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(72) Inventeurs/Inventors:
STAVENHAGEN, JEFFREY, US;
VIJH, SUJATA, US

(73) Propriétaire/Owner:
MACROGENICS, INC., US

(74) Agent: OSLER, HOSKIN & HARCOURT LLP

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(54) Title: IDENTIFICATION AND ENGINEERING OF ANTIBODIES WITH VARIANT FC REGIONS AND METHODS OF
USING SAME

(57) **Abrégé/Abstract:**

The present invention relates to molecules, particularly polypeptides, more particularly immunoglobulins (e.g., antibodies), comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, which variant Fc region binds FcγRIIIA and/or FcγRIIA with a greater affinity, relative to a comparable molecule comprising the wild-type Fc region. The molecules of the invention are particularly useful in preventing, treating, or ameliorating one or more symptoms associated with a disease, disorder, or infection. The molecules of the invention are particularly useful for the treatment or prevention of a disease or disorder where an enhanced efficacy of effector cell function (e.g., ADCC) mediated by FcγR is desired, e.g., cancer, infectious disease, and in enhancing the therapeutic efficacy of therapeutic antibodies the effect of which is mediated by ADCC.

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(71) Applicant (for all designated States except US): **MACRO-
GENICS, INC.** [US/US]; 1500 East Gude Drive,
Rockville, MD 20850-5307 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **STAVENHAGEN,
Jeffrey** [US/US]; 19417 Treadway Road, Brookville, MD
20833 (US). **VIJH, Sujata** [US/US]; 912-100 Orchard
Ridge Drive, Gaithersburg, MD 20878 (US).

(74) Agents: **INSOGNA, Anthony, M.** et al.; Jones Day, 222
East 41st Street, New York, NY 10017-6702 (US).

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(54) Title: IDENTIFICATION AND ENGINEERING OF ANTIBODIES WITH VARIANT Fc REGIONS AND METHODS OF USING SAME

(57) Abstract: The present invention relates to molecules, particularly polypeptides, more particularly immunoglobulins (*e.g.*, antibodies), comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, which variant Fc region binds FcγRIIIA and/or FcγRIIA with a greater affinity, relative to a comparable molecule comprising the wild-type Fc region. The molecules of the invention are particularly useful in preventing, treating, or ameliorating one or more symptoms associated with a disease, disorder, or infection. The molecules of the invention are particularly useful for the treatment or prevention of a disease or disorder where an enhanced efficacy of effector cell function (*e.g.*, ADCC) mediated by FcγR is desired, *e.g.*, cancer, infectious disease, and in enhancing the therapeutic efficacy of therapeutic antibodies the effect of which is mediated by ADCC.



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P,X	US 2004/0002587 A1 (WATKINS et al) 01 January 2004 (01.01.2004), see pages 2, 21-22.	1, 15, 40, 57-58, 60
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IDENTIFICATION AND ENGINEERING OF ANTIBODIES WITH VARIANT Fc REGIONS AND METHODS OF USING SAME

5 1. FIELD OF THE INVENTION

[0002] The present invention relates to molecules, particularly polypeptides, more particularly immunoglobulins (*e.g.*, antibodies), comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, which variant Fc region binds Fc γ RIIIA and/or Fc γ RIIA with a greater affinity,
10 relative to a comparable molecule comprising the wild-type Fc region. The molecules of the invention are particularly useful in preventing, treating, or ameliorating one or more symptoms associated with a disease, disorder, or infection. The molecules of the invention are particularly useful for the treatment or prevention of a disease or disorder where an enhanced efficacy of effector cell function (*e.g.*, ADCC) mediated by Fc γ R is desired, *e.g.*,
15 cancer, infectious disease, and in enhancing the therapeutic efficacy of therapeutic antibodies the effect of which is mediated by ADCC.

2. BACKGROUND OF THE INVENTION

2.1 Fc RECEPTORS AND THEIR ROLES IN THE IMMUNE SYSTEM

[0003] The interaction of antibody-antigen complexes with cells of the immune
20 system results in a wide array of responses, ranging from effector functions such as antibody-dependent cytotoxicity, mast cell degranulation, and phagocytosis to immunomodulatory signals such as regulating lymphocyte proliferation and antibody secretion. All these interactions are initiated through the binding of the Fc domain of antibodies or immune complexes to specialized cell surface receptors on hematopoietic
25 cells. The diversity of cellular responses triggered by antibodies and immune complexes results from the structural heterogeneity of Fc receptors. Fc receptors share structurally related ligand binding domains which presumably mediate intracellular signaling.

[0004] The Fc receptors, members of the immunoglobulin gene superfamily of proteins, are surface glycoproteins that can bind the Fc portion of immunoglobulin
30 molecules. Each member of the family recognizes immunoglobulins of one or more isotypes through a recognition domain on the α chain of the Fc receptor. Fc receptors are defined by their specificity for immunoglobulin subtypes. Fc receptors for IgG are referred

to as Fc γ R, for IgE as Fc ϵ R, and for IgA as Fc α R. Different accessory cells bear Fc receptors for antibodies of different isotype, and the isotype of the antibody determines which accessory cells will be engaged in a given response (reviewed by Ravetch J.V. *et al.* 1991, *Annu. Rev. Immunol.* 9: 457-92; Gerber J.S. *et al.* 2001 *Microbes and Infection*, 3: 131-139; Billadeau D.D. *et al.* 2002, *The Journal of Clinical Investigation*, 2(109): 161-168; Ravetch J.V. *et al.* 2000, *Science*, 290: 84-89; Ravetch J.V. *et al.*, 2001 *Annu. Rev. Immunol.* 19:275-90; Ravetch J.V. 1994, *Cell*, 78(4): 553-60). The different Fc receptors, the cells that express them, and their isotype specificity is summarized in Table 1 (adapted from Immunobiology: The Immune System in Health and Disease, 4th ed. 1999, Elsevier Science Ltd/Garland Publishing, New York).

Fc γ Receptors

[0005] Each member of this family is an integral membrane glycoprotein, possessing extracellular domains related to a C2-set of immunoglobulin-related domains, a single membrane spanning domain and an intracytoplasmic domain of variable length.

15 There are three known Fc γ Rs, designated Fc γ RI(CD64), Fc γ RII(CD32), and Fc γ RIII(CD16). The three receptors are encoded by distinct genes; however, the extensive homology between the three family members suggest they arose from a common progenitor perhaps by gene duplication.

Fc γ RII(CD32)

20 [0006] Fc γ RII proteins are 40KDa integral membrane glycoproteins which bind only the complexed IgG due to a low affinity for monomeric Ig (10^6 M⁻¹). This receptor is the most widely expressed Fc γ R, present on all hematopoietic cells, including monocytes, macrophages, B cells, NK cells, neutrophils, mast cells, and platelets. Fc γ RII has only two immunoglobulin-like regions in its immunoglobulin binding chain and hence a much lower

25 affinity for IgG than Fc γ RI. There are three human Fc γ RII genes (Fc γ RII-A, Fc γ RII-B, Fc γ RII-C), all of which bind IgG in aggregates or immune complexes.

[0007] Distinct differences within the cytoplasmic domains of Fc γ RII-A and Fc γ RII-B create two functionally heterogenous responses to receptor ligation. The fundamental difference is that the A isoform initiates intracellular signaling leading to cell

30 activation such as phagocytosis and respiratory burst, whereas the B isoform initiates inhibitory signals, *e.g.*, inhibiting B-cell activation.

Signaling through FcγRs

[0008] Both activating and inhibitory signals are transduced through the FcγRs following ligation. These diametrically opposing functions result from structural differences among the different receptor isoforms. Two distinct domains within the cytoplasmic signaling domains of the receptor called immunoreceptor tyrosine based activation motifs (ITAMs) or immunoreceptor tyrosine based inhibitory motifs (ITIMs) account for the different responses. The recruitment of different cytoplasmic enzymes to these structures dictates the outcome of the FcγR-mediated cellular responses. ITAM-containing FcγR complexes include FcγRI, FcγRIIA, FcγRIIA, whereas ITIM-containing complexes only include FcγRIIB.

[0009] Human neutrophils express the FcγRIIA gene. FcγRIIA clustering via immune complexes or specific antibody cross-linking serves to aggregate ITAMs along with receptor-associated kinases which facilitate ITAM phosphorylation. ITAM phosphorylation serves as a docking site for Syk kinase, activation of which results in activation of downstream substrates (*e.g.*, PI₃K). Cellular activation leads to release of proinflammatory mediators.

[0010] The FcγRIIB gene is expressed on B lymphocytes; its extracellular domain is 96% identical to FcγRIIA and binds IgG complexes in an indistinguishable manner. The presence of an ITIM in the cytoplasmic domain of FcγRIIB defines this inhibitory subclass of FcγR. Recently the molecular basis of this inhibition was established. When colligated along with an activating FcγR, the ITIM in FcγRIIB becomes phosphorylated and attracts the SH2 domain of the inositol polyphosphate 5'-phosphatase (SHIP), which hydrolyzes phosphoinositol messengers released as a consequence of ITAM-containing FcγR-mediated tyrosine kinase activation, consequently preventing the influx of intracellular Ca⁺⁺. Thus crosslinking of FcγRIIB dampens the activating response to FcγR ligation and inhibits cellular responsiveness. B cell activation, B cell proliferation and antibody secretion is thus aborted.

TABLE 1. Receptors for the Fc Regions of Immunoglobulin Isotypes

Receptor	FcγRI (CD64)	FcγRII-A (CD32)	FcγRII-B2 (CD32)	FcγRII-B1 (CD32)	FcγRIII (CD16)	FcεRI	FcαRI (CD89)
Binding	IgG1 10 ⁸ M ⁻¹	IgG1 2 x 10 ⁶ M ⁻¹	IgG1 2 x 10 ⁶ M ⁻¹	IgG1 2 x 10 ⁶ M ⁻¹	IgG1 5 x 10 ⁵ M ⁻¹	IgE 1010 M ⁻¹	IgA1, IgA2 10 ⁷ M ⁻¹
Cell Type	Macrophages Neutrophils Eosinophils Dendritic cells	Macrophages Neutrophils Eosinophils Dendritic cells Platelets Langerhan cells	Macrophages Neutrophils Eosinophils	B cells Mast cells	NK cells Eosinophil Macrophages Neutrophils Mast Cells	Mast cells Eosinophil Basophils	Macrophages Neutrophils Eosinophils
Effect of Ligation	Uptake Stimulation Activation of respiratory burst Induction of killing	Uptake Granule release	Uptake Inhibition of Stimulation	No uptake Inhibition of Stimulation	Induction of Killing	Secretion of granules	Uptake Induction of killing

2.2 DISEASES OF RELEVANCE

2.2.1 CANCER

[0011] A neoplasm, or tumor, is a neoplastic mass resulting from abnormal uncontrolled cell growth which can be benign or malignant. Benign tumors generally remain localized. Malignant tumors are collectively termed cancers. The term “malignant” generally means that the tumor can invade and destroy neighboring body structures and spread to distant sites to cause death (for review, *see* Robbins and Angell, 1976, *Basic Pathology*, 2d Ed., W.B. Saunders Co., Philadelphia, pp. 68-122). Cancer can arise in many sites of the body and behave differently depending upon its origin. Cancerous cells destroy the part of the body in which they originate and then spread to other part(s) of the body where they start new growth and cause more destruction.

[0012] More than 1.2 million Americans develop cancer each year. Cancer is the second leading cause of death in the United States and if current trends continue, cancer is expected to be the leading cause of the death by the year 2010. Lung and prostate cancer are the top cancer killers for men in the United States. Lung and breast cancer are the top cancer killers for women in the United States. One in two men in the United States will be diagnosed with cancer at some time during his lifetime. One in three women in the United States will be diagnosed with cancer at some time during her lifetime.

[0013] A cure for cancer has yet to be found. Current treatment options, such as surgery, chemotherapy and radiation treatment, are oftentimes either ineffective or present serious side effects.

Cancer Therapy

[0014] Currently, cancer therapy may involve surgery, chemotherapy, hormonal therapy and/or radiation treatment to eradicate neoplastic cells in a patient (*See*, for example, Stockdale, 1998, “Principles of Cancer Patient Management”, in *Scientific American: Medicine*, vol. 3, Rubenstein and Federman, eds., Chapter 12, Section IV). Recently, cancer therapy could also involve biological therapy or immunotherapy. All of these approaches pose significant drawbacks for the patient. Surgery, for example, may be contraindicated due to the health of the patient or may be unacceptable to the patient. Additionally, surgery may not completely remove the neoplastic tissue. Radiation therapy is only effective when the neoplastic tissue exhibits a higher sensitivity to radiation than normal tissue, and radiation therapy can also often elicit serious side effects. Hormonal therapy is rarely given as a single agent and although can be effective, is often used to

prevent or delay recurrence of cancer after other treatments have removed the majority of the cancer cells. Biological therapies/immunotherapies are limited in number and may produce side effects such as rashes or swellings, flu-like symptoms, including fever, chills and fatigue, digestive tract problems or allergic reactions.

5 [0015] With respect to chemotherapy, there are a variety of chemotherapeutic agents available for treatment of cancer. A significant majority of cancer chemotherapeutics act by inhibiting DNA synthesis, either directly, or indirectly by inhibiting the biosynthesis of the deoxyribonucleotide triphosphate precursors, to prevent DNA replication and concomitant cell division (*See*, for example, Gilman *et al.*, Goodman and Gilman's: The
10 Pharmacological Basis of Therapeutics, Eighth Ed. (Pergamom Press, New York, 1990)). These agents, which include alkylating agents, such as nitrosourea, anti-metabolites, such as methotrexate and hydroxyurea, and other agents, such as etoposides, campathecins, bleomycin, doxorubicin, daunorubicin, *etc.*, although not necessarily cell cycle specific, kill cells during S phase because of their effect on DNA replication. Other agents, specifically
15 colchicine and the vinca alkaloids, such as vinblastine and vincristine, interfere with microtubule assembly resulting in mitotic arrest. Chemotherapy protocols generally involve administration of a combination of chemotherapeutic agents to increase the efficacy of treatment.

[0016] Despite the availability of a variety of chemotherapeutic agents,
20 chemotherapy has many drawbacks (*See*, for example, Stockdale, 1998, "Principles Of Cancer Patient Management" in Scientific American Medicine, vol. 3, Rubenstein and Federman, eds., ch. 12, sect. 10). Almost all chemotherapeutic agents are toxic, and chemotherapy causes significant, and often dangerous, side effects, including severe nausea, bone marrow depression, immunosuppression, *etc.* Additionally, even with administration
25 of combinations of chemotherapeutic agents, many tumor cells are resistant or develop resistance to the chemotherapeutic agents. In fact, those cells resistant to the particular chemotherapeutic agents used in the treatment protocol often prove to be resistant to other drugs, even those agents that act by mechanisms different from the mechanisms of action of the drugs used in the specific treatment; this phenomenon is termed pleiotropic drug or
30 multidrug resistance. Thus, because of drug resistance, many cancers prove refractory to standard chemotherapeutic treatment protocols.

[0017] There is a significant need for alternative cancer treatments, particularly for treatment of cancer that has proved refractory to standard cancer treatments, such as surgery, radiation therapy, chemotherapy, and hormonal therapy. A promising alternative is
35 immunotherapy, in which cancer cells are specifically targeted by cancer antigen-specific

antibodies. Major efforts have been directed at harnessing the specificity of the immune response, for example, hybridoma technology has enabled the development of tumor selective monoclonal antibodies (See Green M.C. *et al.*, 2000 *Cancer Treat Rev.*, 26: 269-286; Weiner LM, 1999 *Semin Oncol.* 26(suppl. 14):43-51), and in the past few years, the Food and Drug Administration has approved the first MAbs for cancer therapy: Rituxin (anti-CD20) for non-Hodgkin's Lymphoma and Herceptin [anti-(c-erb-2/HER-2)] for metastatic breast cancer (Suzanne A. Eccles, 2001, *Breast Cancer Res.*, 3: 86-90). However, the potency of antibody effector function, *e.g.*, to mediate antibody dependent cellular cytotoxicity ("ADCC") is an obstacle to such treatment. Methods to improve the efficacy of such immunotherapy are thus needed.

2.2.2 INFLAMMATORY DISEASES AND AUTOIMMUNE DISEASES

[0018] Inflammation is a process by which the body's white blood cells and chemicals protect our bodies from infection by foreign substances, such as bacteria and viruses. It is usually characterized by pain, swelling, warmth and redness of the affected area. Chemicals known as cytokines and prostaglandins control this process, and are released in an ordered and self-limiting cascade into the blood or affected tissues. This release of chemicals increases the blood flow to the area of injury or infection, and may result in the redness and warmth. Some of the chemicals cause a leak of fluid into the tissues, resulting in swelling. This protective process may stimulate nerves and cause pain. These changes, when occurring for a limited period in the relevant area, work to the benefit of the body.

[0019] In autoimmune and/or inflammatory disorders, the immune system triggers an inflammatory response when there are no foreign substances to fight and the body's normally protective immune system causes damage to its own tissues by mistakenly attacking self. There are many different autoimmune disorders which affect the body in different ways. For example, the brain is affected in individuals with multiple sclerosis, the gut is affected in individuals with Crohn's disease, and the synovium, bone and cartilage of various joints are affected in individuals with rheumatoid arthritis. As autoimmune disorders progress destruction of one or more types of body tissues, abnormal growth of an organ, or changes in organ function may result. The autoimmune disorder may affect only one organ or tissue type or may affect multiple organs and tissues. Organs and tissues commonly affected by autoimmune disorders include red blood cells, blood vessels, connective tissues, endocrine glands (*e.g.*, the thyroid or pancreas), muscles, joints, and

skin. Examples of autoimmune disorders include, but are not limited to, Hashimoto's thyroiditis, pernicious anemia, Addison's disease, type 1 diabetes, rheumatoid arthritis, systemic lupus erythematosus, dermatomyositis, Sjogren's syndrome, dermatomyositis, lupus erythematosus, multiple sclerosis, autoimmune inner ear disease myasthenia gravis, Reiter's syndrome, Graves disease, autoimmune hepatitis, familial adenomatous polyposis and ulcerative colitis.

[0020] Rheumatoid arthritis (RA) and juvenile rheumatoid arthritis are types of inflammatory arthritis. Arthritis is a general term that describes inflammation in joints. Some, but not all, types of arthritis are the result of misdirected inflammation. Besides rheumatoid arthritis, other types of arthritis associated with inflammation include the following: psoriatic arthritis, Reiter's syndrome, ankylosing spondylitis arthritis, and gouty arthritis. Rheumatoid arthritis is a type of chronic arthritis that occurs in joints on both sides of the body (such as both hands, wrists or knees). This symmetry helps distinguish rheumatoid arthritis from other types of arthritis. In addition to affecting the joints, rheumatoid arthritis may occasionally affect the skin, eyes, lungs, heart, blood or nerves.

[0021] Rheumatoid arthritis affects about 1% of the world's population and is potentially disabling. There are approximately 2.9 million incidences of rheumatoid arthritis in the United States. Two to three times more women are affected than men. The typical age that rheumatoid arthritis occurs is between 25 and 50. Juvenile rheumatoid arthritis affects 71,000 young Americans (aged eighteen and under), affecting six times as many girls as boys.

[0022] Rheumatoid arthritis is an autoimmune disorder where the body's immune system improperly identifies the synovial membranes that secrete the lubricating fluid in the joints as foreign. Inflammation results, and the cartilage and tissues in and around the joints are damaged or destroyed. In severe cases, this inflammation extends to other joint tissues and surrounding cartilage, where it may erode or destroy bone and cartilage and lead to joint deformities. The body replaces damaged tissue with scar tissue, causing the normal spaces within the joints to become narrow and the bones to fuse together. Rheumatoid arthritis creates stiffness, swelling, fatigue, anemia, weight loss, fever, and often, crippling pain. Some common symptoms of rheumatoid arthritis include joint stiffness upon awakening that lasts an hour or longer; swelling in a specific finger or wrist joints; swelling in the soft tissue around the joints; and swelling on both sides of the joint. Swelling can occur with or without pain, and can worsen progressively or remain the same for years before progressing.

[0023] The diagnosis of rheumatoid arthritis is based on a combination of factors, including: the specific location and symmetry of painful joints, the presence of joint

stiffness in the morning, the presence of bumps and nodules under the skin (rheumatoid nodules), results of X-ray tests that suggest rheumatoid arthritis, and/or positive results of a blood test called the rheumatoid factor. Many, but not all, people with rheumatoid arthritis have the rheumatoid-factor antibody in their blood. The rheumatoid factor may be present
5 in people who do not have rheumatoid arthritis. Other diseases can also cause the rheumatoid factor to be produced in the blood. That is why the diagnosis of rheumatoid arthritis is based on a combination of several factors and not just the presence of the rheumatoid factor in the blood.

[0024] The typical course of the disease is one of persistent but fluctuating joint
10 symptoms, and after about 10 years, 90% of sufferers will show structural damage to bone and cartilage. A small percentage will have a short illness that clears up completely, and another small percentage will have very severe disease with many joint deformities, and occasionally other manifestations of the disease. The inflammatory process causes erosion or destruction of bone and cartilage in the joints. In rheumatoid arthritis, there is an
15 autoimmune cycle of persistent antigen presentation, T-cell stimulation, cytokine secretion, synovial cell activation, and joint destruction. The disease has a major impact on both the individual and society, causing significant pain, impaired function and disability, as well as costing millions of dollars in healthcare expenses and lost wages. (See, for example, the NIH website and the NIAID website).

20 [0025] Currently available therapy for arthritis focuses on reducing inflammation of the joints with anti-inflammatory or immunosuppressive medications. The first line of treatment of any arthritis is usually anti-inflammatories, such as aspirin, ibuprofen and Cox-2 inhibitors such as celecoxib and rofecoxib. "Second line drugs" include gold, methotrexate and steroids. Although these are well-established treatments for arthritis, very
25 few patients remit on these lines of treatment alone. Recent advances in the understanding of the pathogenesis of rheumatoid arthritis have led to the use of methotrexate in combination with antibodies to cytokines or recombinant soluble receptors. For example, recombinant soluble receptors for tumor necrosis factor (TNF)- α have been used in combination with methotrexate in the treatment of arthritis. However, only about 50% of
30 the patients treated with a combination of methotrexate and anti-TNF- α agents such as recombinant soluble receptors for TNF- α show clinically significant improvement. Many patients remain refractory despite treatment. Difficult treatment issues still remain for patients with rheumatoid arthritis. Many current treatments have a high incidence of side effects or cannot completely prevent disease progression. So far, no treatment is ideal, and

there is no cure. Novel therapeutics are needed that more effectively treat rheumatoid arthritis and other autoimmune disorders.

2.2.3 INFECTIOUS DISEASES

[0026] Infectious agents that cause disease fall into five groups: viruses, bacteria, fungi, protozoa, and helminths (worms). The remarkable variety of these pathogens has caused the natural selection of two crucial features of adaptive immunity. First, the advantage of being able to recognize a wide range of different pathogens has driven the development of receptors on B and T cells of equal or greater diversity. Second, the distinct habitats and life cycles of pathogens have to be countered by a range of distinct effector mechanisms. The characteristic features of each pathogen are its mode of transmission, its mechanism of replication, its pathogenesis or the means by which it causes disease, and the response it elicits.

[0027] The record of human suffering and death caused by smallpox, cholera, typhus, dysentery, malaria, etc. establishes the eminence of the infectious diseases. Despite the outstanding successes in control afforded by improved sanitation, immunization, and antimicrobial therapy, the infectious diseases continue to be a common and significant problem of modern medicine. The most common disease of mankind, the common cold, is an infectious disease, as is the feared modern disease AIDS. Some chronic neurological diseases that were thought formerly to be degenerative diseases have proven to be infectious. There is little doubt that the future will continue to reveal the infectious diseases as major medical problems.

[0028] An enormous number of human and animal diseases result from virulent and opportunistic infections from any of the above mentioned infectious agents (*see* Belshe (Ed.) 1984 Textbook of Human Virology, PSG Publishing, Littleton, MA).

25 [0029] One category of infectious diseases are viral infections for example. Viral diseases of a wide array of tissues, including the respiratory tract, CNS, skin, genitourinary tract, eyes, ears, immune system, gastrointestinal tract, and musculoskeletal system, affect a vast number of humans of all ages (*see* Table 328-2 In: Wyngaarden and Smith, 1988, Cecil Textbook of Medicine, 18th Ed., W.B. Saunders Co., Philadelphia, pp.1750-1753).

30 Although considerable effort has been invested in the design of effective anti-viral therapies, viral infections continue to threaten the lives of millions of people worldwide. In general, attempts to develop anti-viral drugs have focused on several stages of viral life cycle (*See e.g.*, Mitsuya *et al.*, 1991, *FASEB J.* 5:2369-2381, discussing HIV). However, a

common drawback associated with using of many current anti-viral drugs is their deleterious side effects, such as toxicity to the host or resistance by certain viral strains.

3. SUMMARY OF THE INVENTION

[0030] The present invention is based, in part, on the identification of mutant human
5 IgG1 heavy chain Fc regions, with altered affinities for Fc γ R receptors (*e.g.*, activating Fc γ Rs, inhibitory Fc γ Rs), using a yeast display system. Accordingly, the invention relates to molecules, preferably polypeptides, and more preferably immunoglobulins (*e.g.*, antibodies), comprising a variant Fc region, having one or more amino acid modifications (*e.g.*, substitutions, but also including insertions or deletions) in one or more regions, which
10 modifications alter, *e.g.*, increase or decrease, the affinity of the variant Fc region for an Fc γ R. Preferably, said one or more amino acid modification increases the affinity of the variant Fc region for Fc γ RIIA and/or Fc γ RIIA. In a preferred embodiment, the molecules of the invention further specifically bind Fc γ RIIB (via the Fc region) with a lower affinity than a comparable molecule (*i.e.*, having the same amino acid sequence as the molecule of
15 the invention except for the one or more amino acid modifications in the Fc region) comprising the wild-type Fc region binds Fc γ RIIB. In some embodiments, the invention encompasses molecules with variant Fc regions, having one or more amino acid modifications, which modifications increase the affinity of the variant Fc region for Fc γ RIIA and/or Fc γ RIIA and enhance the affinity of the variant Fc region for Fc γ RIIB
20 relative to a comparable molecule with a wild type Fc region. In other embodiments, the invention encompasses molecules with variant Fc regions, having one or more amino acid modifications, which modifications increase the affinity of the variant Fc region for Fc γ RIIA and/or Fc γ RIIA but do not alter the affinity of the variant Fc regions for Fc γ RIIB relative to a comparable molecule with a wild type Fc region.

25 [0031] The invention encompasses molecules that are homodimers or heterodimers of Fc regions. Heterodimers comprising Fc regions refer to molecules where the two Fc chains have the same or different sequences. In some embodiments, in the heterodimeric molecules comprising variant Fc regions, each chain has one or more different modifications from the other chain. In other embodiments, in the heterodimeric molecules
30 comprising variant Fc regions, one chain contains the wild-type Fc region and the other chains comprises one or or more modifications. Methods of engineering heterodimeric Fc containing molecules are known in the art and encompassed within the invention.

[0032] In some embodiments, the invention encompasses molecules comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid

modification relative to a wild type Fc region, which variant Fc region does not bind any Fc γ R or binds with a reduced affinity, relative to a comparable molecule comprising the wild-type Fc region, as determined by standard assays (*e.g.*, in vitro assays) known to one skilled in the art. In a specific embodiment, the invention encompasses molecules

5 comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild type Fc region, which variant Fc region only binds one Fc γ R, wherein said Fc γ R is Fc γ IIIA. In another specific embodiment, the invention encompasses molecules comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild type Fc region, which

10 variant Fc region only binds one Fc γ R, wherein said Fc γ R is Fc γ RIIA. In yet another embodiment, the invention encompasses molecules comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild type Fc region, which variant Fc region only binds one Fc γ R, wherein said Fc γ R is Fc γ RIIB.

[0033] The affinities and binding properties of the molecules of the invention for an

15 Fc γ R are initially determined using *in vitro* assays (biochemical or immunological based assays) known in the art for determining Fc-Fc γ R interactions, *i.e.*, specific binding of an Fc region to an Fc γ R including but not limited to ELISA assay, surface plasmon resonance assay, immunoprecipitation assays (*See* Section 5.2.1). Preferably, the binding properties of the molecules of the invention are also characterized by in vitro functional assays for

20 determining one or more Fc γ R mediator effector cell functions (*See* Section 5.2.6). In most preferred embodiments, the molecules of the invention have similar binding properties in in vivo models (such as those described and disclosed herein) as those in in vitro based assays. However, the present invention does not exclude molecules of the invention that do not exhibit the desired phenotype in in vitro based assays but do exhibit the desired phenotype

25 in vivo.

[0034] In a specific embodiment, the invention encompasses a molecule comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ RIIA with a greater affinity than a comparable molecule comprising the wild-type Fc

30 region binds Fc γ RIIA, provided that said variant Fc region does not solely have a substitution at any one of positions 329, 331, or 332, and do not include or are not solely substitution with any one of: alanine at any of positions 256, 290, 298, 312, 333, 334, 359, 360, 326, or 430; a lysine at position 330; a threonine at position 339; a methionine at position 320; a serine at position 326; an asparagine at position 326; an aspartic acid at

35 position 326; a glutamic acid at position 326; a glutamine at position 334; a glutamic acid at

position 334; a methionine at position 334; a histidine at position 334; a valine at position 334; or a leucine at position 334; a lysine at position 335.

[0035] In another specific embodiment, the invention encompasses a molecule comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds FcγRIIA with a greater affinity than a comparable molecule comprising the wild-type Fc region binds FcγRIIA, provided that the one or more amino acid modifications do not include or are not solely substitution with an alanine at any of positions 256, 290, 326, 255, 258, 267, 272, 276, 280, 283, 285, 286, 331, 337, 268, 272, or 430; an asparagine at position 268; a glutamine at position 272; a glutamine, serine, or aspartic acid at position 286; a serine at position 290; a methionine, glutamine, glutamic acid, or arginine at position 320; a glutamic acid at position 322; a serine, glutamic acid, or aspartic acid at position 326; a lysine at position 330; a glutamine at position 335; or a methionine at position 301.

[0036] In a preferred specific embodiment, the invention encompasses a molecule comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said molecule has an altered affinity for an FcγR, provided that said variant Fc region does not have a substitution at positions that make a direct contact with FcγR based on crystallographic and structural analysis of Fc-FcγR interactions such as those disclosed by Sondermann *et al.*, (2000 *Nature*, 406: 267-273 . Examples of positions within the Fc region that make a direct contact with FcγR are amino acids 234-239 (hinge region), amino acids 265-269 (B/C loop), amino acids 297-299 (C'/E loop), and amino acids 327-332 (F/G) loop. In some embodiments, the molecules of the invention comprising variant Fc regions comprise modification of at least one residue that does not make a direct contact with an FcγR based on structural and crystallographic analysis, *e.g.*, is not within the Fc-FcγR binding site.

