comprising an aglycosylated antibody Fc domain capable of binding a human FcR polypeptide, wherein the Fc domain comprises particular amino acid substitutions. In some embodiments there are multiple amino acid substitutions. In additional embodiments, there are up to eight amino acid substitutions relative to the wild-type Fc domain sequence. With substitutions in the human Fc domain, embodiments include a polypeptide with a human Fc domain having an amino acid substitution at amino acids 382 and 428 and at least one additional substitution of any of the following amino acids: 224, 241, 251, 266, 269, 276, 279, 286, 295, 297, 300, 315, 325, 328, 330, 331, 332, 338, 340, 341, 348, 369, 378, 382, 392, 424, 426, 428 and/or 434. In some cases, it is specifically contemplated that the amino acid at 329 of the human Fc domain is the wild-type sequence, that is, a proline. In some embodiments a polypeptide has a human Fc domain substitution at amino acid 382 that is a valine (V) instead of glutamic acid (E) (E382V). Conventional single letter abbreviations for amino acids are employed herein. In additional embodiments, the polypeptide has a human Fc domain substitution at amino acid 428 that is an isoleucine (M428I). In some cases, a polypeptide has both the substitution at amino acid 382 that is a valine (E382V) and the substitution at amino acid 428 that is an isoleucine (M428I) in the human Fc domain. In some embodiments, a polypeptide has a substitution in the human Fc domain at amino acid 328 that is a tryptophan (L328W). In additional embodiments, a polypeptide has a human Fc domain substitution at amino acid 332 that is a tyrosine (I332Y). Other polypeptides include those having a human Fc domain substitution at least at amino acids 328 and 332. The substitution at amino acid 328 may be a tryptophan (L328W) and the substitution at amino acid 332 may be a tyrosine (I332Y). In additional embodiments, a polypeptide has a human Fc domain substitution at amino acid 341, which is a valine (G341V) in further embodiments. More embodiments involve a polypeptide with a human Fc domain substitution at 382 and 428 and at least one additional substitution in the domain in the following group: H224R/Y, F241L, K251F, V266M, E269K, N276D, V279M, N286D, Q295R, N297D, Y300C, N315D, N325S, L328W, A330V/E/I, P331A/S/E, I332Y, K338I/R, K340N/Q, G341V, V348M, V369A, A378D, K392E, S424L, S426I, or N434S/D. Multiple additional substitutions are contemplated in some embodiments.

[0017] In some embodiments, a polypeptide has an aglycosylated human Fc domain with a substitution in amino acids 382 and 428 and also has at least one additional substitution in the upper CH2 region. Some embodiments involve a polypeptide having at least one additional human Fc domain substitution that is of an amino acid in the following part of the upper CH2 region: 234L-239S; 264V-268H; 297N-299T; or 328L-332I.

[0018] In further embodiments, a polypeptide does not have the additional substitution in the human Fc domain of G341V and/or K338R. In other cases, however, the Fc domain may have a substitution of G341V and at least one other substitution selected from the group consisting of: H224Y, F241L, E269K, N276D, N286D, Y300C, N325S, K338R, V348M, V369A, K392E, S424L, and N434D/S. In some embodiments, the Fc domain has multiple other substitution selected from the group. A polypeptide may have an Fc domain substitution that includes a K338R substitution.

[0019] In some embodiments there are polypeptides with a human Fc domain that has a set of substitutions selected from the group consisting of a) K338R and G341V; b) N297D, N315D, and K340N, c) K340N, d) K338I and K340N, e) K340Q and A378D; f) N325S and K340N, g) H224Y, E269K, N325S, and G341V, h) G341V and K392E, i) K338R, G341V, S424L and N434D, j) F241L and G341V, k) G341V, l) N276D and G341V, m) G341V and V369A, n) N286D, G341V and N434S, o) N325S and G341V, p) Y300C and G341V, q) G341V and V348M, r) E382V and M428I, s) V266M; t) A330V, P331A and Q295R, u) A330E, P331E and V279M, v) A330E and P331E, w) A330E, P331V and S426T, x) A330E and P331V, y) A330I and P331E, z) A330E, aa) P331S, and bb) A330V, P331S, H224R and L251F. It is contemplated that in some embodiments there are additional polypeptides that may include an Fc domain from a non-human, in which case the recited substitutions can be implemented in corresponding amino acids.

[0020] Embodiments involve a polypeptide having an aglycosylated Fc domain that is capable of specifically binding one or more particular human FcR polypeptides. In some embodiments, the aglycosylated Fc domain has been mutated so that it can bind one or more of Fc γ RIa, Fc γ RIIa, Fc γ RIIb, Fc γ RIIc, Fc γ RIIIa, Fc γ RIIIb, or Fc α RI. It is contemplated that the binding to one or more of these

particular human FcR polypeptides is within 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100% (or any range derivable therein) of the binding seen with a glycosylated Fc region or that the binding is altered (increased or decreased) by at least or at most 50, 60, 70, 80, 90, or 100 % (or any range derivable therein) relative to a wild-type glycosylated Fc domain. Alternatively, relative binding capabilities between polypeptides having a mutated and aglycosylated Fc domain and polypeptides having a glycosylated and wild-type Fc domain may be expressed in terms of X-fold differences (increased or decreased). For example, there may be at least or at most at least 2-, 3-, 4-, 5-, 6-, 7-, 8-, 9-, or 10-fold difference, or any range derivable therein).

[0021] In some embodiments, a polypeptide with a mutated aglycosylated Fc domain is capable of specifically binding an Fc γ RI polypeptide. In some cases, it binds at a level within 2-fold of the level of binding by a polypeptide having a glycosylated and wild-type Fc domain. In other embodiments, the level of binding is within at least 2-, 3-, 4-, 5-, 6-, 7-, 8-, 9-, or 10-fold a glycosylated and wild-type Fc domain. For example, the K_D value for a particular Fc receptor and either a polypeptide with the aglycosylated Fc domain variant or a polypeptide with a glycosylated and wild-type Fc domain is within at least 2- or 3-fold in embodiments described herein. In some embodiments, a polypeptide has at least a 2-fold reduction in pH-dependent FcRn binding compared to polypeptide with an aglycosylated wild-type antibody Fc domain.

[0022] Polypeptides described herein may include a linker in some embodiments. In further embodiments, the linker is a conjugatable linker. In some embodiments, the polypeptide contains an Fc domain from an antibody. It may contain other regions from an antibody, such as another binding domain. The additional binding domain is not an FcR binding domain in certain embodiments. In some embodiments, it may contain an antigen binding site or domain from an antibody. This would include all or part of the variable region from an antibody. In other embodiments, a polypeptide contains an Fc domain from an antibody but another binding domain that is a non-FcR binding domain. In some embodiments, the non-Fc binding region is not an antigen binding site of an antibody but specifically binds a cell-surface protein. In some cases, a cell-surface protein that the non-Fc binding region recognizes is a receptor. In some embodiments, a cell-surface receptor

is a tyrosine kinase. In additional embodiments, a polypeptide has a non-Fc binding region capable of binding multiple tyrosine kinase receptors. In some embodiments, such a non-Fc binding region is capable of binding one or more of VEGF receptors, PDGF receptors, EGFR receptors, ErbB-2 receptors, EGF receptors, HGF receptors, and other Src-like tyrosine kinase receptors, or a combination thereof. It is also specifically contemplated that polypeptides have an antigen binding region that recognizes one or more of these receptor tyrosine kinases.

[0023] Other polypeptides include those having an aglycosylated Fc domain capable of binding an FcR γ I polypeptide and a second binding domain, wherein the second binding domain is capable of specifically binding a cell-surface molecule. In some embodiments, the second binding domain is an antigen binding domain of an antibody (“antibody antigen binding domain”). In some cases, the second binding domain is not an antibody antigen binding domain. In some embodiments, the second binding domain is capable of specifically binding a cell-surface molecule that is a proteinaceous molecule. The second binding domain may be a ligand for a cell-surface receptor or it may be a receptor for a cell-surface ligand.

[0024] Embodiments also concern a nucleic acid that encodes any of the polypeptides discussed herein. The nucleic acid may be isolated and/or recombinant. It may be a nucleic acid segment that is isolated and/or recombinant. In some embodiments, the nucleic acid is DNA while in others it is RNA. In certain embodiments, the nucleic acid is a DNA segment. In other embodiments, the nucleic acid is an expression vector that is capable of expressing any of the polypeptides having an Fc binding domain with one or more substitutions that specifically binds a human FcR polypeptide. A nucleic acid may encode one or more polypeptides discussed above, which, depending on how the polypeptide is produced may or may not be glycosylated.

[0025] In some embodiments, there are nucleic acids encoding a polypeptide with an Fc domain capable of specifically binding a human FcR polypeptide. The nucleic acid may be placed in a host cell that can express the polypeptide, particularly an aglycosylated version of the polypeptide. The host cell may be a prokaryotic cell, such as a bacterial cell. Alternatively, the host cell may be an eukaryotic cell, such as a mammalian cell. In some embodiments, a host cell contains a first expression

vector, though it may comprises a second expression vector as well. Because some antibodies are made of multiple polypeptides, a host cell that expresses these polypeptides is contemplated in some embodiments. For example, in some embodiments there is a host cell that includes a second expression vector that encodes a polypeptide comprising an immunoglobulin light chain.

[0026] In some embodiments, there is a population of host cells, wherein the population contains a plurality of host cells that express polypeptides having different Fc domains. It is contemplated that the amino acid sequence of any two different Fc domains differs in identity by less than 20%, 15%, 10%, 5% or less.

[0027] In some embodiments there are methods of making the polypeptides described herein (polypeptides having an aglycosylated Fc region) as well as methods of using these polypeptides. Any of these methods may be implemented with respect to any of the polypeptides described herein.

[0028] In some embodiments there are methods for preparing an aglycosylated polypeptide comprising: a) obtaining a host cell capable of expressing an aglycosylated antibody comprising an Fc domain capable of binding an FcR polypeptide, wherein the Fc domain comprises an amino acid substitution at amino acids 382 and 428 and at least one additional substitution of any of the following amino acids: 224, 241, 251, 266, 269, 276, 279, 286, 295, 297, 300, 315, 325, 328, 330, 331, 332, 338, 340, 341, 348, 369, 378, 382, 392, 424, 426, 428 and/or 434; b) incubating the host cell in culture under conditions to promote expression of the aglycosylated antibody; and, c) purifying expressed antibody from the host cell. In some embodiments, the host cell is a prokaryotic cell, such as a bacterial cell. In other embodiments the host cell is a eukaryotic cell and the polypeptide comprises a N297D substitution. In further embodiments, methods involve collecting expressed antibody from the supernatant, which may be done prior to purification.

[0029] In some embodiments methods involve purifying the antibody from the supernatant. This may involve subjecting the antibodies from the supernatant to filtration, HPLC, anion or cation exchange, high performance liquid chromatography (HPLC), affinity chromatography or a combination thereof. In some embodiments, methods involve affinity chromatography using staphylococcal Protein A, which

binds the IgG Fc region. Other purification methods are well known to those of ordinary skill in the art.

[0030] Other embodiments involve methods for inducing an immune response in a subject. Polypeptides having an aglycosylated Fc domain capable of binding an FcR polypeptide may be implemented in such methods. In certain embodiments, an antibody that is aglycosylated and that has an Fc domain capable of binding an Fc γ RI polypeptide is prescribed or administered to a subject. Alternatively, methods may involve treating a subject with such an antibody. Any of the polypeptides described herein may be used. Certain embodiments involve a polypeptide having an aglycosylated human Fc domain that comprises an amino acid substitution at amino acids 382 and 428 and at least one additional substitution of any of the following amino acids: 224, 241, 251, 266, 269, 276, 279, 286, 295, 297, 300, 315, 325, 328, 330, 331, 332, 338, 340, 341, 348, 369, 378, 382, 392, 424, 426, 428 and/or 434.

[0031] In some embodiments, the aglycosylated polypeptide or antibody is capable of specifically binding an activating FcR polypeptide, which refers to an FcR polypeptide that activates one or more immune cells. Activating polypeptides include Fc γ RI, IIa, IIIa, IIb, and IIIc. Fc γ RIIb is an inhibitory FcR polypeptide. In further embodiments, the aglycosylated polypeptide or antibody no longer binds an inhibitory FcR polypeptide at a level comparable to a glycosylated, wild-type Fc domain. In specific embodiments, an aglycosylated polypeptide or antibody specifically binds an Fc γ RI polypeptide. In further embodiments, the aglycosylated polypeptide or antibody has a reduced capability to bind an Fc γ RIIb polypeptide, wherein its affinity is at least 50-fold less than a glycosylated, wild-type version of the polypeptide or antibody. In certain embodiments, the aglycosylated antibody is an aglycosylated version of a therapeutic antibody, which refers to an antibody used in therapy or treatment for a disease or condition. Any antibody or polypeptide discussed herein, including those discussed above, may be used in implementing methods for inducing an immune response. An example of a therapeutic antibody is trastuzumab.

[0032] In some embodiments, there are methods of inducing dendritic cell (DC) mediated cell killing against a target cell expressing a targeted cell surface polypeptide comprising: a) contacting the target cell with a polypeptide comprising a i) mutated and aglycosylated Fc domain capable of specifically binding at least a

dendritic-cell activating FcR and ii) a second binding domain that binds the targeted cell surface polypeptide; and b) exposing the target cell to dendritic cells under conditions that promote killing of the target cell. In some embodiments, the activating FcR is an Fc γ RI polypeptide. In additional embodiments, the polypeptide with an aglycosylated Fc domain specifically binds to an Fc γ RIIB polypeptide at a level that is reduced compared to a polypeptide having a glycosylated wild-type Fc domain. In some embodiments, polypeptides have an Fc domain that comprises at least one amino acid substitution in the following amino acids: 224, 241, 251, 266, 269, 276, 279, 286, 295, 297, 300, 315, 325, 328, 330, 331, 332, 340, 348, 369, 378, 382, 392, 424, 426, 428 and/or 434. Additional polypeptides are discussed above and throughout this application. In some embodiments, the target cell is a cancer cell. Consequently, methods of treating cancer using aglycosylated and mutated Fc domains in place of a glycosylated and wild-type Fc domain in an antibody therapy are contemplated. Treatment of other diseases or conditions involving antibodies that use glycosylated and wild-type Fc domains can be similarly implemented with aglycosylated and Fc variant polypeptides described herein.

[0033] Other embodiments concern methods for screening for an aglycosylated polypeptide having an Fc domain that binds a one or more specific FcR polypeptides comprising: a) obtaining a population of Gram negative bacterial cells, cells of which population express a aglycosylated polypeptide comprising an Fc domain in their periplasm, wherein the population expresses a plurality of different Fc domains; b) contacting the bacterial cells with a first FcR polypeptide under conditions to allow contact between the FcR polypeptide and the aglycosylated Fc domains, wherein the FcR polypeptide is Fc γ RIa, Fc γ RIIa, Fc γ RIIb, Fc γ RIIc, Fc γ RIIIa, Fc γ RIIIb, or Fc α RI; and, c) selecting at least one bacterial cell based on binding of the aglycosylated Fc domain to the first FcR polypeptide. Methods may further involve identifying or isolating the aglycosylated polypeptide from the selected bacterial cell. Also, methods may involve determining whether an aglycosylated polypeptide in selected bacterial cells can bind to other FcR polypeptides. In some embodiments, determining whether an aglycosylated polypeptide in selected bacterial cells can bind to other FcR polypeptides comprises repeating steps a)-c) with a second FcR polypeptide to determine whether the aglycosylated polypeptide also binds the second FcR polypeptide. It is contemplated

that steps a)-c) may be repeated with more than two different FcR polypeptides. In some embodiments, the aglycosylated polypeptide binds multiple FcR polypeptides.

[0034] In some embodiments, methods involve bacterial cells that are *E. coli* cells. In additional embodiments, the Fc domain is an IgG, IgA or IgE Fc domain. In further embodiments, the population of Gram negative bacterial cells comprise a plurality of nucleic acids encoding the plurality of aglycosylated Fc domains. In some cases the plurality of nucleic acids further encodes a membrane secretion signal fused to the plurality of aglycosylated Fc domains. A membrane secretion signal may be PelB or DsbA. Additionally, the aglycosylated Fc domain may include a hinge, CH2 and CH3 region. In certain embodiments, the aglycosylated polypeptide comprises an eukaryotic FcR domain. In some embodiments, there is a polypeptide with an Fc domain that specifically binds one of the polypeptides of Table 1. In certain embodiments, the Fc domain binds human Fc γ RIa, Fc γ RIIa, Fc γ RIIb, Fc γ RIIc, Fc γ RIIIa, Fc γ RIIIb, Fc α RI or C1q. In other embodiments, it has reduced binding affinity for Fc γ RIIb relative to a glycosylated and wild-type version of the Fc domain. Specific methods are disclosed in WO 2008/137475.

[0035] Other embodiments involve methods for optimizing Fc binding to one or more specific FcR polypeptides of an aglycosylated polypeptide having an Fc domain comprising: a) obtaining a population of Gram negative bacterial cells, cells of which population express a aglycosylated polypeptide comprising an Fc domain in their periplasm, wherein the population expresses a plurality of different polypeptides expressing different mutated Fc domains; b) contacting the bacterial cells with a first FcR polypeptide under conditions to allow contact between the FcR polypeptide and the aglycosylated Fc domains, wherein the FcR polypeptide is Fc γ RIa, Fc γ RIIa, Fc γ RIIb, Fc γ RIIc, Fc γ RIIIa, Fc γ RIIIb, or Fc α RI; and c) selecting at least one bacterial cell based on binding of the aglycosylated Fc domain to the first FcR polypeptide. Any of the embodiments discuss above may apply to the implementation of these methods.

[0036] Embodiments discussed in the context of a methods and/or composition of the invention may be employed with respect to any other method or composition described herein. Thus, an embodiment pertaining to one method or

composition may be applied to other methods and compositions of the invention as well.

[0037] As used herein the terms “encode” or “encoding” with reference to a nucleic acid are used to make the invention readily understandable by the skilled artisan however these terms may be used interchangeably with “comprise” or “comprising” respectively.

[0038] As used herein the specification, "a" or "an" may mean one or more. As used herein in the claim(s), when used in conjunction with the word "comprising", the words "a" or "an" may mean one or more than one.

[0039] The use of the term “or” in the claims is used to mean “and/or” unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and “and/or.” As used herein “another” may mean at least a second or more.

[0040] Throughout this application, the term “about” is used to indicate that a value includes the inherent variation of error for the device, the method being employed to determine the value, or the variation that exists among the study subjects.

[0041] Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0042] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

[0043] **FIG. 1:** Mutation points of isolated aglycosylated Fc5 (382E and 428M) represented on the 3D structure of glycosylated IgG1 Fc (PBD Code: 1FC1).

[0044] FIG. 2. Two beta sheets including 382E in β -sheet C and 428M in β -sheet C of CH3 domain represented on the crystal structure of glycosylated IgG. (PDB Code: 1FC1).

[0045] FIG. 3. Error prone PCR library for engineering aglycosylated Fc5 domains.

[0046] FIG. 4. Fluorescence histogram of spheroplasts from different rounds of sorting labeled with Fc γ RIa-FITC.

[0047] FIG. 5. DNA sequences of isolated Fc mutant clones exhibiting higher affinity to Fc γ RIa than Fc5. Mean fluorescence values for the respective clones labeled with Fc γ RIa are shown in parenthesis.

[0048] FIG. 6. Mutation points of isolated aglycosylated Fc601-619 represented on the 3D structure of glycosylated IgG1 Fc (PDB Code: 1FC1).

[0049] FIG. 7. Fluorescence histogram of spheroplasts for wild type Fc, Fc5, and Fc601 labeled with 30 nM of Fc γ RIa-FITC. M: Mean fluorescence intensity.

[0050] FIG. 8. Mutation points of isolated aglycosylated Fc 601 (K338R, G341V, E382V, M428I) represented on the 3D structure of glycosylated IgG1 Fc (PDB Code: 1FC1).

[0051] FIG. 9. Map of plasmid pSTJ4-Herceptin IgG1.

[0052] FIG. 10. Kinetic rates and equilibrium dissociation constants of aglycosylated trastuzumab, trastuzumab-Fc5, trastuzumab-Fc601, and glycosylated trastuzumab determined by BIACore analysis for binding to Fc γ RIa.

[0053] FIG. 11. ELISA assays for binding of trastuzumab antibodies to Fc γ RIIa-GST.

[0054] FIG. 12. ELISA assays for binding of trastuzumab antibodies to Fc γ RIIb-GST.

[0055] FIG. 13. ELISA assays for binding of trastuzumab antibodies to Fc γ RIIIa.

[0056] FIG. 14. ELISA assays for pH dependent binding to FcRn at pH 7.4 and 6.0. Plates were coated with aglycosylated trastuzumab, trastuzumab-Fc5, trastuzumab-Fc601 or commercial glycosylated trastuzumab and the binding of FcRn was detected using anti-GST-HRP.

[0057] FIG. 15. Library for higher affinity to Fc γ RIa than Fc5 and for pH dependent FcRn binding.

[0058] FIG. 16. Gene assembly PCR for the construction of 4 sub-libraries that randomized upper CH2 region.

[0059] FIG. 17. DNA sequences of isolated Fc mutant clones exhibiting higher affinity to Fc γ RIa than Fc5. Mean fluorescence values for the respective clones labeled with Fc γ RIa are shown in parenthesis.

[0060] FIG. 18. Summary of mutations in Fc701 – 709.

[0061] FIG. 19. Fluorescence histogram of spheroplasted cells for wild type Fc, Fc5, Fc701, and Fc702 labeled with 1 nM of Fc γ RIa-FITC. M: Mean fluorescence intensity.

[0062] FIG. 20. Fluorescence histogram of spheroplasted cells for wild type Fc, Fc5, Fc601, and Fc701 labeled with 1 nM of Fc γ RIa-FITC. M: Mean fluorescence intensity.

[0063] FIG. 21. Mutation points of isolated aglycosylated Fc5 (382E and 428M) represented on the 3D structure of glycosylated IgG1 Fc (PDB Code: 1FC1)

[0064] FIG. 22. Kinetic rates and equilibrium dissociation constants of aglycosylated trastuzumab, trastuzumab-Fc5, trastuzumab-Fc601, trastuzumab-Fc701 and glycosylated trastuzumab determined by BIACore analysis for binding to Fc γ RI.

[0065] FIG. 23. ELISA assays for pH dependent binding to FcRn at pH 7.4 and 6.0. Plates were coated with aglycosylated trastuzumab, trastuzumab-Fc5, trastuzumab-Fc601, trastuzumab-Fc701 or commercial glycosylated trastuzumab and the binding of FcRn was detected using anti-GST-HRP.

[0066] FIG. 24. Covalently anchored full length IgG display system.

[0067] FIG. 25. Comparison of FACS signals between 2 plasmid covalently anchored full length IgG display system and dicistronic system. Trastuzumab full length IgGs were expressed using either the 2 plasmid anchored full length IgG display system or dicistronic full length IgG display system. M: Mean fluorescence intensity. Spheroplasts were incubated with 30 nM Fc γ RI-FITC probe for detection.

[0068] FIG. 26. Comparison of FACS signals between the 2 plasmids covalently anchored full length IgG display system and dicistronic system. Trastuzumab full length IgGs were expressed using either the 2 plasmids anchored full length IgG display system or dicistronic full length IgG display system. M: Mean fluorescence intensity. Spheroplasts were incubated with 30 nM Fc γ RIIa-GST and labeled with polyclonal anti-GST-FITC (1:200) probe for detection.

[0069] FIG. 27. FACS analysis of trastuzumab full length IgG using 2 plasmids covalently anchored full length IgG display system and dicistronic system. Spheroplasts expressing trastuzumab full length IgGs were incubated with 30 nM Fc γ RI-FITC probe for detection. M: Mean fluorescence intensity.

[0070] FIG. 28. FACS analysis of trastuzumab full length IgG using 2 plasmids covalently anchored full length IgG display system and dicistronic system. Spheroplasts expressing trastuzumab full length IgGs were incubated with 30 nM Fc γ RIIa-GST and labeled with polyclonal anti-GST-FITC (1:200) probe for detection. M: Mean fluorescence intensity.

[0071] FIG. 29. Library for randomization of upper CH2 region.

[0072] FIG. 30. ADCC assays with PBMC as effector cells and SkBr3 as the target cell. *, P < 0.05.

[0073] FIG. 31. ADCC assays with mDCs as effector cells and SkBr3 as the target cell. *, P < 0.05 ; **, P < 0.01.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0074] The inventors previously overcame several major problems with current immunotherapeutic technologies in providing aglycosylated antibody Fc domains that are able to bind to Fc receptor polypeptides. Additional Fc domains with

engineered properties have been developed. Further embodiments and advantages are described below, though information about Fc libraries and screening methods are provided.

I. Periplasmic Expression

[0075] In some embodiments, polypeptide comprising an antibody Fc domain may be expressed in the periplasmic space of a gram negative bacteria. Furthermore, in some aspects an antibody Fc domain may be anchored to the periplasmic face of the inner membrane. For example, an Fc domain may be directly fused to a membrane spanning or membrane bound polypeptide or may interact (*e.g.*, via protein-protein interactions) with a membrane spanning or membrane bound polypeptide. Such a technique may be termed “Anchored Periplasmic Expression” or “APEX”.

[0076] The periplasmic compartment is contained between the inner and outer membranes of Gram negative cells (see, *e.g.*, Oliver, 1996). As a sub-cellular compartment, it is subject to variations in size, shape and content that accompany the growth and division of the cell. Within a framework of peptidoglycan heteropolymer is a dense milieu of periplasmic proteins and little water, lending a gel-like consistency to the compartment (Hobot *et al.*, 1984; van Wielink and Duine, 1990). The peptidoglycan is polymerized to different extents depending on the proximity to the outer membrane, close-up it forms the murein sacculus that affords cell shape and resistance to osmotic lysis.

[0077] The outer membrane (see Nikaido, 1996) is composed of phospholipids, porin proteins and, extending into the medium, lipopolysaccharide (LPS). The molecular basis of outer membrane integrity resides with LPS ability to bind divalent cations (Mg^{2+} and Ca^{2+}) and link each other electrostatically to form a highly ordered quasi-crystalline ordered “tiled roof” on the surface (Labischinski *et al.*, 1985). The membrane forms a very strict permeability barrier allowing passage of molecules no greater than around 650 Da (Burman *et al.*, 1972; Decad and Nikaido, 1976) via the porins. The large water filled porin channels are primarily responsible for allowing free passage of mono and disaccharides, ions and amino acids in to the periplasm compartment (Nikaido and Nakae, 1979; Nikaido and Vaara, 1985). With such strict physiological regulation of access by molecules to the periplasm it may appear, at first glance, inconceivable that large ligands (*i.e.*, larger than the 650 Da

exclusion limit) could be employed in screening methods. However, the inventors have shown that ligands greater than 2000 Da in size can diffuse into the periplasm without disruption of the periplasmic membrane. Such diffusion can be aided by one or more treatments of a bacterial cell, thereby rendering the outer membrane more permeable, as is described herein below.

[0078] Method for expressing polypeptides and in particular antibodies in the periplasmic space are known in the art for example see U.S. Patent 7,094,571 and U.S. Patent Publ. 20030180937 and 20030219870. In some cases, a gram negative bacterial cell of the invention may be defined as an *E. coli* cell. Furthermore, in some aspects a Gram negative bacterial cell may be defined as a genetically engineered bacterial cell such as a Jude-1 strain of *E. coli*.

II. Permeabilization of the Outer Membrane

[0079] In some embodiments, methods involve disrupting, permeabilizing or removing the outer membrane of bacteria are well known in the art, for example, see U.S. Patent 7,094,571. For instance, prior to contacting the bacterial cells with an FcR polypeptide the outer membrane of the bacterial cell may be treated with hyperosmotic conditions, physical stress, lysozyme, EDTA, a digestive enzyme, a chemical that disrupts the outer membrane, or by infecting the bacterium with a phage or a combination of the foregoing methods. Thus, in some cases, the outer membrane may be disrupted by lysozyme and EDTA treatment. Furthermore, in certain embodiments, the bacterial outer membrane may be removed entirely.

[0080] In one embodiment, methods are employed for increasing the permeability of the outer membrane to one or more labeled ligands. This can allow screening access of labeled ligands otherwise unable to cross the outer membrane. However, certain classes of molecules, for example, hydrophobic antibiotics larger than the 650 Da exclusion limit, can diffuse through the bacterial outer membrane itself, independent of membrane porins (Farmer *et al.*, 1999). The process may actually permeabilize the membrane on so doing (Jouenne and Junter, 1990). Such a mechanism has been adopted to selectively label the periplasmic loops of a cytoplasmic membrane protein *in vivo* with a polymyxin B nonapeptide (Wada *et al.*, 1999). Also, certain long chain phosphate polymers (100 Pi) appear to bypass the

normal molecular sieving activity of the outer membrane altogether (Rao and Torriani, 1988).

[0081] Conditions have been identified that lead to the permeation of ligands into the periplasm without loss of viability or release of the expressed proteins from the cells, but the invention may be carried out without maintenance of the outer membrane. As demonstrated herein Fc domains expressed or anchored candidate binding polypeptides in the periplasmic space the need for maintenance of the outer membrane (as a barrier to prevent the leakage of the binding protein from the cell) to detect bound labeled ligand is removed. As a result, cells expressing binding proteins anchored to the outer (periplasmic) face of the cytoplasmic membrane can be fluorescently labeled simply by incubating with a solution of fluorescently labeled ligand in cells that either have a partially permeabilized membrane or a nearly completely removed outer membrane.

[0082] The permeability of the outer membrane of different strains of bacterial hosts can vary widely. It has been shown previously that increased permeability due to OmpF overexpression was caused by the absence of a histone like protein resulting in a decrease in the amount of a negative regulatory mRNA for OmpF translation (Painbeni *et al.*, 1997). Also, DNA replication and chromosomal segregation is known to rely on intimate contact of the replisome with the inner membrane, which itself contacts the outer membrane at numerous points. A preferred host for library screening applications is *E. coli* ABLEC strain, which additionally has mutations that reduce plasmid copy number.

[0083] Treatments such as hyperosmotic shock can improve labeling significantly. It is known that many agents including, calcium ions (Bukau *et al.*, 1985) and even Tris buffer (Irvin *et al.*, 1981) alter the permeability of the outer-membrane. Further, phage infection stimulates the labeling process. Both the filamentous phage inner membrane protein pIII and the large multimeric outer membrane protein pIV can alter membrane permeability (Boeke *et al.*, 1982) with mutants in pIV known to improve access to maltodextrins normally excluded (Marciano *et al.*, 1999). Using the techniques of the invention, comprising a judicious combination of strain, salt and phage, a high degree of permeability may be achieved (Daugherty *et al.*, 1999). Cells comprising anchored or periplasm-associated

polypeptides bound to fluorescently labeled ligands can then be easily isolated from cells that express binding proteins without affinity for the labeled ligand using flow cytometry or other related techniques. However, in some cases, it will be desired to use less disruptive techniques in order to maintain the viability of cells. EDTA and Lysozyme treatments may also be useful in this regard.

III. Antibody-binding polypeptides

[0084] In certain aspects there are methods for identifying antibody Fc domains with a specific affinity for antibody-binding polypeptide such as an Fc receptor. In some embodiments, an Fc domain is engineered to bind one or more specific Fc receptors. Additionally or alternatively, an Fc domain may be engineered so that it does not specifically bind one or more specific Fc receptors.

[0085] In certain embodiments, there are compositions comprising a proteinaceous molecule that has been modified relative to a native or wild-type protein.

[0086] In some embodiments that proteinaceous compound has been deleted of amino acid residues; in other embodiments, amino acid residues of the proteinaceous compound have been replaced, while in still further embodiments both deletions and replacements of amino acid residues in the proteinaceous compound have been made. Furthermore, a proteinaceous compound may include an amino acid molecule comprising more than one polypeptide entity. As used herein, a “proteinaceous molecule,” “proteinaceous composition,” “proteinaceous compound,” “proteinaceous chain” or “proteinaceous material” generally refers, but is not limited to, a protein of greater than about 200 amino acids or the full length endogenous sequence translated from a gene; a polypeptide of 100 amino acids or greater; and/or a peptide of 3 to 100 amino acids. All the “proteinaceous” terms described above may be used interchangeably herein; however, it is specifically contemplated that embodiments may be limited to a particular type of proteinaceous compound, such as a polypeptide. Furthermore, these terms may be applied to fusion proteins or protein conjugates as well. A protein may include more than one polypeptide. An IgG antibody, for example, has two heavy chain polypeptides and two light chain polypeptides, which are joined to each other through disulfide bonds.

[0087] As used herein a “distinct Fc domain” may be defined as a domain that differs from another Fc by as little as one amino acid. Methods for making a library of distinct antibody Fc domains or nucleic acids that encode antibodies are well known in the art and exemplified herein. For example, in some cases Fc domains may be amplified by error prone PCR as exemplified herein. Furthermore, in certain cases a plurality of antibody Fc domains may comprise a stretch (1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) amino acids that have been randomized. In certain cases specific mutations may be engineered into Fc domains. For example, in some aspects, residues that are normally glycosylated in an antibody Fc domain may be mutated. Furthermore, in certain aspects, residues that are normally glycosylated (or adjacent residues) may be used as a site for an insertion of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acids. An amino acid insertion may be made at, or adjacent to, a residue corresponding to amino acid 384 of the IgG1 Fc (SEQ ID NO:2). In still further cases, a population of gram negative bacteria according to the invention may be defined as comprising at least about 1×10^3 , 1×10^4 , 1×10^5 , 1×10^6 , 1×10^7 , 1×10^8 , or more distinct antibodies Fc domains. In some specific cases, a population of Gram negative bacterial cells may be produced by a method comprising the steps of: (a) preparing a plurality of nucleic acid sequences encoding a plurality of distinct antibody Fc domains; and (b) transforming a population of Gram negative bacteria with said nucleic acids wherein the Gram negative bacteria comprise a plurality of antibody Fc domains expressed in the periplasm.

[0088] A variety of antibody-binding domains (e.g., FcR polypeptides) are known in the art and may be used in the methods and compositions of the invention. For example, in some aspects, an FcR may have specificity for a particular type or subtype of Ig, such as IgA, IgM, IgE or IgG (e.g., IgG1, IgG2a, IgG2b, IgG3 or IgG4). Thus, in some embodiments the antibody-binding domain may be defined as an IgG binding domain. The FcR polypeptide may comprises an eukaryotic, prokaryotic, or synthetic FcR domain. For instance, an antibody Fc-binding domain may be defined as a mammalian, bacterial or synthetic binding domain. Some Fc-binding domains for use in the invention include but are not limited to a binding domain from one of the polypeptides of Table 1. For example, an Fc-binding polypeptide may be encoded by an FCGR2A, FCGR2B, FCGR2C, FCGR3A, FCGR3B, FCGR1A, Fcgr1, FCGR2, FCGR2, Fcgr2, FCGR3, FCGR3, Fcgr3,

FCGR3, Fcgr3, FCGRT, mrp4, spa or spg gene. Preferably, an FcR polypeptide for use according to the invention may be an Fc binding region from human Fc γ RIa, Fc γ RIIa, Fc γ RIIb, Fc γ RIIc, Fc γ RIIIa, Fc γ RIIIb, Fc α RI or C1q.

[0089] In still further embodiments of the invention an Fc polypeptide may be anchored to the inner membrane of a Gram negative bacteria. Methods and compositions for the anchoring of polypeptides to the inner membrane of Gram negative bacterial have previously been described (U.S. Patent 7,094,571 and U.S. Patent Publ. 20050260736). Thus, in some aspects, an Fc domain may be fused to a polypeptide that is associated with or integrated in a bacterial inner membrane. Such a fusion protein may comprise an N terminal or C terminal fusion with an Fc domain and in some case may comprise additional linker amino acids between the membrane anchoring polypeptide and the Fc domain. In certain specific cases, a membrane anchoring polypeptide may be the first six amino acids encoded by the *E. coli* NlpA gene, one or more transmembrane α -helices from an *E. coli* inner membrane protein, a gene III protein of filamentous phage or a fragment thereof, or an inner membrane lipoprotein or fragment thereof. Thus, as an example, a membrane anchoring polypeptide may be an inner membrane lipoprotein or fragment thereof such as from AraH, MglC, MalF, MalG, MalC, MalD, RbsC, RbsC, ArtM, ArtQ, GlnP, ProW, HisM, HisQ, LivH, LivM, LivA, LivE, DppB, DppC, OppB, AmiC, AmiD, BtuC, ThuD, FecC, FecD, FecR, FepD, NikB, NikC, CysT, CysW, UgpA, UgpE, PstA, PstC, PotB, PotC, PotH, Pod, ModB, NosY, PhnM, LacY, SecY, TolC, Dsb, B, DsbD, TouB, TatC, CheY, TraB, ExbD, ExbB or Aas.

[0090] The skilled artisan will understand that methods for selecting cells based upon their interaction (binding) with an FcR are well known in the art. For example, an FcR may be immobilized on a column or bead (e.g., a magnetic bead) and the bacterial cell binding to the FcR separated by repeated washing of the bead (e.g., magnetic separation) or column. Furthermore, in some aspects a target ligand may be labeled such as with a fluorophor, a radioisotope or an enzyme. Thus, bacterial cells may, in some cases, be selected by detecting a label on a bound FcR. For example, a fluorophore may be used to select cells using fluorescence activated cell sorting (FACS). Furthermore, in some aspects, bacterial cells may be selected based on binding or lack of binding two or more FcR polypeptides. For instance,

bacteria may be selected that display antibodies that bind to two FcR polypeptides, wherein each FcR is used to select the bacterial sequentially. Conversely, in certain aspects, bacteria may be selected that display antibody Fc domains that bind to one FcR (such as an FcR comprising a first label) but not to a second FcR (e.g., comprising a second label). The foregoing method maybe used, for example, to identify antibody Fc domains that bind to a specific FcR but not a second specific FcR.

[0091] In certain embodiments the size of the at least one proteinaceous molecule may comprise, but is not limited to, about or at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 275, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000 or greater amino molecule residues, and any range derivable therein. Compounds may include the above-mentioned number of contiguous amino acids from SEQ ID NO:2 (human IgG Fc polypeptide) or from SEQ ID NOs 4-31 and these may be further qualified as having a percent identity or homology to SEQ ID NO:2 or any of SEQ ID NO:4-31 (discussed below). It is contemplated that embodiments with respect to SEQ ID NO:2 may be employed with respect to any other amino acid sequences described herein, and vice versa, if appropriate.

[0092] As used herein, an “amino molecule” refers to any amino acid, amino acid derivative or amino acid mimic as would be known to one of ordinary skill in the art. In certain embodiments, the residues of the proteinaceous molecule are sequential, without any non-amino molecule interrupting the sequence of amino molecule residues. In other embodiments, the sequence may comprise one or more non-amino molecule moieties. In particular embodiments, the sequence of residues of the proteinaceous molecule may be interrupted by one or more non-amino molecule moieties.

A. Modified Proteins and Polypeptides

[0093] Embodiments concerns modified proteins and polypeptides, particularly a modified protein or polypeptide that exhibits at least one functional activity that is comparable to the unmodified version, yet the modified protein or polypeptide possesses an additional advantage over the unmodified version, such as

provoking ADCC, easier or cheaper to produce, eliciting fewer side effects, and/or having better or longer efficacy or bioavailability. Thus, when the present application refers to the function or activity of “modified protein” or a “modified polypeptide” one of ordinary skill in the art would understand that this includes, for example, a protein or polypeptide that 1) performs at least one of the same activities or has at least one of the same specificities as the unmodified protein or polypeptide, but that may have a different level of another activity or specificity; and 2) possesses an additional advantage over the unmodified protein or polypeptide. Determination of activity may be achieved using assays familiar to those of skill in the art, particularly with respect to the protein’s activity, and may include for comparison purposes, for example, the use of native and/or recombinant versions of either the modified or unmodified protein or polypeptide. It is specifically contemplated that embodiments concerning a “modified protein” may be implemented with respect to a “modified polypeptide,” and vice versa. In addition to the modified proteins and polypeptides discussed herein, embodiments may involve domains, polypeptides, and proteins described in WO 2008/137475.

[0094] Modified proteins may possess deletions and/or substitutions of amino acids; thus, a protein with a deletion, a protein with a substitution, and a protein with a deletion and a substitution are modified proteins. In some embodiments these modified proteins may further include insertions or added amino acids, such as with fusion proteins or proteins with linkers, for example. A “modified deleted protein” lacks one or more residues of the native protein, but possesses the specificity and/or activity of the native protein. A “modified deleted protein” may also have reduced immunogenicity or antigenicity. An example of a modified deleted protein is one that has an amino acid residue deleted from at least one antigenic region—that is, a region of the protein determined to be antigenic in a particular organism, such as the type of organism that may be administered the modified protein.

[0095] Substitutional or replacement variants typically contain the exchange of one amino acid for another at one or more sites within the protein and may be designed to modulate one or more properties of the polypeptide, particularly its effector functions and/or bioavailability. Substitutions may or may not be

conservative, that is, one amino acid is replaced with one of similar shape and charge. Conservative substitutions are well known in the art and include, for example, the changes of: alanine to serine; arginine to lysine; asparagine to glutamine or histidine; aspartate to glutamate; cysteine to serine; glutamine to asparagine; glutamate to aspartate; glycine to proline; histidine to asparagine or glutamine; isoleucine to leucine or valine; leucine to valine or isoleucine; lysine to arginine; methionine to leucine or isoleucine; phenylalanine to tyrosine, leucine or methionine; serine to threonine; threonine to serine; tryptophan to tyrosine; tyrosine to tryptophan or phenylalanine; and valine to isoleucine or leucine.

[0096] In addition to a deletion or substitution, a modified protein may possess an insertion of residues, which typically involves the addition of at least one residue in the polypeptide. This may include the insertion of a targeting peptide or polypeptide or simply a single residue. Terminal additions, called fusion proteins, are discussed below.

[0097] The term “biologically functional equivalent” is well understood in the art and is further defined in detail herein. Accordingly, sequences that have between about 70% and about 80%, or between about 81% and about 90%, or even between about 91% and about 99% of amino acids that are identical or functionally equivalent to the amino acids of a native polypeptide are included, provided the biological activity of the protein is maintained. A modified protein may be biologically functionally equivalent to its native counterpart.

[0098] It also will be understood that amino acid and nucleic acid sequences may include additional residues, such as additional N- or C-terminal amino acids or 5' or 3' sequences, and yet still be essentially as set forth in one of the sequences disclosed herein, so long as the sequence meets the criteria set forth above, including the maintenance of biological protein activity where protein expression is concerned. The addition of terminal sequences particularly applies to nucleic acid sequences that may, for example, include various non-coding sequences flanking either of the 5' or 3' portions of the coding region or may include various internal sequences, *i.e.*, introns, which are known to occur within genes.

[0099] The following is a discussion based upon changing of the amino acids of a protein to create an equivalent, or even an improved, second-generation molecule. For example, certain amino acids may be substituted for other amino acids in a protein structure with or without appreciable loss of interactive binding capacity with structures such as, for example, binding sites to substrate molecules. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid substitutions can be made in a protein sequence, and in its underlying DNA coding sequence, and nevertheless produce a protein with like properties. It is thus contemplated by the inventors that various changes may be made in the DNA sequences of genes without appreciable loss of their biological utility or activity, as discussed below. A proteinaceous molecule has "homology" or is considered "homologous" to a second proteinaceous molecule if one of the following "homology criteria" is met: 1) at least 30% of the proteinaceous molecule has sequence identity at the same positions with the second proteinaceous molecule; 2) there is some sequence identity at the same positions with the second proteinaceous molecule and at the nonidentical residues, at least 30% of them are conservative differences, as described herein, with respect to the second proteinaceous molecule; or 3) at least 30% of the proteinaceous molecule has sequence identity with the second proteinaceous molecule, but with possible gaps of nonidentical residues between identical residues. As used herein, the term "homologous" may equally apply to a region of a proteinaceous molecule, instead of the entire molecule. If the term "homology" or "homologous" is qualified by a number, for example, "50% homology" or "50% homologous," then the homology criteria, with respect to 1), 2), and 3), is adjusted from "at least 30%" to "at least 50%." Thus it is contemplated that there may be homology of at least 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or more between two proteinaceous molecules or portions of proteinaceous molecules.

[00100] Alternatively, a modified polypeptide may be characterized as having a certain percentage of identity to an unmodified polypeptide or to any polypeptide sequence disclosed herein, including SEQ ID NO:2 or any of SEQ ID NOs:4-31. The percentage identity may be at most or at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% (or any range derivable therein) between two proteinaceous molecules or portions of proteinaceous

molecules. It is contemplated that percentage of identity discussed above may relate to a particular region of a polypeptide compared to an unmodified region of a polypeptide. For instance, a polypeptide may contain a modified or mutant Fc domain that can be characterized based on the identity of the amino acid sequence of the modified or mutant Fc domain to an unmodified or mutant Fc domain from the same species. A modified or mutant human Fc domain characterized, for example, as having 90% identity to an unmodified Fc domain means that 90% of the amino acids in that domain are identical to the amino acids in the unmodified human Fc domain (SEQ ID NO:2).

[00101] In making such changes, the hydrophobic index of amino acids may be considered. The importance of the hydrophobic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte & Doolittle, 1982). It is accepted that the relative hydrophobic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

[00102] It also is understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U.S. Patent 4,554,101 states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein. As detailed in U.S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 ± 1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

[00103] It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still produce a biologically equivalent and immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those that are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

[00104] As outlined above, amino acid substitutions generally are based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions that take into consideration the various foregoing characteristics are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

[00105] A variety of Fc receptors to which Fc domains bind are well known in the art and some examples of receptors are listed below in Table 1.

Table 1: Selected FcR Polypeptides

Protein name	Gene name	Description	Organisms	Length (aa)	Reference
Fc-gamma RII-a (CD32)	FCGR2A	Low affinity immunoglobulin gamma Fc region receptor II-a precursor	<i>Homo sapiens</i> (Human)	317	(Stuart <i>et al.</i> , 1987)
Fc-gamma RII-a	FCGR2A	Low affinity immunoglobulin gamma Fc region receptor II-a precursor	<i>Pan troglodytes</i> (Chimpanzee)	316	
Fc-gamma RII-b	FCGR2B	Low affinity immunoglobulin gamma Fc region receptor II-b precursor	<i>Homo sapiens</i> (Human)	310	(Stuart <i>et al.</i> , 1989)
Fc-gamma RII-c	FCGR2C	Low affinity immunoglobulin gamma Fc region receptor II-c precursor	<i>Homo sapiens</i> (Human)	323	(Stuart <i>et al.</i> , 1989)
Fc-gamma RIIIa	FCGR3A	Low affinity immunoglobulin gamma Fc region receptor III-A precursor	<i>Homo sapiens</i> (Human)	254	(Ravetch and Perussia, 1989)
Fc-gamma RIIIb	FCGR3B	Low affinity immunoglobulin gamma Fc region receptor III-B precursor	<i>Homo sapiens</i> (Human)	233	(Ravetch and Perussia, 1989)

Protein name	Gene name	Description	Organisms	Length (aa)	Reference
Fc-gamma RI (CD64)	FCGR1A	High affinity immunoglobulin gamma Fc receptor I precursor	<i>Homo sapiens</i> (Human)	374	(Allen and Seed, 1988)
Fc-gamma RI	Fcgr1	High affinity immunoglobulin gamma Fc receptor I precursor	<i>Mus musculus</i> (Mouse)	404	(Sears <i>et al.</i> , 1990)
Fc-gamma RII	FCGR2	Low affinity immunoglobulin gamma Fc region receptor II precursor	<i>Bos taurus</i> (Bovine)	296	(Zhang <i>et al.</i> , 1994)
Fc-gamma RII	FCGR2	Low affinity immunoglobulin gamma Fc region receptor II precursor	<i>Cavia porcellus</i> (Guinea pig)	341	(Tominaga <i>et al.</i> , 1990)
Fc-gamma RII	Fcgr2	Low affinity immunoglobulin gamma Fc region receptor II precursor	<i>Mus musculus</i> (Mouse)	330	(Ravetch <i>et al.</i> , 1986)
Fc-gamma RII	Fcgr2	Low affinity immunoglobulin gamma Fc region receptor II precursor	<i>Rattus norvegicus</i> (Rat)	285	(Bocek and Pecht, 1993)
Fc-gamma RIII	FCGR3	Low affinity immunoglobulin gamma Fc region receptor III precursor	<i>Bos taurus</i> (Bovine)	250	(Collins <i>et al.</i> , 1997)
Fc-gamma RIII	FCGR3	Low affinity immunoglobulin gamma Fc region receptor III precursor	<i>Macaca fascicularis</i> (Crab eating macaque) (<i>Cynomolgus</i> monkey)	254	
Fc-gamma RIII	Fcgr3	Low affinity immunoglobulin gamma Fc region receptor III precursor	<i>Mus musculus</i> (Mouse)	261	(Ravetch <i>et al.</i> , 1986)

Protein name	Gene name	Description	Organisms	Length (aa)	Reference
Fc-gamma RIII	FCGR3	Low affinity immunoglobulin gamma Fc region receptor III precursor	<i>Sus scrofa</i> (Pig)	257	(Halloran <i>et al.</i> , 1994)
Fc-gamma RIII	Fcgr3	Low affinity immunoglobulin gamma Fc region receptor III precursor	<i>Rattus norvegicus</i> (Rat)	267	(Zeger <i>et al.</i> , 1990)
FcRn	FCGRT	IgG receptor transporter FcRn large subunit p51 precursor	<i>Homo sapiens</i> (Human)	365	
FcRn	FCGRT	IgG receptor transporter FcRn large subunit p51 precursor	<i>Macaca fascicularis</i> (Crab eating macaque) (<i>Cynomolgus</i> monkey)	365	
FcRn	Fcgtrt	IgG receptor transporter FcRn large subunit p51 precursor	<i>Mus musculus</i> (Mouse)	365	(Ahouse <i>et al.</i> , 1993)
FcRn	Fcgtrt	IgG receptor transporter FcRn large subunit p51 precursor	<i>Rattus norvegicus</i> (Rat)	366	(Simister and Mostov, 1989)
MRP protein	mrp4	Fibrinogen- and Ig-binding protein precursor	<i>Streptococcus pyogenes</i>	388	(Stenberg <i>et al.</i> , 1992)
Protein B		cAMP factor	<i>Streptococcus agalactiae</i>	226	(Ruhlmann <i>et al.</i> , 1988)
protein A	spa	Immunoglobulin G-binding protein A precursor	<i>Staphylococcus aureus</i> (strain NCTC 8325)	516	(Uhlen <i>et al.</i> , 1984)
protein A	spa	Immunoglobulin G-binding protein A precursor	<i>Staphylococcus aureus</i>	508	(Shuttleworth <i>et al.</i> , 1987)

Protein name	Gene name	Description	Organisms	Length (aa)	Reference
protein A	spa	Immunoglobulin G-binding protein A precursor	<i>Staphylococcus aureus</i> (strain Mu50 / ATCC 700699)	450	(Kuroda <i>et al.</i> , 2001)
protein A	spa	Immunoglobulin G-binding protein A precursor	<i>Staphylococcus aureus</i> (strain N315)	450	(Kuroda <i>et al.</i> , 2001)
protein G	spg	Immunoglobulin G-binding protein G precursor	<i>Streptococcus</i> sp. group G	448	(Fahnestock <i>et al.</i> , 1986)
protein G	spg	Immunoglobulin G-binding protein G precursor	<i>Streptococcus</i> sp. group G	593	(Olsson <i>et al.</i> , 1987)
protein H		Immunoglobulin G-binding protein H precursor	<i>Streptococcus pyogenes</i> serotype M1	376	(Gomi <i>et al.</i> , 1990)
Protein sbi	sbi	Immunoglobulin G-binding protein sbi precursor	<i>Staphylococcus aureus</i> (strain NCTC 8325-4)	436	(Zhang <i>et al.</i> , 1998)
Allergen Asp fl 1		Allergen Asp fl 1 causes an allergic reaction in human. Binds to IgE and IgG	<i>Aspergillus flavus</i>	32	
Allergen Asp fl 2		Allergen Asp fl 2 causes an allergic reaction in human. Binds to IgE and IgG	<i>Aspergillus flavus</i>	20	
Allergen Asp fl 3		Allergen Asp fl 3 causes an allergic reaction in human. Binds to IgE and IgG	<i>Aspergillus flavus</i>	32	
Fc-epsilon RI		IgE receptor displayed on Mast cells, Eosinophils and Basophils	<i>Homo sapiens</i> (Human)		

Protein name	Gene name	Description	Organisms	Length (aa)	Reference
Fc-alpha RI (CD86)		IgA (IgA1, IgA2) receptor displayed on Macrophages	<i>Homo sapiens</i> (Human)		
C1q	C1QA NP_057075.1, C1QB NP_000482.3, C1QC NP_758957.1	C1q is multimeric complex that binds to antibody Fc composed of 6 A chains, 6 B chains and 6 C chains	<i>Homo sapiens</i> (Human)		

[00106] As discussed above, a polypeptide may comprise an aglycosylated antibody Fc domain capable of binding an FcR polypeptide. In some aspects, the aglycosylated Fc domain may be further defined as having a specific affinity for an FcR polypeptide under physiological conditions. For instance an Fc domain may have an equilibrium dissociation constant between about 10^{-6} M to about 10^{-9} M under physiological conditions. Furthermore in some aspects an aglycosylated Fc domain may be defined as comprising one or more amino acid substitution or insertion relative to a wild-type sequence, such as a human wild-type sequence.

[00107] Means of preparing such a polypeptide include those discussed in WO 2008/137475. One can alternatively prepare such polypeptides directly by genetic engineering techniques such as, for example, by introducing selected amino acid substitutions or insertions into a known Fc background, wherein the insertion or substitution provides an improved FcR binding capability to aglycosylated Fc regions. The inventors have identified as particularly preferred substitutions for achieving such improved FcR binding as those at positions 331, 382 and/or 428 of the Fc domain (for example, see Nagaoka and Akaike 2003; such as P331, E382 and/or M428 of the human IgG Fc domain sequence as shown U.S. Patent Publ. US20060173170), and still more preferred are one or more substitutions defined by P331L, E382V, M428I or M428L.

[00108] In addition to substitutions described in Tables 5 and 6 below, a polypeptide may have a substitution that includes one or more of 426, 229, 322, 350, 361, 372, 442, 402, 224, 430, 238, 436, 310, 313, 384, 372, 380 or 331 of the Fc domain, such as S426, C229, K322, T350, N361, F372, S442, G402, H224, E430, P238, Y436, H310, W313, N384, F372, E380 or P331 of the human IgG Fc domain, with the specific preferred examples being a) E382 and M428; b) N361, E382 and M428; c) N361, F372, E382 and M428; d) H310, K322, T350, E382, S426 and S442; e) C229R, E382 and M428; f) W313 and M428; g) E382, N384 and M428; h) E380, E382 and N384; i) N361, E382 and M428; j) E382, M428 and Y436; k) P238, E382, S426, M428 and E430; l) E380, E382, N384, S426, M428 and E430; m) E382, S426, M428 and E430; n) H224, E382, S426, M428 and E430; o) P331; p) S239, I253, Q347, E382; q) E382, G402 and M428; and r) E382, P331 and M428. Particular substitutions include a) E382V and M428I; b) E382V; c) N361D, E382V and M428I; d) N361D, F372L, E382V and M428I; e) H310Y, K322R, T350A, E382V, S426T and S442P; f) C229R, E382V and M428I; g) W313R and M428I; h) E382T, N384D and M428I; i) E380R, E382M and N384E; j) N361S, E382V and M428I; k) E382V, M428I and Y436A; l) P238S, E382V, S426V, M428L and E430H; m) E380D, E382V, N384R, S426V, M428L and E430D; n) E382V, S426I, M428L and E430S; o) H224R, E382V, S426T, M428S and E430P; p) P331L; q) S239L, I253T, Q347L, E382V; r) E382V, G402D and M428I; and s) E382V, P331L and M428I.

[00109] There may be various insertion points in the Fc domain that, upon insertion of additional amino acids, provide improved FcR binding capability. Insertions of 5 to 15 amino acids are contemplated. In some embodiments, 10 amino acids are inserted, such as between amino acids N297 and S298 of an Fc domain, such as a human IgG Fc domain. Particular insertions at this position (as well as substitutions) include a) RTETPVYVMVM (SEQ ID NO:79); b) WQVFNKYTKP (SEQ ID NO:80); c) LGDGSPCKAN (SEQ ID NO:81); d) EVPLVWMWVS (SEQ ID NO:82) together with F241L and K326E; and e) EQWGSQFGCG (SEQ ID NO:83) together with V282A.

[00110] The Fc domain may be a human IgG Fc that comprises an amino acid substitution at an amino acid residue corresponding to E382 of the IgG Fc domain. Furthermore, an aglycosylated Fc domain may comprise an amino acid

sequence insertion (e.g., about 1 to 5 amino acids) adjacent to an amino acid residue corresponding to E382 of the IgG Fc domain. Thus, in some specific aspects an Fc domain may comprise a hydrophobic amino acid substitution at E382 such as an E to V substitution. Furthermore, in some aspects an Fc domain of the invention may comprise an amino acid substitution at a residue corresponding to M428 (e.g., M428 to I), S426, C229, H310, K322, T350, N361, F372 or S442 of the human IgG Fc. In certain specific embodiments, an aglycosylated Fc domain may comprise an amino acid substitution corresponding to those found in the Fc11 as described in WO 2008/137475. Hence in a very specific case an aglycosylated Fc domain may comprise the amino acid sequence of SEQ ID NO:2 (Fc5).

[00111] In some embodiments, an aglycosylated Fc domain comprises a specific binding affinity for an FcR such as human Fc γ RIa, Fc γ RIIa, Fc γ RIIb, Fc γ RIIc, Fc γ RIIIa, Fc γ RIIIb, Fc α RI or C1q. Thus, in some aspects an aglycosylated Fc domain of the invention is defined as an Fc domain with a specific affinity for Fc γ RIa. Furthermore, such an Fc domain may be defined as having an equilibrium dissociation constant, with respect to Fc γ RIa binding, of about 10⁻⁶ M to about 10⁻⁹ M under physiological conditions.

B. Modified Antibodies and Proteinaceous Compounds with Heterologous Regions

[00112] Embodiments concern a proteinaceous compound that may include amino acid sequences from more than one naturally occurring or native polypeptides or proteins. Embodiments discussed above are contemplated to apply to this section, and vice versa. For instance, a modified antibody is one that contains a modified Fc domain with an antigen binding domain. Moreover, the antibody may have two different antigen binding regions, such as a different region on each of the two heavy chains. Alternatively or additionally, in some embodiments, there are polypeptides comprising multiple heterologous peptides and/or polypeptides (“heterologous” meaning they are not derived from the same polypeptide). A proteinaceous compound or molecule, for example, could include a modified Fc domain with a protein binding region that is not from an antibody. In some embodiments, there are polypeptides comprising a modified Fc domain with a protein

binding region that binds a cell-surface receptor. These proteinaceous molecule comprising multiple functional domains may be two or more domains chemically conjugated to one another or it may be a fusion protein of two or more polypeptides encoded by the same nucleic acid molecule. It is contemplated that proteins or polypeptides may include all or part of two or more heterologous polypeptides.

[00113] Thus, a multipolypeptide proteinaceous compound may be comprised of all or part of a first polypeptide and all or part of a second polypeptide, a third polypeptide, a fourth polypeptide, a fifth polypeptide, a sixth polypeptide, a seventh polypeptide, an eight polypeptide, a ninth polypeptide, a tenth polypeptide, or more polypeptides.