[0037] In another preferred embodiment, the invention encompasses a molecule comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said molecule binds an FcγR with an altered affinity relative to a molecule comprising a wild-type Fc region, provided that said at least one amino acid modification do not include or are not solely a substitution at any of positions 255, 256, 258, 267, 268, 269, 270, 272, 276, 278, 280, 283, 285, 286, 289, 290, 292, 293, 294, 295, 296, 298, 300, 301, 303, 305, 307, 309, 312, 320, 322, 326, 329, 330, 332, 331, 333, 334, 335, 337, 338, 339, 340, 359, 360, 373, 376, 416, 419, 430,

434, 435, 437, 438, 439. In a specific embodiment, the invention encompasses a molecule comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said molecule binds an FcγR with an altered affinity relative to a molecule comprising a wild-type Fc region, provided

5 that said variant Fc region does not include or are not solely a substitution at any of positions 255, 258, 267, 269, 270, 276, 278, 280, 283, 285, 289, 292, 293, 294, 295, 296, 300, 303, 305, 307, 309, 322, 329, 332, 331, 337, 338, 340, 373, 376, 416, 419, 434, 435, 437, 438, 439 and does not have an alanine at any of positions 256, 290, 298, 312, 333, 334, 359, 360, 326, or 430; a lysine at position 330; a threonine at position 339; a methionine at

10 position 320; a serine at position 326; an asparagine at position 326; an aspartic acid at position 326; a glutamic acid at position 326; a glutamine at position 334; a glutamic acid at position 334; a methionine at position 334; a histidine at position 334; a valine at position 334; or a leucine at position 334; a lysine at position 335 an asparagine at position 268; a glutamine at position 272; a glutamine, serine, or aspartic acid at position 286; a serine at

15 position 290; a methionine, glutamine, glutamic acid, or arginine at position 320; a glutamic acid at position 322; a serine, glutamic acid, or aspartic acid at position 326; a lysine at position 330; a glutamine at position 335; or a methionine at position 301.

[0038] In a specific embodiment, the invention encompasses a molecule comprising a variant Fc region, wherein said variant Fc region does not include or are not solely a

20 substitution at any of positions 268, 269, 270, 272, 276, 278, 283, 285, 286, 289, 292, 293, 301, 303, 305, 307, 309, 331, 333, 334, 335, 337, 338, 340, 360, 373, 376, 416, 419, 430, 434, 435, 437, 438 or 439 and does not have a histidine, glutamine, or tyrosine at position 280; a serine, glycine, threonine or tyrosine at position 290, a leucine or isoleucine at position 300; an asparagine at position 294, a proline at position 296; a proline, asparagine,

25 aspartic acid, or valine at position 298; a lysine at position 295. In yet another preferred embodiment, the invention encompasses a molecule comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said molecule binds an FcγR with a reduced affinity relative to a molecule comprising a wild-type Fc region provided that said variant Fc region does not

30 have or are not solely have a substitution at any of positions 252, 254, 265, 268, 269, 270, 278, 289, 292, 293, 294, 295, 296, 298, 300, 301, 303, 322, 324, 327, 329, 333, 335, 338, 340, 373, 376, 382, 388, 389, 414, 416, 419, 434, 435, 437, 438, or 439. In yet another preferred embodiment, the invention encompasses a molecule comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification

35 relative to a wild-type Fc region, such that said molecule binds an FcγR with an enhanced

affinity relative to a molecule comprising a wild-type Fc region provided that said variant Fc region does not have or are not solely a substitution at any of positions 280, 283, 285, 286, 290, 294, 295, 298, 300, 301, 305, 307, 309, 312, 315, 331, 333, 334, 337, 340, 360, 378, 398, or 430.

5 [0039] In a specific embodiment, molecules of the invention comprise a variant Fc region having one or more amino acid modifications (*e.g.*, substitutions), which modifications increase the affinity of the variant Fc region for Fc γ RIIA and/or Fc γ RIIA by at least 2-fold, relative to a comparable molecule comprising a wild-type Fc region. In certain embodiments, molecules of the invention comprise a variant Fc region having one or
10 more amino acid modifications (*e.g.*, substitutions), which modifications increase the affinity of the variant Fc region for Fc γ RIIA and/or Fc γ RIIA by greater than 2-fold, at least 4-fold, at least 5-fold, at least 6-fold, at least 8-fold, or at least 10-fold relative to a comparable molecule comprising a wild-type Fc region. In other embodiments of the invention, molecules of the invention comprising a variant Fc region specifically bind
15 Fc γ RIIA and/or Fc γ RIIA with at least 65%, at least 75%, at least 85%, at least 95%, at least 100%, at least 150%, at least 200% greater affinity relative to a molecule comprising a wild-type Fc region. Such measurements are preferably *in vitro* assays.

[0040] The invention encompasses molecules with altered affinities for the activating and/or inhibitory Fc γ receptors. In particular, the invention contemplates
20 molecules with variant Fc regions, having one or more amino acid modifications, which modifications increase the affinity of the variant Fc region for Fc γ RIIB but decrease the affinity of the variant Fc region for Fc γ RIIA and/or Fc γ RIIA, relative to a comparable molecule with a wild-type Fc region. In other embodiments, the invention encompasses molecules with variant Fc regions, having one or more amino acid modifications, which
25 modifications decrease the affinity of the variant Fc region for Fc γ RIIB and also decrease the affinity of the variant Fc regions for Fc γ RIIA and/or Fc γ RIIA relative to a comparable molecule with a wild-type Fc region. In yet other embodiments, the invention encompasses molecules with variant Fc regions, which modifications decrease the affinity of the variant Fc region for Fc γ RIIA and/or Fc γ RIIA but do not alter the affinity of the variant Fc region
30 for Fc γ RIIB relative to a comparable molecule with a wild-type Fc region.

[0041] In a specific embodiment, the molecules of the invention comprise a variant Fc region, having one or more amino acid modifications (*e.g.*, substitutions), which one or more modifications increase the affinity of the variant Fc region for Fc γ RIIA and decrease the affinity of the variant Fc region for Fc γ RIIB, relative to a comparable molecule

comprising a wild-type Fc region which binds Fc γ RIIA and Fc γ RIIB with wild-type affinity. In a certain embodiment, the one or more amino acid modifications are not a substitution with alanine at any of positions 256, 298, 333, or 334.

[0042] In another specific embodiment, the molecules of the invention comprise a variant Fc region, having one or more amino acid modifications (*e.g.*, substitutions), which one or more modifications increase the affinity of the variant Fc region for Fc γ RIIA and decrease the affinity of the variant Fc region for Fc γ RIIB, relative to a comparable molecule comprising a wild-type Fc region which binds Fc γ RIIA and Fc γ RIIB with wild-type affinity. In a certain embodiment, the one or more amino acid modifications is not a substitution with arginine at position 320.

[0043] In most preferred embodiments, the molecules of the invention with altered affinities for activating and/or inhibitory receptors having variant Fc regions, have one or more amino acid modifications, wherein said one or more amino acid modification is a substitution at position 288 with asparagine, at position 330 with serine and at position 396 with leucine (MgFc10) (See Table 5); or a substitution at position 334 with glutamic acid, at position 359 with asparagine, and at position 366 with serine (MgFc13); or a substitution at position 316 with aspartic acid, at position 378 with valine, and at position 399 with glutamic acid (MgFc27); or a substitution at position 392 with threonine, and at position 396 with leucine (MgFc38); or a substitution at position 221 with glutamic acid, at position 270 with glutamic acid, at position 308 with alanine, at position 311 with histidine, at position 396 with leucine, and at position 402 with aspartic acid (MgFc42); or a substitution at position 240 with alanine, and at position 396 with leucine (MgFc52); or a substitution at position 410 with histidine, and at position 396 with leucine (MgFc53); or a substitution at position 243 with leucine, at position 305 with isoleucine, at position 378 with aspartic acid, at position 404 with serine, and at position 396 with leucine (MgFc54); or a substitution at position 255 with isoleucine, and at position 396 with leucine (MgFc55); or a substitution at position 370 with glutamic acid and at position 396 with leucine (MgFc59).

[0044] The preferred method for screening and identifying molecules comprising variant Fc regions with altered Fc γ R affinities (*e.g.*, enhanced Fc γ RIIA affinity) is yeast surface display technology (for review *see* Boder and Wittrup, 2000, *Methods in Enzymology*, 328: 430-444).

Specifically, the yeast surface display is a genetic method whereby polypeptides comprising Fc mutants are expressed on the yeast cell wall in a form accessible for interacting with Fc γ R. Yeast surface display of the mutant Fc containing polypeptides of the invention may

be performed in accordance with any of the techniques known to those skilled in the art or the specific methods described herein.

[0045] One aspect of the invention provides a method for selecting mutant Fc fusion proteins with a desirable binding property, *e.g.*, the ability of the mutant Fc fusion protein to bind Fc γ RIIIA with a greater affinity than a comparable polypeptide comprising a wild-type Fc region binds Fc γ RIIIA. Yeast cells displaying the mutant Fc fusion proteins can be screened and characterized by any biochemical or immunological based assays known to those skilled in the art for assessing binding interactions. In a specific embodiment, screening of mutant Fc fusion proteins is done using one or more biochemical based assays, *e.g.*, an ELISA assay.

[0046] In preferred embodiments, screening and identifying molecules comprising variant Fc regions with altered Fc γ R affinities (*e.g.*, enhanced Fc γ RIIIA affinity) are done using the yeast display technology as described herein in combination with one or more biochemical based assays, preferably in a high throughput manner. The one or more biochemical assays can be any assay known in the art for identifying Fc-Fc γ R interaction, *i.e.*, specific binding of an Fc region to an Fc γ R, including, but not limited to, an ELISA assay, surface plasmon resonance assays, immunoprecipitation assay, affinity chromatography, and equilibrium dialysis. In some embodiments, screening and identifying molecules comprising variant Fc regions with altered Fc γ R affinities (*e.g.*, enhanced Fc γ RIIIA affinity) are done using the yeast display technology as described herein in combination with one or more functional based assays, preferably in a high throughput manner. The functional based assays can be any assay known in the art for characterizing one or more Fc γ R mediated effector cell function such as those described herein in Section 5.2.6. Non-limiting examples of effector cell functions that can be used in accordance with the methods of the invention, include but are not limited to, antibody-dependent cell mediated cytotoxicity (ADCC), antibody-dependent phagocytosis, phagocytosis, opsonization, opsonophagocytosis, cell binding, rosetting, C1q binding, and complement dependent cell mediated cytotoxicity. In some embodiments, screening and identifying molecules comprising variant Fc regions with altered Fc γ R affinities (*e.g.*, enhanced Fc γ RIIIA affinity) are done using the yeast display technology as described herein in combination with one or more biochemical based assays in combination or in parallel with one or more functional based assays, preferably in a high throughput manner.

[0047] A preferred method for measuring the Fc γ R-Fc interaction in accordance with the invention is an assay developed by the inventors, which allows detection and

quantitation of the interaction, despite the inherently weak affinity of the receptor for its ligand, *e.g.*, in the micromolar range for Fc γ RIIB and Fc γ RIIA. The method involves the formation of an Fc γ R complex (*e.g.*, Fc γ RIIA, Fc γ RIIB) that has an improved avidity for an Fc region, relative to an uncomplexed Fc γ R. In a specific embodiment, the invention encompasses a method for producing a tetrameric Fc γ R complex, wherein said tetrameric complex has an enhanced affinity for an Fc region, relative to the affinity of a monomeric Fc γ R for the Fc region, said method comprising: (i) producing a fusion protein, such that a 15 amino acid AVITAG sequence operably linked to the soluble region of Fc γ R; (ii) biotinylating the protein produced using an *E. coli* BirA enzyme; (iii) mixing the biotinylated protein produced with streptavidin-phycoerythrin in an appropriate molar ratio, such that a tetrameric Fc γ R complex is formed.

[0048] In a preferred embodiment of the invention, polypeptides comprising Fc regions bind the tetrameric Fc γ R complexes, formed according to the methods of the invention, with at least an 8-fold higher affinity than they bind the monomeric uncomplexed Fc γ R. The binding of polypeptides comprising Fc regions to the tetrameric Fc γ R complexes may be determined using standard techniques known to those skilled in the art, such as for example, fluorescence activated cell sorting (FACS), radioimmunoassays, ELISA assays, *etc.*

[0049] The invention encompasses the use of the immune complexes formed according to the methods described above for determining the functionality of molecules comprising an Fc region in cell-based or cell-free assays.

[0050] In a specific embodiment, the invention provides modified immunoglobulins comprising a variant Fc region with an enhanced affinity for Fc γ RIIA and/or Fc γ RIIB. Such immunoglobulins include IgG molecules that naturally contain Fc γ R binding regions (*e.g.*, Fc γ RIIA and/or Fc γ RIIB binding regions), or immunoglobulin derivatives that have been engineered to contain an Fc γ R binding region (*e.g.*, Fc γ RIIA and/or Fc γ RIIB binding regions). The modified immunoglobulins of the invention include any immunoglobulin molecule that binds, preferably, immunospecifically, *i.e.*, competes off non-specific binding as determined by immunoassays well known in the art for assaying specific antigen-antibody binding, an antigen and contains an Fc γ R binding region (*e.g.*, a Fc γ RIIA and/or Fc γ RIIB binding region). Such antibodies include, but are not limited to, polyclonal, monoclonal, bi-specific, multi-specific, human, humanized, chimeric antibodies, single chain antibodies, Fab fragments, F(ab')₂ fragments, disulfide-linked Fvs, and fragments containing either a VL or VH domain or even a complementary determining region (CDR)

that specifically binds an antigen, in certain cases, engineered to contain or fused to an Fc γ R binding region.

[0051] In certain embodiment, the invention encompasses immunoglobulins comprising a variant Fc region with an enhanced affinity for Fc γ RIIA and/or Fc γ RIIA such that the immunoglobulin has an enhanced effector function, *e.g.*, antibody dependent cell mediated cytotoxicity. The effector function of the molecules of the invention can be assayed using any assay described herein or known to those skilled in the art. In some embodiments, immunoglobulins comprising a variant Fc region with an enhanced affinity for Fc γ RIIA and/or Fc γ RIIA have an enhanced ADCC activity relative to wild-type by at least 2-fold, at least 4-fold, at least 8-fold, at least 10-fold, at least 50-fold, or at least 100-fold.

[0052] The invention encompasses engineering human or humanized therapeutic antibodies (*e.g.*, tumor specific monoclonal antibodies) in the Fc region by modification (*e.g.*, substitution, insertion, deletion) of one or more amino acid residues, which modifications modulate the affinity of the therapeutic antibody for an Fc γ R activating receptor and/or an Fc γ R inhibitory receptor. In one embodiment, the invention relates to engineering human or humanized therapeutic antibodies (*e.g.*, tumor specific monoclonal antibodies) in the Fc region by modification of one or more amino acid residues, which modifications increase the affinity of the Fc region for Fc γ RIIA and/or Fc γ RIIA. In another embodiment, the invention relates to engineering human or humanized therapeutic antibodies (*e.g.*, tumor specific monoclonal antibodies) in the Fc region by modification of one or more amino acid residues, which modification increases the affinity of the Fc region for Fc γ RIIA and/or Fc γ RIIA and further decreases the affinity of the Fc region for Fc γ RIIB. The engineered therapeutic antibodies may further have an enhanced effector function, *e.g.*, enhanced ADCC activity, phagocytosis activity, *etc.*, as determined by standard assays known to those skilled in the art.

[0053] In a specific embodiment, the invention encompasses engineering a humanized monoclonal antibody specific for Her2/neu protooncogene (*e.g.*, Ab4D5 humanized antibody as disclosed in Carter *et al.*, 1992, *Proc. Natl. Acad. Sci. USA* 89:4285-9) by modification (*e.g.*, substitution, insertion, deletion) of at least one amino acid residue which modification increases the affinity of the Fc region for Fc γ RIIA and/or Fc γ RIIA. In another specific embodiment, modification of the humanized Her2/neu monoclonal antibody may also further decrease the affinity of the Fc region for Fc γ RIIB. In yet another specific embodiment, the engineered humanized monoclonal antibodies specific for

Her2/neu may further have an enhanced effector function as determined by standard assays known in the art and disclosed and exemplified herein.

[0054] In another specific embodiment, the invention encompasses engineering a mouse human chimeric anti-CD20 monoclonal antibody, 2H7 by modification (*e.g.*, substitution, insertion, deletion) of at least one amino acid residue which modification increases the affinity of the Fc region for Fc γ RIIA and/or Fc γ R1A. In another specific embodiment, modification of the anti-CD20 monoclonal antibody, 2H7 may also further decrease the affinity of the Fc region for Fc γ RIIB. In yet another specific embodiment, the engineered anti-CD20 monoclonal antibody, 2H7 may further have an enhanced effector function as determined by standard assays known in the art and disclosed and exemplified herein.

[0055] In another specific embodiment, the invention encompasses engineering an anti-Fc γ RIIB antibody including but not limited to any of the antibodies disclosed in U.S. Provisional Application No. 60/403,266 filed on August 12, 2002 and U.S. Application No. 10/643,857 filed on August 14, 2003, having Attorney Docket No. 011183-010-999, by modification (*e.g.*, substitution, insertion, deletion) of at least one amino acid residue which modification increases the affinity of the Fc region for Fc γ RIIA and/or Fc γ R1A. Examples of anti-Fc γ RIIB antibodies that may be engineered in accordance with the methods of the invention are 2B6 monoclonal antibody having ATCC accession number PTA-4591 and 3H7 having ATCC accession number PTA-4592 (deposited at ATCC, 10801 University Boulevard, Manassas, VA 02209-2011. In another specific embodiment, modification of the anti-Fc γ RIIB antibody may also further decrease the affinity of the Fc region for Fc γ RIIB. In yet another specific embodiment, the engineered anti-Fc γ RIIB antibody may further have an enhanced effector function as determined by standard assays known in the art and disclosed and exemplified herein. In a specific embodiment, the 2B6 monoclonal antibody comprises a modification at position 334 with glutamic acid, at position 359 with asparagine, and at position 366 with serine (MgFc13); or a substitution at position 316 with aspartic acid, at position 378 with valine, and at position 399 with glutamic acid (MgFc27); or a substitution at position 243 with isoleucine, at position 379 with leucine, and at position 420 with valine (MgFc29); or a substitution at position 392 with threonine and at position 396 with leucine (MgFc38); or a substitution at position 221 with glutamic acid, at position 270 with glutamic acid, at position 308 with alanine, at position 311 with histidine, at position 396 with leucine, and at position 402 with aspartic (MgFc42); or a substitution at position 410 with histidine, and at position

396 with leucine (MgFc53); or a substitution at position 243 with leucine, at position 305 with isoleucine, at position 378 with aspartic acid, at position 404 with serine, and at position 396 with leucine (MgFc54); or a substitution at position 255 with isoleucine, and at position 396 with leucine (MgFc55); or a substitution at position 370 with glutamic acid,
5 and at position 396 with leucine (MgFc59).

[0056] The present invention also includes polynucleotides that encode a molecule of the invention, including polypeptides and antibodies, identified by the methods of the invention. The polynucleotides encoding the molecules of the invention may be obtained, and the nucleotide sequence of the polynucleotides determined, by any method known in
10 the art. The invention relates to an isolated nucleic acid encoding a molecule of the invention. The invention also provides a vector comprising said nucleic acid. The invention further provides host cells containing the vectors or polynucleotides of the invention.

[0057] The invention further provides methods for the production of the molecules
15 of the invention. The molecules of the invention, including polypeptides and antibodies, can be produced by any method known to those skilled in the art, in particular, by recombinant expression. In a specific embodiment, the invention relates to a method for recombinantly producing a molecule of the invention, said method comprising: (i) culturing in a medium a host cell comprising a nucleic acid encoding said molecule, under conditions
20 suitable for the expression of said molecule; and (ii) recovery of said molecule from said medium.

[0058] The molecules identified in accordance with the methods of the invention are useful in preventing, treating, or ameliorating one or more symptoms associated with a disease, disorder, or infection. The molecules of the invention are particularly useful for the
25 treatment or prevention of a disease or disorder where an enhanced efficacy of effector cell function (*e.g.*, ADCC) mediated by Fc γ R is desired, *e.g.*, cancer, infectious disease, and in enhancing the therapeutic efficacy of therapeutic antibodies the effect of which is mediated by ADCC.

[0059] In one embodiment, the invention encompasses a method of treating cancer
30 in a patient having a cancer characterized by a cancer antigen, said method comprising administering a therapeutically effective amount of a therapeutic antibody that binds the cancer antigen, which has been engineered in accordance with the methods of the invention. In a specific embodiment, the invention encompasses a method for treating cancer in a patient having a cancer characterized by a cancer antigen, said method comprising
35 administering a therapeutically effective amount of a therapeutic antibody that specifically

binds said cancer antigen, said therapeutic antibody comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said therapeutic antibody specifically binds Fc γ RIIIA with a greater affinity than the therapeutic antibody comprising the wild-type Fc region binds Fc γ RIIIA, provided that said variant Fc region does not have a substitution at positions 329, 331, or 332, and does not have an alanine at any of positions 256, 290, 298, 312, 333, 334, 359, 360, or 430; a lysine at position 330; a threonine at position 339; a methionine at position 320; a serine at position 326; an asparagine at position 326; an aspartic acid at position 326; a glutamic acid at position 326; a glutamine at position 334; a glutamic acid at position 334; a methionine at position 334; a histidine at position 334; a valine at position 334; or a leucine at position 334. In another specific embodiment, the invention encompasses a method for treating cancer in a patient having a cancer characterized by a cancer antigen, said method comprising administering a therapeutically effective amount of a therapeutic antibody that specifically binds a cancer antigen, said therapeutic antibody comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region such that said therapeutic antibody specifically binds Fc γ RIIIA with a greater affinity than a therapeutic antibody comprising the wild-type Fc region binds Fc γ RIIIA, and said therapeutic antibody further specifically binds Fc γ RIIB with a lower affinity than a therapeutic antibody comprising the wild-type Fc region binds Fc γ RIIB, provided that said variant Fc region does not have an alanine at any of positions 256, 298, 333, or 334. The invention encompasses a method for treating cancer in a patient characterized by a cancer antigen, said method comprising administering a therapeutically effective amount of a therapeutic antibody that specifically binds said cancer antigen and said therapeutic antibody comprises a variant Fc region so that the antibody has an enhanced ADCC activity.

[0060] The invention encompasses a method of treating an autoimmune disorder and/or inflammatory disorder in a patient in need thereof, said method comprising administering to said patient a therapeutically effective amount of a molecule comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild type Fc region, such that said molecule specifically binds Fc γ RIIB with a greater affinity than a comparable molecule comprising the wild type Fc region, and said molecule further specifically binds Fc γ RIIIA with a lower affinity than a comparable molecule comprising the wild type Fc region., and said molecule binds an immune complex (e.g., an antigen/antibody complex). The invention encompasses a method of treating an autoimmune disorder and/or inflammatory disorder further

comprising administering one or more additional prophylactic or therapeutic agents, *e.g.*, immunomodulatory agents, anti-inflammatory agents, used for the treatment and/or prevention of such diseases.

[0061] The invention also encompasses methods for treating or preventing an infectious disease in a subject comprising administering a therapeutically or prophylactically effective amount of one or more molecules of the invention that bind an infectious agent or cellular receptor therefor. Infectious diseases that can be treated or prevented by the molecules of the invention are caused by infectious agents including but not limited to viruses, bacteria, fungi, protozoae, and viruses.

[0062] According to one aspect of the invention, molecules of the invention comprising variant Fc regions have an enhanced antibody effector function towards an infectious agent, *e.g.*, a pathogenic protein, relative to a comparable molecule comprising a wild-type Fc region. In a specific embodiment, molecules of the invention enhance the efficacy of treatment of an infectious disease by enhancing phagocytosis and/or opsonization of the infectious agent causing the infectious disease. In another specific embodiment, molecules of the invention enhance the efficacy of treatment of an infectious disease by enhancing ADCC of infected cells causing the infectious disease.

[0063] In some embodiments, the molecules of the invention may be administered in combination with a therapeutically or prophylactically effective amount of one or additional therapeutic agents known to those skilled in the art for the treatment and/or prevention of an infectious disease. The invention contemplates the use of the molecules of the invention in combination with antibiotics known to those skilled in the art for the treatment and or prevention of an infectious disease.

[0064] The invention provides pharmaceutical compositions comprising a molecule of the invention, *e.g.*, a polypeptide comprising a variant Fc region, an immunoglobulin comprising a variant Fc region, a therapeutic antibody engineered in accordance with the invention, and a pharmaceutically acceptable carrier. The invention additionally provides pharmaceutical compositions further comprising one or more additional therapeutic agents, including but not limited to anti-cancer agents, anti-inflammatory agents, immunomodulatory agents.

3.1 DEFINITIONS

[0065] As used herein, the term "Fc region" is used to define a C-terminal region of an IgG heavy chain. Although the boundaries may vary slightly, the human IgG heavy chain Fc region is defined to stretch from Cys226 to the carboxy terminus. The Fc region of

an IgG comprises two constant domains, CH2 and CH3. The CH2 domain of a human IgG Fc region usually extends from amino acids 231 to amino acid 341. The CH3 domain of a human IgG Fc region usually extends from amino acids 342 to 447. The Fc region of an IgG comprises two constant domains, CH2 and CH3. The CH2 domain of a human IgG Fc region (also referred to as “Cγ2” domain) usually extends from amino acid 231-340. The CH2 domain is unique in that it is not closely paired with another domain. Rather, two N-linked branched carbohydrate chains are interposed between the two CH2 domains of an intact native IgG.

[0066] Throughout the present specification, the numbering of the residues in an IgG heavy chain is that of the EU index as in Kabat *et al.*, Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, NH1, MD (1991).

The “EU index as in Kabat” refers to the numbering of the human IgG1 EU antibody.

[0067] The “hinge region” is generally defined as stretching from Glu216 to Pro230 of human IgG1. Hinge regions of other IgG isotypes may be aligned with the IgG1 sequence by placing the first and last cysteine residues forming inter-heavy chain S-S binds in the same positions.

[0068] As used herein, the terms “antibody” and “antibodies” refer to monoclonal antibodies, multispecific antibodies, human antibodies, humanized antibodies, synthetic antibodies, chimeric antibodies, polyclonal antibodies, camelized antibodies, single-chain Fvs (scFv), single chain antibodies, Fab fragments, F(ab’) fragments, disulfide-linked bispecific Fvs (sdFv), intrabodies, and anti-idiotypic (anti-Id) antibodies (including, *e.g.*, anti-Id and anti-anti-Id antibodies to antibodies of the invention), and epitope-binding fragments of any of the above. In particular, antibodies include immunoglobulin molecules and immunologically active fragments of immunoglobulin molecules, *i.e.*, molecules that contain an antigen binding site. Immunoglobulin molecules can be of any type (*e.g.*, IgG, IgE, IgM, IgD, IgA and IgY), class (*e.g.*, IgG₁, IgG₂, IgG₃, IgG₄, IgA₁ and IgA₂) or subclass.

[0069] As used herein, the term “derivative” in the context of polypeptides or proteins refers to a polypeptide or protein that comprises an amino acid sequence which has been altered by the introduction of amino acid residue substitutions, deletions or additions. The term “derivative” as used herein also refers to a polypeptide or protein which has been modified, *i.e.*, by the covalent attachment of any type of molecule to the polypeptide or protein. For example, but not by way of limitation, an antibody may be modified, *e.g.*, by glycosylation, acetylation, pegylation, phosphorylation, amidation, derivatization by known

protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, *etc.* A derivative polypeptide or protein may be produced by chemical modifications using techniques known to those of skill in the art, including, but not limited to specific chemical cleavage, acetylation, formylation, metabolic synthesis of tunicamycin, *etc.* Further, a derivative polypeptide or protein derivative possesses a similar or identical function as the polypeptide or protein from which it was derived.

[0070] As used herein, the term “derivative” in the context of a non-proteinaceous derivative refers to a second organic or inorganic molecule that is formed based upon the structure of a first organic or inorganic molecule. A derivative of an organic molecule includes, but is not limited to, a molecule modified, *e.g.*, by the addition or deletion of a hydroxyl, methyl, ethyl, carboxyl or amine group. An organic molecule may also be esterified, alkylated and/or phosphorylated.

[0071] As used herein, the terms “disorder” and “disease” are used interchangeably to refer to a condition in a subject. In particular, the term “autoimmune disease” is used interchangeably with the term “autoimmune disorder” to refer to a condition in a subject characterized by cellular, tissue and/or organ injury caused by an immunologic reaction of the subject to its own cells, tissues and/or organs. The term “inflammatory disease” is used interchangeably with the term “inflammatory disorder” to refer to a condition in a subject characterized by inflammation, preferably chronic inflammation. Autoimmune disorders may or may not be associated with inflammation. Moreover, inflammation may or may not be caused by an autoimmune disorder. Thus, certain disorders may be characterized as both autoimmune and inflammatory disorders.

[0072] As used herein, the term “cancer” refers to a neoplasm or tumor resulting from abnormal uncontrolled growth of cells. As used herein, cancer explicitly includes, leukemias and lymphomas. In some embodiments, cancer refers to a benign tumor, which has remained localized. In other embodiments, cancer refers to a malignant tumor, which has invaded and destroyed neighboring body structures and spread to distant sites. In some embodiments, the cancer is associated with a specific cancer antigen.

[0073] As used herein, the term “immunomodulatory agent” and variations thereof refer to an agent that modulates a host’s immune system. In certain embodiments, an immunomodulatory agent is an immunosuppressant agent. In certain other embodiments, an immunomodulatory agent is an immunostimulatory agent. Immunomodulatory agents include, but are not limited to, small molecules, peptides, polypeptides, fusion proteins, antibodies, inorganic molecules, mimetic agents, and organic molecules.

[0074] As used herein, the term “epitope” refers to a fragment of a polypeptide or protein or a non-protein molecule having antigenic or immunogenic activity in an animal, preferably in a mammal, and most preferably in a human. An epitope having immunogenic activity is a fragment of a polypeptide or protein that elicits an antibody response in an animal. An epitope having antigenic activity is a fragment of a polypeptide or protein to which an antibody immunospecifically binds as determined by any method well-known to one of skill in the art, for example by immunoassays. Antigenic epitopes need not necessarily be immunogenic.

[0075] As used herein, the term “fragment” refers to a peptide or polypeptide comprising an amino acid sequence of at least 5 contiguous amino acid residues, at least 10 contiguous amino acid residues, at least 15 contiguous amino acid residues, at least 20 contiguous amino acid residues, at least 25 contiguous amino acid residues, at least 40 contiguous amino acid residues, at least 50 contiguous amino acid residues, at least 60 contiguous amino acid residues, at least 70 contiguous amino acid residues, at least contiguous 80 amino acid residues, at least contiguous 90 amino acid residues, at least contiguous 100 amino acid residues, at least contiguous 125 amino acid residues, at least 150 contiguous amino acid residues, at least contiguous 175 amino acid residues, at least contiguous 200 amino acid residues, or at least contiguous 250 amino acid residues of the amino acid sequence of another polypeptide. In a specific embodiment, a fragment of a polypeptide retains at least one function of the polypeptide.

[0076] As used herein, the terms “nucleic acids” and “nucleotide sequences” include DNA molecules (*e.g.*, cDNA or genomic DNA), RNA molecules (*e.g.*, mRNA), combinations of DNA and RNA molecules or hybrid DNA/RNA molecules, and analogs of DNA or RNA molecules. Such analogs can be generated using, for example, nucleotide analogs, which include, but are not limited to, inosine or tritylated bases. Such analogs can also comprise DNA or RNA molecules comprising modified backbones that lend beneficial attributes to the molecules such as, for example, nuclease resistance or an increased ability to cross cellular membranes. The nucleic acids or nucleotide sequences can be single-stranded, double-stranded, may contain both single-stranded and double-stranded portions, and may contain triple-stranded portions, but preferably is double-stranded DNA.