[00114] Polypeptides or proteins (including antibodies) having an antigen binding domain or region of an antibody and an aglycosylated Fc domain can be used against any antigen or epitope, including but not limited to proteins, subunits, domains, motifs, and/or epitopes belonging to the following list of targets: 17-IA, 4-1BB, 4Dc, 6-keto-PGF1a, 8-iso-PGF2a, 8-oxo-dG, A1 Adenosine Receptor, A33, ACE, ACE-2, Activin, Activin A, Activin AB, Activin B, Activin C, Activin RIA, Activin RIA ALK-2, Activin RIB ALK-4, Activin RIIA, Activin RIIB, ADAM, ADAM10, ADAM12, ADAM15, ADAM17/TACE, ADAM8, ADAM9, ADAMTS, ADAMTS4, ADAMTS5, Addressins, aFGF, ALCAM, ALK, ALK-1, ALK-7, alpha-1-antitrypsin, alpha-V/beta-1 antagonist, ANG, Ang, APAF-1, APE, APJ, APP, APRIL, AR, ARC, ART, Artemin, anti-Id, ASPARTIC, Atrial natriuretic factor, av/b3 integrin, Axl, b2M, B7-1, B7-2, B7-H, B-lymphocyte Stimulator (BlyS), BACE, BACE-1, Bad, BAFF, BAFF-R, Bag-1, BAK, Bax, BCA-1, BCAM, Bcl, BCMA, BDNF, b-ECGF, bFGF, BID, Bik, BIM, BLC, BL-CAM, BLK, BMP, BMP-2 BMP-2a, BMP-3 Osteogenin, BMP-4 BMP-2b, BMP-5, BMP-6 Vgr-1, BMP-7 (OP-1), BMP-8 (BMP-8a, OP-2), BMPR, BMPR-IA (ALK-3), BMPR-IB (ALK-6), BRK-2, RPK-1, BMPR-II (BRK-3), BMPs, b-NGF, BOK, Bombesin, Bone-derived neurotrophic factor, BPDE, BPDE-DNA, BTC, complement factor 3 (C3), C3a, C4, C5, C5a, C10, CA125, CAD-8, Calcitonin, cAMP, carcinoembryonic antigen (CEA), carcinoma-associated antigen, Cathepsin A, Cathepsin B, Cathepsin C/DPPI, Cathepsin D, Cathepsin E, Cathepsin H, Cathepsin L, Cathepsin O, Cathepsin S, Cathepsin V, Cathepsin X/ZIP, CBL, CCI, CCK2, CCL, CCL1, CCL11, CCL12,

CCL13, CCL14, CCL15, CCL16, CCL17, CCL18, CCL19, CCL2, CCL20, CCL21, CCL22, CCL23, CCL24, CCL25, CCL26, CCL27, CCL28, CCL3, CCL4, CCL5, CCL6, CCL7, CCL8, CCL9/10, CCR, CCR1, CCR10, CCR10, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9, CD1, CD2, CD3, CD3E, CD4, CD5, CD6, CD7, CD8, CD10, CD11a, CD11b, CD11c, CD13, CD14, CD15, CD16, CD18, CD19, CD20, CD21, CD22, CD23, CD25, CD27L, CD28, CD29, CD30, CD30L, CD32, CD33 (p67 proteins), CD34, CD38, CD40, CD40L, CD44, CD45, CD46, CD49a, CD52, CD54, CD55, CD56, CD61, CD64, CD66e, CD74, CD80 (B7-1), CD89, CD95, CD123, CD137, CD138, CD140a, CD146, CD147, CD148, CD152, CD164, CEACAM5, CFTR, cGMP, CINC, Clostridium botulinum toxin, Clostridium perfringens toxin, CKb8-1, CLC, CMV, CMV UL, CNTF, CNTN-1, COX, C-Ret, CRG-2, CT-1, CTACK, CTGF, CTLA-4, CX3CL1, CX3CR1, CXCL, CXCL1, CXCL2, CXCL3, CXCL4, CXCL5, CXCL6, CXCL7, CXCL8, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, CXCL14, CXCL15, CXCL16, CXCR, CXCR1, CXCR2, CXCR3, CXCR4, CXCR5, CXCR6, cytokeratin tumor-associated antigen, DAN, DCC, DcR3, DC-SIGN, Decay accelerating factor, des(1-3)-IGF-I (brain IGF-1), Dhh, digoxin, DNAM-1, Dnase, Dpp, DPPIV/CD26, Dtk, ECAD, EDA, EDA-A1, EDA-A2, EDAR, EGF, EGFR (ErbB-1), EMA, EMMPRIN, ENA, endothelin receptor, Enkephalinase, eNOS, Eot, ectaxin1, EpCAM, Ephrin B2/EphB4, EPO, ERCC, E-selectin, ET-1, Factor IIa, Factor VII, Factor VIIIc, Factor IX, fibroblast activation protein (FAP), Fas, FcR1, FEN-1, Ferritin, FGF, FGF-19, FGF-2, FGF3, FGF-8, FGFR, FGFR-3, Fibrin, FL, FLIP, Flt-3, Flt-4, Follicle stimulating hormone, Fractalkine, FZD1, FZD2, FZD3, FZD4, FZD5, FZD6, FZD7, FZD8, FZD9, FZD10, G250, Gas 6, GCP-2, GCSF, GD2, GD3, GDF, GDF-1, GDF-3 (Vgr-2), GDF-5 (BMP-14, CDMP-1), GDF-6 (BMP-13, CDMP-2), GDF-7 (BMP-12, CDMP-3), GDF-8 (Myostatin), GDF-9, GDF-15 (MIC-1), GDNF, GDNF, GFAP, GFR α -1, GFR α 1, GFR-alpha2, GFR-alpha3, GITR, Glucagon, Glut 4, glycoprotein IIb/IIIa (GP IIb/IIIa), GM-CSF, gp130, gp72, GRO, Growth hormone releasing factor, Haptene (NP-cap or NIP-cap), HB-EGF, HCC, HCMV gB envelope glycoprotein, HCMV) gH envelope glycoprotein, HCMV UL, Hemopoietic growth factor (HGF), Hep B gp120, heparanase, Her2, Her2/neu (ErbB-2), Her3 (ErbB-3), Her4 (ErbB-4), herpes simplex virus (HSV) gB glycoprotein, HSV gD glycoprotein, HGFA, High molecular weight melanoma-associated antigen (HMW-MM), HIV gp120, HIV IIIB gp120 V3 loop, HLA, HLA-DR, HM1.24, HMFG PEM, HRG, Hrk, human cardiac myosin, human

cytomegalovirus (HCMV), human growth hormone (HGH), HVEM, I-309, IAP, ICAM, ICAM-1, ICAM-3, ICE, ICOS, IFNg, Ig, IgA receptor, IgE, IGF, IGF binding proteins, IGF-1R, IGFBP, IGF-I, IGF-II, IL, IL-1, IL-1R, IL-2, IL-2R, IL-4, IL-4R, IL-5, IL-5R, IL-6, IL-6R, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-18, IL-18R, IL-23, interferon (INF)-alpha, INF-beta, INF-gamma, Inhibin, iNOS, Insulin A-chain, Insulin B-chain, Insulin-like growth factor 1, integrin alpha2, integrin alpha3, integrin alpha4, integrin alpha4/beta1, integrin alpha4/beta7, integrin alpha5 (alphaV), integrin alpha5/beta1, integrin alpha5/beta3, integrin alpha6, integrin beta1, integrin beta2, interferon gamma, IP-10, I-TAC, JE, Kallikrein 2, Kallikrein 5, Kallikrein 6, Kallikrein 11, Kallikrein 12, Kallikrein 14, Kallikrein 15, Kallikrein L1, Kallikrein L2, Kallikrein L3, Kallikrein L4, KC, KDR, Keratinocyte Growth Factor (KGF), laminin 5, LAMP, LAP, LAP (TGF-1), Latent TGF-1, Latent TGF-1 bp1, LBP, LDGF, LECT2, Lefty, Lewis-Y antigen, Lewis-Y related antigen, LFA-1, LFA-3, Lfo, LIF, LIGHT, lipoproteins, LIX, LKN, Lptn, L-Selectin, LT-a, LT-b, LTB4, LTBP-1, Lung surfactant, Luteinizing hormone, Lymphotoxin Beta Receptor, Mac-1, MAAdCAM, MAG, MAP2, MARC, MCAM, MCAM, MCK-2, MCP, M-CSF, MDC, Mer, METALLOPROTEASES, MGDF receptor, MGMT, MHC (HLA-DR), MIF, MIG, MIP, MIP-1-alpha, MK, MMAC1, MMP, MMP-1, MMP-10, MMP-11, MMP-12, MMP-13, MMP-14, MMP-15, MMP-2, MMP-24, MMP-3, MMP-7, MMP-8, MMP-9, MPIF, Mpo, MSK, MSP, mucin (Muc1), MUC18, Muellerian-inhibitin substance, Mug, MuSK, NAIP, NAP, NCAD, N-Cadherin, NCA 90, NCAM, NCAM, Neprilysin, Neurotrophin-3,-4, or -6, Neurturin, Neuronal growth factor (NGF), NGFR, NGF-beta, nNOS, NO, NOS, Npn, NRG-3, NT, NTN, OB, OGG1, OPG, OPN, OSM, OX40L, OX40R, p150, p95, PADPr, Parathyroid hormone, PARC, PARP, PBR, PBSF, PCAD, P-Cadherin, PCNA, PDGF, PDGF, PDK-1, PECAM, PEM, PF4, PGE, PGF, PGI2, PGJ2, PIN, PLA2, placental alkaline phosphatase (PLAP), PIGF, PLP, PP14, Proinsulin, Prorelaxin, Protein C, PS, PSA, PSCA, prostate specific membrane antigen (PSMA), PTEN, PTHrp, Ptk, PTN, R51, RANK, RANKL, RANTES, RANTES, Relaxin A-chain, Relaxin B-chain, renin, respiratory syncytial virus (RSV) F, RSV Fgp, Ret, Rheumatoid factors, RLIP76, RPA2, RSK, S100, SCF/KL, SDF-1, SERINE, Serum albumin, sFRP-3, Shh, SIGIRR, SK-1, SLAM, SLPI, SMAC, SMDF, SMOH, SOD, SPARC, Stat, STEAP, STEAP-II, TACE, TACI, TAG-72 (tumor-associated glycoprotein-72), TARC, TCA-3, T-cell receptors (e.g., T-cell receptor alpha/beta), TdT, TECK, TEM1, TEM5, TEM7,

TEM8, TERT, testicular PLAP-like alkaline phosphatase, TfR, TGF, TGF-alpha, TGF-beta, TGF-beta Pan Specific, TGF-beta RI (ALK-5), TGF-beta RII, TGF-beta RIIb, TGF-beta RIII, TGF-beta1, TGF-beta2, TGF-beta3, TGF-beta4, TGF-beta5, Thrombin, Thymus Ck-1, Thyroid stimulating hormone, Tie, TIMP, TIQ, Tissue Factor, TMEFF2, Tmpo, TMPRSS2, TNF, TNF-alpha, TNF-alpha beta, TNF-beta2, TNFc, TNF-RI, TNF-RII, TNFRSF10A (TRAIL R1 Apo-2, DR4), TNFRSF10B (TRAIL R2 DR5, KILLER, TRICK-2A, TRICK-B), TNFRSF10C (TRAIL R3 DcR1, LIT, TRID), TNFRSF10D (TRAIL R4 DcR2, TRUNDD), TNFRSF11A (RANK ODF R, TRANCE R), TNFRSF11B (OPG OCIF, TR1), TNFRSF12 (TWEAK R FN14), TNFRSF13B (TACI), TNFRSF13C (BAFF R), TNFRSF14 (HVEM ATAR, HveA, LIGHT R, TR2), TNFRSF16 (NGFR p75NTR), TNFRSF17 (BCMA), TNFRSF18 (GITR AITR), TNFRSF19 (TROY TAJ, TRADE), TNFRSF19L (RELT), TNFRSF1A (TNF RI CD120a, p55-60), TNFRSF1B (TNF RII CD120b, p75-80), TNFRSF26 (TNFRH3), TNFRSF3 (LTbR TNF RIII, TNFC R), TNFRSF4 (OX40 ACT35, TXGP1 R), TNFRSF5 (CD40 p50), TNFRSF6 (Fas Apo-1, APT1, CD95), TNFRSF6B (DcR3 M68, TR6), TNFRSF7 (CD27), TNFRSF8 (CD30), TNFRSF9 (4-1BB CD137, ILA), TNFRSF21 (DR6), TNFRSF22 (DcTRAIL R2 TNFRH2), TNFRST23 (DcTRAIL R1 TNFRH1), TNFRSF25 (DR3 Apo-3, LARD, TR-3, TRAMP, WSL-1), TNFSF10 (TRAIL Apo-2 Ligand, TL2), TNFSF11 (TRANCE/RANK Ligand ODF, OPG Ligand), TNFSF12 (TWEAK Apo-3 Ligand, DR3 Ligand), TNFSF13 (APRIL TALL2), TNFSF13B (BAFF BLYS, TALL1, THANK, TNFSF20), TNFSF14 (LIGHT HVEM Ligand, LTg), TNFSF15 (TL1A/VEGI), TNFSF18 (GITR Ligand AITR Ligand, TL6), TNFSF1A (TNF-a Conectin, DIF, TNFSF2), TNFSF1B (TNF-b LTa, TNFSF1), TNFSF3 (LTb TNFC, p33), TNFSF4 (OX40 Ligand gp34, TXGP1), TNFSF5 (CD40 Ligand CD154, gp39, HIGM1, IMD3, TRAP), TNFSF6 (Fas Ligand Apo-1 Ligand, APT1 Ligand), TNFSF7 (CD27 Ligand CD70), TNFSF8 (CD30 Ligand CD153), TNFSF9 (4-1BB Ligand CD137 Ligand), TP-1, t-PA, Tpo, TRAIL, TRAIL R, TRAIL-R1, TRAIL-R2, TRANCE, transferring receptor, TRF, Trk, TROP-2, TSG, TSLP, tumor-associated antigen CA 125, tumor-associated antigen expressing Lewis Y related carbohydrate, TWEAK, TXB2, Ung, uPAR, uPAR-1, Urokinase, VCAM, VCAM-1, VECAD, VE-Cadherin, VE-cadherin-2, VEFGR-1 (flt-1), VEGF, VEGFR, VEGFR-3 (fit-4), VEGI, VIM, Viral antigens, VLA, VLA-1, VLA-4, VNR integrin, von Willebrands factor, WIF-1, WNT1, WNT2, WNT2B/13, WNT3, WNT3A, WNT4, WNT5A,

WNT5B, WNT6, WNT7A, WNT7B, WNT8A, WNT8B, WNT9A, WNT9A, WNT9B, WNT10A, WNT10B, WNT11, WNT16, XCL1, XCL2, XCR1, XCR1, XEDAR, XIAP, XPD, and receptors for hormones and growth factors. In some embodiments, a polypeptide or protein has an antigen binding domain specific for one or more cell surface tumor antigens. Methods and compositions may be employed to target a tumor cell for ADCC.

[00115] Fc domains can bind to an FcR, however, it is contemplated that ADCC can be directed not only through an antigen binding domain on the polypeptide containing the Fc domain, but through some other protein binding domain. Consequently, embodiments concern an Fc domain and a heterologous non-antigen binding domain. In certain embodiments, the non-antigen binding domain bind to the cell surface. Therefore, these agents require either chemical conjugation to or fusion with agents/proteins which are capable of binding to specific target cells. Embodiments further include adjoining all or part of an aglycosylated Fc domain to all or part of any of the proteins listed in Table 2. It is contemplated that embodiments include, but are not limited to, the examples provided in Table 2 and the description herein.

TABLE 2

Protein Genus	Subgenus	Species	Subspecies
1) Antibodies	Polyclonal		
	Monoclonal	non-recombinant	
		recombinant	
			chimeric
			single chain
			diabody
			multimeric
2) Ligands for cell-surface receptors			IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IL-19
	Cytokines/growth factors		
		Cytokines/growth factors for receptor tyrosine kinases	
			GM-CSF, G-CSF, M-CSF, EGF, VEGF, FGF, PDGF, HGF, GDNF, Trk, AXL, LTK, TIE, ROR, DDR, KLG, RYK, MuSK ligands
3) Non-Ab binding protein for cell-surface molecule			
	Binders of cell surface proteins		
		Cluster of differentiation (CD) molecules	

[00116] A ligand for receptor may be employed to target a cell expressing on its surface the receptor for the ligand. Ligands also include, for instance, CD95 ligand, TRAIL, TNF (such as TNF- α or TNF- β), growth factors, including those discussed above, such as VEGF and cytokines, such as interferons or interleukins and variants thereof.

[00117] Embodiments with multiple domains are also contemplated, such as a VEGF Trap fusion protein that includes the second extracellular domain of the VEGF receptor 1 (Flt-1) with the third domain of the VEGF receptor 2 (KDR/FIK-1) and an IgG Fc region.

1. Fusion and Conjugated Proteins

[00118] A specialized kind of insertional variant is the fusion protein. This molecule generally has all or a substantial portion of the native molecule, linked at the N- or C-terminus, to all or a portion of a second polypeptide.

[00119] Embodiments also concern conjugated polypeptides, such as translated proteins, polypeptides and peptides, that are linked to at least one agent to form a modified protein or polypeptide. In order to increase the efficacy of molecules as diagnostic or therapeutic agents, it is conventional to link or covalently bind or complex at least one desired molecule or moiety. Such a molecule or moiety may be, but is not limited to, at least one effector or reporter molecule. Effector molecules comprise molecules having a desired activity, *e.g.*, cytotoxic activity. Non-limiting examples of effector molecules which have been attached to antibodies include toxins, anti-tumor agents, therapeutic enzymes, radio-labeled nucleotides, antiviral agents, chelating agents, cytokines, growth factors, and oligo- or poly-nucleotides. By contrast, a reporter molecule is defined as any moiety that may be detected using an assay. Non-limiting examples of reporter molecules which have been conjugated to antibodies include enzymes, radiolabels, haptens, fluorescent labels, phosphorescent molecules, chemiluminescent molecules, chromophores, luminescent molecules, photoaffinity molecules, colored particles or ligands, such as biotin.

[00120] Any antibody of sufficient selectivity, specificity or affinity may be employed as the basis for an antibody conjugate. Such properties may be evaluated using conventional immunological screening methodology known to those of skill in the art. Sites for binding to biological active molecules in the antibody molecule, in addition to the canonical antigen binding sites, include sites that reside in the variable domain that can bind pathogens, B-cell superantigens, the T cell co-receptor CD4 and the HIV-1 envelope (Sasso *et al.*, 1989; Shorki *et al.*, 1991; Silvermann *et al.*, 1995; Cleary *et al.*, 1994; Lenert *et al.*, 1990; Berberian *et al.*, 1993; Kreier *et al.*, 1991). In addition, the variable domain is involved in antibody

self-binding (Kang *et al.*, 1988), and contains epitopes (idiotopes) recognized by anti-antibodies (Kohler *et al.*, 1989).

[00121] Certain examples of antibody conjugates are those conjugates in which the antibody is linked to a detectable label. “Detectable labels” are compounds and/or elements that can be detected due to their specific functional properties, and/or chemical characteristics, the use of which allows the antibody to which they are attached to be detected, and/or further quantified if desired. Another such example is the formation of a conjugate comprising an antibody linked to a cytotoxic or anti-cellular agent, and may be termed “immunotoxins.”

[00122] Amino acids such as selectively-cleavable linkers, synthetic linkers, or other amino acid sequences may be used to separate proteinaceous moieties.

C. Protein Purification

[00123] While some of the embodiments involve recombinant proteins, embodiments may involve methods and processes for purifying proteins, including modified proteins and recombinant proteins. Generally, these techniques involve, at one level, the crude fractionation of the cellular milieu to polypeptide and non-polypeptide fractions. Having separated the polypeptide from other proteins, the polypeptide of interest may be further purified using chromatographic and electrophoretic techniques to achieve partial or complete purification (or purification to homogeneity). Analytical methods particularly suited to the preparation are ion-exchange chromatography, exclusion chromatography; polyacrylamide gel electrophoresis; isoelectric focusing. A particularly efficient method of purifying peptides is fast protein liquid chromatography or even HPLC. In addition, the conditions under which such techniques are executed may be affect characteristics, such as functional activity, of the purified molecules.

[00124] Certain aspects concern the purification, and in particular embodiments, the substantial purification, of an encoded protein or peptide. The term “purified protein or peptide” as used herein, is intended to refer to a composition, isolatable from other components, wherein the protein or peptide is purified to any degree relative to its naturally-obtainable state. A purified protein or peptide

therefore also refers to a protein or peptide, free from the environment in which it may naturally occur. A “substantially purified” protein or peptide

[00125] Generally, “purified” will refer to a protein or peptide composition that has been subjected to fractionation to remove various other components, and which composition substantially retains its expressed biological activity. Where the term “substantially purified” is used, this designation will refer to a composition in which the protein or peptide forms the major component of the composition, such as constituting about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, about 99.2%, about 99.4%, about 99.6%, about 99.8%, about 99.9% or more of the proteins in the composition.

[00126] Various methods for quantifying the degree of purification of the protein or peptide will be known to those of skill in the art in light of the present disclosure. These include, for example, determining the specific activity of an active fraction, or assessing the amount of polypeptides within a fraction by SDS/PAGE analysis. A preferred method for assessing the purity of a fraction is to calculate the specific activity of the fraction, to compare it to the specific activity of the initial extract, and to thus calculate the degree of purity, herein assessed by a “-fold purification number.” The actual units used to represent the amount of activity will, of course, be dependent upon the particular assay technique chosen to follow the purification and whether or not the expressed protein or peptide exhibits a detectable activity.

[00127] Various techniques suitable for use in protein purification will be well known to those of skill in the art. These include, for example, precipitation with ammonium sulphate, PEG, antibodies and the like or by heat denaturation, followed by centrifugation; chromatography steps such as ion exchange, gel filtration, reverse phase, hydroxylapatite and affinity chromatography; isoelectric focusing; gel electrophoresis; and combinations of such and other techniques. As is generally known in the art, it is believed that the order of conducting the various purification steps may be changed, or that certain steps may be omitted, and still result in a suitable method for the preparation of a substantially purified protein or peptide.

[00128] There is no general requirement that the protein or peptide always be provided in their most purified state. Indeed, it is contemplated that less substantially purified products will have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme. For example, it is appreciated that a cation-exchange column chromatography performed utilizing an HPLC apparatus will generally result in a greater “-fold” purification than the same technique utilizing a low pressure chromatography system. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of protein product, or in maintaining the activity of an expressed protein.

[00129] It is known that the migration of a polypeptide can vary, sometimes significantly, with different conditions of SDS/PAGE (Capaldi *et al.*, 1977). It will therefore be appreciated that under differing electrophoresis conditions, the apparent molecular weights of purified or partially purified expression products may vary.

[00130] The use of a peptide tag in combination with the methods and compositions is also contemplated. A tag takes advantage of an interaction between two polypeptides. A portion of one of the polypeptides that is involved in the interaction may be used as a tag. For instance, the binding region of glutathione S transferase (GST) may be used as a tag such that glutathione beads can be used to enrich for a compound containing the GST tag. An epitope tag, which an amino acid region recognized by an antibody or T cell receptor, may be used. The tag may be encoded by a nucleic acid segment that is operatively linked to a nucleic acid segment encoding a modified protein such that a fusion protein is encoded by the nucleic acid molecule. Other suitable fusion proteins are those with β -galactosidase, ubiquitin, hexahistidine (6xHis), or the like.

IV. Antibody Fc Libraries

[00131] Examples of techniques that could be employed in conjunction with embodiments for creation of diverse antibody Fc domains and/or antibodies comprising such domains may employ techniques similar to those for expression of immunoglobulin heavy chain libraries described in U.S. Patent 5,824,520. Previously employed Fc libraries are discussed in WO 2008/137475.

V. Screening Antibody Fc Domains

[00132] There are embodiments involving methods for identifying molecules capable of binding to a particular FcR. They are described herein, as well as in PCT Application WO 2008/137475. The binding polypeptides screened may comprise a large library of diverse candidate Fc domains, or, alternatively, may comprise particular classes of Fc domains (e.g., engineered point mutations or amino acid insertions) selected with an eye towards structural attributes that are believed to make them more likely to bind the target ligand. In one embodiment, the candidate binding protein is an intact antibody, or a fragment or portion thereto comprising an Fc domain.

[00133] To identify a candidate Fc domain capable of binding a target ligand, one may carry out the steps of: providing a population of Gram negative bacterial cells that express a distinct antibody Fc domain; admixing the bacteria or phages and at least a first labeled or immobilized target ligand (FcR polypeptide) capable of contacting the antibody and identifying at least a first bacterium expressing a molecule capable of binding the target ligand.

[00134] In some aspects of the aforementioned method, the binding between antibody Fc domain and a labeled FcR polypeptide will prevent diffusing out of a bacterial cell. In this way, molecules of the labeled ligand can be retained in the periplasm of the bacterium comprising a permeabilized outer membrane. Alternatively, the periplasm can be removed, whereby the Fc domain will cause retention of the bound candidate molecule since Fc domains are shown to associate with the inner membrane. The labeling may then be used to isolate the cell expressing a binding polypeptide capable of binding the FcR polypeptide, and in this way, the gene encoding the Fc domain polypeptide isolated. The molecule capable of binding the target ligand may then be produced in large quantities using *in vivo* or *ex vivo* expression methods, and then used for any desired application, for example, for diagnostic or therapeutic applications. Furthermore, it will be understood that isolated antibody Fc domains identified may be used to construct an antibody fragment or full-length antibody comprising an antigen binding domain.

[00135] In further embodiments, methods for producing bacteria of the invention, may comprise at least two rounds of selection (step c) wherein the sub-

population of bacterial cells obtained in the first round of selection is subjected to at least a second round of selection based on the binding of the candidate antibody Fc domain to an FcR. Furthermore in some aspects the sub-population of bacterial cells obtained in the first round of selection may be grown under permissive conditions prior to a second selection (to expand the total number of cells). Thus, in some aspects, methods may comprise 2, 3, 4, 5, 6, 7, 8, 9, 10 or more rounds of selection. Furthermore, in some aspects, a sub-population of bacterial cells obtained from each round of selection will be grown under permissive conditions before a subsequent round of selection. Cells isolated following one or more such rounds of selection may be subjected to additional rounds of mutagenesis. In some cases, selection will be performed after removing FcR polypeptide that is not bound to the antibody. Furthermore, in some cases the stringency of selection may be modified by adjusting the pH, salt concentration, or temperature of a solution comprising bacteria that display antibodies. Thus, in some aspects, it may be preferred that a bacterial cell of the invention is grown at a sub-physiological temperature such as at about 25 °C.

[00136] In still further aspects, a method of producing a bacterial cell according to the invention may be further defined as a method of producing a nucleic acid sequence encoding an Fc domain that binds to at least a first FcR. Thus, a bacterial cell produced by the methods herein may be used to clone a nucleic acid sequence encoding the Fc domain having a specific affinity for an FcR polypeptide. Methods for isolating and amplifying such a nucleic acid from a cell for example by PCR are well known in the art and further described below. Thus, a nucleic acid sequence produced by the forgoing methods is included as part of the instant invention. Furthermore, such a sequence may be expressed in a cell to produce an Fc domain having a specific affinity for an FcR. Thus, in some aspects, the invention provides a method for producing an Fc domain having a specific affinity for an FcR. Furthermore, the invention includes antibody Fc domains produced by the methods of the invention. It will be understood however that the antibody Fc domains produced by such a screen may be combined with antibody variable regions that have an affinity for a particular target ligand and these antibodies are also included as part of the invention.

A. Cloning of Fc domain Coding Sequences

[00137] The binding affinity of an antibody Fc or other binding protein can, for example, be determined by the Scatchard analysis of Munson & Pollard (1980). Alternatively, binding affinity can be determined by surface plasmon resonance or any other well known method for determining the kinetics and equilibrium constants for protein:protein interactions. After a bacterial cell is identified that produces molecules of the desired specificity, affinity, and/or activity, the corresponding coding sequence may be cloned. In this manner, DNA encoding the molecule can be isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the antibody or binding protein).

[00138] Once isolated, the antibody Fc domain DNA may be placed into expression vectors, which can then transfected into host cells such as bacteria. The DNA also may be modified, for example, by the addition of sequence for human heavy and light chain variable domains, or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. In that manner, "chimeric" or "hybrid" binding proteins are prepared to have the desired binding specificity. For instance, an identified antibody Fc domain may be fused to a therapeutic polypeptide or a toxin and used to target cells (*in vitro* or *in vivo*) that express a particular FcR.

[00139] Chimeric or hybrid Fc domains also may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, targeted-toxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptoputyrimidate.

[00140] It will be understood by those of skill in the art that nucleic acids may be cloned from viable or inviable cells. In the case of inviable cells, for example, it may be desired to use amplification of the cloned DNA, for example, using PCR. This may also be carried out using viable cells either with or without further growth of cells.

B. Labeled Ligands

[00141] In one embodiment, an Fc domain is isolated which has affinity for a labeled FcR polypeptide. By permeabilization and/or removal of the periplasmic membrane of a Gram negative bacterium in accordance with the invention, labeled ligands of potentially any size may be screened. In the absence of removal of the periplasmic membrane, it will typically be preferable that the labeled ligand is less than 50,000 Da in size in order to allow efficient diffusion of the ligand across the bacterial periplasmic membrane.

[00142] As indicated above, it will typically be desired to provide an FcR polypeptide which has been labeled with one or more detectable agent(s). This can be carried out, for example, by linking the ligand to at least one detectable agent to form a conjugate. For example, it is conventional to link or covalently bind or complex at least one detectable molecule or moiety. A "label" or "detectable label" is a compound and/or element that can be detected due to specific functional properties, and/or chemical characteristics, the use of which allows the ligand to which it is attached to be detected, and/or further quantified if desired. Examples of labels which could be used include, but are not limited to, enzymes, radiolabels, haptens, fluorescent labels, phosphorescent molecules, chemiluminescent molecules, chromophores, luminescent molecules, photoaffinity molecules, colored particles or ligands, such as biotin.

[00143] In one embodiment of the invention, a visually-detectable marker is used such that automated screening of cells for the label can be carried out. In particular, fluorescent labels are beneficial in that they allow use of flow cytometry for isolation of cells expressing a desired binding protein or antibody. Examples of agents that may be detected by visualization with an appropriate instrument are known in the art, as are methods for their attachment to a desired ligand (see, e.g., U.S. Patents 5,021,236; 4,938,948; and 4,472,509). Such agents can include paramagnetic ions; radioactive isotopes; fluorochromes; NMR-detectable substances and substances for X-ray imaging.

[00144] Another type of FcR conjugate is where the ligand is linked to a secondary binding molecule and/or to an enzyme (an enzyme tag) that will generate a colored product upon contact with a chromogenic substrate. Examples of such

enzymes include urease, alkaline phosphatase, (horseradish) hydrogen peroxidase or glucose oxidase. In such instances, it will be desired that cells selected remain viable. Preferred secondary binding ligands are biotin and/or avidin and streptavidin compounds. The use of such labels is well known to those of skill in the art and are described, for example, in U.S. Patents 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149 and 4,366,241.

[00145] Molecules containing azido groups also may be used to form covalent bonds to proteins through reactive nitrene intermediates that are generated by low intensity ultraviolet light (Potter & Haley, 1983). In particular, 2- and 8-azido analogues of purine nucleotides have been used as site-directed photoprobes to identify nucleotide-binding proteins in crude cell extracts (Owens & Haley, 1987; Atherton *et al.*, 1985). The 2- and 8-azido nucleotides have also been used to map nucleotide-binding domains of purified proteins (Khatoon *et al.*, 1989; King *et al.*, 1989; and Dholakia *et al.*, 1989) and may be used as ligand binding agents.

[00146] Labeling can be carried out by any of the techniques well known to those of skill in the art. For instance, FcR polypeptides can be labeled by contacting the ligand with the desired label and a chemical oxidizing agent such as sodium hypochlorite, or an enzymatic oxidizing agent, such as lactoperoxidase. Similarly, a ligand exchange process could be used. Alternatively, direct labeling techniques may be used, *e.g.*, by incubating the label, a reducing agent such as SNCl_2 , a buffer solution such as sodium-potassium phthalate solution, and the ligand. Intermediary functional groups on the ligand could also be used, for example, to bind labels to a ligand in the presence of diethylenetriaminepentaacetic acid (DTPA) or ethylene diaminetetraacetic acid (EDTA).

[00147] Other methods are also known in the art for the attachment or conjugation of a ligand to its conjugate moiety. Some attachment methods involve the use of an organic chelating agent such as diethylenetriaminepentaacetic acid anhydride (DTPA); ethylenetriaminetetraacetic acid; N-chloro-p-toluenesulfonamide; and/or tetrachloro-3 α -6 α -diphenylglycouril-3 attached to the ligand (U.S. Patents 4,472,509 and 4,938,948). FcR polypeptides also may be reacted with an enzyme in the presence of a coupling agent such as glutaraldehyde or periodate. Conjugates with fluorescein markers can be prepared in the presence of these coupling agents or by

reaction with an isothiocyanate. In U.S. Patent 4,938,948, imaging of breast tumors is achieved using monoclonal antibodies and the detectable imaging moieties are bound to the antibody using linkers such as methyl-p-hydroxybenzimidate or N-succinimidyl-3-(4-hydroxyphenyl)propionate. In still further aspects an FcR polypeptide may be fused to a reporter protein such as an enzyme as described *supra* or a fluorescence protein.

[00148] The ability to specifically label periplasmic expressed proteins with appropriate fluorescent ligands also has applications other than library screening. Specifically labeling with fluorescent ligands and flow cytometry can be used for monitoring production of Fc domains during protein manufacturing.

[00149] Once an Fc domain has been isolated, it may be desired to link the molecule to at least one agent to form a conjugate to enhance the utility of that molecule. For example, in order to increase the efficacy of Fc domains or antibody molecules as diagnostic or therapeutic agents, it is conventional to link or covalently bind or complex at least one desired molecule or moiety. Such a molecule or moiety may be, but is not limited to, at least one effector or reporter molecule. Effector molecules comprise molecules having a desired activity, *e.g.*, cytotoxic activity. Non-limiting examples of effector molecules which have been attached to antibodies include toxins, anti-tumor agents, therapeutic enzymes, radio-labeled nucleotides, antiviral agents, chelating agents, cytokines, growth factors, and oligo- or poly-nucleotides. By contrast, a reporter molecule is defined as any moiety which may be detected using an assay. Techniques for labeling such a molecule are known to those of skill in the art and have been described herein above.

[00150] Labeled binding proteins such as Fc domains which have been prepared in accordance with the invention may also then be employed, for example, in immunodetection methods for binding, purifying, removing, quantifying and/or otherwise generally detecting biological components such as protein(s), polypeptide(s) or peptide(s). Some immunodetection methods include enzyme linked immunosorbent assay (ELISA), radioimmunoassay (RIA), immunoradiometric assay, fluoroimmunoassay, chemiluminescent assay, bioluminescent assay, and Western blot to mention a few. The steps of various useful immunodetection methods have been described in the scientific literature, such as, *e.g.*, Doolittle and Ben-Zeev, 1999;

Gulbis and Galand, 1993; and De Jager R *et al.*, 1993. Such techniques include binding assays such as the various types of enzyme linked immunosorbent assays (ELISAs) and/or radioimmunoassays (RIA) known in the art.

[00151] The Fc domain molecules, including antibodies, may be used, for example, in conjunction with both fresh-frozen and/or formalin-fixed, paraffin-embedded tissue blocks prepared for study by immunohistochemistry (IHC). The method of preparing tissue blocks from these particulate specimens has been successfully used in previous IHC studies of various prognostic factors, and/or is well known to those of skill in the art (Abbondanzo *et al.*, 1990).