[0077] As used herein, a “therapeutically effective amount” refers to that amount of the therapeutic agent sufficient to treat or manage a disease or disorder. A therapeutically effective amount may refer to the amount of therapeutic agent sufficient to delay or minimize the onset of disease, *e.g.*, delay or minimize the spread of cancer. A therapeutically effective amount may also refer to the amount of the therapeutic agent that

provides a therapeutic benefit in the treatment or management of a disease. Further, a therapeutically effective amount with respect to a therapeutic agent of the invention means the amount of therapeutic agent alone, or in combination with other therapies, that provides a therapeutic benefit in the treatment or management of a disease.

5 [0078] As used herein, the terms “prophylactic agent” and “prophylactic agents” refer to any agent(s) which can be used in the prevention of a disorder, or prevention of recurrence or spread of a disorder. A prophylactically effective amount may refer to the amount of prophylactic agent sufficient to prevent the recurrence or spread of hyperproliferative disease, particularly cancer, or the occurrence of such in a patient,
10 including but not limited to those predisposed to hyperproliferative disease, for example those genetically predisposed to cancer or previously exposed to carcinogens. A prophylactically effective amount may also refer to the amount of the prophylactic agent that provides a prophylactic benefit in the prevention of disease. Further, a prophylactically effective amount with respect to a prophylactic agent of the invention means that amount of
15 prophylactic agent alone, or in combination with other agents, that provides a prophylactic benefit in the prevention of disease.

[0079] As used herein, the terms “prevent”, “preventing” and “prevention” refer to the prevention of the recurrence or onset of one or more symptoms of a disorder in a subject resulting from the administration of a prophylactic or therapeutic agent.

20 [0080] As used herein, the term “in combination” refers to the use of more than one prophylactic and/or therapeutic agents. The use of the term “in combination” does not restrict the order in which prophylactic and/or therapeutic agents are administered to a subject with a disorder. A first prophylactic or therapeutic agent can be administered prior to (e.g., 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12
25 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks before), concomitantly with, or subsequent to (e.g., 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 24
hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks after) the administration of a second prophylactic or therapeutic agent
30 to a subject with a disorder.

4. BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 SDS-PAGE ANALYSIS OF RECOMBINANT SOLUBLE FcγR

[0081] The purity of recombinant soluble Fc γ R proteins was assessed by 10% polyacrylamide gel electrophoresis. The gels were stained with Coomassie blue. Lane 1: purified recombinant soluble Fc γ RIIA; Lane 2: molecular weight marker; Lane 3: molecular weight marker; Lane 4: purified recombinant soluble Fc γ RIIB. The dashes refer to the molecular weight of the markers, from top to bottom, they correspond to a molecular weight of 98, 50, 36, and 22 KDa respectively.

FIG. 2 ELISA ASSAY OF RECOMBINANT SOLUBLE Fc γ R

[0082] The direct binding of purified recombinant soluble Fc γ RIIA to aggregated and monomeric IgG was determined using an ELISA assay. Binding of aggregated biotinylated IgG (■) and biotinylated monomeric IgG (◆) is compared. 3G8 antibody blocks the binding of aggregated biotinylated IgG to Fc γ RIIA (X).

FIGs. 3 A and B CHARACTERIZATION OF Fc γ RIIA TETRAMERIC COMPLEX USING AN ELISA ASSAY

- A. Soluble tetrameric Fc γ RIIA complex binds soluble monomeric human IgG specifically. Binding of soluble tetrameric Fc γ RIIA to human IgG is blocked by 3G8 (◆), a mouse anti-Fc γ RIIA monoclonal antibody; the 4-4-20 monoclonal antibody harboring the D265A mutation was not able to block the binding of soluble tetrameric Fc γ RIIA to aggregated human IgG (Δ).
- B. Binding of soluble tetrameric Fc γ RIIA complex to soluble monomeric human IgG (■) is compared to the binding of monomeric soluble Fc γ RIIA human IgG ($\square$).

FIGs. 4 A and B CHARACTERIZATION OF Fc γ RIIA TETRAMERIC COMPLEX USING A MAGNETIC BEAD ASSAY

- A. Fc γ RIIA Complex: two Fc γ RIIA (filled shape) are joined by a monoclonal antibody DJ130c (1st Ab); the anti-mouse F(ab)₂ is conjugated to PE (circle).
- B. FACS analysis of Fc γ RIIA bound to Fc coated beads: (a) beads alone; (b) complex without Fc γ RIIA; (c) complex with Fc γ RIIA; (d) complex with Fc γ RIIA and LNK16.

FIG. 5 SCHEMATIC PRESENTATION OF Fc CONTAINING CONSTRUCTS

[0083] A schematic diagram of the IgG1 Fc domains cloned into pYD1 is presented. The open box represents the hinge-CH2-CH3 domains; parallel vertical lines represent the CH1 domain. In the case of the GIF206 and 227 constructs; the N-terminal amino acids are shown. The underlined residues correspond to the hinge region; the * represents the Xpress epitope tag; hatched boxes represent the Gly4-Ser linker, and the stippled boxes represent the Aga2p gene.

FIGS. 6A-H FACS ANALYSIS OF THE Fc FUSION PROTEINS ON THE YEAST CELL WALL

[0084] Cells were incubated with either a PE-conjugated polyclonal goat anti-human Fc antibody (FIGS. 6A-D) or with HP6017 (Sigma), a mouse anti-human IgG1 Fc (CH3) specific monoclonal antibody (FIGS. 6E-H). A and E represent vector alone; Panels B and F represent the CH1-CH3 construct; Panels C and G represent the GIF227; and Panels D and H represent the GIF 206 construct.

FIGS. 7A-C BINDING OF SOLUBLE TETRAMERIC Fc γ RIIIA TO THE SURFACE DISPLAYED Fc FUSION PROTEINS

[0085] Cells containing pYD1-CH1 (A); pYD-CH1-D265A (B); and pYD vector (C) were grown under conditions to express Aga2p fusion proteins on the cell surface. Cells were incubated with Fc γ RIIIA at 0.15 mM, 7.5 mM, and 7.5 mM, respectively, and analyzed by FACS.

FIG. 8 CHARACTERIZATION OF THE BINDING OF SOLUBLE TETRAMERIC Fc γ RIIIA TO THE SURFACE DISPLAYED Fc FUSION PROTEINS

[0086] Binding of Fc γ RIIIA tetrameric complex to Fc fusion proteins on the yeast cell surface was analyzed. PE-conjugated Fc γ RIIIA tetrameric complexes were pre-incubated with different concentrations of 3G8 (◆), LNK (▲) or an irrelevant isotype control (■), and subsequently incubated with the yeast cells. Cells were analyzed by FACS for PE fluorescence. The percent cells that bound the Fc γ RIIIA tetrameric complex were plotted on the y-axis.

FIG. 9 EXAMPLE OF SORT GATE FOR SELECTING Fc MUTANTS WITH INCREASED BINDING TO Fc γ RIIIA

[0087] Cells were stained with PE-conjugated Fc γ RIIIA tetrameric complexes (y-axis) and anti-Fc-FITC conjugated antibody (x-axis). Boxed area represents sort gate set to select ~1.0% of the cell population.

FIGs. 10A-N FACS ANALYSIS OF SOME OF THE Fc MUTANTS IDENTIFIED HAVING AN INCREASED AFFINITY FOR Fc γ RIIIA TETRAMERIC COMPLEXES

[0088] Individual clones harboring the pYD-CH1 plasmid containing independent
 5 Fc mutations were amplified in selective media containing glucose, induced for Fc expression in selective media containing galactose, and subsequently analyzed by FACS. FIGS. 10A and B represent cells harboring wild-type Fc; FIGS. 10C and D represent mutant # 5; FIGS. 10E and F represent mutant # 20; FIGS. 10G and H represent mutant # 21; FIGS. 10 I and J represent mutant # 24; FIGS. 10K and L represent mutant # 25; FIGS. 10M and
 10 N represent mutant # 27. Cells were stained with Fc γ RIIIA tetrameric complex (FIGS. 10 A, C, E, G, I, K, and M) or Fc γ RIIB tetrameric complex (FIGS. 10B, D, F, H, J, L, and N).

FIGs. 11A-B CHARACTERIZATION OF Fc MUTANTS IN THE 4-4-20 MONOCLONAL ANTIBODY BY ELISA

[0089] Fc domains from the pYD-CH1 plasmids were cloned into the heavy chain
 15 of the chimeric 4-4-20 monoclonal antibody. The 4-4-20 monoclonal antibody was expressed in 293 cells and supernatants were collected. ELISA plates were coated with fluoresceine conjugated BSA to capture the chimeric 4-4-20 mutant antibodies. Fc γ RIIIA (Panel A) and Fc γ RIIB (Panel B) receptors were then coated onto the ELISA plates to which the 4-4-20 monoclonal antibodies had been absorbed in order to determine the
 20 relative affinities of the variant receptors to the Fc domains. Mutants # 15 and # 29 were non-binding isolates included as controls.

FIG. 12 ADCC ACTIVITY OF MUTANTS IN THE 4-4-20 MONOCLONAL ANTIBODY

[0090] 4-4-20 antibodies containing mutant Fc regions were assessed for their
 25 ADCC activity, and compared to the ADCC activity of a wild type 4-4-20 antibody. The mutants analyzed are as follows: MGFc-10 (K288N, A330S, P396L), MGFc-26 (D265A), MGFc-27 (G316D, A378V, D399E), MGFc28 (N315I, A379M, D399E), MGFc29 (F243I, V379L, G420V), MGFc30 (F275V), MGFc-31 (P247L, N421K), MGFc-32 (D280E, S354F, A431D, L441I), MGFc-33 (K317N, F423 deleted), MGFc-34 (F241L, E258G),
 30 MGFc-35 (R255Q, K326E), MGFc-36 (K218R, G281D, G385R)

FIGs. 13 A and B ADCC ACTIVITY OF MUTANTS IN THE HER2/NEU HUMANIZED MONOCLONAL ANTIBODY

A. Humanized HER2/neu monoclonal antibodies containing mutant Fc regions were assessed for their ADCC activity and compared to the ADCC activity of a wild type Her2/neu antibody. The mutants analyzed are as follows: MGFc-5 (V379M), MGFc-9 (F243I, V379L), MGFc-10 (K288N, A330S, P396L), MGFc-13 (K334E, T359N, T366S),
 5 MGFc-27 (G316D, A378V, D399E).

B. ADCC activity of additional mutants in the context of the humanized Her2/neu monoclonal antibody MGFc-37 (K248M), MGFc-39 (E293V Q295E, A327T), MGFc-38 (K392T, P396L), MGFc-41 (H268N, P396L), MGFc-23 (K334E, R292L), MGFc-44, MGFc-45.

10 **FIG. 14 CAPTURE OF CH 4-4-20 ANTIBODY ON BSA-FITC SURFACE**

[0091] 6 μ L of antibody at a concentration of approximately 20 μ g/mL was injected at 5 μ L/min over a BSA- fluoroscein isothiocyanate (FITC) surface. BIAcore sensogram of the binding of ch 4-4-20 antibodies with mutant Fc regions on the surface of the BSA-FITC
 15 immobilized sensor ship is shown. The marker was set on wild-type captured antibody response.

FIG. 15 SENSOGRAM OF REAL TIME BIDDING OF Fc γ RIIA TO CH 4-4-20 ANTIBODIES CARRYING VARIANT Fc REGIONS

[0092] Binding of Fc γ RIIA to ch-4-4-20 antibodies carrying variant Fc regions was
 20 analyzed at 200 nM concentration. Responses were normalized at the level of ch-4-4-20 antibody obtained for wild-type.

FIG. 16 ANALYSIS OF KINETIC PARAMETERS OF Fc γ RIIA BINDING TO ANTIBODIES CARRYING VARIANT Fc REGIONS

[0093] Kinetic parameters for Fc γ RIIA binding to antibodies carrying variant Fc
 25 regions were obtained by generating separate best fit curves for 200 nM and 800 nM. Solid line indicates an association fit which was obtained based on the k_{off} values calculated for the dissociation curves in the 32-34 sec interval. K_d and k_{off} values represent the average from two concentrations.

30 **FIG. 17 SENSOGRAM OF REAL TIME BINDING OF Fc γ RIIB-Fc FUSION PROTEINS TO ANTIBODIES CARRYING VARIANT Fc REGIONS**

[0094] Binding of Fc γ RIIB-Fc fusion proteins to ch-4-4-20 antibodies carrying variant Fc regions was analyzed at 200 nM concentration. Responses were normalized at the level of ch-4-4-20 antibody obtained for wild type.

FIG. 18 ANALYSIS OF KINETIC PARAMETERS Fc γ RIIB-Fc FUSION PROTEINS TO ANTIBODIES CARRYING VARIANT Fc REGIONS

[0095] Kinetic parameters for Fc γ RIIB-Fc binding to antibodies carrying variant Fc regions were obtained by generating separate best fit curves for 200 nM and 800 nM. Solid line indicates an association fit which was obtained based on the k_{off} values calculated for the dissociation curves in the 32-34 sec. interval. K_d and K_{off} values represent the average from two concentrations.

FIG. 19 RATIOS OF K_{off} (WT)/ K_{off} (MUT) FOR Fc γ RIIA-Fc PLOTTED AGAINST ADCC DATA

[0096] Numbers higher than one show a decreased dissociation rate for Fc γ RIIA binding and increased dissociation rate for Fc γ RIIB-Fc binding relative to wild-type.

Mutants in the box have lower off rate for Fc γ RIIA binding and higher off rate for Fc γ RIIB-Fc binding.

FIG. 20 COMPETITION WITH UNLABELED Fc γ RIIA

[0097] A kinetic screen was implemented to identify Fc region mutants with improved K_{off} rates for binding Fc γ RIIA. A library of Fc region variants containing P396L mutation was incubated with 0.1 μ M biotinylated Fc γ RIIA-Linker-Avitag for one hour and then washed. Subsequently 0.8 uM unlabeled Fc γ RIIA was incubatd with the labeled yeast for different time points. Yeast was spun down and unlabeled Fc γ RIIA was removed, Receptor bound yease was stained with SA (streptavidin):PE (phycoerythrin) for FACS analysis.

FIG. 21 FACS ANALYSIS BASED ON THE KINETIC SCREEN

[0098] Based on the calculated K_{off} from the data presented in FIG. 20, a one minute time point selection was chosen. A 10-fold excess of library was incubated with 0.1 μ M biotinylated Fc γ RIIA-Linker-Avitag monomer; cells were washed and incubated with unlabeled ligand for one minute; then washed and labeled with SA:PE. The cells were then sorted by FACS, selecting the top 0.3% binders. The nonselected P396L library was compared to the yeast cells selected for improved binding by FACS. The histograms show

the percentage of cells that are costained with both Fc γ RIIIA /PE and goat anti-human Fc/FITC (upper right).

FIG. 22 SELECTION BASED ON SOLID PHASE DEPLETION OF Fc γ RIIB Fc BINDERS

5 [0099] The P396L library was screened based on Fc γ RIIB depletion and Fc γ RIIIA selection using magnetic beads. The Fc γ RIIB depletion by magnetic beads was repeated 5 times. The resulting yeast population was analyzed and found to show greater than 50% cell staining with goat anti-human Fc and a very small percentage of cells stained with Fc γ RIIIA. Subsequently cells were selected twice by FACS using 0.1 μ M biotinylated
10 Fc γ RIIIA linker-avitag. Yeast cells were analyzed for both Fc γ RIIIA and Fc γ RIIB binding after each sort and compared to wild type binding.

FIG. 23 RELATIVE RATES OF SKBR3 TARGET CELL LYSIS MEDIATED BYCHIMERIC 4D5 HARBORING FC MUTANTS

[00100] Relative rate of lysis was calculated for each Fc mutant tested. Lysis rates
15 for 4D5 antibody with Fc mutants were divided by the rate of lysis mediated by wild type 4D5 antibody. Data from at least 2 independent assays were averaged and plotted on the histogram. For each Fc mutant data from two different antibody concentrations are shown. The antibody concentrations were chosen to flank the point along the curve at which lysis was ~50%.

20 **FIG. 24 RELATIVE RATES OF DAUDI CELL LYSIS MEDIATED BY CHIMERIC 2H7 HARBORING FC MUTANTS**

[00101] Relative rate of lysis was calculated for each Fc mutant tested. Lysis rates
for 2H7 antibody with Fc mutants were divided by the rate of lysis mediated by wild type 2H7 antibody. Data from at least 1- 2 independent assays were averaged and plotted on the
25 histogram. For each Fc mutant data from two different antibody concentrations are shown. The antibody concentrations were chosen based on the point along the curve at which lysis was ~50%.

5. DESCRIPTION OF THE PREFERRED EMBODIMENTS

30 [00102] The present invention relates to molecules, preferably polypeptides, and more preferably immunoglobulins (*e.g.*, antibodies), comprising a variant Fc region, having one or more amino acid modifications (*e.g.*, substitutions, but also including insertions or

deletions) in one or more regions, which modifications alter, *e.g.*, increase or decrease, the affinity of the variant Fc region for an Fc γ R. In some embodiments, the invention provides molecules comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, which variant Fc region binds Fc γ RIIA with a greater affinity, relative to a comparable molecule, *i.e.*, being the same as said molecule with a variant Fc region but not having the one or more amino acid modifications, comprising the wild-type Fc region as determined by methods known to one skilled in the art for determining Fc-Fc γ R interactions and methods disclosed herein, for example, an ELISA assay or a surface plasmon resonance assay. In yet other embodiments, the invention encompasses molecules comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, which variant Fc region binds Fc γ RIIA with a reduced affinity relative to a comparable molecule comprising the wild-type Fc region. In a preferred embodiment, the molecules of the invention further specifically bind Fc γ RIIB (via the Fc region) with a lower affinity than a comparable molecule comprising the wild-type Fc region binds Fc γ RIIB. In some embodiments, the invention encompasses molecules comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, which variant Fc region binds Fc γ RIIA and Fc γ RIIB with a greater affinity, relative to a comparable molecule comprising the wild-type Fc region. In other embodiments, the invention encompasses molecules comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, which variant Fc region binds Fc γ RIIB with a greater affinity, relative to a comparable molecule comprising the wild-type Fc region. In other embodiments, the invention encompasses molecules comprising variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, which variant Fc region binds Fc γ RIIB with a reduced affinity, relative to a comparable molecule comprising the wild-type Fc region.

[00103] In some embodiments, the invention encompasses molecules comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild type Fc region, which variant Fc region does not show a detectable binding to any Fc γ R (*e.g.*, does not bind Fc γ RIIA, Fc γ RIIB, or Fc γ RIIA, as determined by, for example, an ELISA assay), relative to a comparable molecule comprising the wild-type Fc region.

[00104] In a specific embodiment, the invention encompasses molecules comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild type Fc region, which variant Fc region only binds one Fc γ R, wherein said Fc γ R is Fc γ IIIA. In another specific embodiment, the invention encompasses molecules comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild type Fc region, which variant Fc region only binds one Fc γ R, wherein said Fc γ R is Fc γ RIIA. In yet another embodiment, the invention encompasses molecules comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild type Fc region, which variant Fc region only binds one Fc γ R, wherein said Fc γ R is Fc γ RIIB. The invention particularly relates to the modification of human or humanized therapeutic antibodies (*e.g.*, tumor specific anti-angiogenic or anti-inflammatory monoclonal antibodies) for enhancing the efficacy of therapeutic antibodies by enhancing, for example, the effector function of the therapeutic antibodies, *e.g.*, enhancing ADCC.

[00105] The affinities and binding properties of the molecules of the invention for an Fc γ R are initially determined using *in vitro* assays (biochemical or immunological based assays) known in the art for determining Fc-Fc γ R interactions, *i.e.*, specific binding of an Fc region to an Fc γ R including but not limited to ELISA assay, surface plasmon resonance assay, immunoprecipitation assays (*See* Section 5.2.1). Preferably, the binding properties of the molecules of the invention are also characterized by *in vitro* functional assays for determining one or more Fc γ R mediator effector cell functions (*See* Section 5.2.6). In most preferred embodiments, the molecules of the invention have similar binding properties in *in vivo* models (such as those described and disclosed herein) as those in *in vitro* based assays. However, the present invention does not exclude molecules of the invention that do not exhibit the desired phenotype in *in vitro* based assays but do exhibit the desired phenotype *in vivo*.

[00106] In some embodiments, the molecules of the invention comprising a variant Fc region comprise at least one amino acid modification in the CH3 domain of the Fc region, which is defined as extending from amino acids 342-447. In other embodiments, the molecules of the invention comprising a variant Fc region comprise at least one amino acid modification in the CH2 domain of the Fc region, which is defined as extending from amino acids 231-341. In some embodiments, the molecules of the invention comprise at least two amino acid modifications, wherein one modification is in the CH3 region and one modification is in the CH2 region. The invention further encompasses amino acid modification in the hinge region. Molecules of the invention with one or more amino acid

modifications in the CH2 and/or CH3 domains have altered affinities for an FcγR as determined using methods described herein or known to one skilled in the art.

[00107] In a preferred specific embodiment, the invention encompasses a molecule comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said molecule has an altered affinity for an FcγR, provided that said variant Fc region does not have a substitution at positions that make a direct contact with FcγR based on crystallographic and structural analysis of Fc-FcγR interactions such as those disclosed by Sondermann *et al.*, 2000 (*Nature*, 406: 267-273). Examples

of positions within the Fc region that make a direct contact with FcγR are amino acids 234-239 (hinge region), amino acids 265-269 (B/C loop), amino acids 297-299 (C'/E loop), and amino acids 327-332 (F/G) loop. In some embodiments, the molecules of the invention comprising variant Fc regions comprise modification of at least one residue that makes a direct contact with an FcγR based on structural and crystallographic analysis.

[00108] In another preferred embodiment, the invention encompasses a molecule comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said molecule binds an FcγR with an altered affinity relative to a molecule comprising a wild-type Fc region, provided that said variant Fc region does not have or are not solely a substitution at any of positions 255, 256, 258, 267, 268, 269, 270, 272, 276, 278, 280, 283, 285, 286, 289, 290, 292, 293, 294, 295, 296, 298, 300, 301, 303, 305, 307, 309, 312, 320, 322, 326, 329, 330, 332, 331, 333, 334, 335, 337, 338, 339, 340, 359, 360, 373, 376, 416, 419, 430, 434, 435, 437, 438, 439. In a specific embodiment, the invention encompasses a molecule comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said molecule binds an FcγR with an altered affinity relative to a molecule comprising a wild-type Fc region, provided that said variant Fc region does not have or are not solely a substitution at any of positions 255, 258, 267, 269, 270, 276, 278, 280, 283, 285, 289, 292, 293, 294, 295, 296, 300, 303, 305, 307, 309, 322, 329, 332, 331, 337, 338, 340, 373, 376, 416, 419, 434, 435, 437, 438, 439 and does not have an alanine at any of positions 256, 290, 298, 312, 333, 334, 359, 360, 326, or 430; a lysine at position 330; a threonine at position 339; a methionine at position 320; a serine at position 326; an asparagine at position 326; an aspartic acid at position 326; a glutamic acid at position 326; a glutamine at position 334; a glutamic acid at position 334; a methionine at position 334; a histidine at position 334; a valine at position 334; or a leucine at position

334; a lysine at position 335 an asparagine at position 268; a glutamine at position 272; a glutamine, serine, or aspartic acid at position 286; a serine at position 290; a methionine, glutamine, glutamic acid, or arginine at position 320; a glutamic acid at position 322; a serine, glutamic acid, or aspartic acid at position 326; a lysine at position 330; a glutamine
 5 at position 335; or a methionine at position 301.

[00109] In a specific embodiment, the invention encompasses a molecule comprising a variant Fc region, wherein said variant Fc region does not have or are not solely a substitution at any of positions 268, 269, 270, 272, 276, 278, 283, 285, 286, 289, 292, 293, 301, 303, 305, 307, 309, 331, 333, 334, 335, 337, 338, 340, 360, 373, 376, 416, 419, 430,
 10 434, 435, 437, 438 or 439 and does not have a histidine, glutamine, or tyrosine at position 280; a serine, glycine, threonine or tyrosine at position 290, a leucine or isoleucine at position 300; an asparagine at position 294, a proline at position 296; a proline, asparagine, aspartic acid, or valine at position 298; a lysine at position 295. In yet another preferred embodiment, the invention encompasses a molecule comprising a variant Fc region,
 15 wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said molecule binds an FcγR with a reduced affinity relative to a molecule comprising a wild-type Fc region provided that said variant Fc region does not have or are not solely a substitution at any of positions 252, 254, 265, 268, 269, 270, 278, 289, 292, 293, 294, 295, 296, 298, 300, 301, 303, 322, 324, 327, 329, 333, 335, 338, 340,
 20 373, 376, 382, 388, 389, 414, 416, 419, 434, 435, 437, 438, or 439. In yet another preferred embodiment, the invention encompasses a molecule comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said molecule binds an FcγR with an enhanced affinity relative to a molecule comprising a wild-type Fc region provided that said variant Fc region
 25 does not have or are not solely a substitution at any of positions 280, 283, 285, 286, 290, 294, 295, 298, 300, 301, 305, 307, 309, 312, 315, 331, 333, 334, 337, 340, 360, 378, 398, or 430.

[00110] In most preferred embodiments, the molecules of the invention with altered affinities for activating and/or inhibitory receptors having variant Fc regions, have one or
 30 more amino acid modifications, wherein said one or more amino acid modification is a substitution at position 288 with asparagine, at position 330 with serine and at position 396 with leucine (MgFc10)(See Table 5); or a substitution at position 334 with glutamic acid, at position 359 with asparagine, and at position 366 with serine (MgFc13); or a substitution at position 316 with aspartic acid, at position 378 with valine, and at position 399 with
 35 glutamic acid (MgFc27); or a substitution at position 392 with threonine, and at position

- 396 with leucine (MgFc38); or a substitution at position 221 with glutamic acid, at position 270 with glutamic acid, at position 308 with alanine, at position 311 with histidine, at position 396 with leucine, and at position 402 with aspartic acid (MgFc42); or a substitution at position 240 with alanine, and at position 396 with leucine (MgFc52); or a substitution at position 410 with histidine, and at position 396 with leucine (MgFc53); or a substitution at position 243 with leucine, at position 305 with isoleucine, at position 378 with aspartic acid, at position 404 with serine, and at position 396 with leucine (MgFc54); or a substitution at position 255 with isoleucine, and at position 396 with leucine (MgFc55); or a substitution at position 370 with glutamic acid and at position 396 with leucine (MgFc59).
- 10 **[00111]** In some embodiments, the invention encompasses molecules comprising a variant Fc region having an amino acid modification at one or more of the following positions: 185, 142, 192, 141, 132, 149, 133, 125, 162, 147, 119, 166, 251, 292, 290, 291, 252, 288, 268, 256, 262, 218, 214, 205, 215, 247, 275, 202, 289, 258, 219, 279, 222, 246, 233, 246, 268, 244, 217, 253, 246, 224, 298, 280, 255, 218, 281, 284, 216, 223, 235, 221, 15 252, 241, 258, 227, 231, 215, 274, 287, 244, 229, 287, 291, 240, 281, 232, 269, 225, 246, 246, 293, 295, 248, 276, 268, 210, 288, 227, 221, 217, 261, 210, 242, 255, 240, 250, 247, 258, 246, 282, 219, 225, 270, 263, 272, 292, 233, 247, 254, 243, 347, 339, 392, 399, 301, 315, 383, 396, 385, 348, 333, 334, 310, 337, 371, 359, 366, 359, 379, 330, 318, 395, 319, 380, 305, 309, 335, 370, 378, 394, 386, 377, 358, 384, 397, 372, 326, 320, 375, 327, 381, 20 354, 385, 335, 387, 353, 375, 383, 397, 345, 375, 389, 335, 394, 316, 399, 315, 394, 382, 390, 369, 377, 304, 323, 313, 388, 339, 317, 365, 367, 340, 311, 312, 398, 343, 352, 362, 303, 308, 327, 307, 344, 328, 393, 355, 360, 306, 361, 355, 415, 408, 409, 407, 424, 401, 402, 435, 421, 431, 441, 440, 435, 431, 442, 400, 422, 406, 411, 422, 433, 406, 423, 420, 412, 447, 443, 414, 433, 428, 446, 402, 419, 410, 404, 427, 417, 433, 436, 438, 416.
- 25 Preferably such mutations result in molecules that have an altered affinity for an FcγR and/or has an altered effector cell mediated function as determined using methods disclosed and exemplified herein and known to one skilled in the art.
- [00112]** The invention encompasses molecules comprising variant Fc regions consisting of or comprising any of the mutations listed in the table below in Table 2.

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TABLE 2. EXEMPLARY MUTATIONS

SINGLE SITE MUTANTS	DOUBLE SITE MUTANTS
K392R	Q347H, A339V
N315I	S415I, L251F
S132I	K290E, L142P
P396L	G285E, P247H
P396H	K409R, S166N

A162V	E334A, K334A
R292L	R292L, K334E
T359N	K288N, A330S
T366S	R255L, E318K
V379L	F243L, E318K
K288N	V279L, P395S
A330S	K246T, Y319F
F243L	F243I, V379L
E318K	K288M, K334E
V379M	K334E, E308D
S219Y	E233D, K334E
V282M	K246T, P396H
D401V	H268D, E318D
K222N	K246I, K334N
K334I	K320E, K326E
K334E	S375C, P396L
I377F	K288N, K326N
P247L	P247L, N421K
F372Y	S298N, W381R
K326E	R255Q, K326E
H224L	V284A, F372L
F275Y	T394M, V397M
L398V	P247L, E389G
K334N	K290T, G371D
S400P	P247L, L398Q
S407I	P247L, I377F
F372Y	K326E, G385E
T366N	S298N, S407R
K414N	E258D, N384K
M352L	F241L, E258G
T225S	K370N, S440N
I377N	K317N, F423-DELETED
K248M	P227S, K290E
R292G	K334E, E380D
S298N	P291S, P353Q
D270E	V240I, V281M
E233G	P232S, S304G
	P247L, L406F
	D399E, M428L
	L251F, F372L
	D399E, G402D
	D399E, M428L
	K392T, P396L
	H268N, P396L
	K326I, P396L
	H268D, P396L
	K210M, P396L
	L358P, P396L
	K334N, P396L
	V379M, P396L

	P227S, P396L
	P217S, P396L
	Q419H, P396L
	K370E, P396L
	L242F, P396L
	R255L, P396L
	V240A, P396L
	T250A, P396L
	P247S, P396L
	L410H, P396L
	Q419L, P396L
	V427A, P396L
	E258D, P396L
	N384K, P396L
	V323I, P396L
	P244H, P396L
	V305L, P396L
	S400F, P396L
	V303I, P396L
	A330V, Q419H
	V263Q, E272D
	K326E, A330T

[00113] In yet other embodiments, the invention encompasses molecules comprising variant Fc regions having more than two amino acid modifications. A non-limiting example of such variants is listed in the table below (Table 3). The invention encompasses mutations listed in Table 3 which further comprise one or more amino acid modifications such as

5 those disclosed herein.

TABLE 3. EXEMPLARY COMBINATION VARIANTS

D399E, R292L, V185M
R301C, M252L, S192T
P291S, K288E, H268L, A141V
S383N, N384K, T256N, V262L, K218E, R214I, K205E, F149Y, K133M
S408I, V215I, V125L
G385E, P247H
V348M, K334N, F275I, Y202M, K147T
H310Y, T289A, Y407V, E258D
R292L, P396L, T359N
F275I, K334N, V348M
F243L, R255L, E318K
K334E, T359N, T366S
T256S, V305I, K334E, N390S
T335N, K370E, A378V, T394M, S424L
K334E, T359N, T366S, Q386R
K288N, A330S, P396L
P244H, L358M, V379M, N384K, V397M
P217S, A378V, S408R
P247L, I253N, K334N

D312E, K327N, I378S
D280E, S354F, A431D, L441I
K218R, G281D, G385R
P247L, A330T, S440G
T355N, P387S, H435Q
P247L, A431V, S442F
P343S, P353L, S375I, S383N
E216D, E345K, S375I
K288N, A330S, P396L
K222N, T335N, K370E, A378V, T394M
G316D, A378V, D399E
N315I, V379M, T394M
K326Q, K334E, T359N, T366S
A378V, N390I, V422I
V282E, V369I, L406F
V397M, T411A, S415N
T223I, T256S, L406F
L235P, V382M, S304G, V305I, V323I
P247L, W313R, E388G
D221Y, M252I, A330G, A339T, T359N, V422I, H433L
F243I, V379L, G420V
A231V, Q386H, V412M
T215P, K274N, A287G, K334N, L365V, P396L
P244A, K326I, C367R, S375I, K447T
R301H, K340E, D399E
C229Y, A287T, V379M, P396L, L443V
E269K, K290N, Q311R, H433Y
E216D, K334R, S375I
T335N, P387S, H435Q
K246I, Q362H, K370E
K334E, E380D, G446V
V303I, V369F, M428L
K246E, V284M, V308A
E293V, Q295E, A327T
Y319F, P352L, P396L
D221E, D270E, V308A, Q311H, P396L, G402D
K290T, N390I, P396L
K288R, T307A, K344E, P396L
V273I, K326E, L328I, P396L
K326I, S408N, P396L
K261N, K210M, P396L
F243L, V305I, A378D, F404S, P396L
K290E, V369A, T393A, P396L
K210N, K222I, K320M, P396L
P217S, V305I, I309L, N390H, P396L
K246N, Q419R, P396L
P217A, T359A, P396L
V215I, K290V, P396L
F275L, Q362H, N384K, P396L
A330V, H433Q, V427M

V263Q, E272D, Q419H
N276Y, T393N, W417R
V282L, A330V, H433Y, T436R
V284M, S298N, K334E, R355W
A330V, G427M, K438R
S219T, T225K, D270E, K360R
K222E, V263Q, S298N
E233G, P247S, L306P
S219T, T225K, D270E
S254T, A330V, N361D, P243L
V284M, S298N, K334E, R355W R416T

[00114] In some embodiments, the molecules, preferably the immunoglobulins of the invention further comprise one or more glycosylation sites, so that one or more carbohydrate moieties are covalently attached to the molecule. Preferably, the antibodies of the invention with one or more glycosylation sites and/or one or more modifications in the Fc region have an enhanced antibody mediated effector function, *e.g.*, enhanced ADCC activity. In some embodiments, the invention further comprises antibodies comprising one or more modifications of amino acids that are directly or indirectly known to interact with a carbohydrate moiety of the antibody, including but not limited to amino acids at positions 241, 243, 244, 245, 249, 256, 258, 260, 262, 264, 265, 296, 299, and 301. Amino acids that directly or indirectly interact with a carbohydrate moiety of an antibody are known in the art, *see, e.g.*, Jefferis *et al.*, 1995 *Immunology Letters*, 44: 111-7.

[00115] The invention encompasses antibodies that have been modified by introducing one or more glycosylation sites into one or more sites of the antibodies, preferably without altering the functionality of the antibody, *e.g.*, binding activity to FcγR. Glycosylation sites may be introduced into the variable and/or constant region of the antibodies of the invention. As used herein, "glycosylation sites" include any specific amino acid sequence in an antibody to which an oligosaccharide (*i.e.*, carbohydrates containing two or more simple sugars linked together) will specifically and covalently attach. Oligosaccharide side chains are typically linked to the backbone of an antibody via either N-or O-linkages. N-linked glycosylation refers to the attachment of an oligosaccharide moiety to the side chain of an asparagine residue. O-linked glycosylation refers to the attachment of an oligosaccharide moiety to a hydroxyamino acid, *e.g.*, serine, threonine. The antibodies of the invention may comprise one or more glycosylation sites, including N-linked and O-linked glycosylation sites. Any glycosylation site for N-linked or O-linked glycosylation known in the art may be used in accordance with the instant

invention. An exemplary N-linked glycosylation site that is useful in accordance with the methods of the present invention, is the amino acid sequence: Asn-X-Thr/Ser, wherein X may be any amino acid and Thr/Ser indicates a threonine or a serine. Such a site or sites may be introduced into an antibody of the invention using methods well known in the art to which this invention pertains. *See, for example, "In Vitro Mutagenesis," Recombinant DNA: A Short Course, J. D. Watson, et al. W.H. Freeman and Company, New York, 1983, chapter 8, pp. 106-116.* An exemplary method for introducing a glycosylation site into an antibody of the invention may comprise: modifying or mutating an amino acid sequence of the antibody so that the desired Asn-X-Thr/Ser sequence is obtained.

[00116] In some embodiments, the invention encompasses methods of modifying the carbohydrate content of an antibody of the invention by adding or deleting a glycosylation site. Methods for modifying the carbohydrate content of antibodies are well known in the art and encompassed within the invention, *see, e.g.,* U.S. Patent No. 6,218,149; EP 0 359 096 B1; U.S. Publication No. US 2002/0028486; WO 03/035835; U.S. Publication No. 2003/0115614; U.S. Patent No. 6,218,149; U.S. Patent No. 6,472,511. In other embodiments, the invention encompasses methods of modifying the carbohydrate content of an antibody of the invention by deleting one or more endogenous carbohydrate moieties of the antibody. In a specific embodiment, the invention encompasses shifting the glycosylation site of the Fc region of an antibody, by modifying positions adjacent to 297. In a specific embodiment, the invention encompasses modifying position 296 so that position 296 and not position 297 is glycosylated.

5.1 POLYPEPTIDES AND ANTIBODIES WITH VARIANT Fc REGIONS

[00117] The present invention is based, in part, on the identification of mutant human IgG1 heavy chain Fc regions, with altered affinities for different Fc γ R receptors, using a yeast display system. Accordingly, the invention relates to molecules, preferably polypeptides, and more preferably immunoglobulins (*e.g.,* antibodies), comprising a variant Fc region, having one or more amino acid modifications (*e.g.,* substitutions, but also including insertions or deletions) in one or more regions, which modifications alter the affinity of the variant Fc region for an Fc γ R. The affinities and binding properties of the molecules of the invention for an Fc γ R are initially determined using *in vitro* assays (biochemical or immunological based assays) known in the art for determining Fc-Fc γ R interactions, *i.e.,* specific binding of an Fc region to an Fc γ R including but not limited to

ELISA assay, surface plasmon resonance assay, immunoprecipitation assays (*See* Section 5.2.1). Preferably, the binding properties of the molecules of the invention are also characterized by in vitro functional assays for determining one or more Fc γ R mediator effector cell functions (*See* Section 5.2.6). In most preferred embodiments, the molecules of the invention have similar binding properties in in vivo models (such as those described and disclosed herein) as those in in vitro based assays. However, the present invention does not exclude molecules of the invention that do not exhibit the desired phenotype in in vitro based assays but do exhibit the desired phenotype in vivo.

A. MUTANTS WITH ENHANCED ALTERED AFFINITIES FOR

Fc γ RIIIA and/or Fc γ RIIA

[00118] The invention encompasses molecules comprising a variant Fc region, having one or more amino acid modifications (*e.g.*, substitutions) in one or more regions, wherein such modifications alter the affinity of the variant Fc region for an activating Fc γ R. In some embodiments, molecules of the invention comprise a variant Fc region, having one or more amino acid modifications (*e.g.*, substitutions) in one or more regions, which modifications increase the affinity of the variant Fc region for Fc γ RIIIA and/or Fc γ RIIA by at least 2-fold, relative to a comparable molecule comprising a wild-type Fc region. In another specific embodiment, molecules of the invention comprise a variant Fc region, having one or more amino acid modifications (*e.g.*, substitutions) in one or more regions, which modifications increase the affinity of the variant Fc region for Fc γ RIIIA and/or Fc γ RIIA by greater than 2 fold, relative to a comparable molecule comprising a wild-type Fc region. In other embodiments of the invention the one or more amino acid modifications increase the affinity of the variant Fc region for Fc γ RIIIA and/or Fc γ RIIA by at least 3-fold, 4-fold, 5-fold, 6-fold, 8-fold, or 10-fold relative to a comparable molecule comprising a wild-type Fc region. In yet other embodiments of the invention the one or more amino acid modifications decrease the affinity of the variant Fc region for Fc γ RIIIA and/or Fc γ RIIA by at least 3-fold, 4-fold, 5-fold, 6-fold, 8-fold, or 10-fold relative to a comparable molecule comprising a wild-type Fc region. Such fold increases are preferably determined by an ELISA or surface plasmon resonance assays. In a specific embodiment, the one or more amino acid modifications do not include or are not solely a substitution at any one of positions 329, 331, or 322 with any amino acid. In certain embodiments, the one or more amino acid modification do not include or are not solely a substitution with any one of alanine at positions 256, 290, 298, 312, 333, 334, 359, 360, or 430; with lysine at position 330; with threonine at position 339; with methionine at position 320; with serine, asparagine, aspartic acid, or glutamic acid at position 326 with glutamine, glutamic acid,

methionine, histidine, valine, or leucine at position 334. In another specific embodiment, the one or more amino acid modifications do not include or are not solely a substitution at any of positions 280, 290, 300, 294, or 295. In another more specific embodiment, the one or more amino acid modification does not include or are not solely a substitution at position 300 with leucine or isoleucine; at position 295 with lysine; at position 294 with asparagine; at position 298 with valine, aspartic acid, proline, asparagine, or valine; at position 280 with histidine, glutamine or tyrosine; at position 290 with serine, glycine, theonine or tyrosine.

[00119] In another specific embodiment, the invention encompasses a molecule comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ R1IA with a greater affinity than a comparable molecule comprising the wild-type Fc region binds Fc γ R1IA, provided that said variant Fc region does not have an alanine at any of positions 256, 290, 326, 255, 258, 267, 272, 276, 280, 283, 285, 286, 331, 337, 268, 272, or 430; an asparagine at position 268; a glutamine at position 272; a glutamine, serine, or aspartic acid at position 286; a serine at position 290; a methionine, glutamine, glutamic acid, or arginine at position 320; a glutamic acid at position 322; a serine, glutamic acid, or aspartic acid at position 326; a lysine at position 330; a glutamine at position 335; or a methionine at position 301. In a specific embodiment, molecules of the invention comprise a variant Fc region, having one or more amino acid modifications (*e.g.*, substitutions) in one or more regions, which modifications increase the affinity of the variant Fc region for Fc γ R1IA by at least 2-fold, relative to a comparable molecule comprising a wild-type Fc region. In another specific embodiment, molecules of the invention comprise a variant Fc region, having one or more amino acid modifications (*e.g.*, substitutions) in one or more regions, which modifications increase the affinity of the variant Fc region for Fc γ R1IA by greater than 2 fold, relative to a comparable molecule comprising a wild-type Fc region. In other embodiments of the invention the one or more amino acid modifications increase the affinity of the variant Fc region for Fc γ R1IA by at least 3-fold, 4-fold, 5-fold, 6-fold, 8-fold, or 10-fold relative to a comparable molecule comprising a wild-type Fc region

[00120] In a specific embodiment, the invention encompasses molecules, preferably polypeptides, and more preferably immunoglobulins (*e.g.*, antibodies), comprising a variant Fc region, having one or more amino acid modifications (*e.g.*, substitutions but also include insertions or deletions), which modifications increase the affinity of the variant Fc region for Fc γ R1IA and/or Fc γ R1IA by at least 65%, at least 70%, at least 75%, at least 85%, at least 90%, at least 95%, at least 99%, at least 100%, at least 150%, and at least 200%, relative to a comparable molecule comprising a wild-type Fc region.

[00121] In a specific embodiment, the one or more amino acid modifications which increase the affinity of the variant Fc region comprise a substitution at position 347 with histidine, and at position 339 with valine; or a substitution at position 425 with isoleucine and at position 215 with phenylalanine; or a substitution at position 408 with isoleucine, at position 215 with isoleucine, and at position 125 with leucine; or a substitution at position 385 with glutamic acid and at position 247 with histidine; or a substitution at position 348 with methionine, at position 334 with asparagine, at position 275 with isoleucine, at position 202 with methionine, and at position 147 with threonine; or a substitution at position 275 with isoleucine, at position 334 with asparagine, and at position 348 with methionine; or a substitution at position 279 with leucine and at position 395 with serine; or a substitution at position 246 with threonine and at position 319 with phenylalanine; or a substitution at position 243 with isoleucine and at position 379 with leucine; or a substitution at position 243 with leucine, at position 255 with leucine and at position 318 with lysine; or a substitution at position 334 with glutamic acid, at position 359 with asparagine, and at position 366 with serine; or a substitution at position 288 with methionine and at position 334 with glutamic acid; or a substitution at position 334 with glutamic acid and at position 380 with aspartic acid; or a substitution at position 256 with serine, at position 305 with isoleucine, at position 334 with glutamic acid and at position 390 with serine; or a substitution at position 335 with asparagine, at position 370 with glutamic acid, at position 378 with valine, at position 394 with methionine, and at position 424 with leucine; or a substitution at position 233 with aspartic acid and at position 334 with glutamic acid; or a substitution at position 334 with glutamic acid, at position 359 with asparagine, at position 366 with serine, and at position 386 with arginine; or a substitution at position 246 with threonine and at position 396 with histidine; or a substitution at position 268 with aspartic acid and at position 318 with aspartic acid; or a substitution at position 288 with asparagine, at position 330 with serine, and at position 396 with leucine; or a substitution at position 244 with histidine, at position 358 with methionine, at position 379 with methionine, at position 384 with lysine and at position 397 with methionine; or a substitution at position 217 with serine, at position 378 with valine, and at position 408 with arginine; or a substitution at position 247 with leucine, at position 253 with asparagine, and at position 334 with asparagine; or a substitution at position 246 with isoleucine, and at position 334 with asparagine; or a substitution at position 320 with glutamic acid and at position 326 with glutamic acid; or a substitution at position 375 with cysteine and at position 396 with leucine. Examples of other amino acid substitutions that results in an enhanced affinity for FcγRIIIA in vitro are disclosed below and summarized in Table 4.

[00122] The invention encompasses a molecule comprising a variant Fc region, wherein said variant Fc region comprises a substitution at position 243 with isoleucine and at position 379 with leucine, such that said molecule binds FcγRIIIA with about a 1.5 fold higher affinity than a comparable molecule comprising the wild type Fc region binds FcγRIIIA, as determined by an ELISA assay. In a specific embodiment, the invention encompasses a molecule comprising a variant Fc region, wherein said variant Fc region comprises a substitution at position 288 with asparagine, at position 330 with serine, and at position 396 with leucine, such that said molecule binds FcγRIIIA with about a 5 fold higher affinity than a comparable molecule comprising the wild type Fc region binds FcγRIIIA, as determined by an ELISA assay. In a specific embodiment, the invention encompasses a molecule comprising a variant Fc region, wherein said variant Fc region comprises a substitution at position 243 with leucine and at position 255 with leucine such that said molecule binds FcγRIIIA with about a 1 fold higher affinity than a comparable molecule comprising the wild type Fc region binds FcγRIIIA, as determined by an ELISA assay. In a specific embodiment, the invention encompasses a molecule comprising a variant Fc region, wherein said variant Fc region comprises a substitution at position 334 with glutamic acid, at position 359 with asparagine, and at position 366 with serine, such that said molecule binds FcγRIIIA with about a 1.5 fold higher affinity than a comparable molecule comprising the wild type Fc region binds FcγRIIIA, as determined by an ELISA assay. In a specific embodiment, the invention encompasses a molecule comprising a variant Fc region, wherein said variant Fc region comprises a substitution at position 288 with methionine and at position 334 with glutamic acid, such that said molecule binds FcγRIIIA with about a 3 fold higher affinity than a comparable molecule comprising the wild type Fc region binds FcγRIIIA, as determined by an ELISA assay. In a specific embodiment, the invention encompasses a molecule comprising a variant Fc region, wherein said variant Fc region comprises a substitution at position 316 with aspartic acid, at position 378 with valine, and at position 399 with glutamic acid, such that said molecule binds FcγRIIIA with about a 1.5 fold higher affinity than a comparable molecule comprising the wild type Fc region binds FcγRIIIA, as determined by an ELISA assay. In a specific embodiment, the invention encompasses a molecule comprising a variant Fc region, wherein said variant Fc region comprises a substitution at position 315 with isoleucine, at position 379 with methionine, and at position 399 with glutamic acid, such that said molecule binds FcγRIIIA with about a 1 fold higher affinity than a comparable molecule comprising the wild type Fc region binds FcγRIIIA, as determined by an ELISA assay. In a

specific embodiment, the invention encompasses a molecule comprising a variant Fc region, wherein said variant Fc region comprises a substitution at position 243 with isoleucine, at position 379 with leucine, and at position 420 with valine, such that said molecule binds FcγRIIIA with about a 2.5 fold higher affinity than a comparable molecule comprising the wild type Fc region binds FcγRIIIA, as determined by an ELISA assay. In a specific embodiment, the invention encompasses a molecule comprising a variant Fc region, wherein said variant Fc region comprises a substitution at position 247 with leucine, and at position 421 with lysine, such that said molecule binds FcγRIIIA with about a 3 fold higher affinity than a comparable molecule comprising the wild type Fc region binds FcγRIIIA, as determined by an ELISA assay. In a specific embodiment, the invention encompasses a molecule comprising a variant Fc region, wherein said variant Fc region comprises a substitution at position 392 with threonine and at position 396 with leucine such that said molecule binds FcγRIIIA with about a 4.5 fold higher affinity than a comparable molecule comprising the wild type Fc region binds FcγRIIIA, as determined by an ELISA assay. In a specific embodiment, the invention encompasses a molecule comprising a variant Fc region, wherein said variant Fc region comprises a substitution at position 293 with valine, at position 295 with glutamic acid, and at position 327 with threonine, such that said molecule binds FcγRIIIA with about a 1.5 fold higher affinity than a comparable molecule comprising the wild type Fc region binds FcγRIIIA, as determined by an ELISA assay. In a specific embodiment, the invention encompasses a molecule comprising a variant Fc region, wherein said variant Fc region comprises a substitution at position 268 with asparagine and at position 396 with leucine, such that said molecule binds FcγRIIIA with about a 2 fold higher affinity than a comparable molecule comprising the wild type Fc region binds FcγRIIIA, as determined by an ELISA assay. In a specific embodiment, the invention encompasses a molecule comprising a variant Fc region, wherein said variant Fc region comprises a substitution at position 319 with phenylalanine, at position 352 with leucine, and at position 396 with leucine, such that said molecule binds FcγRIIIA with about a 2 fold higher affinity than a comparable molecule comprising the wild type Fc region binds FcγRIIIA, as determined by an ELISA assay.

[00123] In a specific embodiment, the invention encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds FcγRIIIA with a greater affinity than a comparable polypeptide comprising the wild-type Fc region, wherein said at least one amino acid modification comprises substitution at

position 396 with histidine. In a specific embodiment, the invention encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ R11A with a greater affinity than a comparable polypeptide comprising the wild-type Fc region, wherein said at least one amino acid modification comprises substitution at position 248 with methionine. The invention encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ R11A with a similar affinity than a comparable polypeptide comprising the wild-type Fc region, wherein said at least one amino acid modification comprises substitution at position 392 with arginine. The invention encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ R11A with a similar affinity than a comparable polypeptide comprising the wild-type Fc region, wherein said at least one amino acid modification comprises substitution at position 315 with isoleucine. The invention encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ R11A with a similar affinity than a comparable polypeptide comprising the wild-type Fc region, wherein said at least one amino acid modification comprises substitution at position 132 with isoleucine. The invention encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ R11A with a similar affinity than a comparable polypeptide comprising the wild-type Fc region, wherein said at least one amino acid modification comprises substitution at position 162 with valine. The invention encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ R11A with a greater affinity than a comparable polypeptide comprising the wild-type Fc region, wherein said at least one amino acid modification comprises substitution at position 396 with leucine. The invention encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ R11A with a greater affinity than a comparable polypeptide comprising the wild-type Fc region, wherein said at least one

amino acid modification comprises substitution at position 379 with methionine. The invention encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ RIIIA with a greater affinity than a comparable polypeptide comprising the wild-type Fc region, wherein said at least one amino acid modification comprises substitution at position 219 with tyrosine. The invention encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ RIIIA with a greater affinity than a comparable polypeptide comprising the wild-type Fc region, wherein said at least one amino acid modification comprises substitution at position 282 with methionine. The invention encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ RIIIA with a greater affinity than a comparable polypeptide comprising the wild-type Fc region, wherein said at least one amino acid modification comprises substitution at position 401 with valine. The invention encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ RIIIA with a greater affinity than a comparable polypeptide comprising the wild-type Fc region, wherein said at least one amino acid modification comprises substitution at position 222 with asparagine. The invention encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ RIIIA with a greater affinity than a comparable polypeptide comprising the wild-type Fc region, wherein said at least one amino acid modification comprises substitution at position 334 with glutamic acid. The invention encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ RIIIA with a greater affinity than a comparable polypeptide comprising the wild-type Fc region, wherein said at least one amino acid modification comprises substitution at position 377 with phenylalanine. The invention encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ RIIIA with a greater affinity than a comparable polypeptide comprising the wild-type Fc region, wherein said at least one

amino acid modification comprises substitution at position 334 with isoleucine. The invention encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ R11A with a greater affinity than a comparable polypeptide comprising the wild-type Fc region, wherein said at least one amino acid modification comprises substitution at position 247 with leucine. The invention encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ R11A with a greater affinity than a comparable polypeptide comprising the wild-type Fc region, wherein said at least one amino acid modification comprises substitution at position 326 with glutamic acid. The invention encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ R11A with a greater affinity than a comparable polypeptide comprising the wild-type Fc region, wherein said at least one amino acid modification comprises substitution at position 372 with tyrosine. The invention encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ R11A with a greater affinity than a comparable polypeptide comprising the wild-type Fc region, wherein said at least one amino acid modification comprises substitution at position 224 with leucine.

[00124] The invention encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ R11A with a greater affinity than a comparable polypeptide comprising the wild-type Fc region, wherein said at least one amino acid modification comprises substitution at position 275 with tyrosine. The invention encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ R11A with a greater affinity than a comparable polypeptide comprising the wild-type Fc region, wherein said at least one amino acid modification comprises substitution at position 398 with valine. The invention encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ R11A with a greater affinity than a comparable polypeptide comprising the wild-type Fc region,

wherein said at least one amino acid modification comprises substitution at position 334 with asparagine. The invention encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ R11A

5 with a greater affinity than a comparable polypeptide comprising the wild-type Fc region, wherein said at least one amino acid modification comprises substitution at position 400 with proline. The invention encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ R11A

10 with a greater affinity than a comparable polypeptide comprising the wild-type Fc region, wherein said at least one amino acid modification comprises substitution at position 407 with isoleucine. The invention encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ R11A

15 with a greater affinity than a comparable polypeptide comprising the wild-type Fc region, wherein said at least one amino acid modification comprises substitution at position 372 with tyrosine. The invention encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ R11A

20 with a similar affinity than a comparable polypeptide comprising the wild-type Fc region, wherein said at least one amino acid modification comprises substitution at position 366 with asparagine. The invention encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ R11A

25 with a reduced affinity than a comparable polypeptide comprising the wild-type Fc region, wherein said at least one amino acid modification comprises substitution at position 414 with asparagine. The invention encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ R11A

30 with a reduced affinity than a comparable polypeptide comprising the wild-type Fc region, wherein said at least one amino acid modification comprises substitution at position 225 with serine. The invention encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ R11A

35 with a reduced affinity than a comparable polypeptide comprising the wild-type Fc region,

wherein said at least one amino acid modification comprises substitution at position 377 with asparagine.

- [00125] In a specific embodiment, the invention encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ RIIIA with about a 2 fold greater affinity than a comparable polypeptide comprising the wild-type Fc region as determined by an ELISA assay, wherein said at least one amino acid modification comprises substitution at position 379 with methionine. In another specific embodiment, the invention encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ RIIIA with about a 1.5 fold greater affinity than a comparable polypeptide comprising the wild-type Fc region as determined by an ELISA assay, wherein said at least one amino acid modification comprises substitution at position 248 with methionine.
- [00126] In some embodiments, the molecules of the invention have an altered affinity for Fc γ RIIIA and/or Fc γ RIIA as determined using *in vitro* assays (biochemical or immunological based assays) known in the art for determining Fc-Fc γ R interactions, *i.e.*, specific binding of an Fc region to an Fc γ R including but not limited to ELISA assay, surface plasmon resonance assay, immunoprecipitation assays (*See* Section 5.2.1).
- Preferably, the binding properties of these molecules with altered affinities for activating Fc γ R receptors are also correlated to their activity as determined by *in vitro* functional assays for determining one or more Fc γ R mediator effector cell functions (*See* Section 5.2.6), *e.g.*, molecules with variant Fc regions with enhanced affinity for Fc γ RIIIA have an enhanced ADCC activity. In most preferred embodiments, the molecules of the invention that have an altered binding property for an activating Fc receptor, *e.g.*, Fc γ RIIIA in an *in vitro* assay also have an altered binding property in *in vivo* models (such as those described and disclosed herein). However, the present invention does not exclude molecules of the invention that do not exhibit an altered Fc γ R binding in *in vitro* based assays but do exhibit the desired phenotype *in vivo*.

30 **B. *MUTANTS WITH ENHANCED AFFINITY FOR Fc γ RIIIA AND REDUCED OR NO AFFINITY FOR Fc γ RIIB***

[00127] In a specific embodiment, the molecules of the invention comprise a variant Fc region, having one or more amino acid modifications (*i.e.*, substitutions) in one or more

regions, which one or more modifications increase the affinity of the variant Fc region for Fc γ RIIA and decreases the affinity of the variant Fc region for Fc γ RIIB, relative to a comparable molecule comprising a wild-type Fc region which binds Fc γ RIIA and Fc γ RIIB with wild-type affinity. In a certain embodiment, the one or more amino acid modifications do not include or are not solely a substitution with alanine at any of positions 256, 298, 333, 334, 280, 290, 294, 298, or 296; or a substitution at position 298 with asparagine, valine, aspartic acid, or proline; or a substitution 290 with serine.. In certain amino embodiments, the one or more amino acid modifications increases the affinity of the variant Fc region for Fc γ RIIA by at least 65%, at least 70%, at least 75%, at least 85%, at least 90%, at least 95%, at least 99%, at least 100%, at least 200%, at least 300%, at least 400% and decreases the affinity of the variant Fc region for Fc γ RIIB by at least 65%, at least 70%, at least 75%, at least 85%, at least 90%, at least 95%, at least 99%, at least 100%, at least 200%, at least 300%, at least 400%.

[00128] In a specific embodiment, the molecule of the invention comprising a variant Fc region with an enhanced affinity for Fc γ RIIA and a lowered affinity or no affinity for Fc γ RIIB, as determined based on an ELISA assay and/or an ADCC based assay using ch-4-4-20 antibody carrying the variant Fc region comprises a substitution at position 275 with isoleucine, at position 334 with asparagine, and at position 348 with methionine; or a substitution at position 279 with leucine and at position 395 with serine; or a substitution at position 246 with threonine and at position 319 with phenylalanine; or a substitution at position 243 with leucine, at position 255 with leucine, and at position 318 with lysine; or a substitution at position 334 with glutamic acid, at position 359 with asparagine and at position 366 with serine; or a substitution at position 334 with glutamic acid and at position 380 with aspartic acid; or a substitution at position 256 with serine, at position 305 with isoleucine, at position 334 with glutamic acid, and at position 390 with serine; or a substitution at position 335 with asparagine, at position 370 with glutamic acid, at position 378 with valine, at position 394 with methionine and at position 424 with leucine; or a substitution at position 233 with aspartic acid and at position 334 with glutamic acid; or a substitution at position 334 with glutamic acid, at position 359 with asparagine, at position 366 with serine and at position 386 with arginine; or a substitution at position 312 with glutamic acid, at position 327 with asparagine, and at position 378 with serine; or a substitution at position 288 with asparagine and at position 326 with asparagine; or a substitution at position 247 with leucine and at position 421 with lysine; or a substitution at position 298 with asparagine and at position 381 with arginine; or a substitution at position 280 with glutamic acid, at position 354 with phenylalanine, at position 431 with aspartic

acid, and at position 441 with isoleucine; or a substitution at position 255 with glutamine and at position 326 with glutamic acid; or a substitution at position 218 with arginine, at position 281 with aspartic acid and at position 385 with arginine; or a substitution at position 247 with leucine, at position 330 with threonine and at position 440 with glycine; or a substitution at position 284 with alanine and at position 372 with leucine; or a substitution at position 335 with asparagine, at position 387 with serine and at position 435 with glutamine; or a substitution at position 247 with leucine, at position 431 with valine and at position 442 with phenylalanine.

[00129] In a specific embodiment, the molecule of the invention comprising a variant Fc region with an enhanced affinity for Fc γ RIIA and a lowered affinity or no affinity for Fc γ RIIB as determined based on an ELISA assay and/or an ADCC based assay using ch-4-4-20 antibody carrying the variant Fc region comprises a substitution at position 379 with methionine; at position 219 with tyrosine; at position 282 with methionine; at position 401 with valine; at position 222 with asparagine; at position 334 with isoleucine; at position 334 with glutamic acid; at position 275 with tyrosine; at position 398 with valine.

[00130] The invention encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ RIIB with about a 3 fold lower affinity than a comparable polypeptide comprising the wild-type Fc region as determined by an ELISA assay, wherein said at least one amino acid modification comprises substitution at position 288 with asparagine, at position 330 with serine, and at position 396 with leucine. The invention encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ RIIB with about a 10-15 fold lower affinity than a comparable polypeptide comprising the wild-type Fc region as determined by an ELISA assay, wherein said at least one amino acid modification comprises substitution at position 316 with aspartic acid, at position 378 with valine, and at position 399 with glutamic acid. The invention encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ RIIB with about a 10 fold lower affinity than a comparable polypeptide comprising the wild-type Fc region as determined by an ELISA assay, wherein said at least one amino acid modification comprises substitution at position 315 with isoleucine, at position 379 with methionine, and at position 399 with glutamic acid. The invention encompasses an isolated polypeptide comprising a variant Fc region,

wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ RIIB with about a 7 fold lower affinity than a comparable polypeptide comprising the wild-type Fc region as determined by an ELISA assay, wherein said at least one amino acid modification

5 comprises substitution at position 243 with isoleucine, at position 379 with leucine, and at position 420 with valine. The invention encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ RIIB with about a 3 fold lower affinity than a comparable polypeptide comprising the

10 wild-type Fc region as determined by an ELISA assay, wherein said at least one amino acid modification comprises substitution at position 392 with threonine and at position 396 with leucine. The invention encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, such that said polypeptide specifically binds Fc γ RIIB with about a 5

15 fold lower affinity than a comparable polypeptide comprising the wild-type Fc region as determined by an ELISA assay, wherein said at least one amino acid modification comprises substitution at position 268 with asparagine and at position 396 with leucine. The invention also encompasses an isolated polypeptide comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a

20 wild-type Fc region, such that said polypeptide specifically binds Fc γ RIIB with about a 2 fold lower affinity than a comparable polypeptide comprising the wild-type Fc region as determined by an ELISA assay, wherein said at least one amino acid modification comprises substitution at position 319 with phenylalanine, at position 352 with leucine, and at position 396 with leucine.