VI. Automated Screening with Flow Cytometry

[00152] In one embodiment of the invention, fluorescence activated cell sorting (FACS) screening or other automated flow cytometric techniques may be used for the efficient isolation of a bacterial cell comprising a labeled ligand bound to an Fc domain. Instruments for carrying out flow cytometry are known to those of skill in the art and are commercially available to the public. Examples of such instruments include FACS Star Plus, FACScan and FACSort instruments from Becton Dickinson (Foster City, Calif.) Epics C from Coulter Epics Division (Hialeah, Fla.) and MOFLOTM from Cytomation (Colorado Springs, Co).

[00153] Flow cytometric techniques in general involve the separation of cells or other particles in a liquid sample. Typically, the purpose of flow cytometry is to analyze the separated particles for one or more characteristics thereof, for example, presence of a labeled ligand or other molecule. The basic steps of flow cytometry involve the direction of a fluid sample through an apparatus such that a liquid stream passes through a sensing region. The particles should pass one at a time by the sensor and are categorized base on size, refraction, light scattering, opacity, roughness, shape, fluorescence, *etc.*

[00154] Rapid quantitative analysis of cells proves useful in biomedical research and medicine. Apparati permit quantitative multiparameter analysis of cellular properties at rates of several thousand cells per second. These instruments provide the ability to differentiate among cell types. Data are often displayed in one-dimensional (histogram) or two-dimensional (contour plot, scatter plot) frequency

distributions of measured variables. The partitioning of multiparameter data files involves consecutive use of the interactive one- or two-dimensional graphics programs.

[00155] Quantitative analysis of multiparameter flow cytometric data for rapid cell detection consists of two stages: cell class characterization and sample processing. In general, the process of cell class characterization partitions the cell feature into cells of interest and not of interest. Then, in sample processing, each cell is classified in one of the two categories according to the region in which it falls. Analysis of the class of cells is very important, as high detection performance may be expected only if an appropriate characteristic of the cells is obtained.

[00156] Not only is cell analysis performed by flow cytometry, but so too is sorting of cells. In U.S. Patent 3,826,364, an apparatus is disclosed which physically separates particles, such as functionally different cell types. In this machine, a laser provides illumination which is focused on the stream of particles by a suitable lens or lens system so that there is highly localized scatter from the particles therein. In addition, high intensity source illumination is directed onto the stream of particles for the excitation of fluorescent particles in the stream. Certain particles in the stream may be selectively charged and then separated by deflecting them into designated receptacles. A classic form of this separation is *via* fluorescent-tagged antibodies, which are used to mark one or more cell types for separation.

[00157] Other examples of methods for flow cytometry that could include, but are not limited to, those described in U.S. Patent Nos. 4,284,412; 4,989,977; 4,498,766; 5,478,722; 4,857,451; 4,774,189; 4,767,206; 4,714,682; 5,160,974; and 4,661,913.

[00158] For the present invention, an important aspect of flow cytometry is that multiple rounds of screening can be carried out sequentially. Cells may be isolated from an initial round of sorting and immediately reintroduced into the flow cytometer and screened again to improve the stringency of the screen. Another advantage known to those of skill in the art is that nonviable cells can be recovered using flow cytometry. Since flow cytometry is essentially a particle sorting technology, the ability of a cell to grow or propagate is not necessary. Techniques for

the recovery of nucleic acids from such non-viable cells are well known in the art and may include, for example, use of template-dependent amplification techniques including PCR.

VII. Automated Screening with Flow Cytometry

[00159] Nucleic acid-based expression systems may find use, in certain embodiments of the invention, for the expression of recombinant proteins. For example, one embodiment of the invention involves transformation of Gram negative bacteria with the coding sequences for an antibody Fc domain, or preferably a plurality of distinct Fc domains.

VIII. Nucleic Acid-Based Expression Systems

[00160] Nucleic acid-based expression systems may find use, in certain embodiments of the invention, for the expression of recombinant proteins. For example, one embodiment of the invention involves transformation of Gram negative bacteria with the coding sequences for an antibody Fc domain, or preferably a plurality of distinct Fc domains.

A. Methods of Nucleic Acid Delivery

[00161] Certain aspects of the invention may comprise delivery of nucleic acids to target cells (e.g., gram negative bacteria). For example, bacterial host cells may be transformed with nucleic acids encoding candidate Fc domains potentially capable binding an FcR. In particular embodiments of the invention, it may be desired to target the expression to the periplasm of the bacteria. Transformation of eukaryotic host cells may similarly find use in the expression of various candidate molecules identified as capable of binding a target ligand.

[00162] Suitable methods for nucleic acid delivery for transformation of a cell are believed to include virtually any method by which a nucleic acid (e.g., DNA) can be introduced into such a cell, or even an organelle thereof. Such methods include, but are not limited to, direct delivery of DNA such as by injection (U.S. Patents 5,994,624, 5,981,274, 5,945,100, 5,780,448, 5,736,524, 5,702,932, 5,656,610, 5,589,466 and 5,580,859), including microinjection (Harland and Weintraub, 1985; U.S. Patent 5,789,215); by electroporation (U.S. Patent 5,384,253); by calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987;

Rippe *et al.*, 1990); by using DEAE-dextran followed by polyethylene glycol (Gopal, 1985); by direct sonic loading (Fechheimer *et al.*, 1987); by liposome mediated transfection (Nicolau and Sene, 1982; Fraley *et al.*, 1979; Nicolau *et al.*, 1987; Wong *et al.*, 1980; Kaneda *et al.*, 1989; Kato *et al.*, 1991); by microprojectile bombardment (PCT Application Nos. WO 94/09699 and 95/06128; U.S. Patents 5,610,042; 5,322,783, 5,563,055, 5,550,318, 5,538,877 and 5,538,880); or by agitation with silicon carbide fibers (Kaepller *et al.*, 1990; U.S. Patents 5,302,523 and 5,464,765); by desiccation/inhibition-mediated DNA uptake (Potrykus *et al.*, 1985). Through the application of techniques such as these, cells may be stably or transiently transformed.

B. Vectors

[00163] Vectors may find use with the current invention, for example, in the transformation of a Gram negative bacterium with a nucleic acid sequence encoding a candidate Fc domain which one wishes to screen for ability to bind a target FcR. In one embodiment of the invention, an entire heterogeneous “library” of nucleic acid sequences encoding target polypeptides may be introduced into a population of bacteria, thereby allowing screening of the entire library. The term “vector” is used to refer to a carrier nucleic acid molecule into which a nucleic acid sequence can be inserted for introduction into a cell where it can be replicated. A nucleic acid sequence can be “exogenous,” or “heterologous”, which means that it is foreign to the cell into which the vector is being introduced or that the sequence is homologous to a sequence in the cell but in a position within the host cell nucleic acid in which the sequence is ordinarily not found. Vectors include plasmids, cosmids and viruses (e.g., bacteriophage). One of skill in the art may construct a vector through standard recombinant techniques, which are described in Maniatis *et al.*, 1988 and Ausubel *et al.*, 1994.

[00164] The term “expression vector” refers to a vector containing a nucleic acid sequence coding for at least part of a gene product capable of being transcribed.

In some cases, RNA molecules are then translated into a protein, polypeptide, or peptide. Expression vectors can contain a variety of “control sequences,” which refer to nucleic acid sequences necessary for the transcription and possibly translation of an operably linked coding sequence in a particular host organism. In addition to control sequences that govern transcription and translation, vectors and expression vectors may contain nucleic acid sequences that serve other functions as well and are described *infra*.

1. Promoters and Enhancers

[00165] A “promoter” is a control sequence that is a region of a nucleic acid sequence at which initiation and rate of transcription are controlled. It may contain genetic elements at which regulatory proteins and molecules may bind such as RNA polymerase and other transcription factors. The phrases “operatively positioned,” “operatively linked,” “under control,” and “under transcriptional control” mean that a promoter is in a correct functional location and/or orientation in relation to a nucleic acid sequence to control transcriptional initiation and/or expression of that sequence. A promoter may or may not be used in conjunction with an “enhancer,” which refers to a *cis*-acting regulatory sequence involved in the transcriptional activation of a nucleic acid sequence.

[00166] A promoter may be one naturally associated with a gene or sequence, as may be obtained by isolating the 5' non-coding sequences located upstream of the coding segment and/or exon. Such a promoter can be referred to as “endogenous.” Similarly, an enhancer may be one naturally associated with a nucleic acid sequence, located either downstream or upstream of that sequence. Alternatively, certain advantages will be gained by positioning the coding nucleic acid segment under the control of a recombinant or heterologous promoter, which refers to a promoter that is not normally associated with a nucleic acid sequence in its natural environment. A recombinant or heterologous enhancer refers also to an enhancer not normally associated with a nucleic acid sequence in its natural environment. Such promoters or enhancers may include promoters or enhancers of other genes, and promoters or enhancers isolated from any other prokaryotic cell, and promoters or enhancers not “naturally occurring,” *i.e.*, containing different elements of different transcriptional regulatory regions, and/or mutations that alter expression. In addition to producing

nucleic acid sequences of promoters and enhancers synthetically, sequences may be produced using recombinant cloning and/or nucleic acid amplification technology, including PCR™, in connection with the compositions disclosed herein (see U.S. Patent 4,683,202, U.S. Patent 5,928,906).

[00167] Naturally, it will be important to employ a promoter and/or enhancer that effectively directs the expression of the DNA segment in the cell type chosen for expression. One example of such promoter that may be used with the invention is the *E. coli* arabinose or T7 promoter. Those of skill in the art of molecular biology generally are familiar with the use of promoters, enhancers, and cell type combinations for protein expression, for example, see Sambrook *et al.* (1989). The promoters employed may be constitutive, tissue-specific, inducible, and/or useful under the appropriate conditions to direct high level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins and/or peptides. The promoter may be heterologous or endogenous.

2. Initiation Signals and Internal Ribosome Binding Sites

[00168] A specific initiation signal also may be required for efficient translation of coding sequences. These signals include the ATG initiation codon or adjacent sequences. Exogenous translational control signals, including the ATG initiation codon, may need to be provided. One of ordinary skill in the art would readily be capable of determining this and providing the necessary signals. It is well known that the initiation codon must be “in-frame” with the reading frame of the desired coding sequence to ensure translation of the entire insert. The exogenous translational control signals and initiation codons can be either natural or synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements.

3. Multiple Cloning Sites

[00169] Vectors can include a multiple cloning site (MCS), which is a nucleic acid region that contains multiple restriction enzyme sites, any of which can be used in conjunction with standard recombinant technology to digest the vector (see Carbonelli *et al.*, 1999, Levenson *et al.*, 1998, and Cocea, 1997.) “Restriction enzyme digestion” refers to catalytic cleavage of a nucleic

acid molecule with an enzyme that functions only at specific locations in a nucleic acid molecule. Many of these restriction enzymes are commercially available. Use of such enzymes is understood by those of skill in the art. Frequently, a vector is linearized or fragmented using a restriction enzyme that cuts within the MCS to enable exogenous sequences to be ligated to the vector. "Ligation" refers to the process of forming phosphodiester bonds between two nucleic acid fragments, which may or may not be contiguous with each other. Techniques involving restriction enzymes and ligation reactions are well known to those of skill in the art of recombinant technology.

4. Termination Signals

[00170] The vectors or constructs prepared in accordance with the present invention will generally comprise at least one termination signal. A "termination signal" or "terminator" is comprised of the DNA sequences involved in specific termination of an RNA transcript by an RNA polymerase. Thus, in certain embodiments, a termination signal that ends the production of an RNA transcript is contemplated. A terminator may be necessary *in vivo* to achieve desirable message levels.

[00171] Terminators contemplated for use in the invention include any known terminator of transcription described herein or known to one of ordinary skill in the art, including but not limited to, for example, rho dependent or rho independent terminators. In certain embodiments, the termination signal may be a lack of transcribable or translatable sequence, such as due to a sequence truncation.

5. Origins of Replication

[00172] In order to propagate a vector in a host cell, it may contain one or more origins of replication sites (often termed "ori"), which is a specific nucleic acid sequence at which replication is initiated.

6. Selectable and Screenable Markers

[00173] In certain embodiments of the invention, cells containing a nucleic acid construct of the present invention may be identified *in vitro* or *in vivo* by including a marker in the expression vector. Such markers would confer an identifiable change to the cell permitting easy identification of cells containing the

expression vector. Generally, a selectable marker is one that confers a property that allows for selection. A positive selectable marker is one in which the presence of the marker allows for its selection, while a negative selectable marker is one in which its presence prevents its selection. An example of a positive selectable marker is a drug resistance marker.

[00174] Usually the inclusion of a drug selection marker aids in the cloning and identification of transformants, for example, genes that confer resistance to neomycin, puromycin, hygromycin, DHFR, GPT, zeocin and histidinol are useful selectable markers. In addition to markers conferring a phenotype that allows for the discrimination of transformants based on the implementation of conditions, other types of markers including screenable markers such as GFP, whose basis is colorimetric analysis, are also contemplated. Alternatively, screenable enzymes such as chloramphenicol acetyltransferase (CAT) may be utilized. One of skill in the art would also know how to employ immunologic markers, possibly in conjunction with FACS analysis. The marker used is not believed to be important, so long as it is capable of being expressed simultaneously with the nucleic acid encoding a gene product. Further examples of selectable and screenable markers are well known to one of skill in the art.

C. Host Cells

[00175] In the context of expressing a heterologous nucleic acid sequence, “host cell” refers to a prokaryotic cell, and it includes any transformable organism that is capable of replicating a vector and/or expressing a heterologous gene encoded by a vector. A host cell can, and has been, used as a recipient for vectors. A host cell may be “transfected” or “transformed,” which refers to a process by which exogenous nucleic acid is transferred or introduced into the host cell. A transformed cell includes the primary subject cell and its progeny.

[00176] In particular embodiments of the invention, a host cell is a Gram negative bacterial cell. These bacteria are suited for use with the invention in that they possess a periplasmic space between the inner and outer membrane and, particularly, the aforementioned inner membrane between the periplasm and cytoplasm, which is also known as the cytoplasmic membrane. As such, any other cell with such a periplasmic space could be used in accordance with the invention. Examples of Gram

to, *E. coli*, *Pseudomonas aeruginosa*, *Vibrio cholera*, *Salmonella typhimurium*, *Shigella flexneri*, *Haemophilus influenza*, *Bordetella pertussi*, *Erwinia amylovora*, *Rhizobium* sp. The Gram negative bacterial cell may be still further defined as bacterial cell which has been transformed with the coding sequence of a fusion polypeptide comprising a candidate binding polypeptide capable of binding a selected ligand. The polypeptide is anchored to the outer face of the cytoplasmic membrane, facing the periplasmic space, and may comprise an antibody coding sequence or another sequence. One means for expression of the polypeptide is by attaching a leader sequence to the polypeptide capable of causing such directing.

[00177] Numerous prokaryotic cell lines and cultures are available for use as a host cell, and they can be obtained through the American Type Culture Collection (ATCC), which is an organization that serves as an archive for living cultures and genetic materials (www.atcc.org). An appropriate host can be determined by one of skill in the art based on the vector backbone and the desired result. A plasmid or cosmid, for example, can be introduced into a prokaryote host cell for replication of many vectors. Bacterial cells used as host cells for vector replication and/or expression include DH5 α , JM109, and KC8, as well as a number of commercially available bacterial hosts such as SURE[®] Competent Cells and SOLOPACK[™] Gold Cells (STRATAGENE[®], La Jolla). Alternatively, bacterial cells such as *E. coli* LE392 could be used as host cells for bacteriophage.

[00178] Many host cells from various cell types and organisms are available and would be known to one of skill in the art. Similarly, a viral vector may be used in conjunction with a prokaryotic host cell, particularly one that is permissive for replication or expression of the vector. Some vectors may employ control sequences that allow it to be replicated and/or expressed in both prokaryotic and eukaryotic cells. One of skill in the art would further understand the conditions under which to incubate all of the above described host cells to maintain them and to permit replication of a vector. Also understood and known are techniques and conditions that would allow large-scale production of vectors, as well as production of the nucleic acids encoded by vectors and their cognate polypeptides, proteins, or peptides.

D. Expression Systems

[00179] Numerous expression systems exist that comprise at least a part or all of the compositions discussed above. Such systems could be used, for example, for the production of a polypeptide product identified in accordance with the invention as capable of binding a particular ligand. Prokaryote-based systems can be employed for use with the present invention to produce nucleic acid sequences, or their cognate polypeptides, proteins and peptides. Many such systems are commercially and widely available. Other examples of expression systems comprise of vectors containing a strong prokaryotic promoter such as T7, Tac, Trc, BAD, lambda pL, Tetracycline or Lac promoters, the pET Expression System and an *E. coli* expression system.

E. Candidate Binding Proteins and Antibodies

[00180] In certain embodiments, antibody Fc domains are expressed on the cytoplasmic or in the periplasmic space membrane of a host bacterial cell. By expression of a heterogeneous population of such Fc domains, those polypeptides having a high affinity for a target ligand (FcR) may be identified. The identified Fc domains may then be used in various diagnostic or therapeutic applications, as described herein.

[00181] As used herein, the term "Fc domain" is intended to refer broadly to any immunoglobulin Fc region such as an IgG, IgM, IgA, IgD or IgE Fc. The techniques for preparing and using various antibody-based constructs and fragments are well known in the art. Means for preparing and characterizing antibodies are also well known in the art (See, e.g., Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988).

[00182] Once an antibody having affinity for a target ligand is identified, the Fc domain may be purified, if desired, using filtration, centrifugation and various chromatographic methods such as HPLC or affinity chromatography. Alternatively, Fc domains, or polypeptides and peptides more generally, can be synthesized using an automated peptide synthesizer.

IX. Examples

[00183] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that

the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1: Combinatorial library construction for engineering Fc5

[00184] All plasmids and primers used in this study are described in Table 3 and Table 4. For the screening of IgG1 Fc fragments exhibiting higher binding affinity to Fc γ RI than the previously isolated Fc fragment (Fc5, containing amino acid substitutions E328V/M428I) (FIG. 1 and 2), the Fc5 gene was subjected to random mutagenesis by error prone PCR. Standard error prone PCR method (Fromant et al., 1995) was employed with the template of the pPelBFLAG-Fc5 and two primers (STJ#196 and STJ#197) synthesized from Integrated DNA Technologies (Coralville, IA). The amplified PCR fragments were ligated into *Sfi*I digested pPelBFLAG. The resulting plasmids were transformed into *E. coli* Jude-1(F' [Tn10(Tet^r) proAB^r lacI^q Δ(lacZ)M15] mcrA Δ(mrr-hsdRMS-mcrBC) φ80dlacZΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara leu)7697 galU galK rpsL endA1 nupG) (Kawasaki et al., 2003). Based on the sequence of 20 library clones randomly selected, the library was 7 × 10⁸ individual transformants with 0.264% error rate per gene (FIG. 3).

Example 2: Spheroplasting and high throughput flow cytometry screening for affinity maturation of Fc5

[00185] The library cells cultured overnight at 37 °C with 250 rpm shaking in Terrific Broth (Becton Dickinson Diagnostic Systems Difco™, Sparks, MD) with 2% (wt/vol) glucose supplemented with chloramphenicol (50 µg/ml) were diluted 1:50 in fresh TB media containing 0.5 M trehalose (Fisher Scientific, Fair Lawn, NJ) and chloramphenicol (40 µg/ml). After 3 h incubation at 37 °C with 250 rpm shaking and then 20 min cooling at 25°C, the expression of Fc fragments was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). After 5 hours culture at 25 °C, 4.5 ml of the culture broth was harvested by centrifugation and washed two times in 1 ml of cold 10 mM Tris-HCl (pH 8.0). After resuspension in 1 ml of cold STE solution (0.5 M Sucrose, 10 mM Tris-HCl, 10 mM EDTA, pH 8.0), the cells were incubated

with rotating mixing at 37°C for 30 min, pelleted by centrifugation at 12,000 x g for 1 min and washed in 1 ml of cold Solution A (0.5 M Sucrose, 20 mM MgCl₂, 10 mM MOPS, pH 6.8). The washed cells were incubated in 1 ml of Solution A with 1 mg/ml of hen egg lysozyme at 37°C for 15 min. After centrifugation at 12,000 × g for 1 min and the resulting spheroplasts pellets were resuspended in 1 ml of cold PBS.

[00186] For library screening, extracellular domain of recombinant glycosylated Fc γ RIa/CD64 (R&D Systems, Minneapolis, MN) was labeled with FITC using FITC protein labeling kit (Invitrogen, Carlsbad, CA). After the labeling reaction, the affinity of FITC labeled Fc γ RI for human IgG Fc was confirmed by fluorescent ELISA displaying high fluorescence in the Fc glycosylated human IgG-Fc coated wells comparing in the BSA coated wells. Spheroplasts were labeled with 30 nM of Fc γ RI-FITC. In the subsequent round sorting, reduced concentration of Fc γ RIa-FITC (10, 3, and 1 nM for the 2nd, 3rd, and 4th round sorting, respectively) were used for labeling of spheroplasts. More than 4 × 10⁸ spheroplasts were sorted by MoFlo (Dako Cytomation, Fort Collins, CO) equipped with a 488 nm argon laser for excitation. In each round the top 3% of the population showing the highest fluorescence due to Fc γ RIa-FITC binding was isolated by sorting and resorting immediately after the initial sorting.

[00187] The Fc genes in the spheroplasts were rescued by PCR amplification using two specific primers (STJ#16 and STJ#220), ligated into pPelBFLAG-Fc using *Sfi*I restriction enzyme site, and transformed in electrocompetent *E. coli* Jude-1 cells. The resulting transformants were selected on chloramphenicol containing media and then grown, spheroplasted as above in preparation for the next round of sorting. High fluorescent clones were enriched as sorting rounds go on (FIG. 4). After the 5th round of sorting, 19 individual clones exhibiting higher fluorescence than Fc5 were isolated (FIG. 5). Except redundant mutations, all of the mutations were in three distinct regions comprising upper CH2 part that might directly contact to Fc γ RIa, linker region located interface of CH2 and CH3 domains, and CH3 region that might contribute conformational change of Fc fragments for the binding to Fc γ RIa (FIG. 6). The highest fluorescent clone was Fc601 that have 2 additional mutations (K338R, G341V) in Fc5 (E382V/M428I) (FIG. 7 and 8).

Example 3: Sequences of selected clones displaying higher affinity binding to Fc_YRIa than Fc5

[00188] Fc5 (Nucleotide Sequence #2 and Protein Sequence #2) have 2 mutations (E382V and M428I) in the sequence of wild type IgG1-Fc (Nucleotide Sequence #1 and Protein Sequence #1). The engineered Fc mutants exhibiting higher affinity to Fc_YRIa than Fc5 have substitution mutations in the sequence of Fc5. Isolated Fc mutants, Fc601-Fc619 (Protein Sequence #3 ~ #21), have substitution mutations in the sequence of Fc5. The isolated mutants are summarized in Table 5.

Example 4: Production and purification of full length IgG1-Fc601

[00189] Trastuzumab (HerceptinTM) has been clinically used for the treatment of breast metastatic carcinoma that overexpress HER2/neu (Erb2) (Sergina and Moasser, 2007). For the clearance of the metastatic carcinoma, trastuzumab antibodies recognize HER2/neu (Erb2) and interact with surface Fc_YRs of immune cells leading to antibody-dependent cell-mediated cytotoxicity (ADCC), an essential effector function mechanism for therapeutic action (Lazar et al., 2006; Sergina and Moasser, 2007). Fc fragment genes engineered for high Fc_YRs affinity were incorporated to full length trastuzumab antibodies. For the construction of pSTJ4-Herceptin IgG1, *E. coli* codon optimized (Hoover and Lubkowski, 2002) V_L and V_H domains of humanized 4D5 (anti-p185HER2) were synthesized by total gene synthesis with overlap extension PCR using 12 oligonucleotides that included 2 external primers (STJ#302 and STJ#313) and 10 internal primers (STJ#303-312) for V_L and 14 primers total 2 external primers (STJ#314 and STJ#327) and 12 internal primers (STJ#315-326) for V_H, respectively. The ligation of the amplified V_L and V_H into pMAZ360-M18.1-Hum-IgG1 using *Nco*I / *Not*I for V_L and *Nhe*I / *Hind*III restriction endonuclease sites for V_H generated pSTJ4-Herceptin IgG1. For pSTJ4-Herceptin-Fc2a-IgG1 and pSTJ4-Herceptin-Fc5-IgG1, Fc5 and Fc2a mutant genes were amplified using the primers (STJ#290 and STJ#291) and the templates, pPelBFLAG-Fc5 or pPelBFLAG-Fc601, ligated into pSTJ4-Herceptin IgG1 digested using *Sal*I / *Eco*RV. For preparative production of aglycosylated trastuzumab and trastuzumab-Fc5, and trastuzumab-Fc601 in *E. coli*, dicistronic plasmids, pSTJ4-Herceptin-IgG1, pSTJ4-Herceptin-Fc5-IgG1, and pSTJ4-Herceptin-Fc601-IgG1 were constructed. These plasmids are under the control of *lac* promoter in a dicistronic operon with PelB leader peptide fusions to both heavy and light chains (FIG. 9).

[00190] After transformation of the plasmids into *E. coli* BL21(DE3) (EMD Chemicals, Gibbstown, NJ), cells were grown in LB complex medium for overnight and then overnight cultured twice for adaptation in R/2 medium (Jeong and Lee, 2003) consisting of: 2 g of $(\text{NH}_4)_2\text{HPO}_4$, 6.75 g of KH_2PO_4 , 0.93 g of citric acid H_2O , 0.34 g of MgSO_4 , 20 g of glucose, 0.05 g of ampicillin and 5 ml of trace metal solution dissolved in 2 N HCl (10 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 2.25 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.35 g of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.23 g of $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, 1.5 g of CaCl_2 , and 0.1 g of $(\text{NH}_4)_6\text{Mo}_7\text{O}_24$ per L). *E. coli* BL21(DE3) harboring pSTJ4-Herceptin-IgG1, pSTJ4-Herceptin-IgG1-Fc5, or pSTJ4-Herceptin-IgG1-Fc601 were cultured in 500 mL baffled-flask with 120 mL R/2 media at 30 °C at 250 rpm for 8 h and then inoculated to 3.3L BioFlo™ 310 fermentor (New Brunswick Scientific Co., Edison, NJ) with 1.2 L R/2 medium. Fed-batch fermentation was performed at 30 °C using pH-stat glucose feeding strategy. The dissolved oxygen (DO) concentration was maintained at 40% of air saturation using automatic cascade control by increasing agitation speed from 100 rpm to 1000 rpm, air flow rate from 1 to 3 SLP (Standard liquid per minute) and pure oxygen flow rate from 0 to 1.5 SLP when required. The initial pH was adjusted to 6.8 and controlled by the addition of 30% (v/v) ammonium hydroxide when it decreased to less than 6.75 and by the supply of feeding solutions, (700 g/L of glucose and 10 g/L of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; before induction) and (500 g/L glucose, 10 g/L of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 100 g/L of yeast extract; after induction), when it increased to more than 6.9. When OD_{600} reached 100, the culture temperature was reduced to 25 °C and 30 min later, protein expression was induced with 1 mM of isopropyl-1-thio-β-D-galactopyranoside (IPTG). The culture broth was harvested 7 h later at an OD_{600} of ~130-140. The yield of aglycosylated tertameric IgG was about 40 mg/L.

[00191] Cells were harvested by centrifugation at $11,000 \times g$ for 30 min and suspended in 1.2 L solution containing 100 mM Tris, 10 mM EDTA (pH 7.4) supplemented with 4 mg of lysozyme (per g of dry cell weight) and 1 mM PMSF. Periplasmic proteins were released by the incubation of the suspended solution with shaking at 250 rpm at 30 °C for 16 h. After centrifugation at $14,000 \times g$ for 30 min, the supernatant was mixed with polyethyleneimine (MP Biomedical, Solon, OH) to a final concentration of 0.2% (w/v) re-centrifuged at $14,000 \times g$ for 30 min, and filtered through 0.2 μm filter. Clear filtrate was mixed with immobilized Protein A agarose

resin pre-equilibrated in 20 mM sodium phosphate buffer (pH 7.0) and incubated at 4 °C for 16 h. After washing with 200 ml of 20 mM sodium phosphate buffer (pH 7.0) and 200 ml of 40 mM sodium citrate (pH 5.0), wild type aglycosylated trastuzumab, aglycosylated trastuzumab-Fc5, and aglycosylated trastuzumab-Fc601 were eluted from the resin using 15 ml of 0.1 M glycine (pH 3.0) and neutralized immediately with 1M Tris (pH 8.0) solution. The eluted samples were concentrated by ultrafiltration through a 10 kDa MW cutoff membrane and the retentate was applied to a Superdex 200 gel filtration column developed with PBS (pH 7.4).

Example 5: Affinity of aglycosylated trastuzumab-Fc601 to Fc receptors

[00192] Affinity of full assembled aglycosylated trastuzumab antibodies to Fc γ RIa was measured by immobilizing glycosylated trastuzumab (Clinical grade, Fox Chase Cancer Center Pharmacy), aglycosylated trastuzumab, and aglycosylated trastuzumab-Fc5, aglycosylated trastuzumab-Fc601 individually on the CM-5 sensor chip. The soluble monomeric Fc γ RIa in HBS-EP (10 mM HEPES pH 7.4, 150 mM NaCl, 3.4 mM EDTA, and 0.005% P20 surfactant) buffer was injected at flow rate of 30 μ l/min for 60 s with dissociation time 300 s. Regeneration of the ligand was performed by single injection of 100 mM citric acid, pH 3.0. Affinities of the soluble monomeric Fc γ RIa with glycosylated trastuzumab, aglycosylated trastuzumab, trastuzumab-Fc5, and trastuzumab-Fc601 were obtained by injection of soluble Fc γ RIa in duplicate at concentrations of 0, 25, 50, 100, 200 nM for 60 s at a flow rate of 30 μ l/min over immobilized glycosylated trastuzumab, trastuzumab, trastuzumab-Fc5, and trastuzumab Fc601. Affinity of Fc γ RIa toward wild type aglycosylated trastuzumab was obtained by Fc γ RIa injections in duplicates at concentrations 0, 200, 300, 400, 500, and 600 nM for 60 s at a flow rate 30 μ l/min over immobilized aglycosylated trastuzumab. Binding curve at zero concentration was subtracted as a blank. Equilibrium dissociation constants (K_D) were determined by fitting of equilibrium responses to steady-state affinity model provided by BIAevaluation™ 3.0 software. As shown in FIG. 10, trastuzumab-Fc601 bound to Fc γ RIa with similar affinity with commercial-grade glycosylated trastuzumab from CHO cells and over 130 fold increased affinity compared with wild type aglycosylated trastuzumab.

[00193] The affinity of the purified IgGs for the extracellular domain of Fc γ RIIa, Fc γ RIIb, Fc γ RIIIa was analyzed by ELISA. 50 μ l of 4 μ g/ml of aglycosylated trastuzumab, trastuzumab-Fc5, or trastuzumab-Fc601 purified from *E.*

coli, glycosylated IgG trastuzumab were diluted in 0.05 M Na₂CO₃ (pH 9.6) buffer and used to coat 96 well polystyrene ELISA wells (Corning, Corning, NY) for 16 hr at 4 °C. After blocking with 1 × PBS (pH 7.4), 0.5% BSA for 2 hr at room temperature, the plate was washed 4 times with PBS containing 0.05% Tween20™, and incubated with serially diluted FcγRIIa, FcγRIIb C-terminal fused to GST (Berntzen et al., 2005), FcγRIIIa (R&D Systems, Minneapolis, MN) at room temperature for 1 h. After washing 4 times with the same buffer, 1:5,000 diluted anti-GST antibody HRP conjugate (Amersham Pharmacia, Piscataway, NJ) for FcγRIIa and FcγRIIb or 1:10,000 diluted anti-polyhistidine antibody HRP conjugate (Sigma-Aldrich, St. Louis, MO) for FcγRIIIa was added and plates were washed and developed as described previously (Mazor et al., 2007). To determine the binding of IgG to FcRn at pH 7.4, 2 µg/ml FcRn preincubated with in PBS (pH 7.4) containing 1:5,000 diluted anti-GST-HRP at room temperature for 1 h as previously described (Andersen et al., 2006) was added to plates coated with trastuzumab antibodies. To evaluate binding at pH 6.0, ELISAs were carried out as above except 20 mM MES was added to washing buffer and sample dilution buffers and pH was adjusted to 6.0. As expected, the aglycosylated trastuzumab exhibited low affinity to FcγRIIa or FcγRIIb (EC₅₀ ≥ 1000-fold and 100-fold higher for GST fused FcγRIIa and FcγRIIb, respectively, FIG. 3 C and D) (FIG. 11 and FIG. 12), FcγRIIIa (FIG. 13). Trastuzumab-Fc601 antibody exhibited only slightly higher affinity for FcγRIIb. The neonatal FcRn receptor binding to the interface of CH2 domain and the CH3 domain is responsible for the endosomal recycling of IgG in plasma (Ghetie and Ward, 2000). Trastuzumab-Fc5 did show its pH-dependent binding (high affinity binding at pH 6.0 and its low binding at pH 7.4) to the neonatal FcRn. However, trastuzumab-Fc601 exhibited much reduced binding affinity to FcRn at pH 6.0 (FIG. 14).

Example 6: Library construction for higher affinity to FcγRIa than Fc5 and for pH dependent FcRn binding

[00194] Human FcRn has high affinity to human IgG under slightly acidic pH condition and low affinity at neutral or basic pH (Ober et al., 2004a; Ober et al., 2004b; Raghavan and Bjorkman, 1996; Rodewald, 1976). The FcRn binding sites are located at the interface of CH2 and CH3 domains, similar binding sites for staphylococcal protein A (SpA) (Kim et al., 1994; Shields et al., 2001). Fc601 showed improved FcγRIa binding affinity than Fc5. However, two additional mutations

(K338R, G341V) of Fc601 in lower CH2 region of IgG1 impaired the pH dependent FcRn binding that is critical for the regulation of serum IgG concentration by allowing pinocytosed IgGs to make strong IgG-FcRn complex in acidified endosomes for recycling to blood across vascular endothelial cell membrane instead of degradation in lysosomes (Ghetie and Ward, 2000).

[00195] To isolate engineered Fc fragments showing higher affinity to FcγRIa than Fc5 and retaining the pH dependent FcRn binding, new combinatorial libraries consisting of random amino acids in upper CH2 region were constructed. The libraries are composed of 4 sub-libraries. Four parts of upper CH2 region (234L-239S, 264V-268H, 297N-299T, 328L-332I) (Kabat et al., 1991) were substituted by random amino acids using NNS degenerate codons (FIG. 15 and 16). For the first sub-library, DNA fragments were amplified using the primers (STJ#465 and STJ#220) and the template, pPelBFLAG-Fc5. The N-terminal sequence extension of the PCR amplified fragments using the primer STJ#473 generated the sub-library replacing 5 amino acids in the region 234L-239S with random amino acids. Gene assembly PCR products using DNA fragments amplified by the primers (STJ#467 and STJ#220) and DNA fragments amplified by the primers (STJ#473 and STJ#468) generated the second sub-library that randomized 5 amino acid residues for 264V-268H. The third sub-library randomized 297N-299T was generated using the primer pairs (STJ#473/STJ#470 and STJ#469/STJ#220) and the fourth sub-library (328L-332I) was generated using the primer pairs (STJ#473/STJ#470 and STJ#469/STJ#220) using the same PCR template plasmid, pPelBFLAG-Fc5. Based on the number of possible mutations, the same amount of DNA from three sub-libraries (234L-239S; 264V-268H; 328L-332I) that randomized 5 amino acid residues were mixed with and 20³/20⁵ fold amount of DNA from the third sub-library (297N-299T) that randomized 3 amino acid residues. Each of the three sub-libraries was subcloned into *Sfi*I digested pPelBFLAG. The resulting plasmids were transformed into *E. coli* Jude-1(F' [Tn10(Tet^r) proAB⁺ lacI^q Δ(lacZ)M15] mcrA Δ(mrr-hsdRMS-mcrBC) φ80dlacZΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara leu)7697 galU galK rpsL endA1 nupG) (Kawasaki et al., 2003).

Example 7: Screening of Fc mutants exhibiting higher affinity to Fc γ RIa than Fc5 and for pH dependent FcRn binding

[00196] The library cells composed of 4 sub-libraries were converted to spheroplasts by the methods described in EXAMPLE 2. Over 4×10^8 spheroplasts were sorted by MoFlo flow cytometry (Dako Cytomation, Fort Collins, CO) equipped with an argon laser. Following labeling of 10 nM (3 nM for the 2nd round, 1 nM for the 3rd round, 0.3 nM for the 4th round) of Fc γ RIa-FITC for 1 hr at room temperature, spheroplasts were sorted with selectively gating the top 3% of the population showing the highest fluorescence due to Fc γ RIa-FITC binding. After the initial sorting, collected spheroplasts were immediately resorted. The Fc encoding genes were rescued by PCR using two specific primers (STJ#16 and STJ#220) and ligated into *Sfi*I digested pPelBFLAG plasmid. The ligation mixture was transformed into *E. coli* Jude-1. Transformants selected on chloramphenicol containing media were grown, spheroplasted as above, and sorted. After the 4th round of sorting, 8 individual clones exhibiting higher fluorescence than Fc5 were isolated (FIG. 17). All of the clones have consensus mutations in L328W and I332Y mutations. Also, the amino acid residue 329P was well conserved suggesting the critical role of the specific amino acid residue in the binding of Fc γ RIa (FIG. 18). The highest fluorescent clone was Fc701 that have L328W, A330V, P331A, I332Y mutations in 328L-332I region and one additional Q295R mutation (FIG. 19-21).

Example 8: Sequences of selected clones displaying high affinity binding to Fc γ RIa screened from upper CH2 randomization library

[00197] The engineered Fc mutants exhibiting higher affinity to Fc γ RIa than Fc5 have substitution mutations in the sequence of Fc5. Isolated Fc mutants Fc701-Fc708 (Protein Sequence #22 ~ #29) have mutations in the sequence of Fc5. Isolated mutant showing higher affinity to Fc γ RI than Fc5 are summarized in Table 6.

Example 9: Characterization of full length trastuzumab-Fc701 IgG1

[00198] Full length trastuzumab-Fc701 IgG1 was produced using the fed batch fermentation and purified using Protein A affinity chromatography followed by gel filtration chromatography as described in EXAMPLE 4. To obtain kinetic rate constants for the binding of full length trastuzumab-Fc701 to Fc γ RIa, purified trastuzumab-Fc701 was immobilized on CM5 sensor chip using amine coupling

method. The interaction between trastuzumab-Fc701 and Fc γ RIa was analyzed using the condition described in EXAMPLE 5. Trastuzumab-Fc701 bound to Fc γ RIa with similar affinity with trastuzumab Fc601 (FIG. 22). pH dependent FcRn binding was analyzed using ELISA at pH 6.0 and at pH 7.4 as described in EXAMPLE 5. As expected, all the trastuzumab antibodies including trastuzumab-Fc701 did not show significant binding affinity to FcRn at neutral pH 7.4. On the other hand, trastuzumab Fc701 showed higher affinity binding to FcRn at pH 6.0 than wild type aglycosylated or glycosylated trastuzumab antibodies (FIG. 23).

Table 3. Plasmids used in this study.

Plasmids	Relevant characteristics	Reference or source
pMoPac1	Cm ^r , lac promoter, <i>tetA</i> gene, C-terminal polyhistidine tag and c-myc tag	(Hayhurst et al., 2003)
pMoPac12	Ap ^r , lac promoter, <i>tetA</i> gene, <i>skp</i> gene, C-terminal polyhistidine tag and c-myc tag	(Hayhurst et al., 2003)
pMoPac1-FLAG-M18	NlpA fused <i>M18 scFv</i> gene, C-terminal FLAG tag in pMoPac1	(Jung et al., 2007)
pPelBFLAG	Cm ^r , lac promoter, <i>tetA</i> gene, <i>skp</i> gene, C-terminal FLAG tag	This study
pPelBFLAG-Fc	<i>IgGl-Fc</i> gene in pPelBFLAG	This study
pPelBFLAG-Fc5	<i>IgGl-Fc5</i> gene in pPelBFLAG	This study
pPelBFLAG-Fc601	<i>IgGl-Fc601</i> gene in pPelBFLAG	This study
pPelBFLAG-Fc701	<i>IgGl-Fc701</i> gene in pPelBFLAG	This study
pMAZ360-M18.1-Hum-IgG	<i>M18.1 humanized IgGl</i> gene in pMAZ360	(Mazor et al.)
pSTJ4-Herceptin IgG1	<i>Herceptin IgGl</i> gene in pMAZ360-M18.1-Hum-IgG1	This study
pSTJ4-Herceptin-Fc5-IgG1	<i>Herceptin IgGl-Fc5</i> gene in pMAZ360-M18.1-Hum-IgG1	This study
pSTJ4-Herceptin-Fc601-IgG1	<i>Herceptin IgGl-601</i> gene in pMAZ360-M18.1-Hum-IgG1	This study
pSTJ4-Herceptin-Fc701-IgG1	<i>Herceptin IgGl-701</i> gene in pMAZ360-M18.1-Hum-IgG1	This study

Table 4. Primers used in this study.

Primer Name	Seq ID No.	Primer nucleotide sequence (5' à 3')
STJ#16	32	TTGTGAGGGATAAACATTTC
STJ#196	33	CGCAGCGAGGCCAGCCGCCATGGCG
STJ#197	34	CGCAATTTCGAATTGGCCCCGAGGCC
STJ#220	35	CAATTGGTCAAGCCCTGAGCAGAAC
STJ#302	36	GCGGAATTCCCATGGGGATTCATAATGACCC
STJ#303	37	CAGACGGCTTAAGAAAGACGGGCTTGGGICATTGAATAATCCGCCATG
STJ#304	38	CGTCTTCCTTAAGCGCTCTGTCGGTGATCGCGTGAACCATCACGTGTCGT
STJ#305	39	AGGCCACCGCCGTTAACATCCTGGCTGCACGACACGTGATGGTCACCG
STJ#306	40	GTTAATACGGGGTGGCTGGTATCAACAAAAACGGGTAAGGCCCGAA
STJ#307	41	GAGTACAGAAAGCTGGCGTGTAGATAAACAGCTICGGGGTTCACCCGG
STJ#308	42	CAGGCCAGCTTCTGTACTCTGGCTCCCGAGCGCTTCTGGCAGGCC
STJ#309	43	TGCTTAATGGCTAGCGTGAAGTCCGTACCGCTGGCTGGCAGAAAAGCGG
STJ#310	44	ACTTCACGCTGACCATAGGAGCCCTGAGCGGGAGGATTTGCCACCTAT
STJ#311	45	TGGGGGGTGGTLAGTGTGTGCTGACAATAATAGCTGGCGAAATTCCTCCG
STJ#312	46	ACTACACCACCCGCCAACCTTGGCCAGGGTAGAAAGTGGAGATTAA
STJ#313	47	GACAGATGGTGGCCCGTGGCTTAATCTCAACTTGTACCCCTGG
STJ#314	48	ATTGTTATGCTAGGGCTAGGCCAAATGGCAGGGCAATGGCG
STJ#315	49	ACCAGACCACCCGCAAGATCCACTAAATGAAACCTCCGCATGGGGC
STJ#316	50	TCTGGGGGCTCTGGCTGGCAGCCAGGGGTAGCTTACGTTCTGAGCTGTGC
STJ#317	51	AGGTATCTTGTATGTTGAAGCCAGGGCTGCACAGCTCAAGCTAAGCTA
STJ#318	52	TCTGGCTCAACATCAAAGATAACCTACATCATGGTTCGCAAGCC
STJ#319	53	ATAGATACTGGCCACCCACTCCAGGGCTTACCTGGGCTTGGGAACCC
STJ#320	54	GAGTGGGGCCGTATCTATCCAACCAATGGCTACACGGCTTATGCAGA
STJ#321	55	GCGCTAATGGTGAAGGGGCTTICACAGAGTCTGATAACGGCTGTAGGC
STJ#322	56	CCGCTTCACCAATTAGCGCCGACACCTCTAAGAACACCCGATATTACAGA
STJ#323	57	GTCTCTGCGCTAAAGAGTCTACCTGTAATAATGGGGTGTCTTAGAGG

Table 5. Mutations in Fc conferring higher affinity to Fc γ RI than Fc5

Fc mutants	Mutations
Fc601	K338R, G341V, E382V, M428I
Fc602	N297D, N315D, K340N, E382V, M428I
Fc603	K340N, E382V, M428I
Fc604	K338I, K340N, E382V, M428I
Fc605	K340Q, A378D, E382V, M428I
Fc606	N325S, K340N, E382V, M428I
Fc607	H224Y, E269K, N325S, G341V, E382V, M428I
Fc608	G341V, E382V, K392E, M428I
Fc609	K338R, G341V, E382V, S424L, M428I, N434D
Fc610	F241L, G341V, E382V, M428I
Fc611	G341V, E382V, M428I
Fc612	N276D, G341V, E382V, M428I
Fc613	G341V, V369A, E382V, M428I
Fc614	N286D, G341V, E382V, M428I, N434S
Fc615	N325S, G341V, E382V, M428I
Fc616	Y300C, G341V, E382V, M428I
Fc617	G341V, V348M, E382V, M428I
Fc618	E382V, M428I, N434S
Fc619	V266M, E382V, M428I

Table 6. Mutations conferring higher affinity to FcγRI than Fc5 isolated from upper CH2 region randomization library

Fc mutants	Mutations (in addition to E382V and M428I)
Fc701	L328W, A330V, P331A, I332Y, Q295R
Fc702	L328W, A330E, P331E, I332Y, V279M
Fc703	L328W, A330E, P331E, I332Y
Fc704	L328W, A330E, P331V, I332Y, S426T
Fc705	L328W, A330E, P331V, I332Y
Fc706	L328W, A330I, P331E, I332Y
Fc707	L328W, A330E, I332Y
Fc708	L328W, P331S, I332Y
Fc709	L328W, A330V, P331S, I332Y, H224R, L251F

Example 10: Detailed Construction of plasmids for covalently anchored full length IgG display system.

[00199] Subcloning of PCR amplified and *Sfi*I digested Fc gene encoding human IgG1-Fc fragment, hinge, CH2 and CH3 region of human IgG1 heavy chain (GeneBank Accession No. AF237583) into *Sfi*I digested pPelBFLAG generated pPelBFLAG-Fc. pBADNlpAHis-M18 was performed by ligating *Xba*I-*Hind*III digested NlpA fused M18 scFv gene from pMoPac1-FLAG-M18 into pBAD30-KmR digested with same restriction endonucleases. Ligation of *Sfi*I digested trastuzumab VL-Ck amplified using the primers (STJ#475 and STJ#476) and the template, pSTJ4-Herceptin IgG1 into *Sfi*I digested pBADNlpAHis-M18 generated pBADNlpA-VL-Ck-His. PelB leader peptide fused trastuzumab VL-Ck was amplified using the primers (STJ#16 and STJ#340) and the template (pSTJ4-Herceptin IgG1), digested by *Xba*I / *Hind*III endonucleases and ligated into pBAD-NlpA-VL-Ck-His digested with same endonucleases to generate pBADPeIB-VL-Ck. pBADPeIB-VL-Ck-NlpA-VL-Ck-His was constructed by ligating *Xba*I digested PCR fragments amplified using the primers (STJ#70 and STJ#332) and the template (pBADPeIB-VL-Ck) into pBADNlpA-VL-Ck-His digested using the same endonuclease. Trastuzumab heavy chains were amplified using the primers (STJ#474 and STJ#67) and the template pSTJ4-Herceptin IgG1 for pPeIB-Herceptin(H)-FLAG, pPeIB-Herceptin(H)-Fc5-FLAG, and pPeIB-Herceptin(H)-Fc2a-FLAG, respectively. Fc2a is an aglycosylated antibody variants optimized for Fc γ RII binding by two mutations (S298G/T299A) in the upper CH2 region; it has been reported that IgG containing Fc2a displays Fc γ RIIa binding and effector functions comparable to those of glycosylated antibodies (Sazinsky et al., 2008). For the expression of correctly assembled, homodimeric wild type Fc and Fc2a in the periplasmic space of *E. coli*, the plasmids pDsbA-Fc-FLAG and pDsbA-Fc2a-FLAG were constructed for the export of Fc via the DsbA signal peptide. The PCR amplified fragments were digested with *Sfi*I, ligated into pPeIBFLAG digested with the same endonuclease to generate pPeIB-Herceptin(H)-FLAG, pPeIB-Herceptin(H)-Fc5-FLAG and pPeIB-Herceptin(H)-Fc2a-FLAG.

[00200] Tables 7 and 8 summarize the plasmids and primers used for Examples 10-14.

Example 11. Preparation of spheroplasts and FACS analysis for the covalently anchored full length IgG display system to engineer IgG heavy chain

[00201] To use bacterial full length IgG display system for library screening, four factors should be considered, Firstly, IgG heavy chains and light chains must be well expressed. Secondly, the heavy and light chains should be assembled well in *E. coli*. Thirdly, binding ligands should be accessible to the full length IgG in bacterial cells. Finally, fourth, the anchoring of the displayed full length IgG should be robust during library screening.

[00202] Two plasmid co-expression plasmids were used for stable, covalent anchoring of full length IgG (FIG. 24). The pBADPelB-VL-Ck-NlpA-VL-Ck-His plasmid enables the expression of the NlpA leader peptide fused IgG light chain (VL-Ck) and the PelB leader peptide fused IgG light chain (VL-Ck). Thus a portion of the light chain becomes anchored on the periplasmic side of the inner membrane where it associates with heavy chain to produce tetrameric full length, IgG. pPelB-Herceptin(H)-FLAG is a high copy number plasmid encoding the IgG heavy chain under the control of the *lac* promoter. The plasmid pBADPelB-VL-Ck-NlpA-VL-Ck-His was transformed with pPelB-Herceptin(H)-FLAG, pPelB-Herceptin(H)-Fc5-FLAG, or pPelB-Herceptin(H)-Fc2a-FLAG for wild type trastuzumab, trazumab-Fc5, or trastuzumab-Fc2a, respectively into *E. coli* Juds-1(F^r [Tn10(Tet^r) proAB⁺ lacI^q Δ(lacZ)M15] mcrA Δ(mrr-hsdRMS-mcrBC) φ80dlacZΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara leu)7697 galU galK rpsL endA1 nupG) (Kawasaki et al., 2003). The transformed *E. coli* cells were cultured overnight at 37 °C with 250 rpm shaking in Terrific Broth (Becton Dickinson Diagnostic Systems Difco™, Sparks, MD) with 2% (wt/vol) glucose supplemented with chloramphenicol (50 µg/ml) and kanamycin (50 g/ml).

[00203] The overnight cultured cells were diluted 1:100 in fresh 7 ml of TB medium with chloramphenicol (50 µg/ml) and kanamycin (50 g/ml) in 125 ml Erlenmeyer flask. After incubation at 37°C for 2 h and cooling at 25°C for 20 min with 250 rpm shaking, protein expression was induced with 1 mM of isopropyl-1-thio-D-galactopyranoside (IPTG). 20 h after IPTG induction, 6 ml of the culture broth was harvested by centrifugation and washed two times in 1 ml of cold 10 mM Tris-HCl (pH 8.0). After resuspension in 1 ml of cold STE solution (0.5 M Sucrose, 10

mM Tris-HCl, 10 mM EDTA, pH 8.0), the cells were incubated with rotating mixing at 37°C for 30 min, pelleted by centrifugation at 12,000 x g for 1 min and washed in 1 ml of cold Solution A (0.5 M Sucrose, 20 mM MgCl₂, 10 mM MOPS, pH 6.8). The washed cells were incubated in 1 ml Solution A with 1 mg/ml of hen egg lysozyme at 37°C for 15 min. After centrifugation at 12,000 x g for 1 min the resulting spheroplast pellets were resuspended in 1 ml of cold PBS. 300 µl of the spheroplasts were further diluted in 700 µl of PBS was labeled with 30 nM Fc_γRI-FITC to analyze the binding of Fc_γRIa. For the FACS analysis of Fc_γRIIa binding, spheroplasts were incubated with 90 nM Fc_γRIIa C-terminal fused to GST (Berntzen et al., 2005), washed in 1 ml of PBS, and labeled with polyclonal goat anti-GST-FITC (Abcam, Cambridge, MA) diluted 1:200 in 1 ml of PBS. After incubation for 1 h with vigorous shaking at 25°C in dark condition, the mixture was pelleted by centrifugation at centrifuged at 12,000 x g for 1 min and resuspended in 1 ml of PBS. The fluorescently labeled spheroplasts were diluted in 2.5 ml of PBS and analyzed on BD FACSCalibur™ (BD Bioscience, San Jose, CA).

Example 12: FACS analysis

[00204] For affinity maturation using FACS sorting method based on gating selective fluorescence and scattering regions, it is required to get distinguishable high or low fluorescence signal comparing a negative control with low coefficient of variation (CV = [Standard Deviation/Mean Value] × 100). The fluorescence for the 2 plasmids covalently anchored full length IgG display systems was compared with that for the dicistronic plasmids, pSTJ4-Herceptin IgG, pSTJ4-Herceptin-IgG1-Fc5 or pSTJ4-Herceptin-IgG1-Fc2a.

[00205] The fluorescent profile of spheroplasts expressing inner membrane anchored (via the NlpA-VL-Ck polypeptide) wild type full length IgG trastuzumab and spheroplasts expressing soluble IgG from a dicistronic vector system (Mazor et al., 2007) were compared. The 2 plasmids anchored full length IgG display system clearly exhibited dramatically improved signal intensity and CV value upon labeling with Fc_γRIa-FITC. The fluorescence signal for the anchored full length IgG display system was tested with cells cultured at 12 °C or 25 °C in TB. Spheroplasts generated from trastuzumab-Fc5 displaying cells using the 2 plasmids covalently anchored full length IgG display system cultured at 25 °C exhibited much higher fluorescence and

improved CV upon labeling with Fc γ RI-FITC relative to spheroplasts expressing a wild type trastuzumab (FIG. 25). Also, in the FACS analysis to measure the affinity of spheroplasts for Fc γ RIIa-GST (FIG. 26), 2 plasmids covalently anchored full length IgG display system cultured at 25 °C in TB showed surprisingly improved signal intensity and CV providing a selective display system for real affinity maturation of full length IgG (FIG. 27 and FIG. 28).

Example 13. Construction of error prone PCR library for IgG Fc engineering

[00206] An error prone PCR library of the CH2-CH3 region in anchored IgG was constructed by standard error prone PCR (Fromant et al., 1995) using the wt Fc as the template and two primers (STJ# 196 and STJ#197). The amplified PCR fragments were ligated into pPelBFLAG with *Sfi*I restriction sites for error prone PCR library. The library Fc fragments were amplified using the primers (STJ#479 and STJ#67). For trastuzumab heavy chain (VH-CH1-Hinge-CH2-CH3) library with randomized Fc region, VH-CH1 fragments were amplified using the primers (STJ#474 and STJ#480) from the template, pSTJ4-Herceptin IgG. Gene assembly PCR from 2 fragments, Hinge-CH2-CH3 regions and VH-CH1 regions using the primer (STJ#474 and STJ#67) generated trastuzumab heavy chain (VH-CH1-Hinge-CH2-CH3) library that randomized Fc region. The gene assembled PCR fragments were ligated into pPelBFLAG with *Sfi*I restriction sites. The resulting plasmids were transformed into *E. coli* Jude-1(F' [Tn10(Tet^r) proAB⁺ lacI^q Δ(lacZ)M15] mcrA Δ(mrr-hsdRMS-mcrBC) 80dlacZΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara leu)7697 galU galK rpsL endA1 nupG) (Kawasaki et al., 2003). The library consisted 9.2×10^8 individual transformants with 0.49% error rate per gene based on the sequencing of 20 library clones randomly selected.

Example 14. Construction of upper CH2 region randomization library for IgG Fc engineering

[00207] These libraries are composed of 4 sub-libraries. Four parts of upper CH2 region (234L-239S, 264V-268H, 297N-299T, 328L-332I) (Kabat et al., 1991) were substituted by random amino acids using NNS degenerate codons (FIG. 29). For the first sub-library, DNA fragments were amplified using the primers (STJ#465 and STJ#220) and the template, pPelBFLAG-Fc. A 5' sequence extension using the primer STJ#473 was used to generate a sub-library replacing 5 amino acids in the

region 234L-239S with random amino acids. Gene assembly PCR products using DNA fragments amplified using the primers (STJ#467 and STJ#220) and DNA fragments amplified using the primers (STJ#473 and STJ#468) generated the second sub-library that randomized 5 amino acid residues for 264V-268H. In the third sub-library residues 297N-299T were randomized using the primer pairs (STJ#473/STJ#470 and STJ#469/STJ#220) and the fourth sub-library (328L-332I) was generated using the primer pairs (STJ#473/STJ#470 and STJ#469/STJ#220) using the same PCR template plasmid, pPelBFLAG-Fc5. Based on the number of possible mutations, the same amount of DNA from three sub-libraries (234L-239S; 264V-268H; 328L-332I) that randomized 5 amino acid residues were mixed with and $20^3/20^5$ fold amount of DNA from the third sub-library (297N-299T) that randomized 3 amino acid residues. Each of the three sub-libraries was subcloned into *Sfi*I digested pPelBFLAG. For trastuzumab heavy chain (VH-CH1-Hinge-CH2-CH3) library that randomized upper CH2 region, VH1-CH1 fragments were amplified using the primers (STJ#474 and STJ#480) from the template, pSTJ4-Herceptin IgG. Gene assembly PCR from 2 fragments, Hinge-CH2-CH3 regions and VH1-CH1 regions using the primer (STJ#474 and STJ#67) generated trastuzumab heavy chain (VH-CH1-Hinge-CH2-CH3) library that randomized upper CH2 region. The gene assembled PCR fragments were ligated into pPelBFLAG with *Sfi*I restriction sites. The resulting plasmids were transformed into *E. coli* Jude-1. The constructed library size was over 3×10^8 individual transformants based on the sequence of 20 library clones randomly selected.

Table 7. Plasmids used in this study.

Plasmids	Relevant characteristics	Reference or source
pMoPac1	Cm ^r , lac promoter, <i>tetA</i> gene, C-terminal polyhistidinc tag and c-myc tag	(Hayhurst et al., 2003)
pMoPac12	Ap ^r , lac promoter, <i>tetA</i> gene, <i>skp</i> gene, C-terminal polyhistidine tag and c-myc tag	(Hayhurst et al., 2003)
pMoPac1-FLAG-M18	NlpA fused <i>M18 scFv</i> gene, C-terminal FLAG tag in pMoPac1	(Jung et al., 2007)
pPelBFLAG-M18	Cm ^r , lac promoter, <i>tetA</i> gene, <i>skp</i> gene, C-terminal FLAG tag	This study
pPelBFLAG-Fc	<i>IgG1-Fc</i> gene in pPelBFLAG	This study
pPelBFLAG-Fc5	<i>IgG1-Fc5</i> gene in pPelBFLAG	This study
pPelBFLAG-Fc2a	<i>IgG1-Fc2a</i> gene in pPelBFLAG	This study
pMAZ360-M18.1-Hum-IgG	<i>M18.1</i> humanized <i>IgG1</i> gene in pMAZ360	(Mazor et al.)
pSTJ4-Herceptin IgG1	<i>Trastuzumab IgG1</i> gene in pMAZ360-M18.1-Hum-IgG1	This study
pSTJ4-Herceptin IgG1-Fc5	<i>Trastuzumab IgG1-Fc5</i> gene in pMAZ360-M18.1-Hum-IgG1	This study
pSTJ4-Herceptin IgG1-Fc2a	<i>Trastuzumab IgG1-Fc2a</i> gene in pMAZ360-M18.1-Hum-IgG1	This study
pPelB-Herceptin(H)-FLAG	<i>IgG1</i> heavy chain gene in pPelBFLAG	This study
pPelB-Herceptin(H)-Fc5-FLAG	<i>IgG1-Fc5</i> heavy chain gene in pPelBFLAG	This study
pPelB-Herceptin(H)-Fc2a-FLAG	<i>IgG1-Fc2a</i> heavy chain gene in pPelBFLAG	This study
pMAZ360-M18.1-Hum-IgG	<i>M18.1</i> humanized <i>IgG1</i> gcnc in pMAZ360	(Mazor et al.)
pSTJ4-Herceptin IgG1	<i>Trastuzumab IgG1</i> gene in pMAZ360-M18.1-Hum-IgG1	This study
pSTJ4-Herceptin IgG1-Fc5	<i>Trastuzumab IgG1-Fc5</i> gene in pMAZ360-M18.1-Hum-IgG1	This study
pSTJ4-Herceptin IgG1-Fc2a	<i>Trastuzumab IgG1-Fc2a</i> gene in pMAZ360-M18.1-Hum-IgG1	This study
pDsbA	<i>DsbA</i> signal sequencc gcnc in pTrc99A	This study
pDsbA-Fc-FLAG	<i>DsbA</i> fused <i>IgG1-Fc</i> gene, C-terminal FLAG tag in pTrc99A	This study
pDsbA-Fc5-FLAG	<i>DsbA</i> fused <i>IgG1-Fc5</i> gene, C-terminal FLAG tag in pTrc99A	This study
pDsbA-Fc2a-FLAG	<i>DsbA</i> fused <i>IgG1-Fc2a</i> gene, C-terminal FLAG tag in pTrc99A	This study

pBAD30	Apr, BAD promoter	(Guzman et al., 1995)
pBAD30-KmR	Km ^r , BAD promoter	(Jung et al., 2007)
pBADNlpAHis-M18	NlpA fused <i>M18 scFv</i> , C-terminal polyhistidine tag in pBAD30	This study
pBAD-PelB-VL-Ck-His	PelB fused trastuzumab VL-Ck domain, C-terminal polyhistidine tag and c-myc tag in pBAD30-KmR	This study
pBAD-PclB-VL-Ck-NlpA-VL-Ck-His	PclB fused trastuzumab VL-Ck domain and NlpA fused trastuzumab VL-Ck-His in pBAD30-KmR	This study

Table 8. Primers used in this study.