25 C. **MUTANTS WITH ENHANCED AFFINITY TO Fc γ RIIA AND Fc γ RIIB**

[00131] The invention encompasses molecules comprising variant Fc regions, having one or more amino acid modifications, which modifications increase the affinity of the variant Fc region for Fc γ RIIA and Fc γ RIIB by at least 65%, at least 70%, at least 75%, at

30 least 85%, at least 90%, at least 95%, at least 99%, at least 100%, at least 200%, at least 300%, at least 400% and decreases the affinity of the variant Fc region for Fc γ RIIB by at least 65%, at least 70%, at least 75%, at least 85%, at least 90%, at least 95%, at least 99%, at least 100%, at least 200%, at least 300%, at least 400%. In a specific embodiment, the molecule of the invention comprising a variant Fc region with an enhanced affinity for

35 Fc γ RIIA and an enhanced affinity for Fc γ RIIB (as determined based on an ELISA assay

and/or an ADCC based assay using ch-4-4-20 antibody carrying the variant Fc region as described herein) comprises a substitution at position 415 with isoleucine and at position 251 with phenylalanine; or a substitution at position 399 with glutamic acid, at position 292 with leucine, and at position 185 with methionine; or a substitution at position 408 with isoleucine, at position 215 with isoleucine, and at position 125 with leucine; or a substitution at position 385 with glutamic acid and at position 247 with histidine; or a substitution at position 348 with methionine, at position 334 with asparagine, at position 275 with isoleucine, at position 202 with methionine and at position 147 with threonine; or a substitution at position 246 with threonine and at position 396 with histidine; or a substitution at position 268 with aspartic acid and at position 318 with aspartic acid; or a substitution at position 288 with asparagine, at position 330 with serine and at position 396 with leucine; or a substitution at position 244 with histidine, at position 358 with methionine, at position 379 with methionine, at position 384 with lysine and at position 397 with methionine; or a substitution at position 217 with serine, at position 378 with valine, and at position 408 with arginine; or a substitution at position 247 with leucine, at position 253 with asparagine, and at position 334 with asparagine; or a substitution at position 246 with isoleucine and at position 334 with asparagine; or a substitution at position 320 with glutamic acid and at position 326 with glutamic acid; or a substitution at position 375 with cysteine and at position 396 with leucine; or a substitution at position 343 with serine, at position 353 with leucine, at position 375 with isoleucine, at position 383 with asparagine; or a substitution at position 394 with methionine and at position 397 with methionine; or a substitution at position 216 with aspartic acid, at position 345 with lysine and at position 375 with isoleucine; or a substitution at position 288 with asparagine, at position 330 with serine, and at position 396 with leucine; or a substitution at position 247 with leucine and at position 389 with glycine; or a substitution at position 222 with asparagine, at position 335 with asparagine, at position 370 with glutamic acid, at position 378 with valine and at position 394 with methionine; or a substitution at position 316 with aspartic acid, at position 378 with valine and at position 399 with glutamic acid; or a substitution at position 315 with isoleucine, at position 379 with methionine, and at position 394 with methionine; or a substitution at position 290 with threonine and at position 371 with aspartic acid; or a substitution at position 247 with leucine and at position 398 with glutamine; or a substitution at position 326 with glutamine; at position 334 with glutamic acid, at position 359 with asparagine, and at position 366 with serine; or a substitution at position 247 with leucine and at position 377 with phenylalanine; or a substitution at position 378 with valine, at position 390 with isoleucine and at position 422 with isoleucine; or a substitution

at position 326 with glutamic acid and at position 385 with glutamic acid; or a substitution
 at position 282 with glutamic acid, at position 369 with isoleucine and at position 406 with
 phenylalanine; or a substitution at position 397 with methionine; at position 411 with
 alanine and at position 415 with asparagine; or a substitution at position 223 with
 5 isoleucine, at position 256 with serine and at position 406 with phenylalanine; or a
 substitution at position 298 with asparagine and at position 407 with arginine; or a
 substitution at position 246 with arginine, at position 298 with asparagine, and at position
 377 with phenylalanine; or a substitution at position 235 with proline, at position 382 with
 methionine, at position 304 with glycine, at position 305 with isoleucine, and at position 323
 10 with isoleucine; or a substitution at position 247 with leucine, at position 313 with arginine,
 and at position 388 with glycine; or a substitution at position 221 with tyrosine, at position
 252 with isoleucine, at position 330 with glycine, at position 339 with threonine, at position
 359 with asparagine, at position 422 with isoleucine, and at position 433 with leucine; or a
 substitution at position 258 with aspartic acid, and at position 384 with lysine; or a
 15 substitution at position 241 with leucine and at position 258 with glycine; or a substitution
 at position 370 with asparagine and at position 440 with asparagine; or a substitution at
 position 317 with asparagine and a deletion at position 423; or a substitution at position 243
 with isoleucine, at position 379 with leucine and at position 420 with valine; or a
 substitution at position 227 with serine and at position 290 with glutamic acid; or a
 20 substitution at position 231 with valine, at position 386 with histidine, and at position 412
 with methionine; or a substitution at positions 215 with proline, at position 274 with
 asparagine, at position 287 with glycine, at position 334 with asparagine, at position 365
 with valine and at position 396 with leucine; or a substitution at position 293 with valine, at
 position 295 with glutamic acid and at position 327 with threonine; or a substitution at
 25 position 319 with phenylalanine, at position 352 with leucine, and at position 396 with
 leucine; or a substitution at position 392 with threonine and at position 396 with leucine; at
 a substitution at position 268 with asparagine and at position 396 with leucine; or a
 substitution at position 290 with threonine, at position 390 with isoleucine, and at position
 396 with leucine; or a substitution at position 326 with isoleucine and at position 396 with
 30 leucine; or a substitution at position 268 with aspartic acid and at position 396 with leucine;
 or a substitution at position 210 with methionine and at position 396 with leucine; or a
 substitution at position 358 with proline and at position 396 with leucine; or a substitution at
 position 288 with arginine, at position 307 with alanine, at position 344 with glutamic acid,
 and at position 396 with leucine; or a substitution at position 273 with isoleucine, at position
 35 326 with glutamic acid, at position 328 with isoleucine and at position 396 with leucine; or

a substitution at position 326 with isoleucine, at position 408 with asparagine and at position 396 with leucine; or a substitution at position 334 with asparagine and at position 396 with leucine; or a substitution at position 379 with methionine and at position 396 with leucine; or a substitution at position 227 with serine and at position 396 with leucine; or a substitution at position 217 with serine and at position 396 with leucine; or a substitution at position 261 with asparagine, at position 210 with methionine and at position 396 with leucine; or a substitution at position 419 with histidine and at position 396 with leucine; or a substitution at position 370 with glutamic acid and at position 396 with leucine; or a substitution at position 242 with phenylalanine and at position 396 with leucine; or a substitution at position 255 with leucine and at position 396 with leucine; or a substitution at position 240 with alanine and at position 396 with leucine; or a substitution at position 250 with serine and at position 396 with leucine; or a substitution at position 247 with serine and at position 396 with leucine; or a substitution at position 410 with histidine and at position 396 with leucine; or a substitution at position 419 with leucine and at position 396 with leucine; or a substitution at position 427 with alanine and at position 396 with leucine; or a substitution at position 258 with aspartic acid and at position 396 with leucine; or a substitution at position 384 with lysine and at position 396 with leucine; or a substitution at position 323 with isoleucine and at position 396 with leucine; or a substitution at position 244 with histidine and at position 396 with leucine; or a substitution at position 305 with leucine and at position 396 with leucine; or a substitution at position 400 with phenylalanine and at position 396 with leucine; or a substitution at position 303 with isoleucine and at position 396 with leucine; or a substitution at position 243 with leucine, at position 305 with isoleucine, at position 378 with aspartic acid, at position 404 with serine and at position 396 with leucine; or a substitution at position 290 with glutamic acid, at position 369 with alanine, at position 393 with alanine and at position 396 with leucine; or a substitution at position 210 with asparagine, at position 222 with isoleucine, at position 320 with methionine and at position 396 with leucine; or a substitution at position 217 with serine, at position 305 with isoleucine, at position 309 with leucine, at position 390 with histidine and at position 396 with leucine; or a substitution at position 246 with asparagine; at position 419 with arginine and at position 396 with leucine; or a substitution at position 217 with alanine, at position 359 with alanine and at position 396 with leucine; or a substitution at position 215 with isoleucine, at position 290 with valine and at position 396 with leucine; or a substitution at position 275 with leucine, at position 362 with histidine, at position 384 with lysine and at position 396 with leucine; or a substitution at position 334 with asparagine; or a substitution at position 400 with proline; or a substitution at position 407

with isoleucine; or a substitution at position 372 with tyrosine; or a substitution at position 366 with asparagine; or a substitution at position 414 with asparagine; or a substitution at position 352 with leucine; or a substitution at position 225 with serine; or a substitution at position 377 with asparagine; or a substitution at position 248 with methionine.

5 ***D.. MUTANTS THAT DO NOT BIND ANY FcγR***

[00132] In some embodiments, the invention encompasses molecules comprising a variant Fc region, wherein said variant Fc region comprises at least one amino acid modification relative to a wild-type Fc region, which variant Fc region does not bind any FcγR, as determined by standard assays known in the art and disclosed herein, relative to a comparable molecule comprising the wild type Fc region. In a specific embodiment, the one or more amino acid modifications which abolish binding to all FcγRs comprise a substitution at position 232 with serine and at position 304 with glycine; or a substitution at position 269 with lysine, at position 290 with asparagine, at position 311 with arginine, and at position 433 with tyrosine; or a substitution at position 252 with leucine; or a substitution at position 216 with aspartic acid, at position 334 with arginine, and at position 375 with isoleucine; or a substitution at position 247 with leucine and at position 406 with phenylalanine, or a substitution at position 335 with asparagine, at position 387 with serine, and at position 435 with glutamine; or a substitution at position 334 with glutamic acid, at position 380 with aspartic acid, and at position 446 with valine; or a substitution at position 303 with isoleucine, at position 369 with phenylalanine, and at position 428 with leucine; or a substitution at position 251 with phenylalanine and at position 372 with leucine; or a substitution at position 246 with glutamic acid, at position 284 with methionine and at position 308 with alanine; or a substitution at position 399 with glutamic acid and at position 402 with aspartic acid; or a substitution at position 399 with glutamic acid and at position 428 with leucine.

D. MUTANTS WITH ALTERED FcγR-MEDIATED EFFECTOR FUNCTIONS

30 [00133] In a specific embodiment, the immunoglobulins of the invention with enhanced affinity for FcγRIIIA and/or FcγRIIA have an enhanced FcγR-mediated effector function as determined using ADCC activity assays disclosed herein. Examples of effector functions that could be mediated by the molecules of the invention include, but are not limited to, C1q binding, complement-dependent cytotoxicity, antibody-dependent cell mediate cytotoxicity (ADCC), phagocytosis, *etc.* The effector functions of the molecules of

the invention can be assayed using standard methods known in the art, examples of which are disclosed in Section 5.2.6. In a specific embodiment, the immunoglobulins of the invention comprising a variant Fc region with enhanced affinity for Fc γ RIIA and/or Fc γ RIIA mediate antibody dependent cell mediated cytotoxicity (ADCC) 2- fold more effectively, than an immunoglobulin comprising a wild-type Fc region. In other embodiments, the immunoglobulins of the invention comprising a variant Fc region with enhanced affinity for Fc γ RIIA and/or Fc γ RIIA mediate antibody dependent cell mediated cytotoxicity (ADCC) at least 4- fold, at least 8-fold, at least 10-fold, at least 100-fold, at least 1000-fold, at least 10⁴-fold, at least 10⁵-fold more effectively, than an immunoglobulin comprising a wild-type Fc region. In another specific embodiment, the immunoglobulins of the invention with enhanced affinity for Fc γ RIIA and/or Fc γ RIIA have altered C1q binding activity. In some embodiments, the immunoglobulins of the invention with enhanced affinity for Fc γ RIIA and/or Fc γ RIIA have at least 2-fold, at least 4- fold, at least 8-fold, at least 10-fold, at least 100-fold, at least 1000-fold, at least 10⁴-fold, at least 10⁵-fold higher C1q binding activity than an immunoglobulin comprising a wild-type Fc region. In yet another specific embodiment, the immunoglobulins of the invention with enhanced affinity for Fc γ RIIA and/or Fc γ RIIA have altered complement dependent cytotoxicity. In yet another specific embodiment, the immunoglobulins of the invention with enhanced affinity for Fc γ RIIA and/or Fc γ RIIA have an enhanced complement dependent cytotoxicity than an immunoglobulin comprising a wild-type Fc region. In some embodiments, the immunoglobulins of the invention with enhanced affinity for Fc γ RIIA and/or Fc γ RIIA have at least 2-fold, at least 4- fold, at least 8-fold, at least 10-fold, at least 100-fold, at least 1000-fold, at least 10⁴-fold, at least 10⁵-fold higher complement dependent cytotoxicity than an immunoglobulin comprising a wild-type Fc region.

[00134] In other embodiments, immunoglobulins of the invention with enhanced affinity for Fc γ RIIA and/or Fc γ RIIA have enhanced phagocytosis activity relative to an immunoglobulin comprising a wild-type Fc region, as determined by standard assays known to one skilled in the art or disclosed herein. In some embodiments, the immunoglobulins of the invention with enhanced affinity for Fc γ RIIA and/or Fc γ RIIA have at least 2-fold, at least 4- fold, at least 8-fold, at least 10-fold higher phagocytosis activity relative to an immunoglobulin comprising a wild-type Fc region.

[00135] In a specific embodiment, the invention encompasses an immunoglobulin comprising a variant Fc region with one or more amino acid modifications, with an enhanced affinity for Fc γ RIIA and/or Fc γ RIIA such that the immunoglobulin has an enhanced effector function, e.g., antibody dependent cell mediated cytotoxicity, or

phagocytosis. In a specific embodiment, the one or more amino acid modifications which increase the affinity of the variant Fc region for FcγRIIIA and/or FcγRIIA and increase the ADCC activity of the immunoglobulin comprise a substitution at position 379 with methionine; or a substitution at position 243 with isoleucine and at position 379 with leucine; or a substitution at position 288 with asparagine, at position 330 with serine, and at position 396 with leucine; or a substitution at position 243 leucine and at position 255 with leucine; or a substitution at position 334 with glutamic acid, at position 359 with asparagine, and at position 366 with serine; or a substitution at position 288 with methionine and at position 334 with glutamic acid; or a substitution at position 334 with glutamic acid and at position 292 with leucine; or a substitution at position 316 with aspartic acid, at position 378 with valine, and at position 399 with glutamic acid; or a substitution at position 315 with isoleucine, at position 379 with methionine, and at position 399 with glutamic acid; or a substitution at position 243 with isoleucine, at position 379 with leucine, and at position 420 with valine; or a substitution at position 247 with leucine and at position 421 with lysine; or a substitution at position 248 with methionine; or a substitution at position 392 with threonine and at position 396 with leucine; or a substitution at position 293 with valine, at position 295 with glutamic acid, and at position 327 with threonine; or a substitution at position 268 with asparagine and at position 396 with leucine; or a substitution at position 319 with phenylalanine, at position 352 with leucine, and at position 396 with leucine.

[00136] In another specific embodiment, the one or more amino acid modifications which increase the ADCC activity of the immunoglobulin is any of the mutations listed below, in table 4.

TABLE 4.

E333A, K334A
R292L, K334E
V379M
S219Y
V282M
K222N
F243I, V379L
F243L, R255L, E318K
K334I
K334E, T359N, T366S
K288M, K334E
K288N, A330S, P396L
K326E
G316D, A378V, D399E
N315I, V379M, T394M
F243I, V379L, G420V

E293V, Q295E, A327T
Y319F, P352L, P396L
K392T, P396L
K248M
H268N, P396L
K290T, N390I, P396L
K326I, P396L
H268D, P396L
K210M, P396L
L358P, P396L
K288R, T307A, K344E, P396L
V273I, K326E, L328I, P396L
K326I, S408N, P396L
K334N, P396L
V379M, P396L
P227S, P396L
P217S, P396L
K261N, K210M, P396L
Q419H, P396L
K370E, P396L
L242F, P396L
F243L, V305I, A378D, F404S, P396L
R255L, P396L
V240A, P396L
T250S, P396L
P247S, P396L
K290E, V369A, T393A, P396L
K210N, K222I, K320M, P396L
L410H, P396L
Q419L, P396L
V427A, P396L
P217S, V305I, I309L, N390H, P396L
E258D, P396L
N384K, P396L
V323I, P396L
K246N, Q419R, P396L
P217A, T359A, P396L
P244H, P396L
V215I, K290V, P396L
F275L, Q362H, N384K, P396L
V305L, P396L
S400F, P396L
V303I, P396L

[00137] The invention encompasses specific variants of the Fc region that have been identified using the methods of the invention from a yeast library of mutants after 2nd-4th-round of sorting are listed in Table 5. Table 5 summarizes the various mutants that were

5 identified using the methods of the invention. The mutants were assayed using an ELISA

assay for determining binding to FcγRIIIA and FcγRIIB. The mutants were also tested in an ADCC assay, by cloning the Fc variants into a ch 4-4-20 antibody using methods disclosed and exemplified herein. Bolded items refer to experiments, in which the ch4-4-20 were purified prior the ADCC assay. The antibody concentration used was in the range 0.5
5 μg/mL - 1.0 μg/mL.

TABLE 5: MUTATIONS IDENTIFIED IN THE Fc REGION

Mutations	Domain	Binding to FcγRIIIA (ELISA)	Binding to FcγRIIB (ELISA)	4-4-20 ADCC
pYD-CH1 library FACS screen with 3A tetramer				
Q347H; A339V	CH3	↑0.5x	NT	
S415I; L251F	CH2,CH3	↑0.5x	↑.75x	0.82
K392R	CH3	N/C	NT	
D399E; R292L; V185M	CH1,CH2,CH3	N/C	↑0.5x	0.65 0.9
K290E; L142P	CH1, CH2	N/C	NT	
R301C; M252L; S192T	CH1,CH2	↓.5x	NT	
P291S; K288E; H268L; A141V	CH1,CH2	↓.5x	NT	
N315I	CH2	N/C	↑.75x	
S132I	CH1	N/C	NT	
S383N; N384K; T256N; V262L; K218E; R214I; K205E; F149Y; K133M	All	↑0.5x	NT	
S408I; V215I; V125L	CH1,CH2,CH3	↑0.5x	↑.75x	0.62
P396L	CH3	↑1x	↑1x	0.55
G385E; P247H;	CH2, CH3	↑1x	↑.75x	0.44
P396H	CH3	↑1x	↑1x	0.58
A162V	CH1	N/C	NT	
V348M; K334N; F275I; Y202M; K147T	CH1,CH2,CH3	↑0.5x	↑.75x	0.33
H310Y; T289A; G337E	CH2	↑.5x	NT	
S119F; G371S; Y407V; E258D	CH1,CH2,CH3	N/C	N/C	0.29
K409R; S166N	CH1, CH3	N/C	NT	
in vitro Site Directed mutants				
R292L	CH2	NT	NT	0.82
T359N	CH3	NT	NT	1.06

Mutations	Domain	Binding to FcγRIIIA (ELISA)	Binding to FcγRIIB (ELISA)	4-4-20 ADCC
T366S	CH3	NT	NT	0.93
E333A, K334A	CH2	NT	NT	1.41
R292L, K334E	CH2	NT	NT	1.41; 1.64
R292L, P396L, T359N	CH2, CH3	NT	NT	0.89; 1.15
V379L	CH3	NT	NT	0.83
K288N	CH2	NT	NT	0.78
A330S	CH2	NT	NT	0.52
F243L	CH2	NT	NT	0.38
E318K	CH2	NT	NT	0.86
K288N, A330S	CH2	NT	NT	0.08
R255L, E318K	CH2	NT	NT	0.82
F243L, E318K	CH2	NT	NT	0.07
Mutants in 4-4-20 mini-library Increased FcγRIIIA binding, decreased or no change to FcγRIIB binding				
V379M	CH3	↑2x	N/C	1.47
S219Y	Hinge	↑1x	↓ or N/B	1.28
V282M	CH2	↑1x	↓ or N/B	1.25; 1
F275L, K334N, V348M	CH2	↑0.5x	N/C	
D401V	CH3	↑0.5x	N/C	
V279L, P395S	CH2	↑1x	N/C	
K222N	Hinge	↑1x	↓ or N/B	1.33; 0.63
K246T, Y319F	CH2	↑1x	N/C	
F243I, V379L	CH2, CH3	↑1.5x	↓ or N/B	1.86; 1.35
F243L, R255L, E318K	CH2	↑1x	↓ or N/B	1.81; 1.45
K334I	CH2	↑1x	N/C	2.1; 1.97
K334E, T359N, T366S	CH2, CH3	↑1.5x	N/C	1.49; 1.45

Mutations	Domain	Binding to FcγRIIIA (ELISA)	Binding to FcγRIIB (ELISA)	4-4-20 ADCC
K288M, K334E	CH2	↑ 3x	↓ or N/B	1.61; 1.69
K334E, E380D	CH2, CH3	↑ 1.5x	N/C	
T256S, V305I, K334E, N390S	CH2, CH3	↑ 1.5x	N/C	
K334E	CH2	↑ 2.5x	N/C	1.75; 2.18
T335N, K370E, A378V, T394M, S424L	CH2, CH3	↑ 0.5x	N/C	
E233D, K334E	CH2	↑ 1.5x	N/C	0.94; 1.02
K334E, T359N, T366S, Q386R	CH2	↑ 1x	N/C	
Increased Binding to FcγRIIIA and FcγRIIB				
K246T, P396H	CH2, CH3	↑ 1x	↑ 2.5x	
H268D, E318D	CH2	↑ 1.5x	↑ 5x	
K288N, A330S, P396L	CH2, CH3	↑ 5x	↑ 3x	2.34; 1.66; 2.54
I377F	CH3	↑ 1.5x	↑ 0.5x	
P244H, L358M, V379M, N384K, V397M	CH2, CH3	↑ 1.75x	↑ 1.5x	
P217S, A378V, S408R	Hinge, CH3	↑ 2x	↑ 4.5x	
P247L, I253N, K334N	CH2	↑ 3x	↑ 2.5x	
P247L	CH2	↑ 0.5x	↑ 4x	0.91; 0.84
F372Y	CH3	↑ 0.75x	↑ 5.5x	0.88; 0.59
K326E	CH2	↑ 2x	↑ 3.5x	1.63; 2
K246I, K334N	CH2	↑ 0.5x	↑ 4x	0.66; 0.6
K320E, K326E	CH2	↑ 1x	↑ 1x	
H224L	Hinge	↑ 0.5x	↑ 5x	0.55; 0.53
S375C, P396L	CH3	↑ 1.5x	↑ 4.5x	
Increased FcγRIIIA binding, decreased or no change to FcγRIIB binding				

Mutations	Domain	Binding to FcγRIIIA (ELISA)	Binding to FcγRIIB (ELISA)	4-4-20 ADCC
D312E,K327N,I378S	CH2,CH3	↑0.5x	N/C	
K288N, K326N	CH2	↑ 1x	N/C	
F275Y	CH2	↑ 3x	N/C	0.64
P247L,N421K	CH2,CH3	↑ 3x	N/C	2.0
S298N,W381R	CH2,CH3	↑ 2x	N/C	
D280E,S354F,A431D,L441I	CH2,CH3	↑ 3x	N/C	0.62
R255Q,K326E	CH2	↑ 2x	N/C	0.79
K218R,G281D,G385R	H,CH2,CH3	↑3.5x	N/C	0.67
L398V	CH3	↑1.5x	N/C	
P247L,A330T,S440G	CH2,CH3	↑0.75x	↓ 0.25x	
V284A,F372L	CH2,CH3	1x	N/C	
T335N,P387S,H435Q	CH2,CH3	1.25x	N/C	
P247L,A431V,S442F	CH2,CH3	1x	N/C	
Increased Binding to FcγRIIIA and FcγRIIB				
P343S,P353L,S375I,S383N	CH3	↑ 0.5x	↑ 6x	
T394M,V397M	CH3	↑0.5x	↑ 3x	
E216D,E345K,S375I	H, CH2,CH3	↑ 0.5x	↑ 4x	
K334N,	CH2	↑0.5x	↑ 2x	
K288N,A330S,P396L	CH2,CH3	↑0.5x	↑ 9x	
P247L,E389G	CH2,CH3	↑1.5x	↑ 9x	
K222N,T335N,K370E,A378V,T394M	H, CH2,CH3	↑ 1x	↑ 7x	
G316D,A378V,D399E	CH2,CH3	↑1.5x	↑ 14x	2.24
N315I,V379M,T394M	CH2,CH3	↑ 1x	↑ 9x	1.37
K290T,G371D,	CH2,CH3	↑ 0.25x	↑ 6x	
P247L,L398Q	CH2,CH3	↑ 1.25x	↑ 10x	

Mutations	Domain	Binding to FcγRIIIA (ELISA)	Binding to FcγRIIB (ELISA)	4-4-20 ADCC
K326Q,K334E,T359N,T366S	CH2,CH3	↑ 1.5x	↑ 5x	
S400P	CH3	↑ 1x	↑ 6x	
P247L,I377F	CH2,CH3	↑ 1x	↑ 5x	
A378V,N390I,V422I	CH3	↑ 0.5x	↑ 5x	
K326E,G385E	CH2,CH3	↑ 0.5x	↑ 15x	
V282E,V369I,L406F	CH2,CH3	↑ 0.5x	↑ 7x	
V397M,T411A,S415N	CH3	↑ 0.25x	↑ 5x	
T223I,T256S,L406F	H, CH2,CH3	↑ 0.25x	↑ 6x	
S298N,S407R	CH2,CH3	↑ 0.5x	↑ 7x	
K246R,S298N,I377F	CH2,CH3	↑ 1x	↑ 5x	
S407I	CH3	↑ 0.5x	↑ 4x	
F372Y	CH3	↑ 0.5x	↑ 4x	
L235P,V382M,S304G,V305I,V323I	CH2,CH3	↑ 2x	↑ 2x	
P247L,W313R,E388G	CH2,CH3	↑ 1.5x	↑ 1x	
D221Y,M252I,A330G,A339T,T359N,V422I,H433L	H, CH2,CH3	↑ 2.5x	↑ 6x	
E258D,N384K	CH2,CH3	↑ 1.25x	↑ 4x	
F241L,E258G	CH2	↑ 2x	↑ 2.5x	-0.08
K370N,S440N	CH3	↑ 1x	↑ 3.5x	
K317N,F423-deleted	CH2,CH3	↑ 2.5x	↑ 7x	0.18
F243I,V379L,G420V	CH2,CH3	↑ 2.5x	↑ 3.5x	1.35
P227S,K290E	H, CH2	↑ 1x	↑ 0.5x	
A231V,Q386H,V412M	CH2,CH3	↑ 1.5x	↑ 6x	
T215P,K274N,A287G,K334N,L365V,P396L	H, CH2,CH3	↑ 2x	↑ 4x	
Increased Binding to FcγRIIB but not FcγRIIIA				

Mutations	Domain	Binding to FcγRIIIA (ELISA)	Binding to FcγRIIB (ELISA)	4-4-20 ADCC
K334E, E380D	CH2, CH3	N/C	↑4.5x	
T366N	CH3	N/C	↑5x	
P244A, K326L, C367R, S375I, K447T	CH2, CH3	N/C	↑3x	
C229Y, A287T, V379M, P396L, L443V	H, CH2, CH3	↓0.25x	↑10x	
Decreased binding to FcγRIIIA and FcγRIIB				
R301H, K340E, D399E	CH2, CH3	↓0.50x	↓0.25x	
K414N	CH3	↓0.25x	N/B	
P291S, P353Q	CH2, CH3	↓0.50x	↓0.25x	
V240I, V281M	CH2	↓0.25x	↓0.25x	
P232S, S304G	CH2	N/B	N/B	
E269K, K290N, Q311R, H433Y	CH2, CH3	N/B	N/B	
M352L	CH3	N/B	N/B	
E216D, K334R, S375I	H, CH2, CH3	N/B	N/B	
P247L, L406F	CH2, CH3	N/B	N/B	
T335N, P387S, H435Q	CH2, CH3	N/B	N/B	
T225S	CH2	↓0.25x	↓0.50x	
D399E, M428L	CH3	↓0.50x	↓0.50x	
K246I, Q362H, K370E	CH2, CH3	N/B	↓0.50x	
K334E, E380D, G446V	CH2, CH3	N/B	N/B	
I377N	CH3	↓0.50x	N/B	
V303I, V369F, M428L	CH2, CH3	N/B	N/B	
L251F, F372L	CH2, CH3	N/B	N/B	
K246E, V284M, V308A	CH2, CH3	N/B	N/B	
D399E, G402D	CH3	N/B	N/B	

Mutations	Domain	Binding to FcγRIIIA (ELISA)	Binding to FcγRIIB (ELISA)	4-4-20 ADCC
D399E,M428L	CH3	N/B	N/B	
FcγRIIB depletion/FcγRIIIA selection: Naive Fc library.				
E293V,Q295E,A327T	CH2	↑0.4x	↓ or N/B	4.29
Y319F,P352L,P396L	CH2,CH3	↑3.4x	↑2x	1.09
K392T,P396L	CH3	↑4.5x	↑2.5x	3.07
K248M	CH2	↑0.4x	↓ or N/B	4.03
H268N,P396L	CH2,CH3	↑2.2x	↑4.5x	2.24
Solution competition 40X FcγRIIB-G2:P396L Library				
D221E, D270E, V308A, Q311H, P396L, G402D		↑3.6x	↑0.1x	3.17
Equilibrium Screen: 0.8 μM FcγRIIIA monomer: P396L library				
K290T, N390I, P396L	CH2, CH3	↑2.8x	↑6.1x	1.93
K326L, P396L	CH2, CH3	↑2.9x	↑5.9x	1.16
H268D, P396L	CH2, CH3	↑3.8x	↑13.7x	2.15
K210M, P396L	CH1, CH3	↑1.9x	↑4.6x	2.02
L358P, P396L	CH3	↑1.9x	↑4.2x	1.58
K288R, T307A, K344E,P396L	CH2, CH3	↑4.1x	↑2.3x	3.3
V273I, K326E, L328I, P396L	CH2, CH3	↑1.3x	↑10.8x	0.78
K326I, S408N, P396L	CH2, CH3	↑4x	↑9.3x	1.65
K334N, P396L	CH2, CH3	↑3.1x	↑3x	2.43
V379M, P396L	CH3	↑1.9x	↑5.6x	2.01
P227S, P396L	CH2, CH3	↑1.5x	↑4x	2.01
P217S, P396L	H, CH3	↑1.6x	↑4.5x	2.04
K261N, K210M, P396L	CH2, CH3	↑2x	↑4.2x	2.06
Kinetic Screen: 0.8 μM, 1' with cold 8 μM FcγRIIIA: P396L Library				

Mutations	Domain	Binding to FcγRIIA (ELISA)	Binding to FcγRIIB (ELISA)	4-4-20 ADCC
term is M, P396L	CH3	↑1.9x	↑7.2x	3.09
Q419H, P396L	CH3	↑2x	↑6.9x	2.24
K370E, P396L	CH3	↑2x	↑6.6x	2.47
L242F, P396L	CH2, CH3	↑2.5x	↑4.1x	2.4
F243L, V305I, A378D, F404S, P396L	CH2, CH3	↑1.6x	↑5.4x	3.59
R255L, P396L	CH2, CH3	↑1.8x	↑6x	2.79
V240A, P396L	CH2, CH3	↑1.3x	↑4.2x	2.35
T250S, P396L	CH2, CH3	↑1.5x	↑6.8x	1.60
P247S, P396L	CH2, CH3	↑1.2x	↑4.2x	2.10
K290E, V369A, T393A, P396L	CH2, CH3	↑1.3x	↑6.7x	1.55
K210N, K222I, K320M, P396L	H, CH2, CH3	↑2.7x	↑8.7x	1.88
L410H, P396L	CH3	↑1.7x	↑4.5x	2.00
Q419L, P396L	CH3	↑2.2x	↑6.1x	1.70
V427A, P396L	CH3	↑1.9x	↑4.7x	1.67
P217S, V305I, I309L, N390H, P396L	H, CH2, CH3	↑2x	↑7x	1.54
E258D, P396L	CH2, CH3	↑1.9x	↑4.9x	1.54
N384K, P396L	CH3	↑2.2x	↑5.2x	1.49
V323I, P396L	CH2, CH3	↑1.1x	↑8.2x	1.29
K246N, Q419R, P396L	CH2, CH3	↑1.1x	↑4.8x	1.10
P217A, T359A, P396L	H, CH2, CH3	↑1.5x	↑4.8x	1.17
P244H, P396L	CH2, CH3	↑2.5x	↑4x	1.40
V215I, K290V, P396L	H, CH2, CH3	↑2.2x	↑4.6x	1.74
F275L, Q362H, N384K, P396L	CH2, CH3	↑2.2x	↑3.7x	1.51
V305L, P396L	CH2, CH3	↑1.3x	↑5.5x	1.50
S400F, P396L	CH3	↑1.5x	↑4.7x	1.19
V303I, P396L	CH3	↑1.1x	↑4x	1.01
FcγRIIB depletion FcγRIIA 158V solid phase selection: Naïve Library				

Mutations	Domain	Binding to FcγRIIIA (ELISA)	Binding to FcγRIIB (ELISA)	4-4-20 ADCC
A330V, H433Q, V427M	CH2,CH3	NT	NT	NT
V263Q, E272D, Q419H	CH2,CH3	NT	NT	NT
N276Y, T393N, W417R	CH2,CH3	NT	NT	NT
V282L, A330V, H433Y, T436R	CH2,CH3	NT	NT	NT
A330V, Q419H	CH2,CH3	NT	NT	NT
V284M, S298N, K334E, R355W	CH2,CH3	NT	NT	NT
A330V, G427M, K438R	CH2,CH3	NT	NT	NT
S219T, T225K, D270E, K360R	CH2,CH3	NT	NT	NT
K222E, V263Q, S298N	CH2	NT	NT	NT
V263Q, E272D	CH2	NT	NT	NT
R292G	CH2	NT	NT	NT
S298N	CH2	NT	NT	NT
E233G, P247S, L306P	CH2	NT	NT	NT
D270E	CH2	NT	NT	NT
S219T, T225K, D270E	CH2	NT	NT	NT
K326E, A330T	CH2	NT	NT	NT
E233G	CH2	NT	NT	NT
S254T, A330V, N361D, P243L	CH2,CH3	NT	NT	NT
FcγRIIB depletion FcγRIIIA 158F solid phase selection:Naïve Library				
158F by FACS top 0.2%				
V284M, S298N, K334E, R355W R416T	CH2,CH3	NT	NT	

[00138] In preferred embodiments, the invention provides modified immunoglobulin molecules (*e.g.*, antibodies) with variant Fc regions, having one or more amino acid modifications, which one or more amino acid modifications increase the affinity of the molecule for Fc γ RIIA and/or Fc γ RIIB. Such immunoglobulins include IgG molecules that naturally contain Fc γ R binding regions (*e.g.*, Fc γ RIIA and/or Fc γ RIIB binding region), or immunoglobulin derivatives that have been engineered to contain an Fc γ R binding region (*e.g.*, Fc γ RIIA and/or Fc γ RIIB binding region). The modified immunoglobulins of the invention include any immunoglobulin molecule that binds, preferably, immunospecifically, *i.e.*, competes off non-specific binding as determined by immunoassays well known in the art for assaying specific antigen-antibody binding, an antigen and contains an Fc γ R binding region (*e.g.*, a Fc γ RIIA and/or Fc γ RIIB binding region). Such antibodies include, but are not limited to, polyclonal, monoclonal, bi-specific, multi-specific, human, humanized, chimeric antibodies, single chain antibodies, Fab fragments, F(ab')₂ fragments, disulfide-linked Fvs, and fragments containing either a VL or VH domain or even a complementary determining region (CDR) that specifically binds an antigen, in certain cases, engineered to contain or fused to an Fc γ R binding region.

[00139] In some embodiments, the molecules of the invention comprise portions of an Fc region. As used herein the term "portion of an Fc region" refers to fragments of the Fc region, preferably a portion with effector activity and/or Fc γ R binding activity (or a comparable region of a mutant lacking such activity). The fragment of an Fc region may range in size from 5 amino acids to the entire Fc region minus one amino acids. The portion of an Fc region may be missing up to 10, up to 20, up to 30 amino acids from the N-terminus or C-terminus.

[00140] The IgG molecules of the invention are preferably IgG1 subclass of IgGs, but may also be any other IgG subclasses of given animals. For example, in humans, the IgG class includes IgG1, IgG2, IgG3, and IgG4; and mouse IgG includes IgG1, IgG2a, IgG2b, IgG2c and IgG3.

[00141] The immunoglobulins (and other polypeptides used herein) may be from any animal origin including birds and mammals. Preferably, the antibodies are human, rodent (*e.g.*, mouse and rat), donkey, sheep, rabbit, goat, guinea pig, camel, horse, or chicken. As used herein, "human" antibodies include antibodies having the amino acid sequence of a human immunoglobulin and include antibodies isolated from human immunoglobulin libraries or from animals transgenic for one or more human immunoglobulin and that do not

express endogenous immunoglobulins, as described *infra* and, for example, in U.S. Patent No. 5,939,598 by Kucherlapati *et al.*

[00142] The antibodies of the present invention may be monospecific, bispecific, trispecific or of greater multispecificity. Multispecific antibodies may be specific for
 5 different epitopes of a polypeptide or may be specific for heterologous epitopes, such as a heterologous polypeptide or solid support material. *See, e.g.*, PCT publications WO 93/17715; WO 92/08802; WO 91/00360; WO 92/05793; Tutt, *et al.*, *J. Immunol.*, 147:60-69, 1991; U.S. Patent Nos. 4,474,893; 4,714,681; 4,925,648; 5,573,920; 5,601,819; Kostelny *et al.*, *J. Immunol.*, 148:1547-1553, 1992.

10 [00143] The antibodies of the invention include derivatives that are otherwise modified, *i.e.*, by the covalent attachment of any type of molecule to the antibody such that covalent attachment does not prevent the antibody from binding antigen and/or generating an anti-idiotypic response. For example, but not by way of limitation, the antibody
 15 derivatives include antibodies that have been modified, *e.g.*, by glycosylation, acetylation, pegylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, etc. Any of numerous chemical modifications may be carried out by known techniques, including, but not limited to, specific chemical cleavage, acetylation, formylation, metabolic synthesis of
 20 tunicamycin, etc. Additionally, the derivative may contain one or more non-classical amino acids.

[00144] For some uses, including *in vivo* use of antibodies in humans and *in vitro* detection assays, it may be preferable to use chimeric, humanized, or human antibodies. A chimeric antibody is a molecule in which different portions of the antibody are derived from different animal species, such as antibodies having a variable region derived from a murine
 25 monoclonal antibody and a constant region derived from a human immunoglobulin. Methods for producing chimeric antibodies are known in the art. *See e.g.*, Morrison, *Science*, 229:1202, 1985; Oi *et al.*, *BioTechniques*, 4:214 1986; Gillies *et al.*, *J. Immunol. Methods*, 125:191-202, 1989; U.S. Patent Nos. 5,807,715; 4,816,567; and 4,816,397. Humanized antibodies are antibody
 30 molecules from non-human species that bind the desired antigen having one or more complementarity determining regions (CDRs) from the non-human species and framework regions and constant domains from a human immunoglobulin molecule. Often, framework residues in the human framework regions will be substituted with the corresponding residue from the CDR donor antibody to alter, preferably improve, antigen binding. These
 35 framework substitutions are identified by methods well known in the art, *e.g.*, by modeling

of the interactions of the CDR and framework residues to identify framework residues important for antigen binding and sequence comparison to identify unusual framework residues at particular positions. *See, e.g.,* Queen *et al.*, U.S. Patent No. 5,585,089; Riechmann *et al.*, *Nature*, 332:323, 1988.

5 Antibodies can be humanized using a variety of techniques known in the art including, for example, CDR-grafting (EP 239,400; PCT publication WO 91/09967; U.S. Patent Nos. 5,225,539; 5,530,101 and 5,585,089), veneering or resurfacing (EP 592,106; EP 519,596; Padlan, *Molecular Immunology*, 28(4/5):489-498, 1991; Studnicka *et al.*, *Protein Engineering*, 7(6):805-814, 1994; Roguska *et al.*, *Proc Natl. Acad. Sci. USA*, 91:969-973, 10 1994), and chain shuffling (U.S. Patent No. 5,565,332).

[00145] Completely human antibodies are particularly desirable for therapeutic treatment of human patients. Human antibodies can be made by a variety of methods known in the art including phage display methods described above using antibody libraries 15 derived from human immunoglobulin sequences. *See* U.S. Patent Nos. 4,444,887 and 4,716,111; and PCT publications WO 98/46645; WO 98/50433; WO 98/24893; WO 98/16654; WO 96/34096; WO 96/33735; and WO 91/10741.

[00146] Human antibodies can also be produced using transgenic mice which are 20 incapable of expressing functional endogenous immunoglobulins, but which can express human immunoglobulin genes. For an overview of this technology for producing human antibodies, *see* Lonberg and Huszar, *Int. Rev. Immunol.*, 13:65-93, 1995. For a detailed discussion of this technology for producing human antibodies and human monoclonal antibodies and protocols for producing such antibodies, *see, e.g.,* PCT publications WO 25 98/24893; WO 92/01047; WO 96/34096; WO 96/33735; European Patent No. 0 598 877; U.S. Patent Nos. 5,413,923; 5,625,126; 5,633,425; 5,569,825; 5,661,016; 5,545,806; 5,814,318; 5,885,793; 5,916,771; and 5,939,598.

In addition, companies such as Abgenix, Inc. (Freemont, CA), Medarex (NJ) and Genpharm (San Jose, CA) can be engaged to provide human antibodies directed 30 against a selected antigen using technology similar to that described above.

[00147] Completely human antibodies which recognize a selected epitope can be generated using a technique referred to as "guided selection." In this approach a selected non-human monoclonal antibody, *e.g.,* a mouse antibody, is used to guide the selection of a completely human antibody recognizing the same epitope (Jespers *et al.*, *Bio/technology*, 35 12:899-903, 1988).

[00148] The invention encompasses engineering human or humanized therapeutic antibodies (*e.g.*, tumor specific monoclonal antibodies) in the Fc region, by modification (*e.g.*, substitution, insertion, deletion) of at least one amino acid residue, which modification increases the affinity of the Fc region for Fc γ RIIIA and/or Fc γ RIIA. In another
5 embodiment, the invention relates to engineering human or humanized therapeutic antibodies (*e.g.*, tumor specific monoclonal antibodies) in the Fc region, by modification of at least one amino acid residue, which modification increases the affinity of the Fc region for Fc γ RIIIA and/or Fc γ RIIA and further decreases the affinity of the Fc region for Fc γ RIIB. The engineered therapeutic antibodies may further have an enhanced effector
10 function, *e.g.*, enhanced ADCC activity, phagocytosis activity, *etc.*, as determined by standard assays known to those skilled in the art.

[00149] In a specific embodiment, the invention encompasses engineering a humanized monoclonal antibody specific for Her2/neu protooncogene (*e.g.*, Ab4D5 humanized antibody as disclosed in Carter *et al.*, 1992, *Proc. Natl. Acad. Sci. USA* 89:4285-
15 9) by modification (*e.g.*, substitution, insertion, deletion) of at least one amino acid residue which modification increases the affinity of the Fc region for Fc γ RIIIA and/or Fc γ RIIA. In another specific embodiment, modification of the humanized Her2/neu monoclonal antibody may also further decrease the affinity of the Fc region for Fc γ RIIB. In yet another specific embodiment, the engineered humanized monoclonal antibodies specific for
20 Her2/neu may further have an enhanced effector function as determined by standard assays known in the art and disclosed and exemplified herein.

[00150] In another specific embodiment, the invention encompasses engineering a mouse human chimeric anti-CD20 monoclonal antibody, 2H7 by modification (*e.g.*, substitution, insertion, deletion) of at least one amino acid residue which modification
25 increases the affinity of the Fc region for Fc γ RIIIA and/or Fc γ RIIA. In another specific embodiment, modification of the anti-CD20 monoclonal antibody, 2H7 may also further decrease the affinity of the Fc region for Fc γ RIIB. In yet another specific embodiment, the engineered anti-CD20 monoclonal antibody, 2H7 may further have an enhanced effector function as determined by standard assays known in the art and disclosed and exemplified
30 herein.

[00151] In another specific embodiment, the invention encompasses engineering an anti-Fc γ RIIB antibody including but not limited to any of the antibodies disclosed in U.S. Provisional Application No. 60/403,266 filed on August 12, 2002 and U.S. Application No. 10/643,857 filed on August 14, 2003, having Attorney Docket No. 011183-010-999, by
35 modification (*e.g.*, substitution, insertion, deletion) of at least one amino acid residue which

modification increases the affinity of the Fc region for FcγRIIIA and/or FcγRIIA. Examples of anti-FcγRIIB antibodies that may be engineered in accordance with the methods of the invention are 2B6 monoclonal antibody having ATCC accession number PTA-4591 and 3H7 having ATCC accession number PTA-4592 (deposited at 10801 University Boulevard, Manassas, VA 02209-2011 . In another specific embodiment, modification of the anti-FcγRIIB antibody may also further decrease the affinity of the Fc region for FcγRIIB. In yet another specific embodiment, the engineered anti-FcγRIIB antibody may further have an enhanced effector function as determined by standard assays known in the art and disclosed and exemplified herein. In a specific embodiment, the 2B6 monoclonal antibody comprises a modification at position 334 with glutamic acid, at position 359 with asparagine, and at position 366 with serine (MgFc13); or a substitution at position 316 with aspartic acid, at position 378 with valine, and at position 399 with glutamic acid (MgFc27); or a substitution at position 243 with isoleucine, at position 379 with leucine, and at position 420 with valine (MgFc29); or a substitution at position 392 with threonine and at position 396 with leucine (MgFc38); or a substitution at position 221 with glutamic acid, at position 270 with glutamic acid, at position 308 with alanine, at position 311 with histidine, at position 396 with leucine, and at position 402 with aspartic (MgFc42); or a substitution at position 410 with histidine, and at position 396 with leucine (MgFc53); or a substitution at position 243 with leucine, at position 305 with isoleucine, at position 378 with aspartic acid, at position 404 with serine, and at position 396 with leucine (MgFc54); or a substitution at position 255 with isoleucine, and at position 396 with leucine (MgFc55); or a substitution at position 370 with glutamic acid, and at position 396 with leucine (MgFc59) (See Table 5).

5.1.1 POLYPEPTIDE AND ANTIBODY CONJUGATES

[00152] Molecules of the invention (*i.e.*, polypeptides, antibodies) comprising variant Fc regions may be recombinantly fused or chemically conjugated (including both covalently and non-covalently conjugations) to heterologous polypeptides (*i.e.*, an unrelated polypeptide; or portion thereof, preferably at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90 or at least 100 amino acids of the polypeptide) to generate fusion proteins. The fusion does not necessarily need to be direct, but may occur through linker sequences.

[00153] Further, molecules of the invention (*i.e.*, polypeptides, antibodies) comprising variant Fc regions may be conjugated to a therapeutic agent or a drug moiety that modifies a given biological response. Therapeutic agents or drug moieties are not to be

construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, a toxin such as abrin, ricin A, pseudomonas exotoxin (*i.e.*, PE-40), or diphtheria toxin, ricin, gelonin, and pokeweed antiviral protein, a protein such as tumor
 5 necrosis factor, interferons including, but not limited to, α -interferon (IFN- α), β -interferon (IFN- β), nerve growth factor (NGF), platelet derived growth factor (PDGF), tissue plasminogen activator (TPA), an apoptotic agent (*e.g.*, TNF- α , TNF- β , AIM I as disclosed in PCT Publication No. WO 97/33899), AIM II (*see*, PCT Publication No. WO 97/34911), Fas Ligand (Takahashi *et al.*, *J. Immunol.*, 6:1567-1574, 1994), and VEGI (PCT Publication
 10 No. WO 99/23105), a thrombotic agent or an anti-angiogenic agent (*e.g.*, angiostatin or endostatin), or a biological response modifier such as, for example, a lymphokine (*e.g.*, interleukin-1 ("IL-1"), interleukin-2 ("IL-2"), interleukin-6 ("IL-6"), granulocyte macrophage colony stimulating factor ("GM-CSF"), and granulocyte colony stimulating factor ("G-CSF"), macrophage colony stimulating factor, ("M-CSF"), or a growth factor
 15 (*e.g.*, growth hormone ("GH"); proteases, or ribonucleases.

[00154] Molecules of the invention (*i.e.*, polypeptides, antibodies) can be fused to marker sequences, such as a peptide to facilitate purification. In preferred embodiments, the marker amino acid sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (QIAGEN, Inc., 9259 Eton Avenue, Chatsworth, CA, 91311), among others, many of
 20 which are commercially available. As described in Gentz *et al.*, 1989, *Proc. Natl. Acad. Sci. USA*, 86:821-824, for instance, hexa-histidine provides for convenient purification of the fusion protein. Other peptide tags useful for purification include, but are not limited to, the hemagglutinin "HA" tag, which corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson *et al.*, *Cell*, 37:767 1984) and the "flag" tag (Knappik *et al.*,
 25 *Biotechniques*, 17(4):754-761, 1994).

[00155] Additional fusion proteins may be generated through the techniques of gene-shuffling, motif-shuffling, exon-shuffling, and/or codon-shuffling (collectively referred to as "DNA shuffling"). DNA shuffling may be employed to alter the activities of molecules of the invention (*e.g.*, antibodies with higher affinities and lower dissociation rates). *See*,
 30 generally, U.S. Patent Nos. 5,605,793; 5,811,238; 5,830,721; 5,834,252; and 5,837,458, and Patten *et al.*, 1997, *Curr. Opinion Biotechnol.* 8:724-33; Harayama, 1998, *Trends Biotechnol.* 16:76; Hansson, *et al.*, 1999, *J. Mol. Biol.* 287:265; and Lorenzo and Blasco, 1998, *BioTechniques* 24:308.

Molecules of the invention comprising variant Fc regions, or
 35 the nucleic acids encoding the molecules of the invention, may be further altered by being

subjected to random mutagenesis by error-prone PCR, random nucleotide insertion or other methods prior to recombination. One or more portions of a polynucleotide encoding a molecule of the invention, may be recombined with one or more components, motifs, sections, parts, domains, fragments, *etc.* of one or more heterologous molecules.

- 5 [00156] The present invention also encompasses molecules of the invention comprising variant Fc regions (*i.e.*, antibodies, polypeptides) conjugated to a diagnostic or therapeutic agent or any other molecule for which serum half-life is desired to be increased and/or targeted to a particular subset of cells. The molecules of the invention can be used
- 10 diagnostically to, for example, monitor the development or progression of a disease, disorder or infection as part of a clinical testing procedure to, *e.g.*, determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling the molecules of the invention to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, radioactive materials, positron emitting metals, and nonradioactive paramagnetic
- 15 metal ions. The detectable substance may be coupled or conjugated either directly to the molecules of the invention or indirectly, through an intermediate (such as, for example, a linker known in the art) using techniques known in the art. *See*, for example, U.S. Patent No. 4,741,900 for metal ions which can be conjugated to antibodies for use as diagnostics according to the present invention. Such diagnosis and detection can be accomplished by
- 20 coupling the molecules of the invention to detectable substances including, but not limited to, various enzymes, enzymes including, but not limited to, horseradish peroxidase, alkaline phosphatase, beta-galactosidase, or acetylcholinesterase; prosthetic group complexes such as, but not limited to, streptavidin/biotin and avidin/biotin; fluorescent materials such as, but not limited to, umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine,
- 25 dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; luminescent material such as, but not limited to, luminol; bioluminescent materials such as, but not limited to, luciferase, luciferin, and aequorin; radioactive material such as, but not limited to, bismuth (^{213}Bi), carbon (^{14}C), chromium (^{51}Cr), cobalt (^{57}Co), fluorine (^{18}F), gadolinium (^{153}Gd , ^{159}Gd), gallium (^{68}Ga , ^{67}Ga), germanium (^{68}Ge), holmium (^{166}Ho), indium (^{115}In , ^{113}In , ^{112}In , ^{111}In), iodine (^{131}I , ^{125}I , ^{123}I , ^{121}I), lanthanum (^{140}La), lutetium (^{177}Lu), manganese (^{54}Mn),
- 30 molybdenum (^{99}Mo), palladium (^{103}Pd), phosphorous (^{32}P), praseodymium (^{142}Pr), promethium (^{149}Pm), rhenium (^{186}Re , ^{188}Re), rhodium (^{105}Rh), ruthenium (^{97}Ru), samarium (^{153}Sm), scandium (^{47}Sc), selenium (^{75}Se), strontium (^{85}Sr), sulfur (^{35}S), technetium (^{99}Tc), thallium (^{201}Tl), tin (^{113}Sn , ^{117}Sn), tritium (^3H), xenon (^{133}Xe), ytterbium (^{169}Yb , ^{175}Yb),

yttrium (^{90}Y), zinc (^{65}Zn); positron emitting metals using various positron emission tomographies, and nonradioactive paramagnetic metal ions.

- [00157] Molecules of the invention (*i.e.*, antibodies, polypeptides) comprising a variant Fc region may be conjugated to a therapeutic moiety such as a cytotoxin (*e.g.*, a
 5 cytostatic or cytocidal agent), a therapeutic agent or a radioactive element (*e.g.*, alpha-emitters, gamma-emitters, *etc.*). Cytotoxins or cytotoxic agents include any agent that is detrimental to cells. Examples include paclitaxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin,
 10 actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Therapeutic agents include, but are not limited to, antimetabolites (*e.g.*, methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (*e.g.*, mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclophosphamide,
 15 busulfan, dibromomannitol, streptozotocin, mitomycin C, and cisdichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (*e.g.*, daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (*e.g.*, dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC), and anti-mitotic agents (*e.g.*, vincristine and vinblastine).
- 20 [00158] Moreover, a molecule of the invention can be conjugated to therapeutic moieties such as a radioactive materials or macrocyclic chelators useful for conjugating radiometal ions (*see above* for examples of radioactive materials). In certain embodiments, the macrocyclic chelator is 1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid (DOTA) which can be attached to the antibody via a linker molecule. Such linker
 25 molecules are commonly known in the art and described in Denardo *et al.*, 1998, *Clin Cancer Res.* 4:2483-90; Peterson *et al.*, 1999, *Bioconjug. Chem.* 10:553; and Zimmerman *et al.*, 1999, *Nucl. Med. Biol.* 26:943-50.

- [00159] Techniques for conjugating such therapeutic moieties to antibodies are well
 30 known; *see, e.g.*, Arnon *et al.*, "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in *Monoclonal Antibodies And Cancer Therapy*, Reisfeld *et al.* (eds.), 1985, pp. 243-56, Alan R. Liss, Inc.); Hellstrom *et al.*, "Antibodies For Drug Delivery", in *Controlled Drug Delivery (2nd Ed.)*, Robinson *et al.* (eds.), 1987, pp. 623-53, Marcel Dekker, Inc.); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A
 35 Review", in *Monoclonal Antibodies '84: Biological And Clinical Applications*, Pinchera *et*

al. (eds.), 1985, pp. 475-506); "Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy", in *Monoclonal Antibodies For Cancer Detection And Therapy*, Baldwin *et al.* (eds.), 1985, pp. 303-16, Academic Press; and Thorpe *et al.*, *Immunol. Rev.*, 62:119-58, 1982.

5 [00160] In one embodiment, where the molecule of the invention is an antibody comprising a variant Fc region, it can be administered with or without a therapeutic moiety conjugated to it, administered alone, or in combination with cytotoxic factor(s) and/or cytokine(s) for use as a therapeutic treatment. Alternatively, an antibody of the invention can be conjugated to a second antibody to form an antibody heteroconjugate as described by
10 Segal in U.S. Patent No. 4,676,980, which is incorporated herein by reference in its entirety. Antibodies of the invention may also be attached to solid supports, which are particularly useful for immunoassays or purification of the target antigen. Such solid supports include, but are not limited to, glass, cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene.

15 **5.2 SCREENING OF MOLECULES WITH VARIANT Fc REGIONS FOR ENHANCED FcγRIII BINDING AND CHARACTERIZATION OF SAME**

[00161] In preferred embodiments, screening and identifying molecules comprising variant Fc regions with altered FcγR affinities (*e.g.*, enhanced FcγRIIIA affinity) are done
20 using the yeast display technology as described herein in combination with one or more biochemical based assays, preferably in a high throughput manner. The one or more biochemical assays can be any assay known in the art for identifying Fc-FcγR interaction, *i.e.*, specific binding of an Fc region to an FcγR, including, but not limited to, an ELISA assay, surface plasmon resonance assays, immunoprecipitation assay, affinity
25 chromatography, and equilibrium dialysis. In some embodiments, screening and identifying molecules comprising variant Fc regions with altered FcγR affinities (*e.g.*, enhanced FcγRIIIA affinity) are done using the yeast display technology as described herein in combination with one or more functional based assays, preferably in a high throughput manner. The functional based assays can be any assay known in the art for characterizing
30 one or more FcγR mediated effector cell functions such as those described herein in Section 5.2.6. Non-limiting examples of effector cell functions that can be used in accordance with the methods of the invention, include but are not limited to, antibody-dependent cell mediated cytotoxicity (ADCC), antibody-dependent phagocytosis, phagocytosis, opsonization, opsonophagocytosis, cell binding, rosetting, C1q binding, and complement

dependent cell mediated cytotoxicity. In some embodiments, screening and identifying molecules comprising variant Fc regions with altered FcγR affinities (e.g., enhanced FcγRIIIA affinity) are done using the yeast display technology as described herein in combination with one or more biochemical based assays in combination or in parallel with one or more functional based assays, preferably in a high throughput manner.

[00162] The term "specific binding" of an Fc region to an FcγR refers to an interaction of the Fc region and a particular FcγR which has an affinity constant of at least about 150 nM, in the case of monomeric FcγRIIIA and at least about 60 nM in the case of dimeric FcγRIIB as determined using, for example, an ELISA or surface plasmon resonance assay (e.g., a BIAcore™). The affinity constant of an Fc region for monomeric FcγRIIIA may be 150 nM, 200 nM or 300nM. The affinity constant of an Fc region for dimeric FcγRIIB may be 60 nM, 80 nM, 90 nM, or 100 nM. Dimeric FcγRIIB for use in the methods of the invention may be generated using methods known to one skilled in the art. Typically, the extracellular region of FcγRIIB is covalently linked to a heterologous polypeptide which is capable of dimerization, so that the resulting fusion protein is a dimer, e.g., see, U.S. Application No. 60/439,709 filed on January 13, 2003 (Attorney Docket No. 11183-005-888). A specific interaction generally is stable under physiological conditions, including, for example, conditions that occur in a living individual such as a human or other vertebrate or invertebrate, as well as conditions that occur in a cell culture such conditions as used for maintaining and culturing mammalian cells or cells from another vertebrate organism or an invertebrate organism.

[00163] In a specific embodiment, screening for and identifying molecules comprising variant Fc regions and altered FcγR affinities comprise: displaying the molecule comprising a variant Fc region on the yeast surface; and characterizing the binding of the molecule comprising the variant Fc region to a FcγR (one or more), using a biochemical assay for determining Fc-FcγR interaction, preferably, an ELISA based assay. Once the molecule comprising a variant Fc region has been characterized for its interaction with one or more FcγRs and determined to have an altered affinity for one or more FcγRs, by at least one biochemical based assay, e.g., an ELISA assay, the molecule maybe engineered into a complete immunoglobulin, using standard recombinant DNA technology methods known in the art, and the immunoglobulin comprising the variant Fc region expressed in mammalian cells for further biochemical characterization. The immunoglobulin into which a variant Fc region of the invention is introduced (e.g., replacing the Fc region of the immunoglobulin)

can be any immunoglobulin including, but not limited to, polyclonal antibodies, monoclonal antibodies, bispecific antibodies, multi-specific antibodies, humanized antibodies, and chimeric antibodies. In preferred embodiments, a variant Fc region is introduced into an immunoglobulin specific for a cell surface receptor, a tumor antigen, or a cancer antigen.