Primer Name	Primer nucleotide sequence (5' → 3')
STJ#16	TGTAGCGGGATAACAATTTC
STJ#67	AATTGGGCCCCCGAGGCCCTTACCCGGGACAGGGAGAGGCTCTCTGCGTG
STJ#70	CTACCTGACGCTTTATCGC
STJ#144	TTTTAGGGTCGACGACAAACTCACACATGCCACCGTG
STJ#145	TTTAAGGGAAGCTCTATTAGCGCGCCCTTGTATCG
STJ#147	GGCAAATTCTGTTTATCAGACCGCTTCTG
STJ#196	CGCAGCGAGGCCAGCCGGCATGGCG
STJ#197	CGCAATTCAATTGGCCCCCGAGGGCCC
STJ#220	CAATTTCAGCCGCTGAGCAGAAG
STJ#290	TTTTAGGGTCGACAAGAAAGTTGAGCCAAATCTTGTGACAAAACACACATGCCACCG
STJ#291	GGCCACCGGATATCTTATTTACCCGGGACAGGGAGAGG
STJ#302	GCGGAATTCCCATTGGCGGATATTCAAATGACCC
STJ#303	CAGACCGCTTAAAGAAGACGGGCTTGGGTATTGAATATCCGCCATG
STJ#304	CGTCTCTTTAACCGCGCTGTCGGTATCGCGTACCGTGTCTG
STJ#305	AGGCCACCGCCGTATTAACATCTTGGCTCGCACGACACGTGATGGTCAGC
STJ#306	GTAAATACGGCGGTGGCCTGGTATCAACAAAACGGGTAAGCCCCGAA
STJ#307	GAGTACAGAAAGCTGGCGCTGTAGATTAAACAGCTTCCGGGCTTACCCGG
STJ#308	CAGCGCCAGTTCTGTACTCTGGCGTCCCGAGCCGCTTCTGGCAGCC
STJ#309	TGCTAATGGTCAGCGTAAGTCCGTACCGCTGCGGTGCCCCAGAAAAGCGG
STJ#310	ACTTCACGCTGACCATTAGCAGCCTGCAGCCGGAGGATTTCGCCACCTAT
STJ#311	GGCGGGGTGGTGTAGTGTGCTGACAATAATAGGTGGCAGAACCTCCCG
STJ#312	ACTACACCACCCGCCAACCTTGGCCAGGGTACGAAAGTGGAGATTAAA
STJ#313	GACAGATGGTGCGGCCGCGTTAACCTCCACCGTACCCGTTACCTGG
STJ#314	ATTGTTATTGCTAGCGGCTCAGCGGCAATGGCG
STJ#315	ACCAGACCACCGCCAGATTCCAACCTTGGCCATTGCCCGCTG
STJ#316	TCTGGCGTGGTCTGGTGCAGCCAGGGCGTAGCTTACGTCTGAGCTGTGC
STJ#317	AGGTATCTTGTATGGTAAAGCCAGCCTGCACAGCTCAGACGTAAGCTA
STJ#318	TCTGGCTCAACATCAAAGATACTTACATTGTTGGCTGCCAACCCCC
STJ#319	ATAGATACGGGCCACCCACTCCAGGCCTTACCTGGGGCTTGGCAACCC
STJ#320	GAGTGGTGGCCGTATCTATCCAACCAATGGCTACACGCGTTATGCAGA
STJ#321	GCGCTAATGGTAAGCGGCCCTCACAGAGTCTGCATAACGCGTGTAGCC
STJ#322	CCGCTTCACCATAGCGCCGACACCTCTAAGAACACCGCATATTACAGA
STJ#323	GTCCTCTGCGCTAAAGAGTTCATCTGAAATATGGGTGTTCTTAGAGG
STJ#324	AACTCTTACGCGCAGAGGACACGGCGGTGTACTACTGCTCTCGTTGGGG
STJ#325	AGTAGTCATCGCGTAGAAACCGTCACCGCCCCAACGAGAGCAGTAGTAC
STJ#326	GGTTTCTACGCGATGGACTACTGGGTCAAGGTACCGCTGGTCACGGTCAG
STJ#327	GCCCTGAAAGCTTGCAGAGCTGACCGTGACCGCGT
STJ#332	GGGAATTCTAGACTATTAGCACTCTCCCCGTGAAAGCTTTG
STJ#340	TTTAAGGGAAGCTCTATTAGCACTCTCCCCGTGAAAGCTTTG
STJ#422	CTAGGGAGCCGGGGAGGAGCAGTACAACGGCGTACCGTGTGGTCAGCGTCCTC
STJ#465	CCCACCGTCCCCAGCACCTGAANNSNNSSNGANNSNNSSGTCTCCCTTCCCCCCCCAAACCC
STJ#466	GGGTTTGGGGGAAGAGGAAGACSNNSNNTCCSNNSNNSSNTTACGGTGCTGGGACCGGTGGG
STJ#467	CCTGAGGTACATCGCTGGTNNSNNSNNSSNNSGAAGACCTGAGGTCAAGTCAACTGG
STJ#468	CCAGTTGAACCTGACCTCAGGGTCTCSNNSNNSNNSNNSNNACCGCATGTGACCTCAGG
STJ#469	GCCGCGGGAGGAGCAGTACNNSNNSNSTACCGTGTGGTCAGCGTCCTC
STJ#470	GAGGACGCTGACCAACCGTASNNSNNSNNGTACTGCTCCTCCCGCGGC
STJ#471	CAAGTCAAGGTCTCCAACAAAGCCNNNSNNSNNSNNSGAGAAAACATCTCCAAAGCCAAAGGG
STJ#472	CCCTTGGCTTGGAGATGGTTCTCSNNNSNNSNNSNNNGCTTGTGAGACCTTGCACCTG
STJ#473	CGCAGCGAGGCCAGCCGGCATGGCGACAAAACACACATGCCACCGTGCCAGCACCTG
STJ#474	CGCAGCGAGGCCAGCCGGCATGGCGAGGTCAATTAGTGGAACTG

STJ#475	CGCAGCGAGGCCAGCCGGCATGGCGGATATTCAAATGACCCAAAGCCCCG
STJ#476	CGCAATTGGCCCCCGAGGCCCGCACTCTCCCTGTTGAAGCTTTG
STJ#479	GACAAAACTCACACATGCCACCGTGCC
STJ#480	GGCACGGTGGGCATGTGTGAGTTTGTC

Example 15: Isolation and differentiation of human monocyte-derived dendritic cells (mDCs) for antibody-dependent cytotoxicity assays

[00208] Buffy coats (Gulf Coast Blood Center, Galveston, TX) was added to histopaque solution (Sigma) at 1:1 volume, avoiding mixing of the contents. The blood-histopaque solution was centrifuged at 1600 RPM for 30 minutes at 23°C without centrifugation braking. The peripheral blood mononuclear cell layer was isolated following gradient centrifugation, and washed twice through centrifugation with wash buffer (PBS, 2.5% Fetal Bovine Serum (FBS), 1mM ethylenediaminetetraacetic acid (EDTA)). Cells were then resuspended in Iscove's Modified Dulbecco's Medium (IMDM, Cambrex) and added to a 24 well plate and incubated at 37°C for 2h to allow monocytes to adhere to the plate. Typically, PBMCs from a 50 ml volume of blood was resuspended in 24 ml of IMDM, and plated at 1 ml/well. Media and non-adherent cells were then aspirated and adherent cells were washed 5 times with wash buffer. Cells were then resuspended with 1ml/well of growth media consisting of IMDM (Cambrex), 10% FBS, and recombinant cytokines Interleukin-4 (IL-4, R&D systems) at 200 ng/ml and granulocyte macrophage colony stimulating factor (GM-CSF, R&D systems) at 200 ng/ml. Additional IL-4 and GM-CSF were added at 200 ng/ml each on day 2 and 5 without changing media. DC differentiation was measured by flow cytometry by staining with a fluorescent antibody against the DC-specific surface marker CD11c (eBioscience).

Example 16: Antibody dependent cellular cytotoxicity (ADCC) assays

[00209] The breast cancer cell line SkBr3 that expresses high levels of Her2 was used as the target for ADCC assays. Cells were labeled with the isotope Na⁵¹CrO₄ (Perkin Elmer Life Sciences) at 100 uCi/10⁶ cells for 1 h at 37°C. Cells were then washed twice with PBS and resuspended in Roswell Park Memorial Institute medium-1640 with glutamax (RPMI) and added to a 96 well plate at 10⁴ cells/well. Aglycosylated wildtype trastuzumab, trastuzumab-Fc5, and trastuzumab-Fc601 (prepared as described in Example 4) and glycosylated trastumab (Clinical grade, Genentech) and relevant controls were added to the target cells in triplicate wells and incubated at 37°C for 1h. The plate was then centrifuged at 2000 RPM for 1 minute and washed with PBS. Effector cells, either fully differentiated mDCs (day 7) or freshly isolated PBMCs, were resuspended in RPMI, 2% low IgG FBS (Invitrogen), lipopolysaccharide (LPS) at 250 ng/10⁶ cells and added to the wells at

various ratios. Target cells and mDCs were incubated at 37°C for 24h. The isotope levels present in cell media were then measured in a liquid scintillation counter for chromium 51. Incubation of target cells with SDS was used as a positive control for maximum lysis and incubation with no effector cells was used as background lysis. When mDCs are used as the effector cells, aglycosylated trastuzumab-Fc5 and trastuzumab-601 show very high levels of ADCC and glycosylated trastuzumab induces very low ADCC (FIG. 31). Presumably this is because Fc-601 and Fc-5 bind only to Fc γ RI and not to the inhibitor receptor Fc γ RIIb which is also expressed on the surface of monocyted derived DCs. On the other hand, Herceptin displays binding to all Fc γ R receptors including Fc γ RIIb and binding to the latter receptor likely inhibits target cell activation and killing. With PBMCs as the effectors cells, glycosylated trastuzumab that can engage all the Fc receptors and can activate NK cells demonstrates high ADCC. In contrast, aglycosylated trastuzumab-Fc5 and trastuzumab-601 show low ADCC (FIG. 30).

* * *

[00210] All of the methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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PCT Appln. WO 90/07641

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CLAIMS

1. A polypeptide comprising an aglycosylated antibody Fc domain capable of binding a human Fc γ RI polypeptide, wherein the Fc domain comprises amino acid substitutions E382V and M428I, according to the Kabat numbering system, together with a substitution set selected from the group consisting of a) K338R and G341V; b) N297D, N315D, and K340N, c) K340N, d) K338I and K340N, e) K340Q and A378D; f) N325S and K340N, g) H224Y, E269K, N325S, and G341V, h) G341V and K392E, i) K338R, G341V, S424L and N434D, j) F241L and G341V, k) G341V, l) N276D and G341V, m) G341V and V369A, n) N286D, G341V and N434S, o) N325S and G341V, p) Y300C and G341V, q) G341V and V348M, r) V266M; s) L328W, A330V, P331A, 1332Y and Q295R, t) L328W, A330E, P331E, 1332Y, and V279M, u) L328W, A330E, P331E, and 1332Y, v) L328W, A330E, P331 V, I332Y, and S426T, w) L328W, A330E, P331V, and 1332Y, x) L328W, A330I, P331E, and 1332Y, y) L328W, A330E, and I332Y, z) L328W, P331S, and 1332Y, and aa) L328W, A330V, P331S, 1332Y, H224R, and L251F.
2. The polypeptide of claim 1, wherein the substitution set is a) K338R and G341V.
3. The polypeptide of claim 1, wherein the substitution set is b) N297D, N315D, and K340N.
4. The polypeptide of claim 1, wherein the substitution set is c) K340N.
5. The polypeptide of claim 1, wherein the substitution set is d) K338I and K340N.
6. The polypeptide of claim 1, wherein the substitution set is e) K340Q and A378D.
7. The polypeptide of claim 1, wherein the substitution set is f) N325S and K340N.
8. The polypeptide of claim 1, wherein the substitution set is g) H224Y, E269K, N325S, and G341V.
9. The polypeptide of claim 1, wherein the substitution set is h) G341V and K392E.

10. The polypeptide of claim 1, wherein the substitution set is i) K338R, G341V, S424L and N434D.
11. The polypeptide of claim 1, wherein the substitution set is j) F241L and G341V.
12. The polypeptide of claim 1, wherein the substitution set is k) G341V.
13. The polypeptide of claim 1, wherein the substitution set is l) N276D and G341V.
14. The polypeptide of claim 1, wherein the substitution set is m) G341V and V369A.
15. The polypeptide of claim 1, wherein the substitution set is n) N286D, G341V and N434S.
16. The polypeptide of claim 1, wherein the substitution set is o) N325S and G341V.
17. The polypeptide of claim 1, wherein the substitution set is p) Y300C and G341V.
18. The polypeptide of claim 1, wherein the substitution set is q) G341V and V348M.
19. The polypeptide of claim 1, wherein the substitution set is r) V266M.
20. The polypeptide of claim 1, wherein the substitution set is s) L328W, A330V, P331A, I332Y and Q295R.
21. The polypeptide of claim 1, wherein the substitution set is t) L328W, A330E, P331E, I332Y, and V279M.
22. The polypeptide of claim 1, wherein the substitution set is u) L328W, A330E, P331E, and I332Y.
23. The polypeptide of claim 1, wherein the substitution set is v) L328W, A330E, P331V, I332Y, and S426T.
24. The polypeptide of claim 1, wherein the substitution set is w) L328W, A330E, P331V, and I332Y.

25. The polypeptide of claim 1, wherein the substitution set is x) L328W, A330I, P331E, and I332Y.
26. The polypeptide of claim 1, wherein the substitution set is y) L328W, A330E, and I332Y.
27. The polypeptide of claim 1, wherein the substitution set is z) L328W, P331S, and I332Y.
28. The polypeptide of claim 1, wherein the substitution set is aa) L328W, A330V, P331S, I332Y, H224R, and L251 F.
29. The polypeptide of any one of claims 1-28, wherein the polypeptide has at least a 2-fold reduction in pH-dependent Fc γ RI binding compared to a polypeptide with an aglycosylated wild-type antibody Fc domain.
30. The polypeptide of claim 12, further comprising a conjugatable linker.
31. The polypeptide of claim 12 further comprising a non-FcR binding domain.
32. The polypeptide of claim 31, wherein the non-FcR binding domain is an antigen binding site of an antibody.
33. The polypeptide of claim 31, wherein the non-Fc binding region is not an antigen binding site of an antibody.
34. The polypeptide of claim 33, wherein the non-Fc binding region binds a cell-surface protein.
35. The polypeptide of claim 34, wherein the cell-surface protein is a receptor.
36. The polypeptide of claim 35, wherein the receptor is a tyrosine kinase.
37. The polypeptide of claim 36, wherein the non-Fc binding region binds multiple tyrosine kinase receptors.
38. A nucleic acid encoding the polypeptide of any one of claims 1-37.
39. The nucleic acid of claim 38, wherein the nucleic acid is a DNA segment.
40. The nucleic acid of claim 38, wherein the nucleic acid is an expression vector.
41. A host cell comprising the nucleic acid of any one of claims 38-40.
42. The host cell of claim 41, wherein the nucleic acid is in a first expression vector.

43. The host cell of claim 42, further comprising a second expression vector.
44. The host cell of claim 43, wherein the second expression vector encodes a polypeptide comprising an immunoglobulin light chain.
45. A population of host cells of claim 44, wherein the population contains a plurality of host cells that express different Fc domains.
46. The host cell population of claim 45, wherein the amino acid sequence of any two different Fc domains differs in identity by less than 20%.

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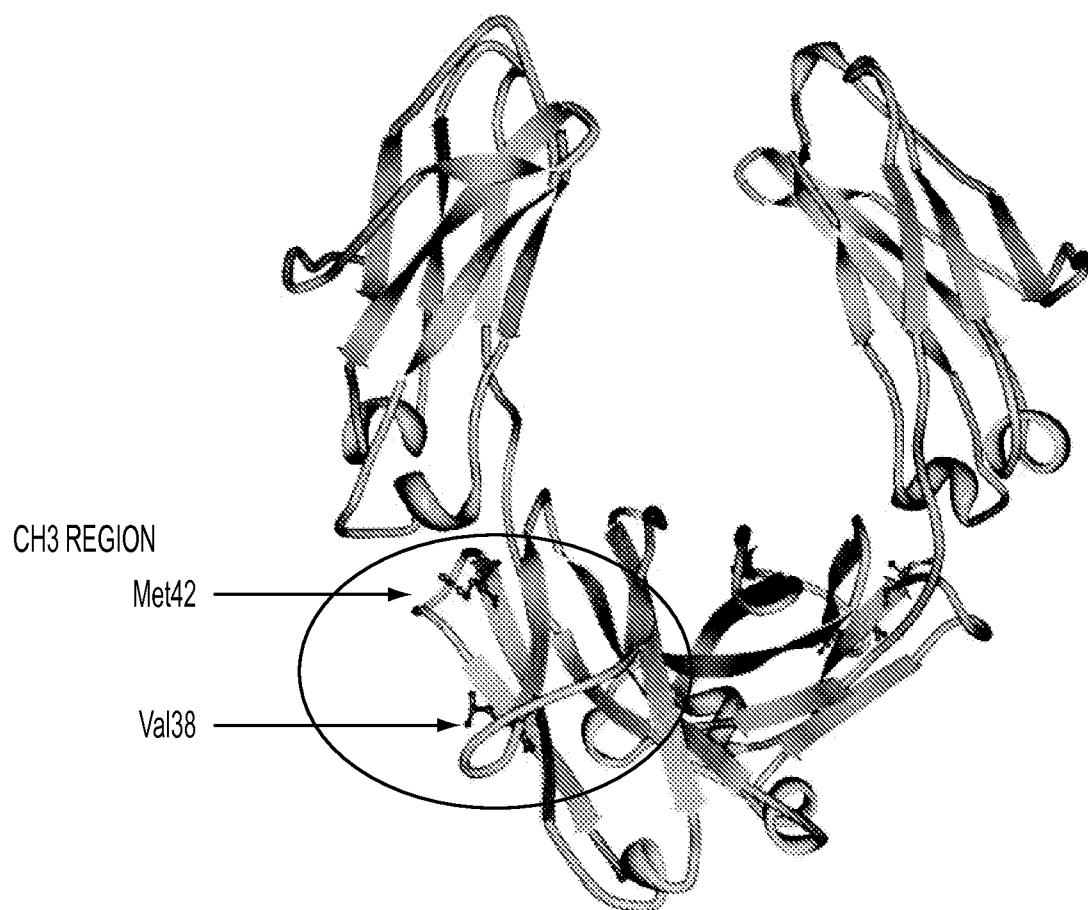


FIG. 1

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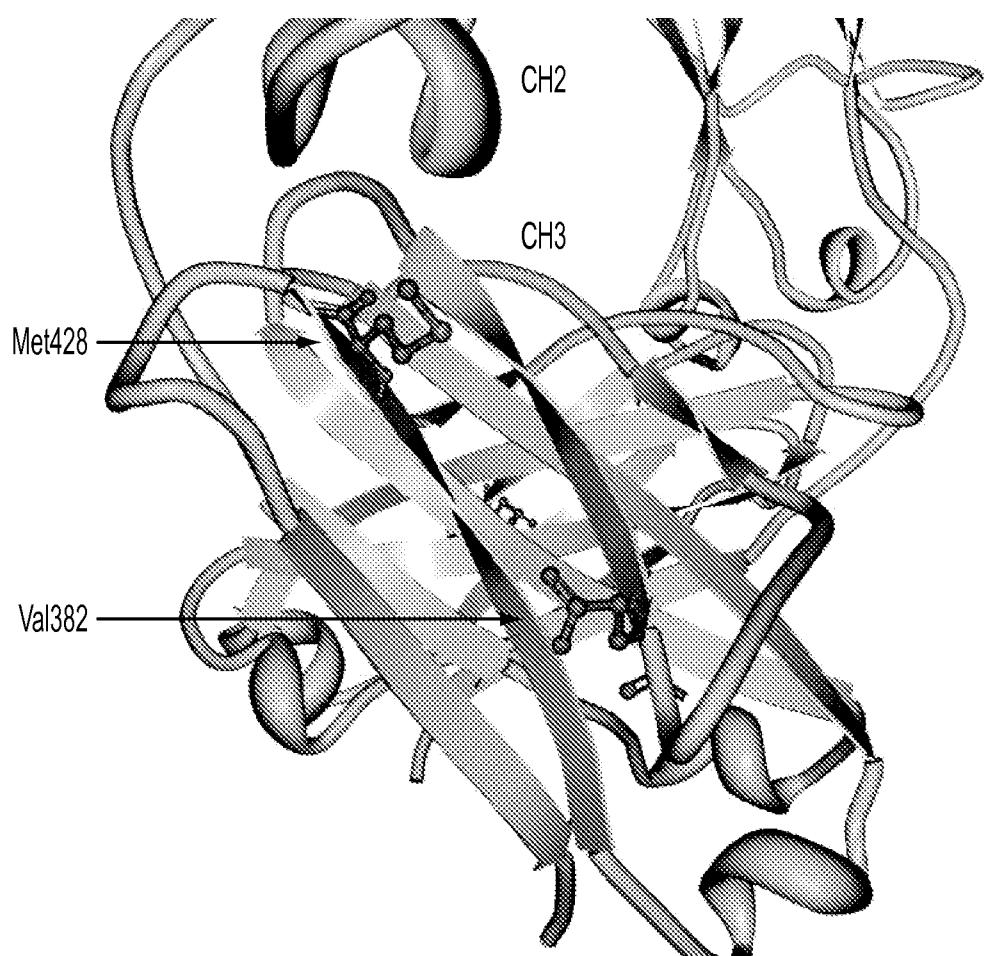


FIG. 2

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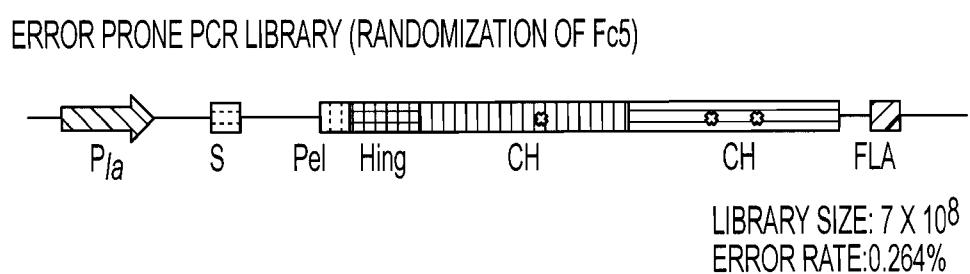


FIG. 3

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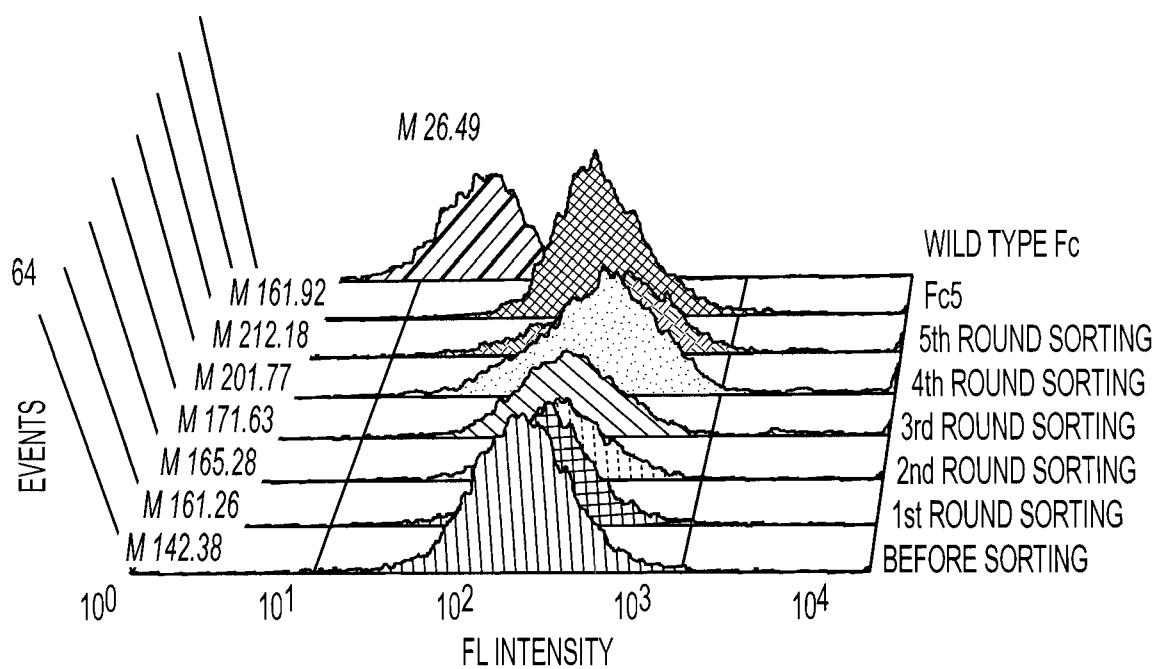


FIG. 4

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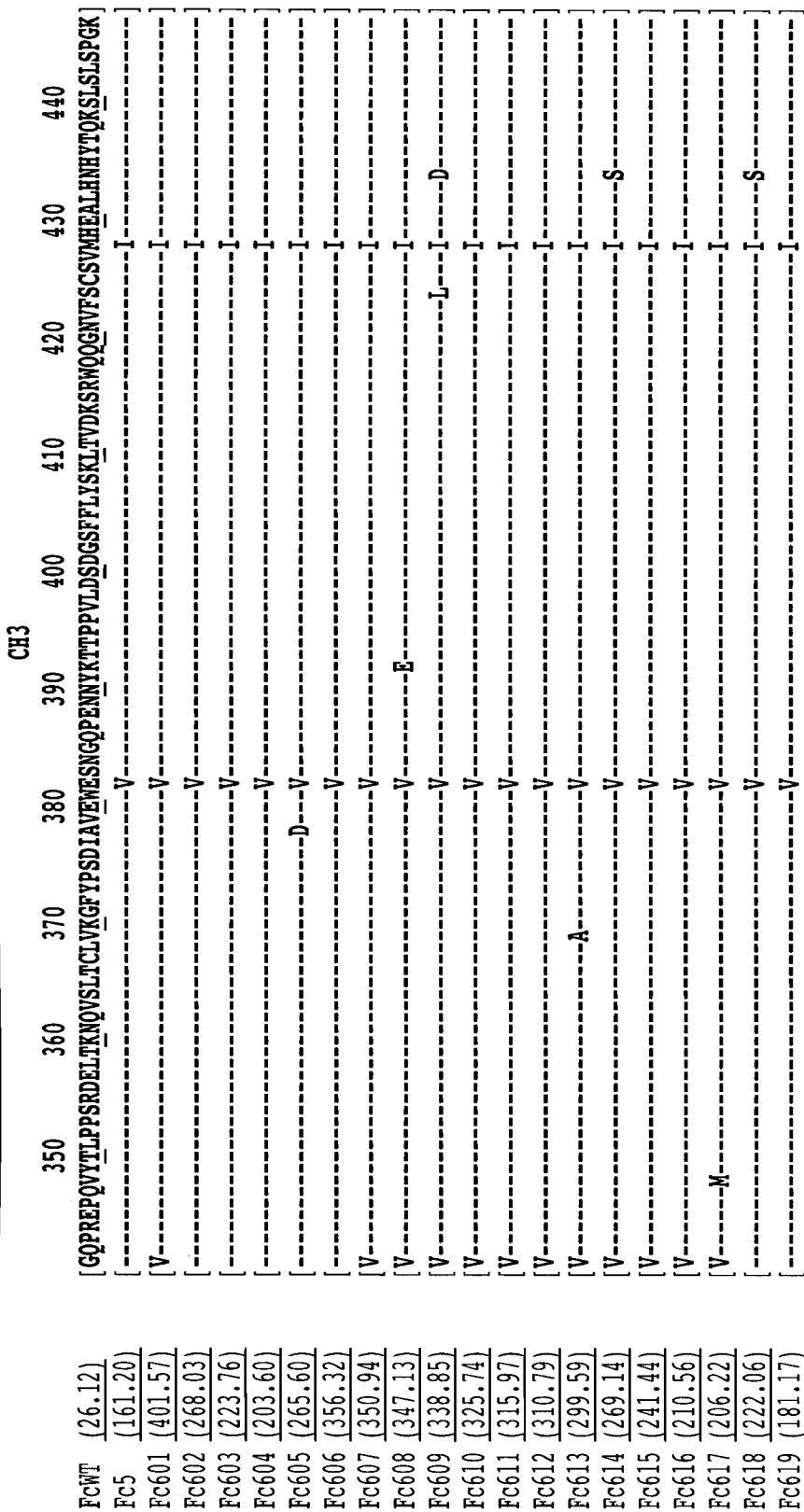
		Ring	230	240	250	260	270	280	290	300	310	320	330	340
FCWT	(26.12)	[DKTHTCPPC]PAPELGGPSVFLFPPKPKDTMISRTPIVTCVWVVDYSHEDPEVKFNWYDGVEVHNAKTKPREEQYNSTYRWSVLTILQDWLNGKEYKCKVSNKALPAPIEKTKAK												
FC5	(161.20)													
FC601	(401.57)													
FC602	(268.03)													
FC603	(223.76)													
FC604	(203.60)													
FC605	(265.60)													
FC606	(356.32)													
FC607	(350.94)													
FC608	(347.13)													
FC609	(338.85)													
FC610	(325.74)													
FC611	(315.97)													
FC612	(310.79)													
FC613	(299.59)													
FC614	(269.14)													
FC615	(241.44)													
FC616	(210.56)													
FC617	(206.22)													
FC618	(222.06)													
FC619	(181.17)													

SUBSTITUTE SHEET (RULE 26)

TO FIG. 5B

FIG. 5A

FROM FIG. 5A



1) FACS mean values are indicated in the parenthesis

FIG. 5B

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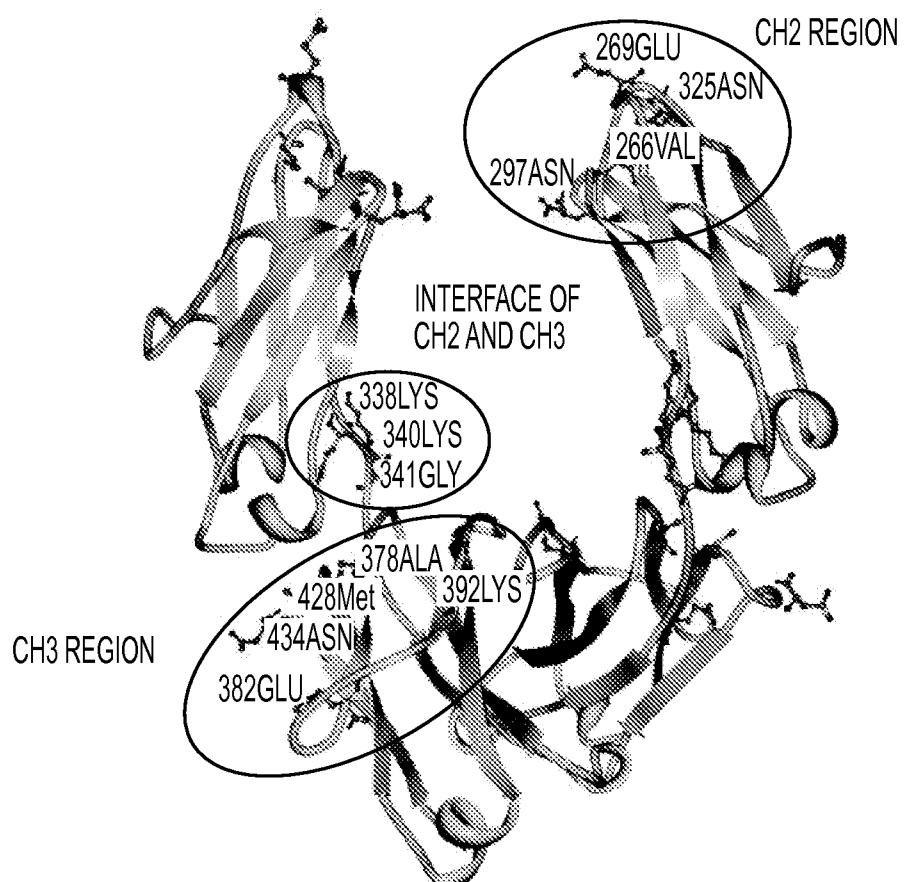


FIG. 6

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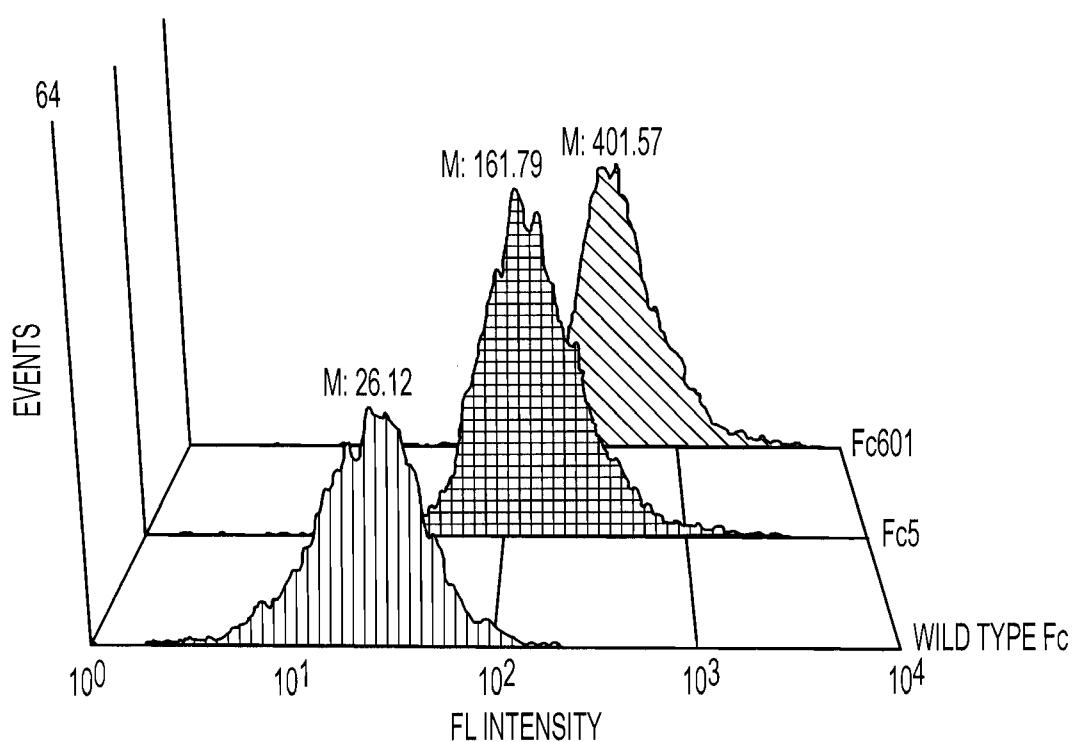