5 The immunoglobulin into which a variant Fc region of the invention is introduced may specifically bind a cancer or tumor antigen for example, including, but not limited to, KS 1/4 pan-carcinoma antigen (Perez and Walker, 1990, *J. Immunol.* 142: 3662-3667; Bumal, 1988, *Hybridoma* 7(4): 407-415), ovarian carcinoma antigen (CA125) (Yu *et al.*, 1991, *Cancer Res.* 51(2): 468-475), prostatic acid phosphatase (Taylor *et al.*, 1990, *Nucl. Acids Res.* 18(16): 4928), prostate specific antigen (Henttu and Vihko, 1989, *Biochem. Biophys. Res. Comm.* 160(2): 903-910; Israeli *et al.*, 1993, *Cancer Res.* 53: 227-230), melanoma-associated antigen p97 (Estlin *et al.*, 1989, *J. Natl. Cancer Instit.* 81(6): 445-446), melanoma antigen gp75 (Vijayasardahl *et al.*, 1990, *J. Exp. Med.* 171(4): 1375-1380), high molecular weight melanoma antigen (HMW-MAA) (Natali *et al.*, 1987, *Cancer* 59: 55-63; Mittelman *et al.*, 1990, *J. Clin. Invest.* 86: 2136-2144), prostate specific membrane antigen, carcinoembryonic antigen (CEA) (Foon *et al.*, 1994, *Proc. Am. Soc. Clin. Oncol.* 13: 294), polymorphic epithelial mucin antigen, human milk fat globule antigen, colorectal tumor-associated antigens such as: CEA, TAG-72 (Yokata *et al.*, 1992, *Cancer Res.* 52: 3402-3408), CO17-1A (Ragnhammar *et al.*, 1993, *Int. J. Cancer* 53: 751-758); GICA 19-9 (Herlyn *et al.*, 1982, *J. Clin. Immunol.* 2: 135), CTA-1 and LEA, Burkitt's lymphoma antigen-38.13, CD19 (Ghetie *et al.*, 1994, *Blood* 83: 1329-1336), human B-lymphoma antigen-CD20 (Reff *et al.*, 1994, *Blood* 83:435-445), CD33 (Sgouros *et al.*, 1993, *J. Nucl. Med.* 34:422-430), melanoma specific antigens such as ganglioside GD2 (Saleh *et al.*, 1993, *J. Immunol.*, 151, 3390-3398), ganglioside GD3 (Shitara *et al.*, 1993, *Cancer Immunol. Immunother.* 36:373-380), ganglioside GM2 (Livingston *et al.*, 1994, *J. Clin. Oncol.* 12: 1036-1044), ganglioside GM3 (Hoon *et al.*, 1993, *Cancer Res.* 53: 5244-5250), tumor-specific transplantation type of cell-surface antigen (TSTA) such as virally-induced tumor antigens including T-antigen DNA tumor viruses and Envelope antigens of RNA tumor viruses, oncofetal antigen-alpha-fetoprotein such as CEA of colon, bladder tumor oncofetal antigen (Hellstrom *et al.*, 1985, *Cancer Res.* 45:2210-2188), differentiation antigen such as human lung carcinoma antigen L6, L20 (Hellstrom *et al.*, 1986, *Cancer Res.* 46: 3917-3923), antigens of fibrosarcoma, human leukemia T cell antigen-Gp37 (Bhattacharya-Chatterjee *et al.*, 1988, *J. of Immun.* 141:1398-1403), neoglycoprotein, sphingolipids, breast cancer antigen such as EGFR (Epidermal growth factor receptor), HER2 antigen (p185^{HER2}), polymorphic epithelial mucin (PEM) (Hilkens *et al.*, 1992, *Trends in Bio.*

Chem. Sci. 17:359), malignant human lymphocyte antigen-APO-1 (Bernhard *et al.*, 1989, *Science* 245: 301-304), differentiation antigen (Feizi, 1985, *Nature* 314: 53-57) such as I antigen found in fetal erythrocytes, primary endoderm I antigen found in adult erythrocytes, preimplantation embryos, I(Ma) found in gastric adenocarcinomas, M18, M39 found in breast epithelium, SSEA-1 found in myeloid cells, VEP8, VEP9, Myl, VIM-D5, D₁₅₆₋₂₂ found in colorectal cancer, TRA-1-85 (blood group H), C14 found in colonic adenocarcinoma, F3 found in lung adenocarcinoma, AH6 found in gastric cancer, Y hapten, Le^y found in embryonal carcinoma cells, TL5 (blood group A), EGF receptor found in A431 cells, E₁ series (blood group B) found in pancreatic cancer, FC10.2 found in embryonal carcinoma cells, gastric adenocarcinoma antigen, CO-514 (blood group Le^a) found in Adenocarcinoma, NS-10 found in adenocarcinomas, CO-43 (blood group Le^b), G49 found in EGF receptor of A431 cells, MH2 (blood group ALe^b/Le^y) found in colonic adenocarcinoma, 19.9 found in colon cancer, gastric cancer mucins, T_{5A7} found in myeloid cells, R₂₄ found in melanoma, 4.2, G_{D3}, D1.1, OFA-1, G_{M2}, OFA-2, G_{D2}, and M1:22:25:8 found in embryonal carcinoma cells, and SSEA-3 and SSEA-4 found in 4 to 8-cell stage embryos. In one embodiment, the antigen is a T cell receptor derived peptide from a Cutaneous Tcell Lymphoma (*see*, Edelson, 1998, *The Cancer Journal* 4:62).

[00164] In some embodiments, a variant Fc region of the invention is introduced into an anti-fluoresceine monoclonal antibody, 4-4-20 (Kranz *et al.*, 1982 *J. Biol. Chem.* 257(12): 6987-6995). In other embodiments, a variant Fc region of the invention is introduced into a mouse-human chimeric anti-CD20 monoclonal antibody 2H7, which recognizes the CD20 cell surface phosphoprotein on B cells (Liu *et al.*, 1987, *Journal of Immunology*, 139: 3521-6).

In yet other embodiments, a variant Fc region of the invention is introduced into a humanized antibody (Ab4D5) against the human epidermal growth factor receptor 2 (p185 HER2) as described by Carter *et al.* (1992, *Proc. Natl. Acad. Sci. USA* 89: 4285-9).

In yet other embodiments, a variant Fc region of the invention is introduced into a humanized anti-TAG72 antibody (CC49) (Sha *et al.*, 1994 *Cancer Biother.* 9(4): 341-9). In other embodiments, a variant Fc region of the invention is introduced into Rituxan which is used for treating lymphomas.

[00165] In another specific embodiment, the invention encompasses engineering an anti-FcγRIIB antibody, by

modification (*e.g.*, substitution, insertion, deletion) of at least one amino acid residue which modification increases the affinity of the Fc region for Fc γ RIIA and/or Fc γ RIIA. Examples of anti-Fc γ RIIB antibodies that may be engineered in accordance with the methods of the invention are 2B6 monoclonal antibody having ATCC accession number PTA-4591 and

5 3H7 having ATCC accession number PTA-4592. In another specific embodiment, modification of the anti-Fc γ RIIB antibody may also further decrease the affinity of the Fc region for Fc γ RIIB. In yet another specific embodiment, the engineered anti-Fc γ RIIB antibody may further have an enhanced effector function as determined by standard assays known in the art and disclosed and exemplified herein. In some embodiments, a variant Fc

10 region of the invention is introduced into a therapeutic monoclonal antibody specific for a cancer antigen or cell surface receptor including but not limited to, ErbituxTM (also known as IMC-C225) (ImClone Systems Inc.), a chimerized monoclonal antibody against EGFR; HERCEPTIN[®] (Trastuzumab) (Genentech, CA) which is a humanized anti-HER2 monoclonal antibody for the treatment of patients with metastatic breast cancer; REOPRO[®]

15 (abciximab) (Centocor) which is an anti-glycoprotein IIb/IIIa receptor on the platelets for the prevention of clot formation; ZENAPAX[®] (daclizumab) (Roche Pharmaceuticals, Switzerland) which is an immunosuppressive, humanized anti-CD25 monoclonal antibody for the prevention of acute renal allograft rejection. Other examples are a humanized anti-CD18 F(ab')₂ (Genentech); CDP860 which is a humanized anti-CD18 F(ab')₂ (Celltech,

20 UK); PRO542 which is an anti-HIV gp120 antibody fused with CD4 (Progenics/Genzyme Transgenics); C14 which is an anti-CD14 antibody (ICOS Pharm); a humanized anti-VEGF IgG1 antibody (Genentech); OVAREXTM which is a murine anti-CA 125 antibody (Altarex); PANOREXTM which is a murine anti-17-IA cell surface antigen IgG2a antibody (Glaxo Wellcome/Centocor); IMC-C225 which is a chimeric anti-EGFR IgG antibody

25 (ImClone System); VITAXINTM which is a humanized anti- α V β 3 integrin antibody (Applied Molecular Evolution/MedImmune); Campath 1H/LDP-03 which is a humanized anti CD52 IgG1 antibody (Leukosite); Smart M195 which is a humanized anti-CD33 IgG antibody (Protein Design Lab/Kanebo); RITUXANTM which is a chimeric anti-CD20 IgG1 antibody (IDEC Pharm/Genentech, Roche/Zettyaku); LYMPHOCIDETM which is a

30 humanized anti-CD22 IgG antibody (Immunomedics); Smart ID10 which is a humanized anti-HLA antibody (Protein Design Lab); ONCOLYMTM (Lym-1) is a radiolabelled murine anti-HLA DR antibody (Techniclone); anti-CD11a is a humanized IgG1 antibody (Genetech/Xoma); ICM3 is a humanized anti-ICAM3 antibody (ICOS Pharm); IDEC-114 is a primatized anti-CD80 antibody (IDEC Pharm/Mitsubishi); ZEVALINTM is a radiolabelled

murine anti-CD20 antibody (IDEC/Schering AG); IDEC-131 is a humanized anti-CD40L antibody (IDEC/Eisai); IDEC-151 is a primatized anti-CD4 antibody (IDEC); IDEC-152 is a primatized anti-CD23 antibody (IDEC/Seikagaku); SMART anti-CD3 is a humanized anti-CD3 IgG (Protein Design Lab); 5G1.1 is a humanized anti-complement factor 5 (C5) antibody (Alexion Pharm); IDEC-151 is a primatized anti-CD4 IgG1 antibody (IDEC Pharm/SmithKline Beecham); MDX-CD4 is a human anti-CD4 IgG antibody (Medarex/Eisai/Genmab); CDP571 is a humanized anti-TNF- α IgG4 antibody (Celltech); LDP-02 is a humanized anti- $\alpha 4\beta 7$ antibody (LeukoSite/Genentech); OrthoClone OKT4A is a humanized anti-CD4 IgG antibody (Ortho Biotech); ANTOVA™ is a humanized anti-CD40L IgG antibody (Biogen); ANTEGREN™ is a humanized anti-VLA-4 IgG antibody (Elan); MDX-33 is a human anti-CD64 (Fc γ R) antibody (Medarex/Centex); rhuMab-E25 is a humanized anti-IgE IgG1 antibody (Genentech/Norvartis/Tanox Biosystems); IDEC-152 is a primatized anti-CD23 antibody (IDEC Pharm); ABX-CBL is a murine anti CD-147 IgM antibody (Abgenix); BTI-322 is a rat anti-CD2 IgG antibody (Medimmune/Bio Transplant); Orthoclone/OKT3 is a murine anti-CD3 IgG2a antibody (ortho Biotech); SIMULECT™ is a chimeric anti-CD25 IgG1 antibody (Novartis Pharm); LDP-01 is a humanized anti- β_2 -integrin IgG antibody (LeukoSite); Anti-LFA-1 is a murine anti CD18 F(ab')₂ (Pasteur-Merieux/Immunotech); CAT-152 is a human anti-TGF- β_2 antibody (Cambridge Ab Tech); and Corsevin M is a chimeric anti-Factor VII antibody (Centocor).

[00166] The variant Fc regions of the invention, preferably in the context of an immunoglobulin, can be further characterized using one or more biochemical assays and/or one or more functional assays, preferably in a high throughput manner. In some alternate embodiments, the variant Fc regions of the inventions are not introduced into an immunoglobulin and are further characterized using one or more biochemical based assays and/or one or more functional assays, preferably in a high throughput manner. The one or more biochemical assays can be any assay known in the art for identifying Fc-Fc γ R interactions, including, but not limited to, an ELISA assay, and surface plasmon resonance-based assay for determining the kinetic parameters of Fc-Fc γ R interaction, *e.g.*, BIAcore assay. The one or more functional assays can be any assay known in the art for characterizing one or more Fc γ R mediated effector cell function as known to one skilled in the art or described herein. In specific embodiments, the immunoglobulins comprising the variant Fc regions are assayed in an ELISA assay for binding to one or more Fc γ Rs, *e.g.*, Fc γ RIIA, Fc γ RIIA, Fc γ RIIA; followed by one or more ADCC assays. In some embodiments, the immunoglobulins comprising the variant Fc regions are assayed further

using a surface plasmon resonance-based assay, *e.g.*, BIAcore. Surface plasmon resonance-based assays are well known in the art, and are further discussed in Section 5.2.7, and exemplified herein in Example 6.8.

[00167] An exemplary high throughput assay for characterizing immunoglobulins comprising variant Fc regions may comprise: introducing a variant Fc region of the invention, *e.g.*, by standard recombinant DNA technology methods, in a 4-4-20 antibody; characterizing the specific binding of the 4-4-20 antibody comprising the variant Fc region to an Fc γ R (*e.g.*, Fc γ RIIA, Fc γ RIIB) in an ELISA assay; characterizing the 4-4-20 antibody comprising the variant Fc region in an ADCC assay (using methods disclosed herein) wherein the target cells are opsonized with the 4-4-20 antibody comprising the variant Fc region; the variant Fc region may then be cloned into a second immunoglobulin, *e.g.*, 4D5, 2H7, and that second immunoglobulin characterized in an ADCC assay, wherein the target cells are opsonized with the second antibody comprising the variant Fc region. The second antibody comprising the variant Fc region is then further analyzed using an ELISA-based assay to confirm the specific binding to an Fc γ R.

[00168] Preferably, a variant Fc region of the invention binds Fc γ RIIA and/or Fc γ RIIA with a higher affinity than a wild type Fc region as determined in an ELISA assay. Most preferably, a variant Fc region of the invention binds Fc γ RIIA and/or Fc γ RIIA with a higher affinity and binds Fc γ RIIB with a lower affinity than a wild type Fc region as determined in an ELISA assay. In some embodiments, the variant Fc region binds Fc γ RIIA and/or Fc γ RIIA with at least 2-fold higher, at least 4-fold higher, more preferably at least 6-fold higher, most preferably at least 8 to 10-fold higher affinity than a wild type Fc region binds Fc γ RIIA and/or Fc γ RIIA and binds Fc γ RIIB with at least 2-fold lower, at least 4-fold lower, more preferably at least 6-fold lower, most preferably at least 8 to 10-fold lower affinity than a wild type Fc region binds Fc γ RIIB as determined in an ELISA assay.

[00169] The immunoglobulin comprising the variant Fc regions may be analyzed at any point using a surface plasmon based resonance based assay, *e.g.*, BIAcore, for defining the kinetic parameters of the Fc-Fc γ R interaction, using methods disclosed herein and known to those of skill in the art. Preferably, the K_d of a variant Fc region of the invention for binding to a monomeric Fc γ RIIA and/or Fc γ RIIA as determined by BIAcore analysis is about 100 nM, preferably about 70 nM, most preferably about 40 nM.; and the K_d of the variant Fc region of the invention for binding a dimeric Fc γ RIIB is about 80 nM, about 100 nM, more preferably about 200 nM.

[00170] In most preferred embodiments, the immunoglobulin comprising the variant Fc regions is further characterized in an animal model for interaction with an Fc γ R. Preferred animal models for use in the methods of the invention are, for example, transgenic mice expressing human Fc γ Rs, *e.g.*, any mouse model described in U.S. Patent No.

5 5,877,397, which is incorporated herein by reference in its entirety. Transgenic mice for use in the methods of the invention include, but are not limited to, nude knockout Fc γ RIIA mice carrying human Fc γ RIIA; nude knockout Fc γ RIIA mice carrying human Fc γ RIIA; nude knockout Fc γ RIIA mice carrying human Fc γ RIIB and human Fc γ RIIA; nude knockout Fc γ RIIA mice carrying human Fc γ RIIB and human Fc γ RIIA.

10 5.2.1 Fc γ R-Fc BINDING ASSAY

[00171] An Fc γ R-Fc binding assay was developed for determining the binding of the molecules of the invention comprising variant Fc regions to Fc γ R, which allowed detection and quantitation of the interaction, despite the inherently weak affinity of the receptor for its ligand, *e.g.*, in the micromolar range for Fc γ RIIB and Fc γ RIIA. The method involves the
15 formation of an Fc γ R complex that has an improved avidity for an Fc region, relative to an uncomplexed Fc γ R. According to the invention, the preferred molecular complex is a tetrameric immune complex, comprising: (a) the soluble region of Fc γ R (*e.g.*, the soluble region of Fc γ RIIA, Fc γ RIIA or Fc γ RIIB); (b) a biotinylated 15 amino acid AVITAG sequence (AVITAG) operably linked to the C-terminus of the soluble region of Fc γ R (*e.g.*,
20 the soluble region of Fc γ RIIA, Fc γ RIIA or Fc γ RIIB); and (c) streptavidin-phycoerythrin (SA-PE); in a molar ratio to form a tetrameric Fc γ R complex (preferably in a 5:1 molar ratio). According to a preferred embodiment of the invention, the fusion protein is biotinylated enzymatically, using for example, the *E.coli* Bir A enzyme, a biotin ligase which specifically biotinylates a lysine residue in the 15 amino acid AVITAG sequence. In
25 a specific embodiment of the invention, 85% of the fusion protein is biotinylated, as determined by standard methods known to those skilled in the art, including but not limited to streptavidin shift assay. According to preferred embodiments of the invention, the biotinylated soluble Fc γ R proteins are mixed with SA-PE in a 1X SA-PE:5X biotinylated soluble Fc γ R molar ratio to form a tetrameric Fc γ R complex.

30 [00172] In a preferred embodiment of the invention, polypeptides comprising Fc regions bind the tetrameric Fc γ R complexes, formed according to the methods of the invention, with at least an 8-fold higher affinity than the monomeric uncomplexed Fc γ R. The binding of polypeptides comprising Fc regions to the tetrameric Fc γ R complexes may be determined using standard techniques known to those skilled in the art, such as for

example, fluorescence activated cell sorting (FACS), radioimmunoassays, ELISA assays, *etc.*

[00173] The invention encompasses the use of the immune complexes formed according to the methods described above, for determining the functionality of molecules comprising an Fc region in cell-based or cell-free assays.

[00174] As a matter of convenience, the reagents may be provided in an assay kit, *i.e.*, a packaged combination of reagents for assaying the ability of molecules comprising variant Fc regions to bind Fc γ R tetrameric complexes. Other forms of molecular complexes for use in determining Fc-Fc γ R interactions are also contemplated for use in the methods of the invention, *e.g.*, fusion proteins formed as described in U.S. Provisional Application 60/439,709, filed on January 13, 2003 (Attorney Docket No. 11183-005-888).

5.2.2 MUTAGENESIS AND CONSTRUCTION OF YEAST DISPLAY LIBRARIES

[00175] An initial library of molecules comprising variant Fc regions is produced using any random based mutagenesis techniques known in the art. It will be appreciated by one of skill in the art that amino acid sequence variants of Fc regions may be obtained by any mutagenesis technique known to those skilled in the art. Some of these techniques are briefly described herein, however, it will be recognized that alternative procedures may produce an equivalent result. In a preferred embodiment molecules of the invention comprising variant Fc regions are prepared by error-prone PCR as exemplified in Example 6, *infra* (See Leung *et al.*, 1989, *Technique*, 1:11). It is especially preferred to have error rates of 2-3 bp/Kb for use in the methods of the invention. In one embodiment, using error prone PCR a mutation frequency of 2-3 mutations/kb is obtained.

[00176] Mutagenesis may be performed in accordance with any of the techniques known in the art including, but not limited to, synthesizing an oligonucleotide having one or more modifications within the sequence of the Fc region of an antibody or a polypeptide comprising an Fc region (*e.g.*, the CH2 or CH3 domain) to be modified. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 30 to about 45 nucleotides or more in length is preferred, with about 10 to about 25 or more residues on both sides of the junction of the

sequence being altered. A number of such primers introducing a variety of different mutations at one or more positions may be used to generated a library of mutants.

[00177] The technique of site-specific mutagenesis is well known in the art, as exemplified by various publications (*see, e.g., Kunkel et al., Methods Enzymol.*, 5 154:367-82, 1987). In general, site-directed mutagenesis is performed by first obtaining a single-stranded vector or melting apart of two strands of a double stranded vector which includes within its sequence a DNA sequence which encodes the desired peptide. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically. This primer is then annealed with the 10 single-stranded vector, and subjected to DNA polymerizing enzymes such as T7 DNA polymerase, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform or transfect appropriate cells, such as *E. coli* cells, and clones are selected which 15 include recombinant vectors bearing the mutated sequence arrangement. As will be appreciated, the technique typically employs a phage vector which exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage are readily commercially available and their use is generally well known to those skilled in the art. Double stranded plasmids are 20 also routinely employed in site directed mutagenesis which eliminates the step of transferring the gene of interest from a plasmid to a phage.

[00178] Alternatively, the use of PCRTM with commercially available thermostable enzymes such as *Taq* DNA polymerase may be used to incorporate a mutagenic oligonucleotide primer into an amplified DNA fragment that can then be cloned into an 25 appropriate cloning or expression vector. *See, e.g., Tomic et al., Nucleic Acids Res.*, 18(6):1656, 1987, and Upender *et al., Biotechniques*, 18(1):29-30, 32, 1995, for PCRTM - mediated mutagenesis procedures. PCRTM employing a thermostable ligase in addition to a thermostable polymerase may also be used to incorporate a phosphorylated mutagenic oligonucleotide into an amplified DNA fragment 30 that may then be cloned into an appropriate cloning or expression vector (*see e.g., Michael, Biotechniques*, 16(3):410-2, 1994).

[00179] Another method for preparing variants for use in the invention, is cassette mutagenesis based on the technique described by Wells *et al.* (1985, *Gene*, 34: 315). The starting material is the plasmid comprising the desired DNA encoding the protein to be 35 mutated (*e.g., the DNA encoding a polypeptide comprising an Fc region*). The codon(s) in

the DNA sequence to be mutated are identified; there must be a unique restriction endonuclease site on each side of the identified mutations site(s). If no such restriction site exists, it may be generated by oligonucleotide directed mutagenesis. After the restriction sites have been introduced into the plasmid, the plasmid is cut at these sites and linearized.

- 5 A double-stranded oligonucleotide encoding the sequence of the DNA between the restriction sites but containing the mutation is synthesized using standard procedures known to those skilled in the art. The double stranded oligonucleotide is referred to as the cassette. This cassette is designed to have 3' and 5' ends that are compatible with the ends of the linearized plasmid, such that it can be directly ligated to the plasmid.
- 10 [00180] Other methods known to those of skill in the art for producing sequence variants of the Fc region of an antibody or polypeptides comprising an Fc region can be used. For example, recombinant vectors encoding the amino acid sequence of the constant domain of an antibody or a fragment thereof may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants.
- 15 [00181] Once a mutant library is produced according to the methods described, the mutagenized library is transformed into a yeast strain, preferably EBY100 (Invitrogen), *MATa ura3-52 trp1 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1 GAL::GAL-AGA1* using a standard lithium acetate transformation protocol known to those skilled in the art (ref).
- [00182] It will be appreciated by one of skill in the art, that once molecules of the invention with desired binding properties (*e.g.*, molecules with variant Fc regions with at least one amino acid modification, which modification enhances the affinity of the variant Fc region for FcγRIIIA relative to a comparable molecule, comprising a wild-type Fc region) have been identified (*See* Section 5.1 and Table 2) according to the methods of the invention, other molecules (*i.e.*, therapeutic antibodies) may be engineered using standard
- 20 recombinant DNA techniques and any known mutagenesis techniques, as described in this section to produce engineered molecules carrying the identified mutation sites.

5.2.3 YEAST SURFACE DISPLAY

- [00183] The preferred method for screening and identifying molecules comprising variant Fc regions with altered FcγR affinities (*i.e.*, enhanced FcγRIIIA affinity and/or
- 30 FcγRIIA) is yeast surface display technology (for review *see* Boder and Wittrup, 2000, *Methods in Enzymology*, 328: 430-444)
- which addresses the deficiency in the prior art for screening binding interactions of extracellular post-translationally modified proteins. Specifically, the yeast surface display is a genetic method whereby polypeptides comprising Fc mutants are expressed on the yeast

cell wall in a form accessible for interacting with FcγR. Yeast surface display of the mutant Fc containing polypeptides of the invention may be performed in accordance with any of the techniques known to those skilled in the art. *See* U.S. Patent No.'s 6,423,538; 6,114,147; and 6,300,065.

- 5 *See Boder et al.*, 1997 *Nat. Biotechnol.*, 15:553-7; *Boder et al.*, 1998 *Biotechnol. Prog.*, 14:55-62; *Boder et al.*, 2000 *Methods Enzymol.*, 328:430-44; *Boder et al.*, 2000 *Proc. Natl. Acad. Sci. U.S.A.*, 2000, 97:10701-5; *Shusta et al.*, 1998 *Nat. Biotechnol.*, 1998, 16:773-7; *Shusta et al.*, 1999 *J. Mol. Biol.*, 292:949-56; *Shusta et al.*, 1999 *Curr. Opin. Biotechnol.*, 10:117-22; *Shusta et al.*, 2000 *Nat. Biotechnol.*, 18:754-9; *Wittrup et al.*, 1994 *Ann. N.Y. Acad. Sci.*, 745:321-30; *Wittrup et al.*, 1994 *Cytometry*, 16:206-13; *Wittrup*, 1995 *Curr. Opin. Biotechnol.*, 6:203-8; *Wittrup*, 1999 *Trends Biotechnol.*, 17:423-4; *Wittrup*, 2000 *Nat. Biotechnol.*, 18:1039-40; *Wittrup*, 2001 *Curr. Opin. Biotechnol.*, 12:395-9.

- [00184] The invention provides methods for constructing an Fc mutant library in yeast for displaying molecules comprising Fc regions, which have been mutated as
- 15 described in Section 5.2.2. Preferably, the Fc mutant libraries for use in the methods of the invention contain at least 10^7 cells, up to 10^9 cells. One exemplary method for constructing a Fc library for use in the methods of the invention comprises the following: nucleic acids encoding molecules comprising Fc regions are cloned into the multiple cloning site of a vector derived from a yeast replicating vector, *e.g.*, pCT302; such that the Fc encoding
- 20 nucleic acids are expressed under the control of the GAL1 galactose-inducible promoter and in-frame with a nucleotide sequence encoding Aga2p, the mating agglutinin cell wall protein. In a preferred embodiment, nucleic acids encoding molecules comprising Fc regions are cloned C-terminal to the Aga2p coding region, such that a Fc-region Aga2p fusion protein is encoded. A fusion protein comprising the Aga2p protein and polypeptides
- 25 comprising Fc regions will be secreted extracellularly and displayed on the cell wall via disulfide linkage to the Aga1p protein, an integral cell wall protein, using the preferred construct of the invention. In an alternative embodiment, the constructs may further comprise nucleotide sequences encoding epitope tags. Any epitope tag nucleotide coding sequence known to those skilled in the art can be used in accordance with the invention,
- 30 including, but not limited to nucleotide sequences encoding hemagglutinin (HA), c-myc Xpress TAG, His - TAG, or V5TAG. The presence of the fusion protein on the yeast cell surface may be detected using FACS analysis, confocal fluorescence microscopy or standard immunostaining methods, all of which are known to those skilled in the art. In one embodiment, the presence of the Fc fusion proteins of the invention on the yeast cell surface
- 35 are detected using Fc-specific monoclonal antibodies (CH3 specific), including but not

limited to IgG1 Fc-specific monoclonal antibody, HP6017 (Sigma), JL512 (Immunotech), and any antibody disclosed in Partridge *et al.*, 1986, *Molecular Immunology*, 23 (12): 1365-72. In another embodiment, the presence of the Fc fusion proteins of the invention are detected by immunofluorescent labeling of epitope tags using techniques known to those skilled in the art. It is particularly useful in the methods of the invention, to use nucleotide sequences encoding epitope tags to flank the nucleic acids encoding the Fc fusion proteins, as an internal control, to detect if the fusion proteins are displayed on the cell wall in a partially proteolyzed form.

5.2.4 SCREENING OF YEAST DISPLAY LIBRARIES

- 10 [00185] The invention encompasses screening the yeast display libraries using immunological based assays including but not limited to cell based assays, solution based assays, and solid phase based assays.
- [00186] In some embodiments, the invention encompasses identification of Fc mutants with altered FcγR affinities using affinity maturation methods which are known to those skilled in the art and encompassed herein. Briefly, affinity maturation creates novel alleles by randomly recombining individual mutations present in a mutant library, *see, e.g.*, Hawkins *et al.*, 1992, *J. Mol. Biol.* 226: 889-896; Stemmer *et al.*, 1994 *Nature*, 370: 389-91. It has been used successfully to increase the affinity of antibodies, T cell receptors and other proteins. The invention encompasses using mutations that show increased FcγR binding as a baseline to construct new mutant libraries with enhanced phenotypes. Using the methods of the invention, a population of IgG1 Fc mutants enriched by yeast surface display for increased binding to an FcγR, *e.g.*, FcγRIIIA, may be selected. Following DNA preparation, Fc regions can be amplified by PCR using flanking primers that selectively amplify the mutated region of the Fc, which is about ~700 bp using methods known to one skilled in the art and exemplified or disclosed herein. Novel mutants can thus be constructed by reshuffling of mutations in the Fc region for example via DNaseI treatment of the amplified DNA and isolation of fragments using methods such as those disclosed by Stemmer *et al.*, 1994 *Proc. Natl. Acad. Sci. USA* 91: 10747-51.
- 30 Fragments can then be religated, PCR amplified with nested primers and cloned into the yeast display vector, *e.g.*, pYD1 using methods known to one skilled in the art. The recombined library can then be reselected in the yeast Fc display screen. As the K_D decreases, below 10 nM, conditions can be established to allow for further increases in affinity based on the reduction of the off rate of the FcγRIIIA ligand from the Fc receptor

using methods known in the art such as those disclosed in Boder *et al.*, 1998, *Biotechnol. Prog.* 14: 55-62, which is incorporated herein by reference in its entirety. The invention encompasses a kinetic screen of the yeast library. A kinetic screen may be established by labeling of the Fc displaying cells to saturation with a labeled ligand, e.g., a fluorescent ligand followed by incubation with an excess of non-labeled ligand for a predetermined period. After termination of the reaction by the addition of excess buffer (e.g., 1X PBS, 0.5 mg/ml BSA) cells will be analyzed by FACS and sort gates set for selection. After each round of enrichment individual mutants can be tested for fold increases in affinity and sequenced for diversity. The *in vitro* recombination process can be repeated. In some embodiments, the *in vitro* is repeated at least 3 times.

[00187] Selection of the Fc variants of the invention may be done using any FcγR including but not limited to polymorphic variants of FcγR. In some embodiments, selection of the Fc variants is done using a polymorphic variant of FcγRIIA which contains a phenylalanine at position 158. In other embodiments, selection of the Fc variants is done using a polymorphic variant of FcγRIIA which contains a valine at position 158. FcγRIIA 158V displays a higher affinity for IgG1 than 158F and an increased ADCC activity (*see, e.g., Koene et al.*, 1997, *Blood*, 90:1109-14; *Wu et al.*, 1997, *J. Clin. Invest.* 100: 1059-70); this residue in fact

directly interacts with the lower hinge region of IgG1 as recently shown by IgG1-FcγRIIA co-crystallization studies, *see, e.g., Sonderman et al.*, 2000, *Nature*, 100: 1059-70.

Studies have shown that in some cases therapeutic antibodies have improved efficacy in FcγRIIA-158V homozygous patients. For example, humanized anti-CD20 monoclonal antibody Rituximab was therapeutically more effective in FcγRIIA158V homozygous patients compared to FcγRIIA 158F homozygous patients (*See, e.g., Cartron et al.*, 2002 *Blood*, 99(3): 754-8). Although not intending to be bound by a particular mechanism of action, selection of Fc variants of the invention with FcγRIIA 158F allotype may provide for variants that once engineered into therapeutic antibodies will be clinically more efficacious for FcγRIIA 158F homozygous patients.

[00188] The invention encompasses screening yeast libraries based on FcγRIIB depletion and FcγRIIA selection so that Fc mutants are selected that not only have an enhanced affinity for FcγRIIA but also have a reduced affinity for FcγRIIB. Yeast libraries may be enriched for clones that have a reduced affinity for FcγRIIB by sequential depletion methods, for example, by incubating the yeast library with magnetic beads coated with FcγRIIB. FcγRIIB depletion is preferably carried out sequentially so that the library is

enriched in clones that have a reduced affinity for Fc γ RIIB. In some embodiments, the Fc γ RIIB depletion step results in a population of cells so that only 30% , preferably only 10%, more preferably only 5%, most preferably less than 1% bind Fc γ RIIB. In some
 5 6 cycles. The Fc γ RIIB depletion step is preferably combined with an Fc γ RIIIA selection step, for example using FACS sorting so that Fc variants with an enhanced affinity for Fc γ RIIIA are selected.