FIG. 7

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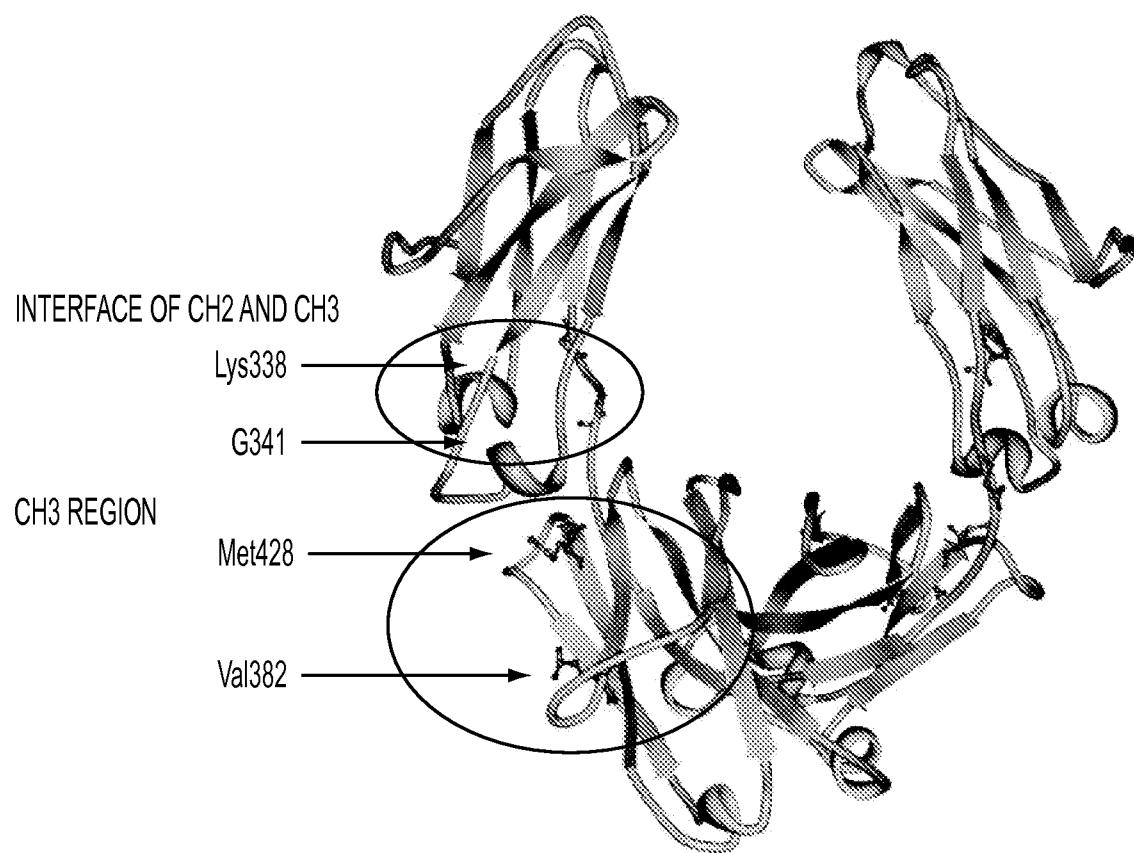


FIG. 8

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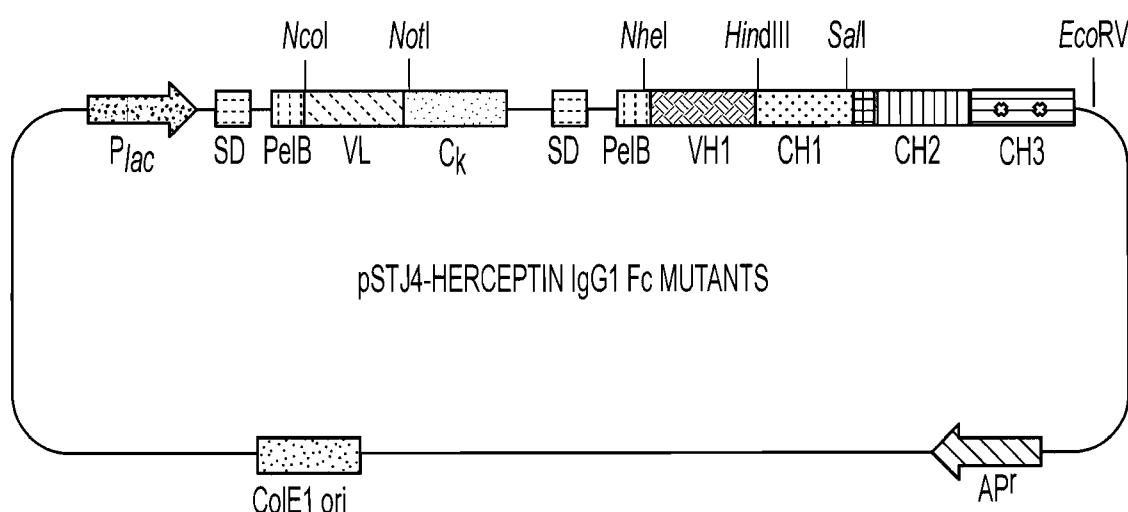


FIG. 9

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	$K_{ON} (M^{-1} \text{ sec}^{-1})$	$K_{OFF} (\text{sec}^{-1})$	$K_D (\text{nM})$	Chi^2
AGLYCOSYLATED WILD TYPE TRASTUZUMAB	1.31×10^5	5.02×10^{-2}	382	0.78
AGLYCOSYLATED TRASTUZUMAB-Fc5	2.17×10^5	1.23×10^{-3}	5.69	0.78
AGLYCOSYLATED TRASTUZUMAB-FC601	1.34×10^5	3.92×10^{-4}	2.92	1.42
GLYCOSYLATED HERCEPTIN	1.77×10^5	2.66×10^{-4}	1.5	2.74

FIG. 10A

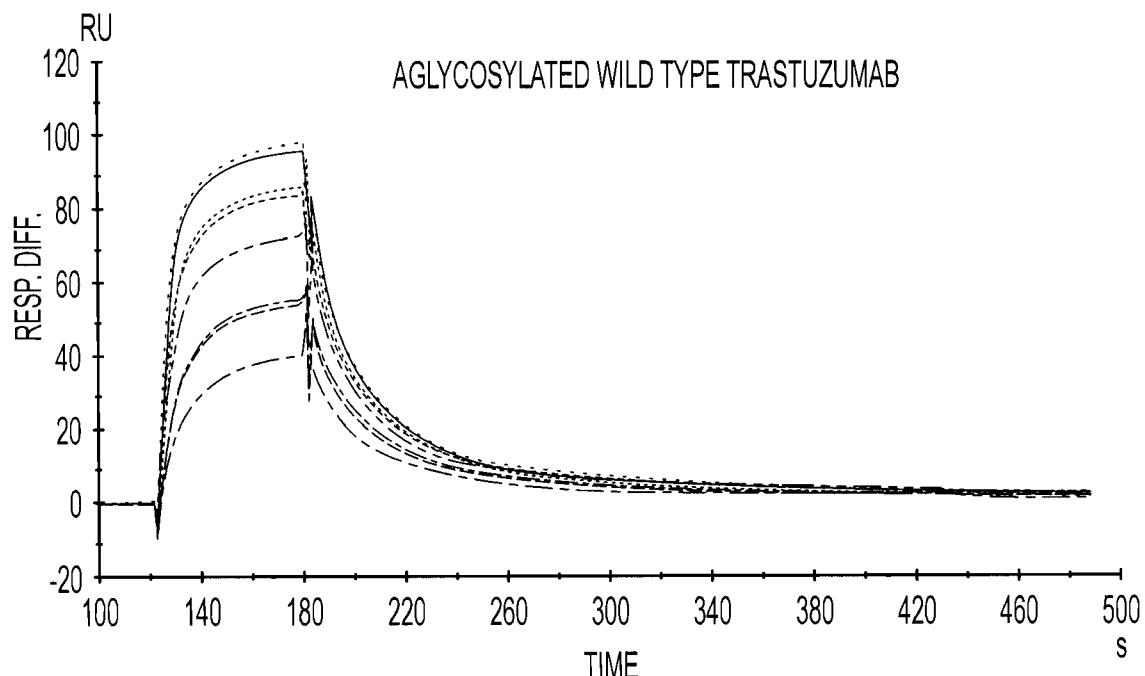


FIG. 10B

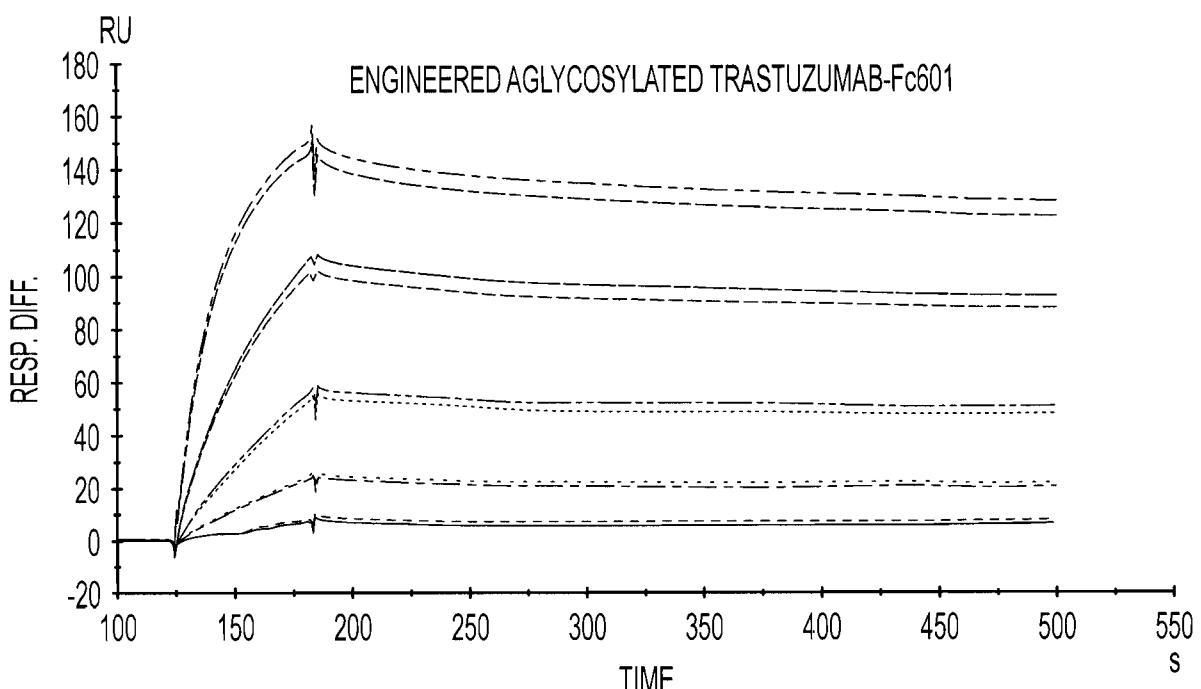


FIG. 10C
SUBSTITUTE SHEET (RULE 26)

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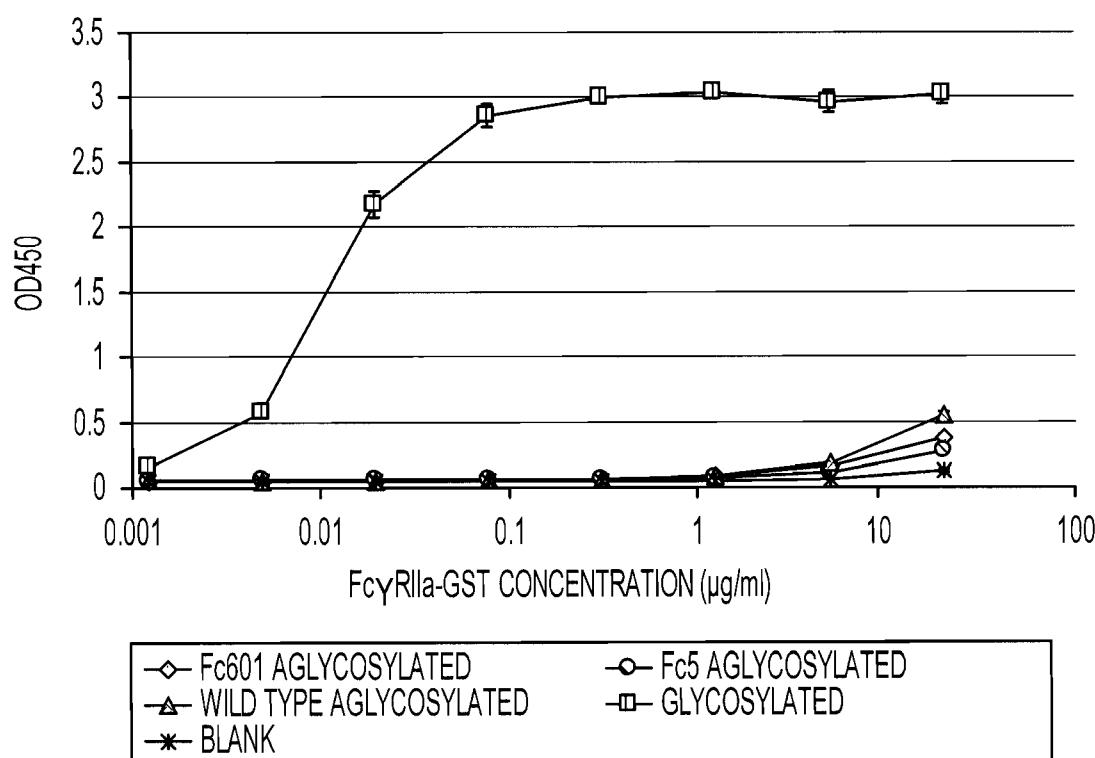


FIG. 11

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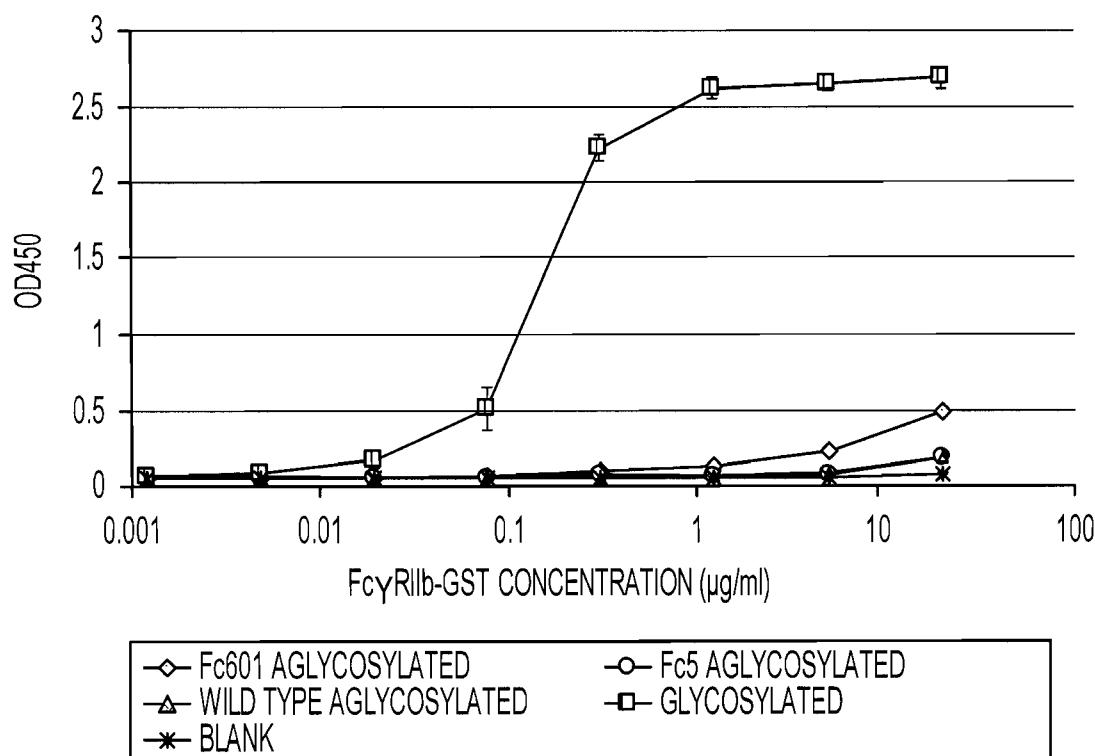


FIG. 12

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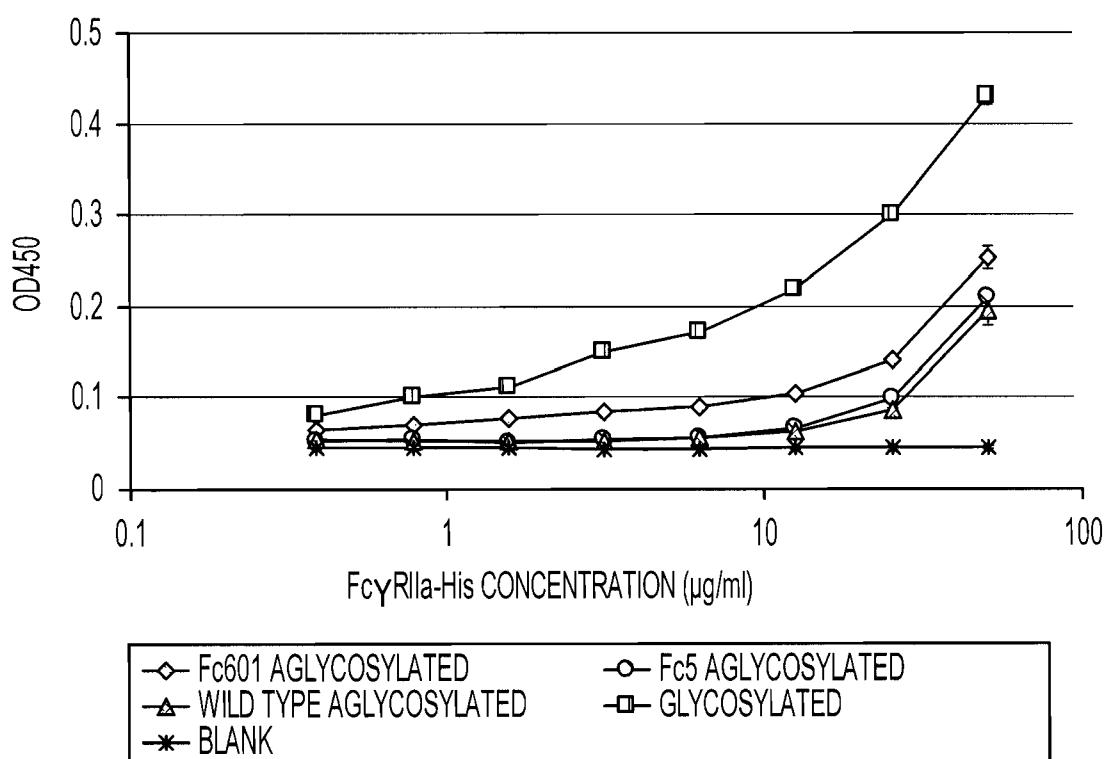


FIG. 13

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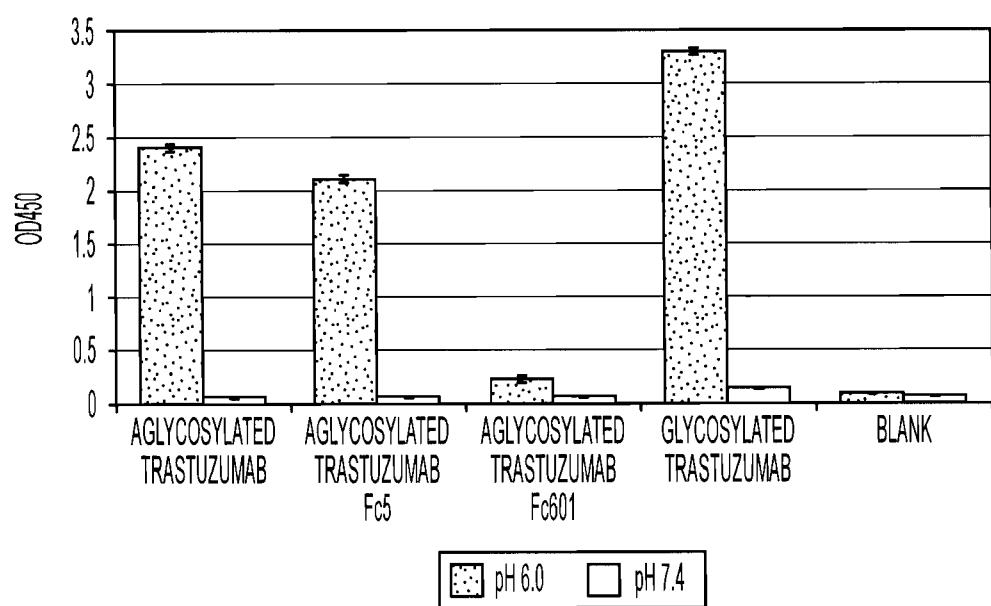


FIG. 14

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234 239
ELLGGPSV

264 268
WVDSHE

297 299
YNSTY

328 332
ALPAPIE

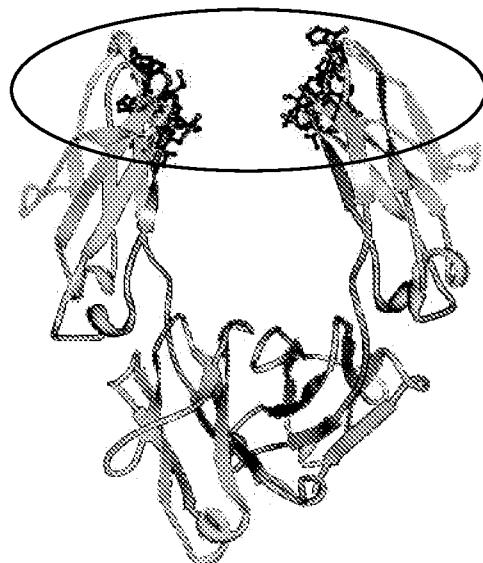


FIG. 15

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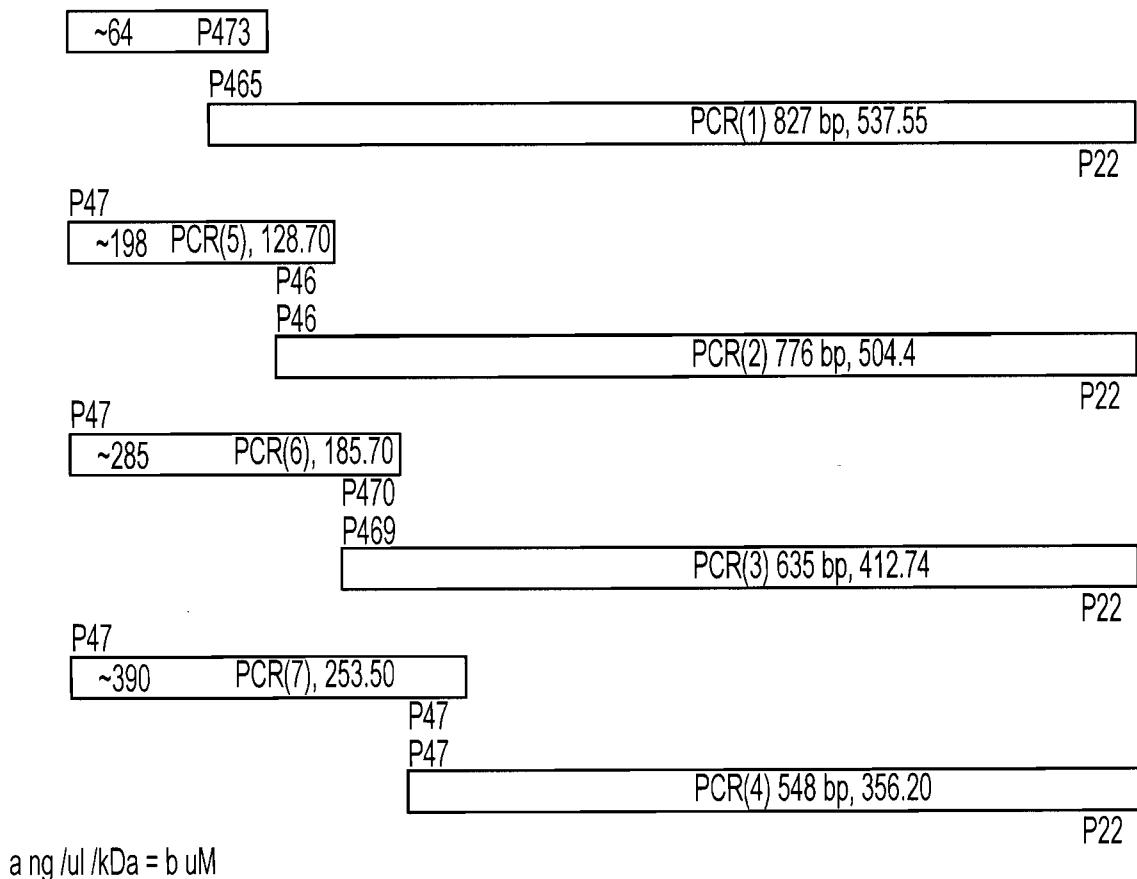
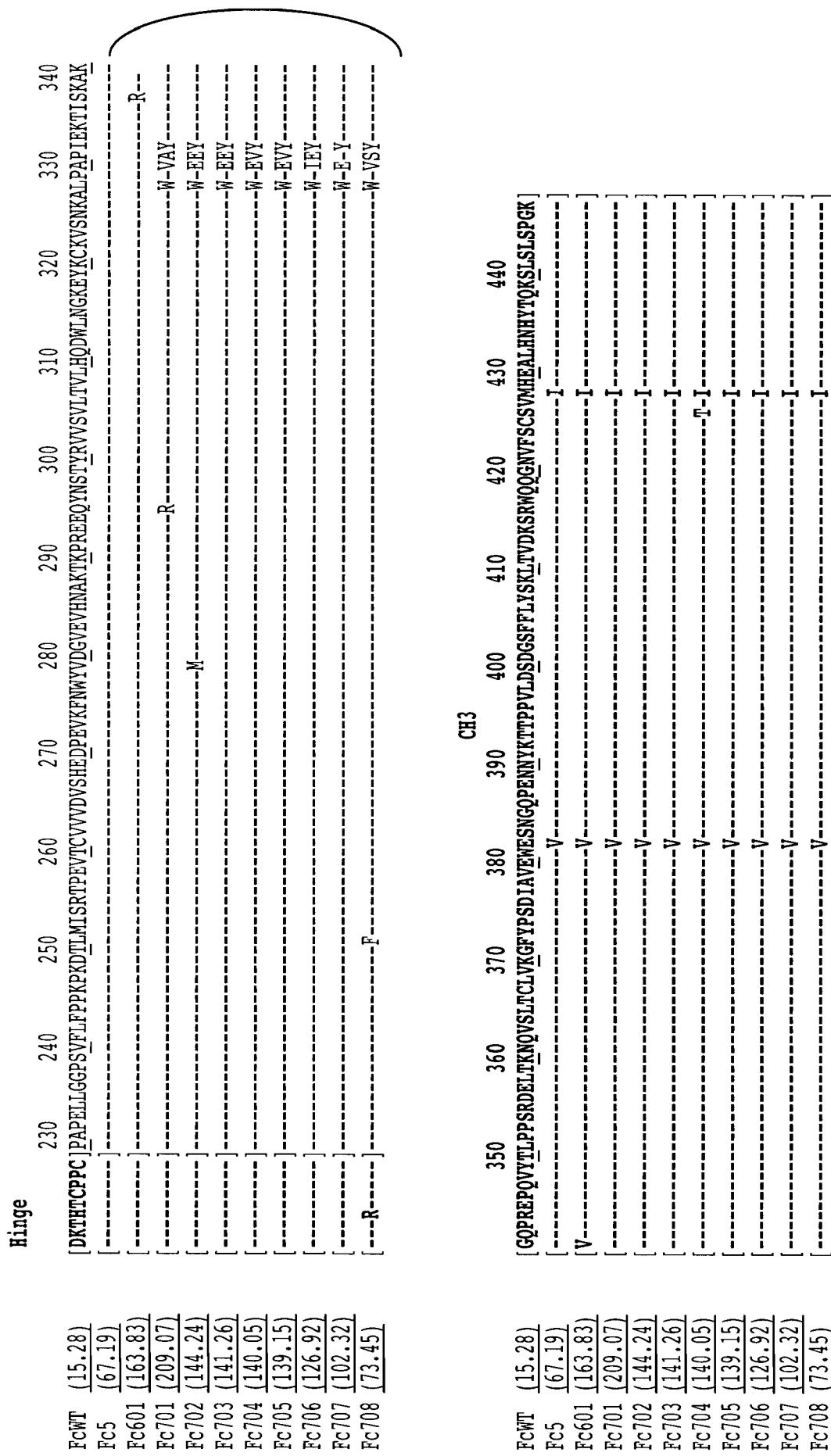


FIG. 16

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SUBSTITUTE SHEET (RULE 26)

1) FACS mean values are indicated in the parenthesis

FIG. 17

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FcWT 32 LPAPI 33

Fc701	W-VAY	Q295R
Fc702	W-EEY	V279M
Fc703	W-EEY	
Fc704	W-EVY	S426T
Fc705	W-EVY	
Fc706	W-IEY	
Fc707	W-EPY	
Fc708	W-VSY	H224R, L251F

FIG. 18

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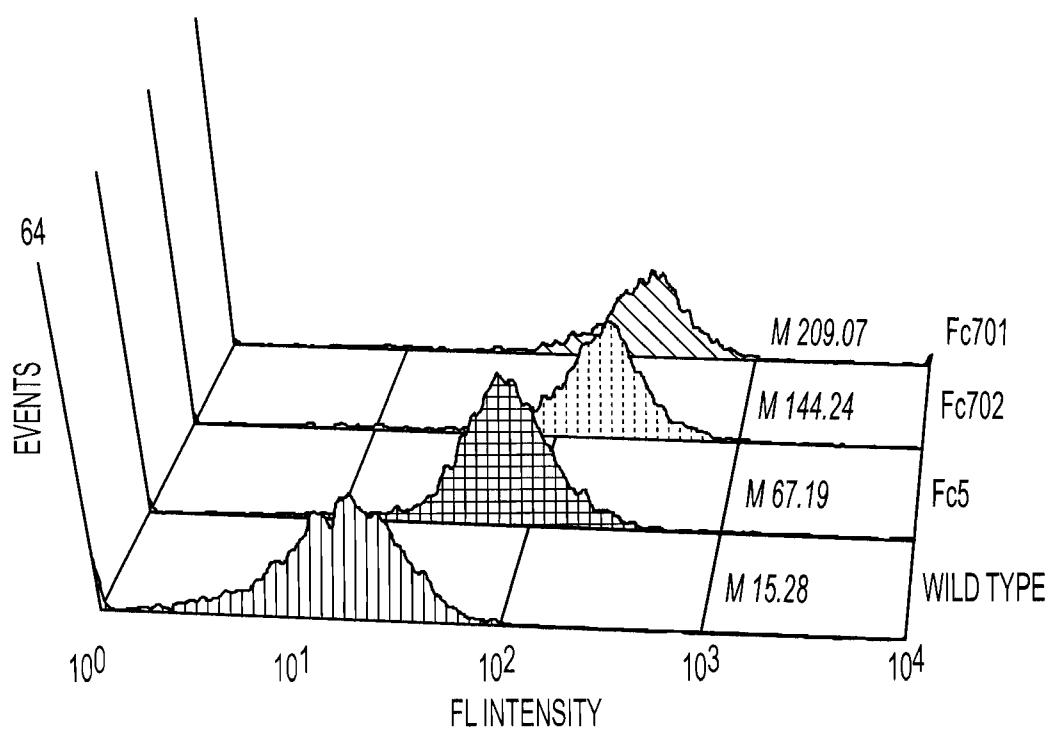


FIG. 19

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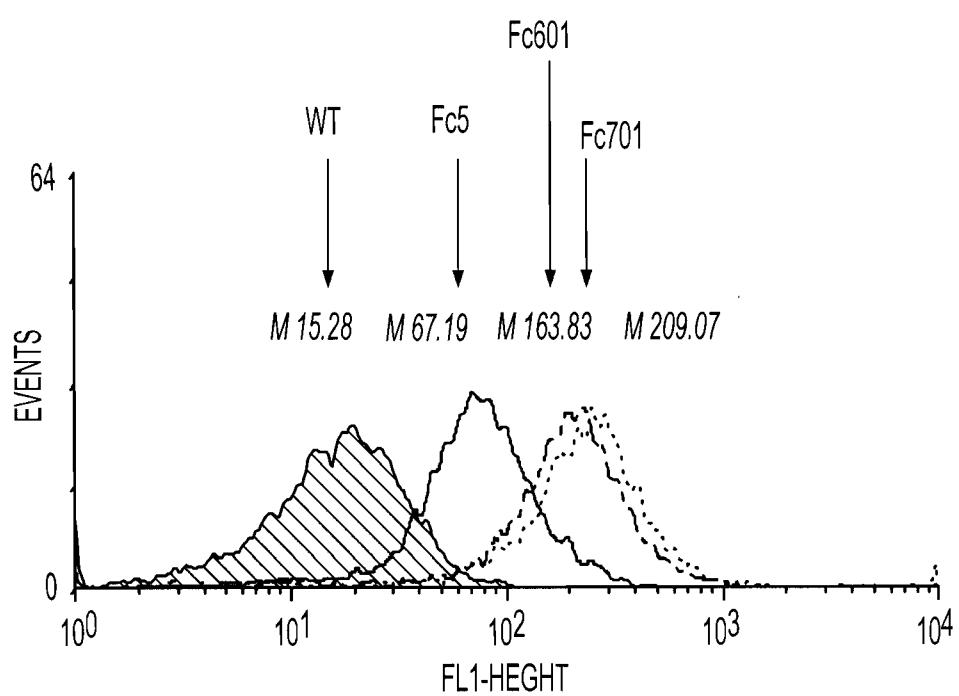


FIG. 20

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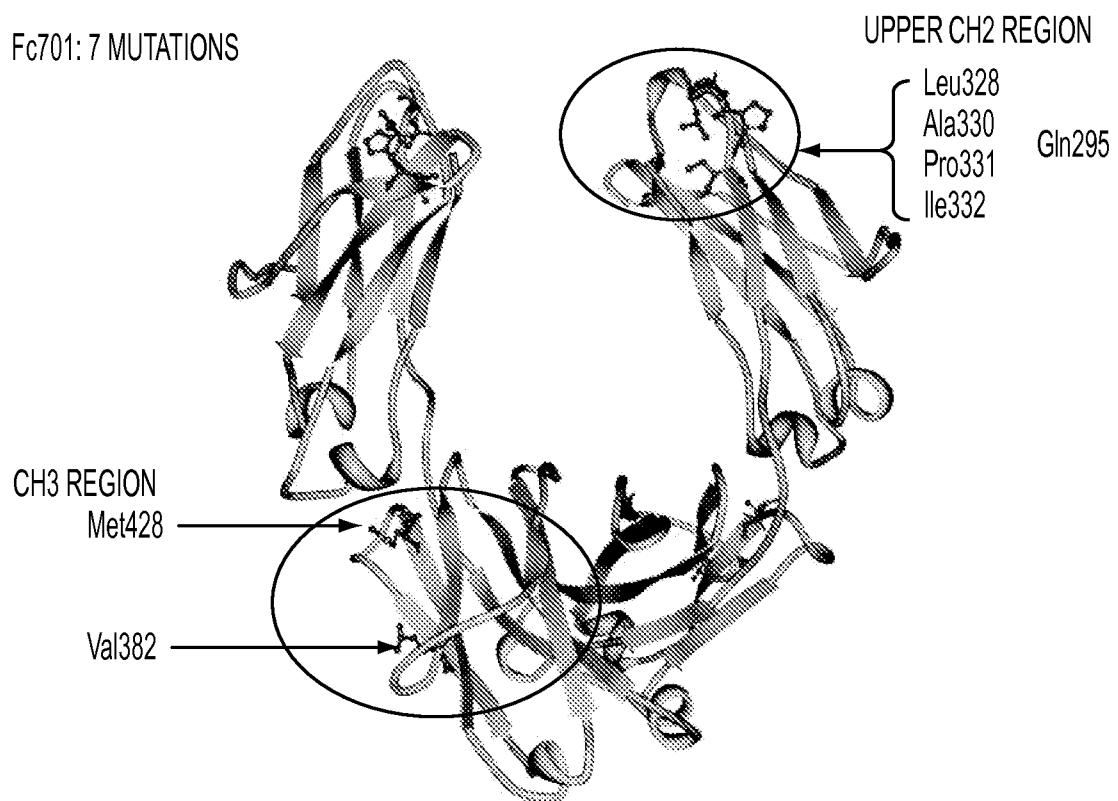


FIG. 21

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	K _{ON} (M ⁻¹ sec ⁻¹)	K _{OFF} (sec ⁻¹)	K _D (nM)	Chi ²
AGLYCOSYLATED WILD TYPE TRASTUZUMAB	1.31 x 10 ⁵	5.02 x 10 ⁻²	382	0.78
AGLYCOSYLATED TRASTUZUMAB-Fc5	2.17 x 10 ⁵	1.23 x 10 ⁻³	5.69	0.78
AGLYCOSYLATED TRASTUZUMAB-Fc601	1.34 x 10 ⁵	3.92 x 10 ⁻⁴	2.92	1.42
AGLYCOSYLATED TRASTUZUMAB-Fc701	2.96 x 10 ⁵	9.25 x 10 ⁻⁴	3.12	0.24
GLYCOSYLATED HERCEPTIN	1.77 x 10 ⁵	2.66 x 10 ⁻⁴	1.5	2.74

FIG. 22A

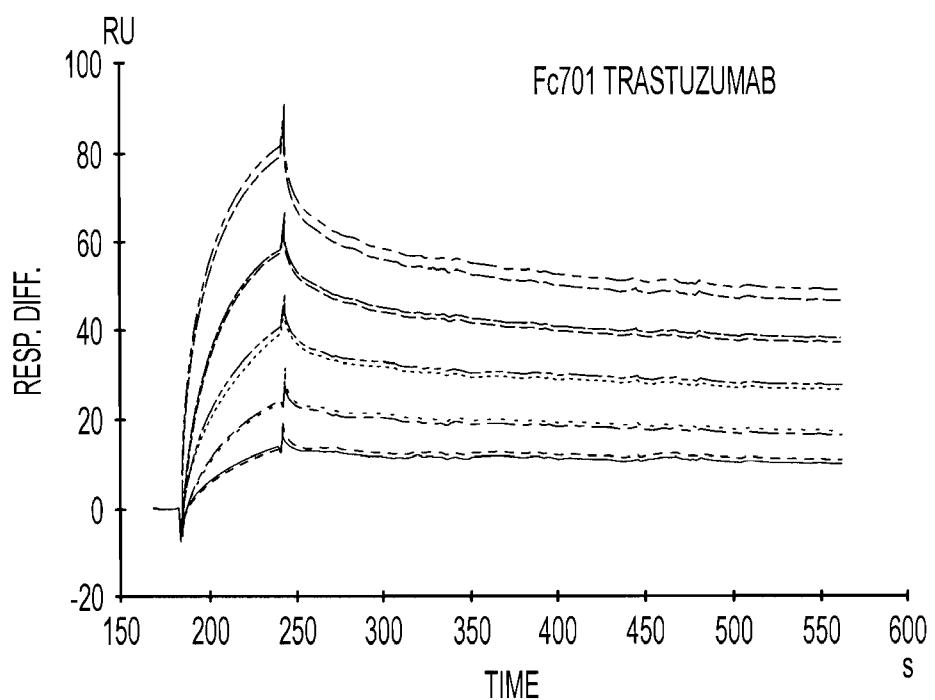


FIG. 22B

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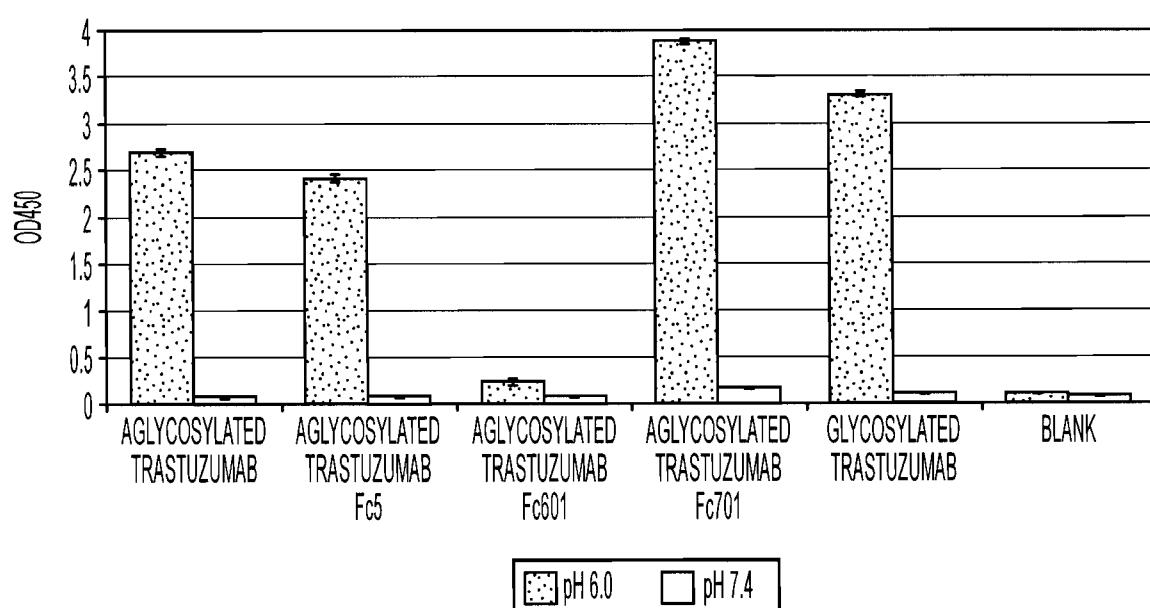


FIG. 23

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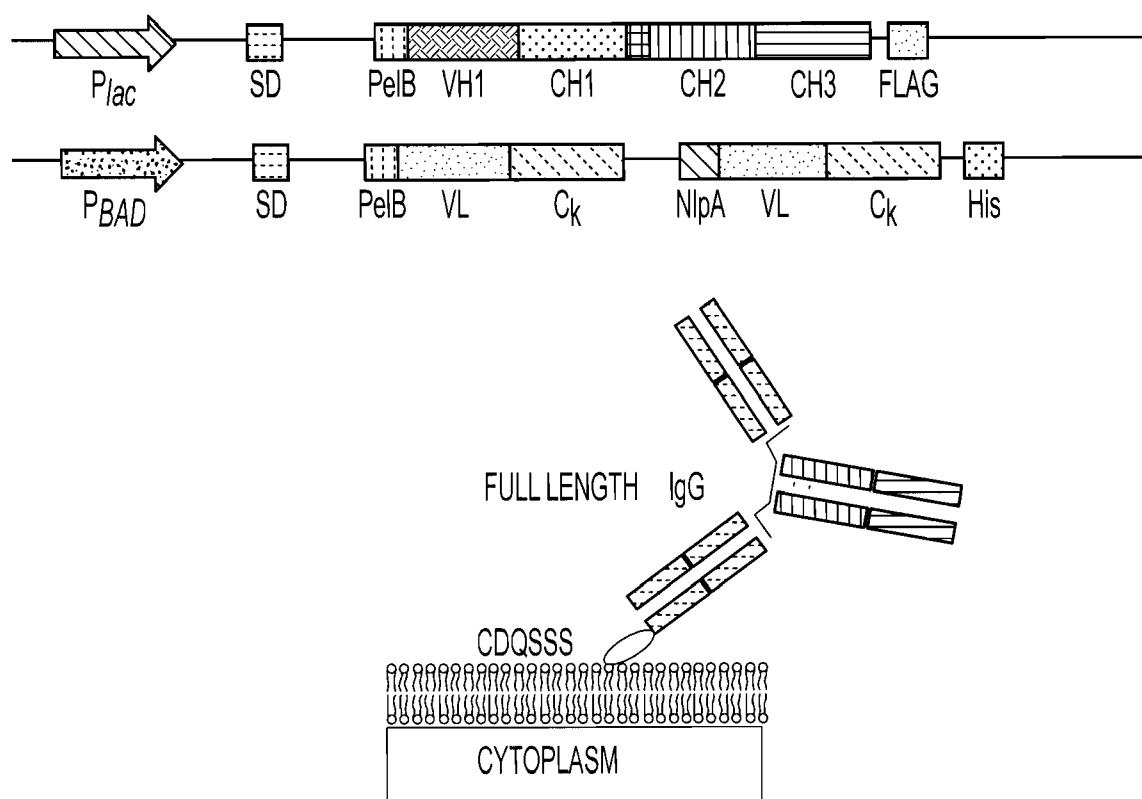


FIG. 24

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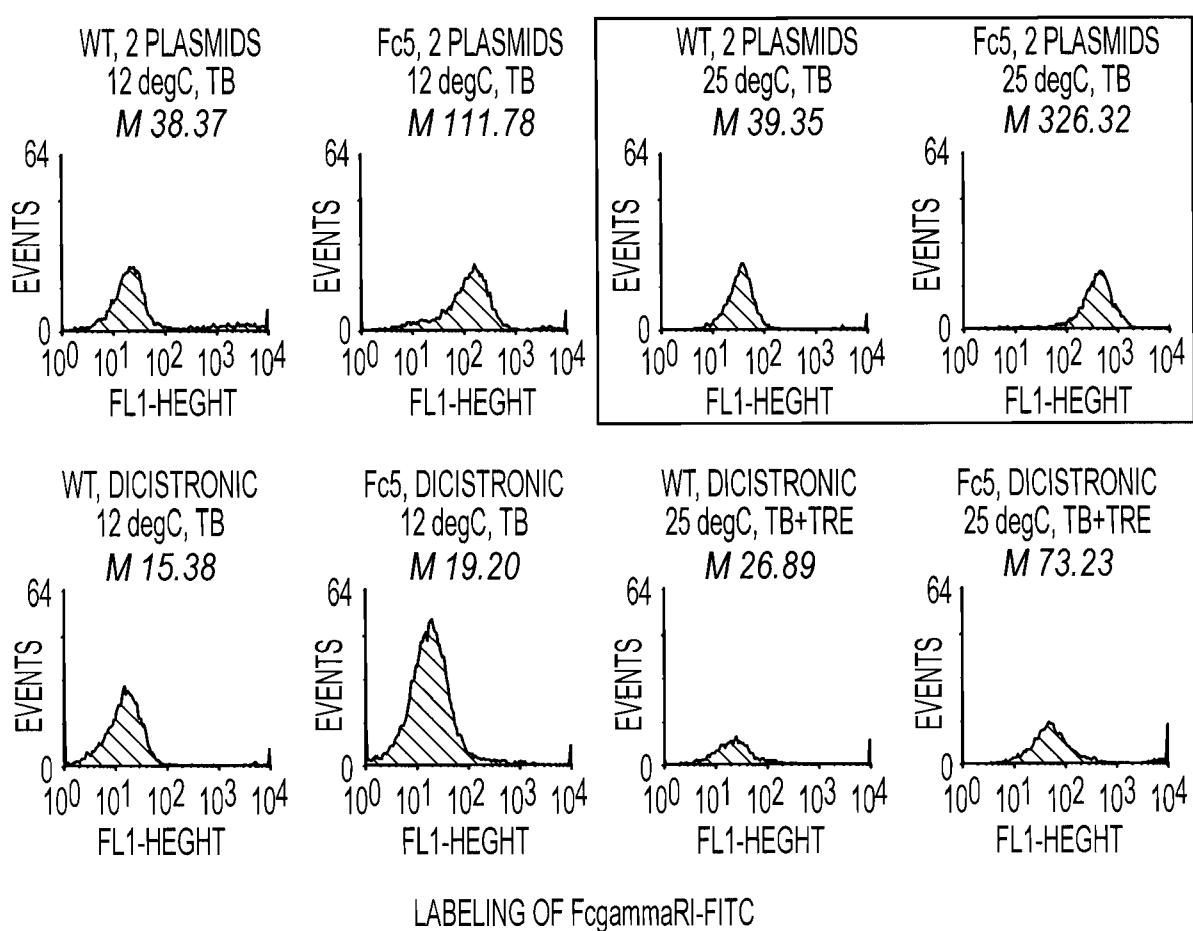


FIG. 25

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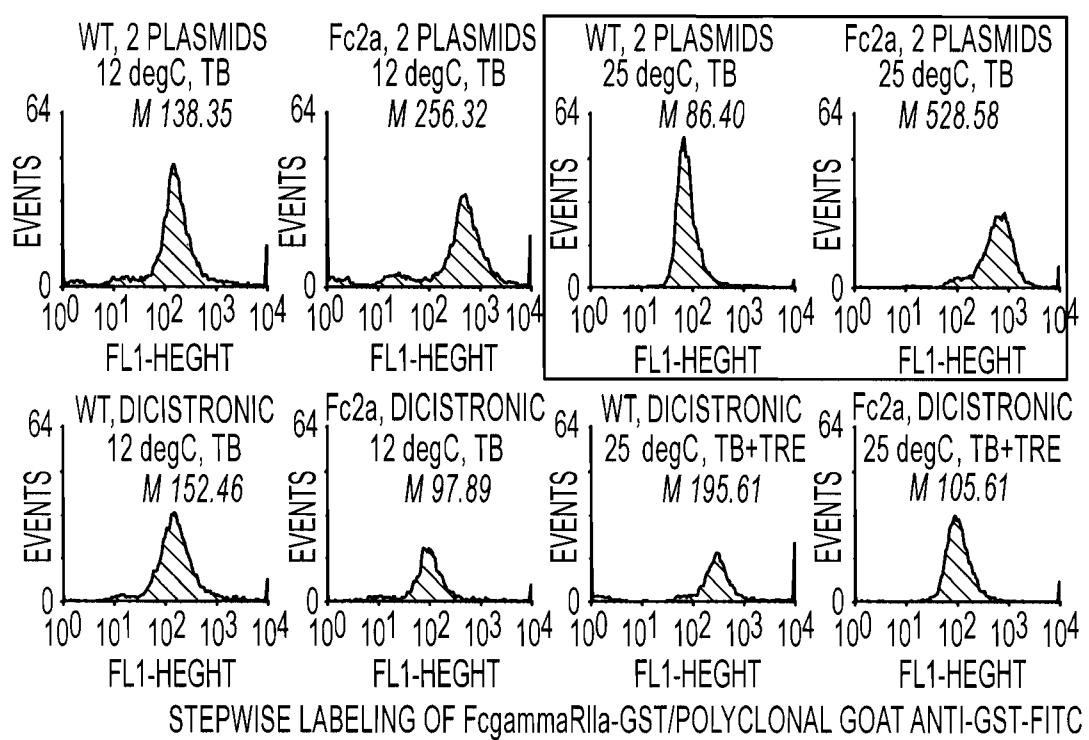


FIG. 26

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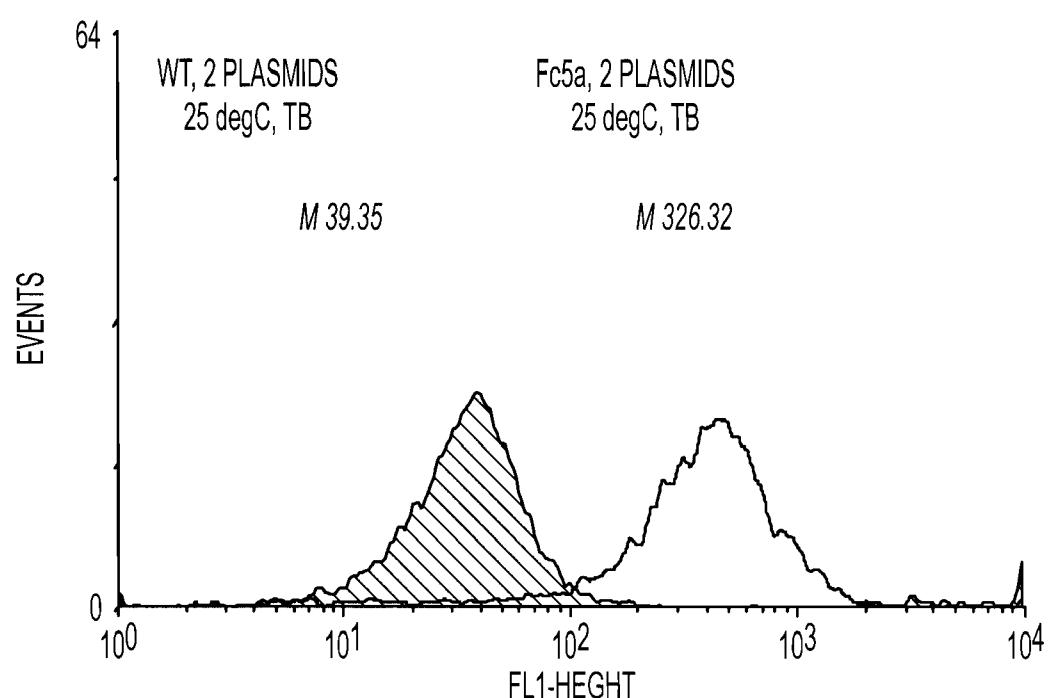


FIG. 27

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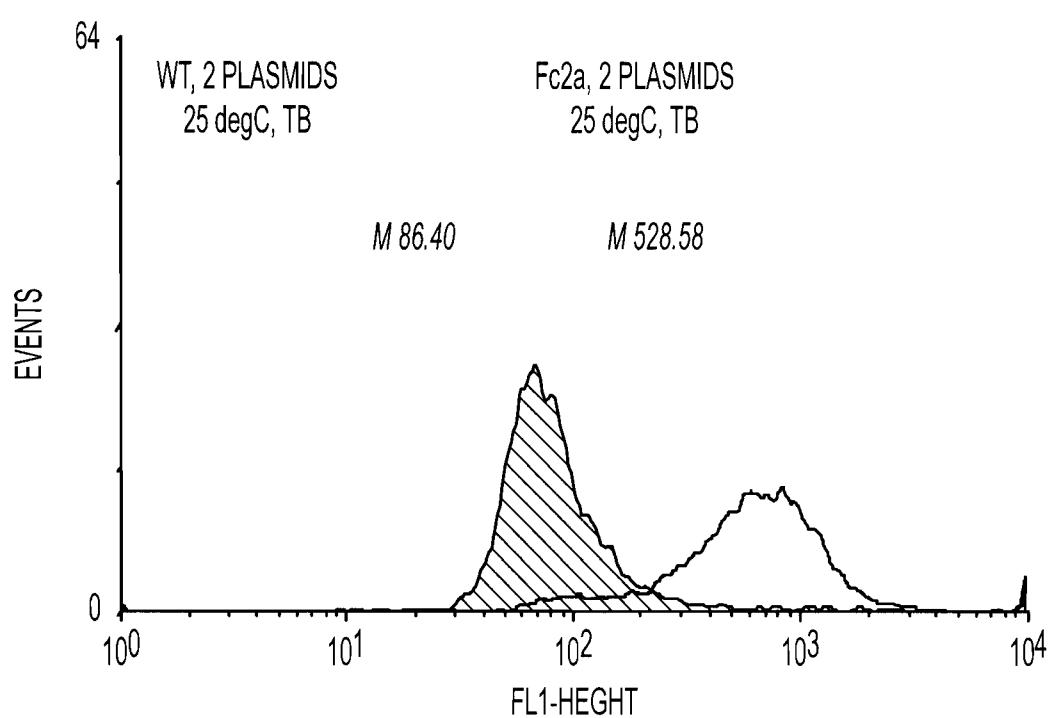


FIG. 28

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234 239
ELLGGPSV

264 268
VVDVSH

297 299
YNSTY

328 332
ALPAPIE

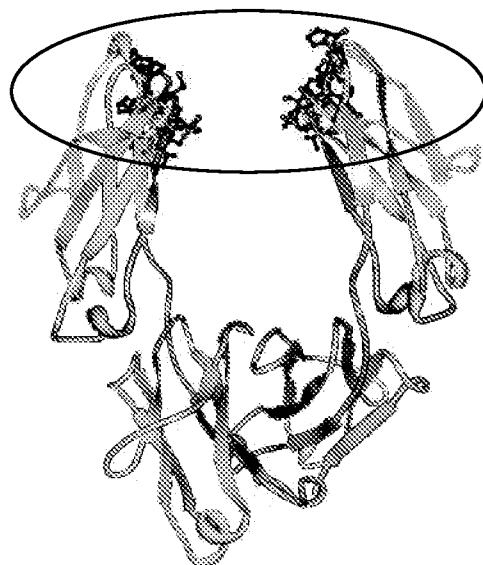


FIG. 29

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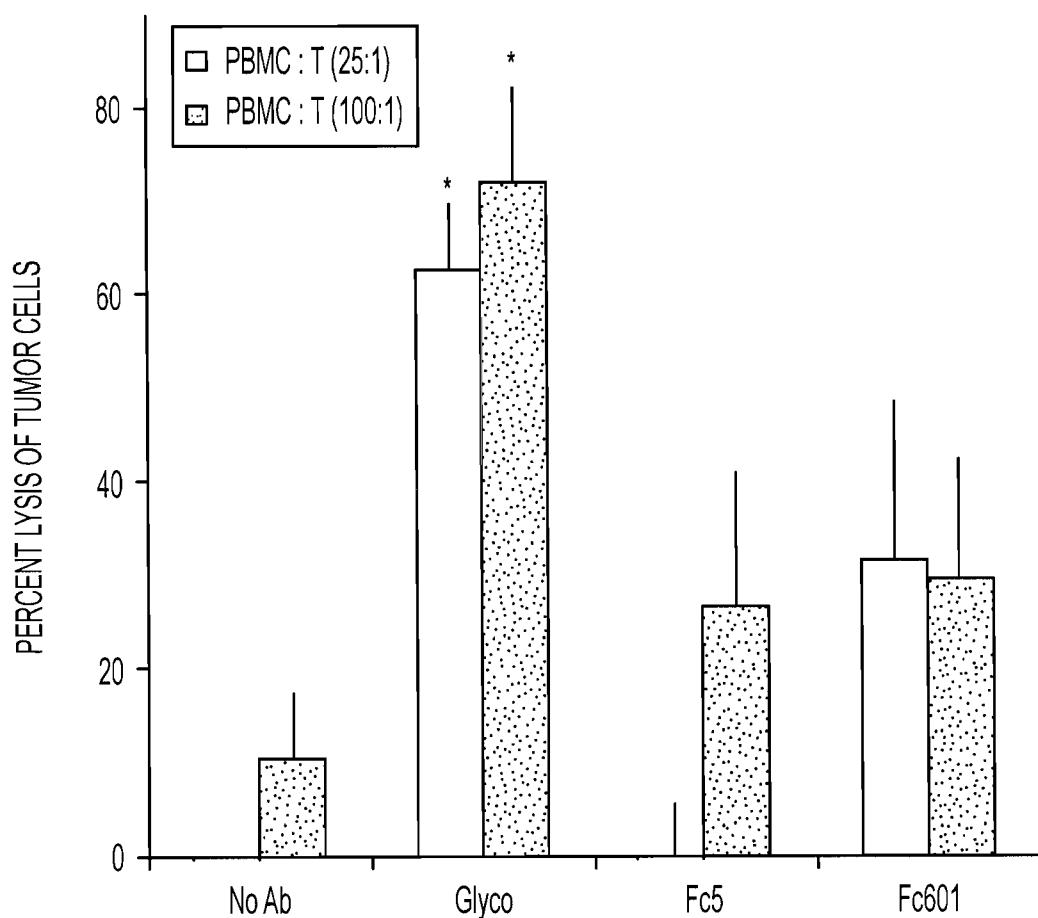


FIG. 30

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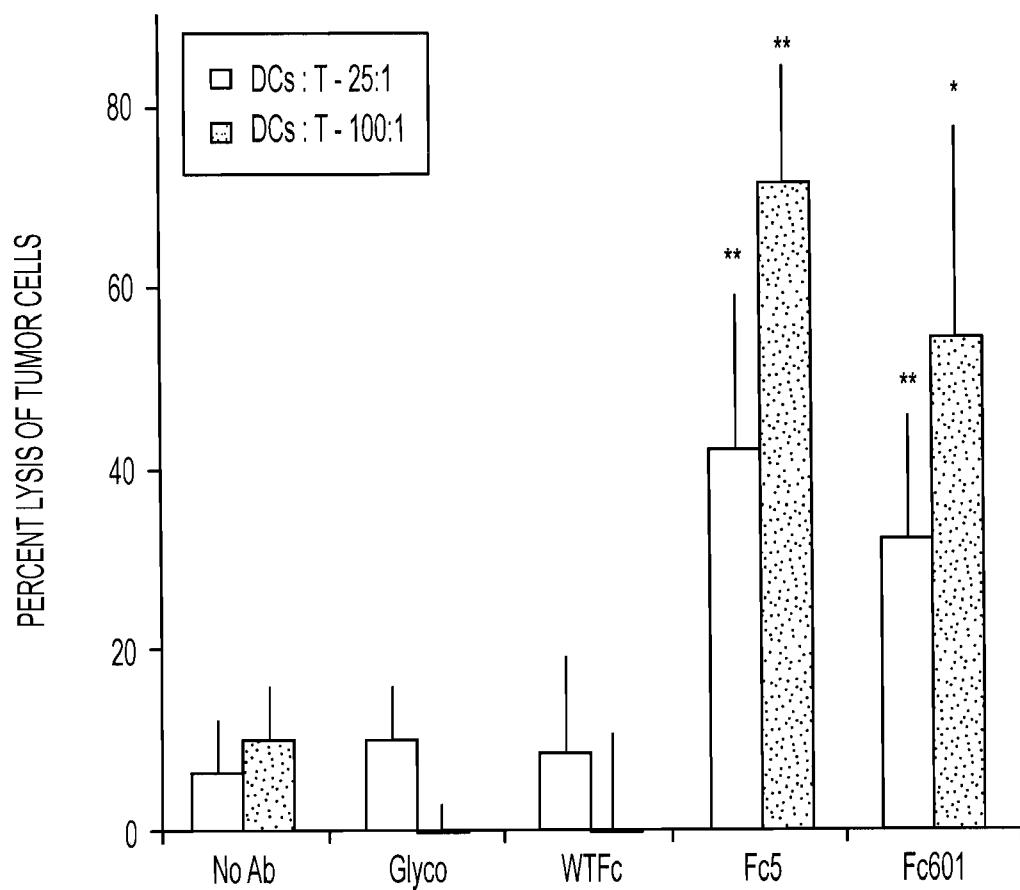


FIG. 31