5.2.4.1 FACS ASSAYS; SOLID PHASED ASSAYS AND IMMUNOLOGICAL BASED ASSAYS

10 [00189] The invention encompasses characterization of the mutant Fc fusion proteins that are displayed on the yeast surface cell wall, according to the methods described in Section 5.2.3. One aspect of the invention provides a method for selecting mutant Fc fusion proteins with a desirable binding property, specifically, the ability of the mutant Fc fusion
 15 polypeptide comprising a wild-type Fc region binds Fc γ RIIIA and/or Fc γ RIIA. In another embodiment, the invention provides a method for selecting mutant Fc fusion proteins with a desirable binding property, specifically, the ability of the mutant Fc fusion protein to bind Fc γ RIIIA and/or Fc γ RIIA with a greater affinity than a comparable polypeptide comprising a wild-type Fc region binds Fc γ RIIIA and/or Fc γ RIIA, and further the ability of the mutant
 20 Fc fusion protein to bind Fc γ RIIB with a lower affinity than a comparable polypeptide comprising a wild-type Fc region binds Fc γ RIIB. It will be appreciated by one skilled in the art, that the methods of the invention can be used for identifying and screening any mutations in the Fc regions of molecules, with any desired binding characteristic.

[00190] Yeast cells displaying the mutant Fc fusion proteins can be screened and
 25 characterized by any biochemical or immunological based assays known to those skilled in the art for assessing binding interactions.

[00191] Preferably, fluorescence activated cell sorting (FACS), using any of the techniques known to those skilled in the art, is used for screening the mutant Fc fusion proteins displayed on the yeast cell surface for binding Fc γ RIIIA, preferably the Fc γ RIIIA
 30 tetrameric complex, or optionally Fc γ RIIB. Flow sorters are capable of rapidly examining a large number of individual cells that contain library inserts (e.g., 10-100 million cells per hour) (Shapiro *et al.*, *Practical Flow Cytometry*, 1995). Additionally, specific parameters used for optimization including, but not limited to, ligand concentration (*i.e.*, Fc γ RIIIA tetrameric complex), kinetic competition time, or FACS stringency may be varied in order

to select for the cells which display Fc fusion proteins with specific binding properties, *e.g.*, higher affinity for FcγRIIIA compared to a comparable polypeptide comprising a wild-type Fc region. Flow cytometers for sorting and examining biological cells are well known in the art. Known flow cytometers are described, for example, in U.S. Patent Nos. 4,347,935;
 5 5,464,581; 5,483,469; 5,602,039; 5,643,796; and 6,211,477.

Other known flow cytometers are the FACS Vantage™ system manufactured by Becton Dickinson and Company, and the COPAS™ system manufactured by Union Biometrica.

[00192] According to a preferred embodiment of the invention, yeast cells are
 10 analyzed by fluorescence activated cell sorting (FACS). In most preferred embodiments, the FACS analysis of the yeast cells is done in an iterative manner, at least twice, at least three times, or at least 5 times. Between each round of selection cells are regrown and induced so the Fc regions are displayed on the maximum number of yeast cell surfaces. Although not intending to be bound by a particular mode of action, this iterative process
 15 helps enrich the population of the cells with a particular phenotype, *e.g.*, high binding to FcγRIIIA.

[00193] In preferred embodiments, screening for Fc variants of the invention comprises a selection process that has multiple rounds of screening, *e.g.*, at least two rounds of screening. In one embodiment, screening for Fc variants that have an enhanced affinity
 20 for FcγRIIIA may comprise the following steps: in the first round of screening, a library of yeast cells, *e.g.*, a naïve library of 10^7 cells is enriched by FACS, preferably in an iterative manner, using for example labeled tetrameric FcγRIIIA to select for Fc variants that have an enhanced affinity for FcγRIIIA; the variant Fc region that is selected with the desired phenotype, *e.g.*, enhanced binding to FcγRIIIA, is then introduced into an antibody, *e.g.*, a
 25 4-4-20 antibody, and the engineered antibody is assayed using a secondary screen, *e.g.*, ELISA for binding to an FcγR. In the second round of screening, a single mutation library may be generated based on the first screen so that the Fc region harbors the variant displaying the enhanced affinity for FcγRIIIA; and enriched by FACS using for example labeled monomeric FcγRIIIA in both the presence and absence of unlabeled receptor; and
 30 the variant Fc region is then introduced into an antibody, *e.g.*, a 4-4-20 antibody, and the engineered antibody is assayed using a secondary screen, *e.g.*, ELISA for binding to an FcγR. In some embodiments, the secondary screen may further comprise characterizing the antibodies comprising Fc variants in an ADCC or BIAcore based assay using methods disclosed herein

[00194] The invention encompasses FACS screening of the mutant yeast library under equilibrium or kinetic conditions. When the screening is performed under equilibrium conditions, an excess of the yeast library carrying Fc mutants is incubated with Fc γ RIIIA, preferably labeled Fc γ RIIIA at a concentration 5-10 fold below the K_d, for at least one hour to allow binding of Fc mutants to Fc γ RIIIA under equilibrium conditions. When the screening is performed under kinetic conditions, the mutant yeast library is incubated with labeled Fc γ RIIIA; the cells are then incubated with equimolar unlabeled Fc γ RIIIA for a pre-selected time, bound Fc γ RIIIA is then monitored.

[00195] One exemplary method of analyzing the yeast cells expressing mutant Fc fusion proteins with FACS is costaining the cells with Fc γ RIIIA-tetrameric complex which has been labeled with a fluorescent label such as, PE and an anti-Fc antibody, such as F(ab)₂ anti-Fc which has been fluorescently labeled. Fluorescence measurements of a yeast library produced according to the methods of the invention preferably involves comparisons with controls; for example, yeast cells that lack the insert encoding molecules comprising an Fc region (negative control). The flow sorter has the ability not only to measure fluorescence signals in cells at a rapid rate, but also to collect cells that have specified fluorescent properties. This feature may be employed in a preferred embodiment of the invention to enrich the initial library population for cells expressing Fc fusion proteins with specific binding characteristics, *e.g.*, higher affinity for Fc γ RIIIA compared to a comparable polypeptide comprising a wild-type Fc region. In a preferred embodiment of the invention, yeast cells are analyzed by FACS and sort gates established to select for cells that show the highest affinity for Fc γ RIIIA relative to the amount of Fc expression on the yeast cell surface. According to a preferred embodiment, four consecutive sorts are established, wherein the gates for each successive sort is 5.5%, 1%, 0.2%, and 0.1%. It is preferred that the yeast display library formed according to the methods of the invention be over-sampled by at least 10-fold to improve the probability of isolating rare clones (*e.g.*, analyze $\sim 10^8$ cells from a library of 10^7 clones). Alternatively, 2-5 sorts are established to select for cells of the desired phenotype. Sort gates can be established empirically by one skilled in the art.

[00196] In other preferred embodiments, mutant Fc fusion proteins displayed on the yeast cell surface are screened using solid phase based assays, for example assays using magnetic beads, *e.g.*, supplied by Dynal, preferably in a high through put manner for binding to an Fc γ R, *e.g.*, Fc γ RIIIA. In one embodiment, magnetic bead assays may be used to identify mutants with enhanced affinity for Fc γ RIIIA and/or reduced affinity for Fc γ RIIB. An exemplary assay to identify mutants with enhanced affinity for Fc γ RIIIA and reduced

affinity for FcγRIIB may comprise selecting mutants by a sequential solid phase depletion using magnetic beads coated with FcγRIIB followed by selection with magnetic beads coated with FcγRIIA. For example one assay may comprise the following steps:
 incubating the library of yeast cells generated in accordance with the methods of the
 5 invention with magnetic beads coated with FcγRIIB; separating yeast cells bound to beads from the non bound fraction by placing the mixture in a magnetic field, removing the non-bound yeast cells and placing them in a fresh media; binding the yeast cells to beads coated with FcγRIIA, separating yeast cells bound to beads from the non bound fraction by placing the mixture in a magnetic field, removing the non-bound yeast cells; removing the
 10 bound cells by rigorous vortexing; growing the recovered cells in glucose containing media; re-inducing in selective media containing galactose. The selection process is repeated at least once. Inserts containing the Fc domain are then amplified using common methodologies known in the art, *e.g.*, PCR, and introduced into an antibody by methods already described for further characterization.

15 [00197] In an alternative embodiment, a non-yeast based system is used to characterize the binding properties of the molecules of the invention. One exemplary system for characterizing the molecules of the invention comprises a mammalian expression vector containing the heavy chain of the anti-fluorescein monoclonal antibody 4-4-20, into which the nucleic acids encoding the molecules of the invention with variant Fc regions are
 20 cloned. The resulting recombinant clone is expressed in a mammalian host cell line (*i.e.*, human kidney cell line 293H), and the resulting recombinant immunoglobulin is analyzed for binding to FcγR using any standard assay known to those in the art, including but not limited to ELISA and FACS.

[00198] Molecules of the present invention (*e.g.*, antibodies, fusion proteins,
 25 conjugated molecules) may be characterized in a variety of ways. In particular, molecules of the invention comprising modified Fc regions may be assayed for the ability to immunospecifically bind to a ligand, *e.g.*, FcγRIIA tetrameric complex. Such an assay may be performed in solution (*e.g.*, Houghten, *Bio/Techniques*, 13:412-421, 1992), on beads (Lam, *Nature*, 354:82-84, 1991, on chips (Fodor, *Nature*, 364:555-556, 1993), on bacteria
 30 (U.S. Patent No. 5,223,409), on spores (U.S. Patent Nos. 5,571,698; 5,403,484; and 5,223,409), on plasmids (Cull *et al.*, *Proc. Natl. Acad. Sci. USA*, 89:1865-1869, 1992) or on phage (Scott and Smith, *Science*, 249:386-390, 1990; Devlin, *Science*, 249:404-406, 1990; Cwirla *et al.*, *Proc. Natl. Acad. Sci. USA*, 87:6378-6382, 1990; and Felici, *J. Mol. Biol.*, 222:301-310, 1991).

entirety). Molecules that have been identified to immunospecifically bind to an ligand, *e.g.*, Fc γ RIIIA can then be assayed for their specificity and affinity for the ligand.

[00199] Molecules of the invention that have been engineered to comprise modified Fc regions (*e.g.*, therapeutic antibodies) or have been identified in the yeast display system
 5 to have the desired phenotype (*see* Section 5.1) may be assayed for immunospecific binding to an antigen (*e.g.*, cancer antigen and cross-reactivity with other antigens (*e.g.*, Fc γ R) by any method known in the art. Immunoassays which can be used to analyze immunospecific binding and cross-reactivity include, but are not limited to, competitive and non-competitive assay systems using techniques such as western blots, radioimmunoassays, ELISA (enzyme
 10 linked immunosorbent assay), “sandwich” immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, to name but a few. Such assays are routine and well known in the art (*see, e.g.*, Ausubel *et al.*, eds, 1994, Current Protocols in Molecular
 15 Biology, Vol. 1, John Wiley & Sons, Inc., New York).

[00200] The binding affinity of the molecules of the present invention comprising modified Fc regions to a ligand, *e.g.*, Fc γ R tetrameric complex and the off-rate of the interaction can be determined by competitive binding assays. One example of a competitive
 20 binding assay is a radioimmunoassay comprising the incubation of labeled ligand, such as tetrameric Fc γ R (*e.g.*, ^3H or ^{125}I) with a molecule of interest (*e.g.*, molecules of the present invention comprising modified Fc regions) in the presence of increasing amounts of unlabeled ligand, such as tetrameric Fc γ R, and the detection of the molecule bound to the labeled ligand. The affinity of the molecule of the present invention for the ligand and the
 25 binding off-rates can be determined from the saturation data by scatchard analysis.

[00201] In a preferred embodiment, BIAcore kinetic analysis is used to determine the binding on and off rates of molecules of the present invention to a ligand such as Fc γ R. BIAcore kinetic analysis comprises analyzing the binding and dissociation of a ligand from
 30 chips with immobilized molecules (*e.g.*, molecules comprising modified Fc regions) on their surface.

5.2.5 SEQUENCING OF MUTANTS

[00202] Any of a variety of sequencing reactions known in the art can be used to directly sequence the molecules of the invention comprising variant Fc regions. Examples of sequencing reactions include those based on techniques developed by Maxim and Gilbert

(*Proc. Natl. Acad. Sci. USA*, 74:560, 1977) or Sanger (*Proc. Natl. Acad. Sci. USA*, 74:5463, 1977). It is also contemplated that any of a variety of automated sequencing procedures can be utilized (*Bio/Techniques*, 19:448, 1995), including sequencing by mass spectrometry (see, e.g., PCT Publication No. WO 94/16101, Cohen *et al.*, *Adv. Chromatogr.*, 36:127-162, 1996, and Griffin *et al.*, *Appl. Biochem. Biotechnol.*, 38:147-159, 1993).

5.2.6 FUNCTIONAL ASSAYS OF MOLECULES WITH VARIANT Fc REGIONS

[00203] The invention encompasses characterization of the molecules of the invention (e.g., an antibody comprising a variant Fc region identified by the yeast display technology described *supra*; or therapeutic monoclonal antibodies engineered according to the methods of the invention) using assays known to those skilled in the art for identifying the effector cell function of the molecules. In particular, the invention encompasses characterizing the molecules of the invention for Fc γ R-mediated effector cell function. Examples of effector cell functions that can be assayed in accordance with the invention, include but are not limited to, antibody-dependent cell mediated cytotoxicity, phagocytosis, opsonization, opsonophagocytosis, C1q binding, and complement dependent cell mediated cytotoxicity. Any cell-based or cell free assay known to those skilled in the art for determining effector cell function activity can be used (For effector cell assays, see Perussia *et al.*, 2000, *Methods Mol. Biol.* 121: 179-92; Baggiolini *et al.*, 1998 *Experientia*, 44(10): 841-8; Lehmann *et al.*, 2000 *J. Immunol. Methods*, 243(1-2): 229-42; Brown EJ. 1994, *Methods Cell Biol.*, 45: 147-64; Munn *et al.*, 1990 *J. Exp. Med.*, 172: 231-237, Abdul-Majid *et al.*, 2002 *Scand. J. Immunol.* 55: 70-81; Ding *et al.*, 1998, *Immunity* 8:403-411).

[00204] In one embodiment, the molecules of the invention can be assayed for Fc γ R-mediated phagocytosis in human monocytes. Alternatively, the Fc γ R-mediated phagocytosis of the molecules of the invention may be assayed in other phagocytes, e.g., neutrophils (polymorphonuclear leukocytes; PMN); human peripheral blood monocytes, monocyte-derived macrophages, which can be obtained using standard procedures known to those skilled in the art (e.g., see Brown EJ. 1994, *Methods Cell Biol.*, 45: 147-164). In one embodiment, the function of the molecules of the invention is characterized by measuring the ability of THP-1 cells to phagocytose fluoresceinated IgG-opsonized sheep red blood cells (SRBC) by methods previously described (Tridandapani *et al.*, 2000, *J. Biol. Chem.* 275: 20480-7). For example, an exemplary assay for measuring phagocytosis of the molecules of the invention comprising variant Fc regions with enhanced affinities for

Fc γ RIIA, comprises of: treating THP-1 cells with a molecule of the invention or with a control antibody that does not bind to Fc γ RIIA, comparing the activity levels of said cells, wherein a difference in the activities of the cells (*e.g.*, rosetting activity (the number of THP-1 cells binding IgG-coated SRBC), adherence activity (the total number of SRBC bound to THP-1 cells), and phagocytic rate) would indicate the functionality of the molecule of the invention. It can be appreciated by one skilled in the art that this exemplary assay can be used to assay any of the molecules identified by the methods of the invention.

[00205] Another exemplary assay for determining the phagocytosis of the molecules of the invention is an antibody-dependent opsonophagocytosis assay (ADCP) which can comprise the following: coating a target bioparticle such as *Escherichia coli*-labeled FITC (Molecular Probes) or *Staphylococcus aureus*-FITC with (i) wild-type 4-4-20 antibody, an antibody to fluorescein (*See* Bedzyk *et al.*, 1989, *J. Biol. Chem.*, 264(3): 1565-1569, which is incorporated herein by reference in its entirety), as the control antibody for Fc γ R-dependent ADCP; or (ii) 4-4-20 antibody harboring the D265A mutation that knocks out binding to Fc γ RIII, as a background control for Fc γ R-dependent ADCP (iii) 4-4-20 antibody carrying variant Fc regions identified by the methods of the invention and produced as exemplified in Example 6.6; and forming the opsonized particle; adding any of the opsonized particles described (i-iii) to THP-1 effector cells (a monocytic cell line available from ATCC) in a 60:1 ratio to allow Fc γ R-mediated phagocytosis to occur; preferably incubating the cells and *E. coli*-FITC/antibody at 37°C for 1.5 hour; adding trypan blue after incubation (preferably at room temperature for 2-3 min.) to the cells to quench the fluorescence of the bacteria that are adhered to the outside of the cell surface without being internalized; transferring cells into a FACS buffer (*e.g.*, 0.1%, BSA in PBS, 0.1%, sodium azide), analyzing the fluorescence of the THP1 cells using FACS (*e.g.*, BD FACS Calibur). Preferably, the THP-1 cells used in the assay are analyzed by FACS for expression of Fc γ R on the cell surface. THP-1 cells express both CD32A and CD64. CD64 is a high affinity Fc γ R that is blocked in conducting the ADCP assay in accordance with the methods of the invention. The THP-1 cells are preferably blocked with 100 μ g/mL soluble IgG1 or 10% human serum. To analyze the extent of ADCP, the gate is preferably set on THP-1 cells and median fluorescence intensity is measured. The ADCP activity for individual mutants is calculated and reported as a normalized value to the wild type chMab 4-4-20 obtained. The opsonized particles are added to THP-1 cells such that the ratio of the opsonized particles to THP-1 cells is 30:1 or 60:1. In most preferred embodiments, the ADCP assay is conducted with controls, such as *E. coli*-FITC in medium, *E. coli*-FITC and THP-1 cells (to serve as Fc γ R-independent ADCP activity), *E. coli*-FITC, THP-1 cells and

wild-type 4-4-20 antibody (to serve as FcγR-dependent ADCP activity), *E coli*-FITC, THP-1 cells, 4-4-20 D265A (to serve as the background control for FcγR-dependent ADCP activity).

[00206] In another embodiment, the molecules of the invention can be assayed for FcγR-mediated ADCC activity in effector cells, *e.g.*, natural killer cells, using any of the standard methods known to those skilled in the art (*See e.g.*, Perussia *et al.*, 2000, *Methods Mol. Biol.* 121: 179-92). An exemplary assay for determining ADCC activity of the molecules of the invention is based on a ⁵¹Cr release assay comprising of: labeling target cells with [⁵¹Cr]Na₂CrO₄ (this cell-membrane permeable molecule is commonly used for labeling since it binds cytoplasmic proteins and although spontaneously released from the cells with slow kinetics, it is released massively following target cell necrosis); opsonizing the target cells with the molecules of the invention comprising variant Fc regions; combining the opsonized radiolabeled target cells with effector cells in a microtitre plate at an appropriate ratio of target cells to effector cells; incubating the mixture of cells for 16-18 hours at 37°C; collecting supernatants; and analyzing radioactivity. The cytotoxicity of the molecules of the invention can then be determined, for example using the following formula: % lysis = (experimental cpm - target leak cpm)/(detergent lysis cpm - target leak cpm) x 100%. Alternatively, % lysis =(ADCC-AICC)/(maximum release-spontaneous release). Specific lysis can be calculated using the formula: specific lysis = % lysis with the molecules of the invention - % lysis in the absence of the molecules of the invention. A graph can be generated by varying either the target: effector cell ratio or antibody concentration.

[00207] In yet another embodiment, the molecules of the invention are characterized for antibody dependent cellular cytotoxicity (ADCC) *see, e.g.*, Ding *et al.*, *Immunity*, 1998, 8:403-11.

[00208] Preferably, the effector cells used in the ADCC assays of the invention are peripheral blood mononuclear cells (PBMC) that are preferably purified from normal human blood, using standard methods known to one skilled in the art, *e.g.*, using Ficoll-Paque density gradient centrifugation. Preferred effector cells for use in the methods of the invention express different FcγR activating receptors. The invention encompasses, effector cells, THP-1, expressing FcγRI, FcγRIIA and FcγRIIB, and monocyte derived primary macrophages derived from whole human blood expressing both FcγRIIA and FcγRIIB, to determine if Fc antibody mutants show increased ADCC activity and phagocytosis relative to wild type IgG1 antibodies.

[00209] The human monocyte cell line, THP-1, activates phagocytosis through expression of the high affinity receptor FcγRI and the low affinity receptor FcγRIIA (Fleit et al., 1991, *J. Leuk. Biol.* 49: 556). THP-1 cells do not constitutively express FcγRIIA or FcγRIIB. Stimulation of these cells with cytokines effects the FcR expression pattern
5 (Pricop et al., 2000 *J. Immunol.* 166: 531-7). Growth of THP-1 cells in the presence of the cytokine IL4 induces FcγRIIB expression and causes a reduction in FcγRIIA and FcγRI expression. FcγRIIB expression can also be enhanced by increased cell density (Tridandapani *et al.*, 2002, *J. Biol Chem.* 277: 5082-9). In contrast, it has been reported that IFNγ can lead to expression of FcγRIIA (Pearse *et al.*, 1993 *PNAS USA* 90: 4314-8). The
10 presence or absence of receptors on the cell surface can be determined by FACS using common methods known to one skilled in the art. Cytokine induced expression of FcγR on the cell surface provides a system to test both activation and inhibition in the presence of FcγRIIB. If THP-1 cells are unable to express the FcγRIIB the invention also encompasses another human monocyte cell line, U937. These cells have been shown to terminally
15 differentiate into macrophages in the presence of IFNγ and TNF (Koren *et al.*, 1979, *Nature* 279: 328-331).

[00210] FcγR dependent tumor cell killing is mediated by macrophage and NK cells in mouse tumor models (Clynes *et al.*, 1998, *PNAS USA* 95: 652-656). The invention encompasses the use of elutriated monocytes from donors as effector cells to analyze the
20 efficiency Fc mutants to trigger cell cytotoxicity of target cells in both phagocytosis and ADCC assays. Expression patterns of FcγRI, FcγRIIA, and FcγRIIB are affected by different growth conditions. FcγR expression from frozen elutriated monocytes, fresh elutriated monocytes, monocytes maintained in 10% FBS, and monocytes cultured in FBS + GM-CSF and or in human serum may be determined using common methods known to
25 those skilled in the art. For example, cells can be stained with FcγR specific antibodies and analyzed by FACS to determine FcR profiles. Conditions that best mimic macrophage *in vivo* FcγR expression is then used for the methods of the invention.

[00211] In some embodiments, the invention encompasses the use of mouse cells especially when human cells with the right FcγR profiles are unable to be obtained. In some
30 embodiments, the invention encompasses the mouse macrophage cell line RAW264.7(ATCC) which can be transfected with human FcγRIIA and stable transfectants isolated using methods known in the art, *see, e.g.*, Ralph *et al.*, *J. Immunol.* 119: 950-4). Transfectants can be quantitated for FcγRIIA expression by FACS analysis using routine experimentation and high expressors can be used in the ADCC assays of the invention. In

other embodiments, the invention encompasses isolation of spleen peritoneal macrophage expressing human FcγR from knockout transgenic mice such as those disclosed herein.

[00212] Lymphocytes may be harvested from peripheral blood of donors (PBM) using a Ficoll-Paque gradient (Pharmacia). Within the isolated mononuclear population of cells the majority of the ADCC activity occurs via the natural killer cells (NK) containing FcγRIIIA but not FcγRIIB on their surface. Results with these cells indicate the efficacy of the mutants on triggering NK cell ADCC and establish the reagents to test with elutriated monocytes.

[00213] Target cells used in the ADCC assays of the invention include, but are not limited to, breast cancer cell lines, *e.g.*, SK-BR-3 with ATCC accession number HTB-30 (*see, e.g.*, Tremp *et al.*, 1976, *Cancer Res.* 33-41); B-lymphocytes; cells derived from Burkitts lymphoma, *e.g.*, Raji cells with ATCC accession number CCL-86 (*see, e.g.*, Epstein *et al.*, 1965, *J. Natl. Cancer Inst.* 34: 231-240), and Daudi cells with ATCC accession number CCL-213 (*see, e.g.*, Klein *et al.*, 1968, *Cancer Res.* 28: 1300-10). The target cells must be recognized by the antigen binding site of the immunoglobulin to be assayed.

[00214] The ADCC assay is based on the ability of NK cells to mediate cell death via an apoptotic pathway. NK cells mediate cell death in part by FcγRIIIA's recognition of IgG bound to an antigen on a cell surface. The ADCC assays used in accordance with the methods of the invention may be radioactive based assays or fluorescence based assays. The ADCC assay used to characterize the molecules of the invention comprising variant Fc regions comprises labeling target cells, *e.g.*, SK-BR-3, MCF-7, OVCAR3, Raji, Daudi cells, opsonizing target cells with an antibody that recognizes a cell surface receptor on the target cell via its antigen binding site; combining the labeled opsonized target cells and the effector cells at an appropriate ratio, which can be determined by routine experimentation; harvesting the cells; detecting the label in the supernatant of the lysed target cells, using an appropriate detection scheme based on the label used. The target cells may be labeled either with a radioactive label or a fluorescent label, using standard methods known in the art. For example the labels include, but are not limited to, [⁵¹Cr]Na₂CrO₄; and the acetoxymethyl ester of the fluorescence enhancing ligand, 2,2':6',2''-terpyridine-6-6''-dicarboxylate (TDA).

[00215] In a specific preferred embodiment, a time resolved fluorimetric assay is used for measuring ADCC activity against target cells that have been labeled with the acetoxymethyl ester of the fluorescence enhancing ligand, 2,2':6',2''-terpyridine-6-6''-dicarboxylate (TDA). Such fluorimetric assays are known in the art, *e.g.*, *see*, Blomberg

et al., 1996, *Journal of Immunological Methods*, 193: 199-206.

Briefly, target cells are labeled with the membrane permeable acetoxymethyl diester of TDA (bis(acetoxymethyl) 2,2':6',2''-terpyridine-6-6''-dicarboxylate, (BATDA), which rapidly diffuses across the cell membrane of viable cells. Intracellular esterases split off the ester groups and the regenerated membrane impermeable TDA molecule is trapped inside the cell. After incubation of effector and target cells, *e.g.*, for at least two hours, up to 3.5 hours, at 37°C, under 5% CO₂, the TDA released from the lysed target cells is chelated with Eu³⁺ and the fluorescence of the Europium-TDA chelates formed is quantitated in a time-resolved fluorometer (*e.g.*, Victor 1420, Perkin Elmer/Wallac).

[00216] In another specific embodiment, the ADCC assay used to characterize the molecules of the invention comprising variant Fc regions comprises the following steps: Preferably 4-5x10⁶ target cells (*e.g.*, SK-BR-3, MCF-7, OVCAR3, Raji cells) are labeled with bis(acetoxymethyl) 2,2':6',2''-terpyridine-t-6''-dicarboxylate (DELFLIA BATDA Reagent, Perkin Elmer/Wallac). For optimal labeling efficiency, the number of target cells used in the ADCC assay should preferably not exceed 5x10⁶. BATDA reagent is added to the cells and the mixture is incubated at 37°C preferably under 5% CO₂, for at least 30 minutes. The cells are then washed with a physiological buffer, *e.g.*, PBS with 0.125 mM sulfinpyrazole, and media containing 0.125 mM sulfinpyrazole. The labeled target cells are then opsonized (coated) with a molecule of the invention comprising a variant Fc region, *i.e.*, an immunoglobulin comprising a variant Fc region of the invention, including, but not limited to, a polyclonal antibody, a monoclonal antibody, a bispecific antibody, a multi-specific antibody, a humanized antibody, or a chimeric antibody. In preferred embodiments, the immunoglobulin comprising a variant Fc region used in the ADCC assay is specific for a cell surface receptor, a tumor antigen, or a cancer antigen. The immunoglobulin into which a variant Fc region of the invention is introduced may specifically bind any cancer or tumor antigen, such as those listed in section 5.4. Additionally, the immunoglobulin into which a variant Fc region of the invention is introduced may be any therapeutic antibody specific for a cancer antigen, such as those listed in section 5.4. In some embodiments, the immunoglobulin comprising a variant Fc region used in the ADCC assay is an anti-fluoresceine monoclonal antibody, 4-4-20 (Kranz *et al.*, 1982 *J. Biol. Chem.* 257(12): 6987-6995) a mouse-human chimeric anti-CD20 monoclonal antibody 2H7 (Liu *et al.*, 1987, *Journal of Immunology*, 139: 3521-6); or a humanized antibody (Ab4D5) against the human epidermal growth factor receptor 2 (p185 HER2) (Carter *et al.* (1992, *Proc. Natl. Acad. Sci. USA* 89: 4285-9). The target cells in the

ADCC assay are chosen according to the immunoglobulin into which a variant Fc region of the invention has been introduced so that the immunoglobulin binds a cell surface receptor of the target cell specifically. Preferably, the ADCC assays of the invention are performed using more than one engineered antibody, *e.g.*, anti Her2/neu, 4-4-20, 2B6, Rituxan, and 2H7, harboring the Fc variants of the invention. In a most preferred embodiment, the Fc variants of the invention are introduced into at least 3 antibodies and their ADCC activities is tested. Although not intending to be bound by a particular mechanism of action, examining at least 3 antibodies in these functional assays will diminish the chance of eliminating a viable Fc mutation erroneously.

10 [00217] Opsonized target cells are added to effector cells, *e.g.*, PBMC, to produce effector:target ratios of approximately 50:1, 75:1, or 100:1. In a specific embodiment, when the immunoglobulin comprising a variant Fc region has the variable domain of 4-4-20, the effector:target is 75:1. The effector and target cells are incubated for at least two hours, up to 3.5 hours, at 37°C, under 5% CO₂. Cell supernatants are harvested and added to an

15 acidic europium solution (*e.g.*, DELFIA Europium Solution, Perkin Elmer/Wallac). The fluorescence of the Europium-TDA chelates formed is quantitated in a time-resolved fluorometer (*e.g.*, Victor 1420, Perkin Elmer/Wallac). Maximal release (MR) and spontaneous release (SR) are determined by incubation of target cells with 1% TX-100 and media alone, respectively. Antibody independent cellular cytotoxicity (AICC) is measured

20 by incubation of target and effector cells in the absence of antibody. Each assay is preferably performed in triplicate. The mean percentage specific lysis is calculated as: Experimental release (ADCC) - AICC)/(MR-SR) x 100.

5.2.7 OTHER ASSAYS

[00218] The molecules of the invention comprising variant Fc regions may also be

25 assayed using any surface plasmon resonance based assays known in the art for characterizing the kinetic parameters of Fc-FcγR interaction binding. Any SPR instrument commercially available including, but not limited to, BIAcore Instruments, available from Biacore AB (Uppsala, Sweden); IAsys instruments available from Affinity Sensors (Franklin, MA.); IBIS system available from Windsor Scientific Limited (Berks, UK), SPR-

30 CELLIA systems available from Nippon Laser and Electronics Lab (Hokkaido, Japan), and SPR Detector Spreeta available from Texas Instruments (Dallas, TX) can be used in the instant invention. For a review of SPR-based technology *see* Mullet *et al.*, 2000, *Methods* 22: 77-91; Dong *et al.*, 2002, *Review in Mol. Biotech.*, 82: 303-23; Fivash *et al.*, 1998, *Current Opinion in Biotechnology* 9: 97-101; Rich *et al.*, 2000, *Current Opinion in*

Biotechnology 11: 54-61.

Additionally, any of the SPR instruments and SPR based methods for measuring protein-protein interactions described in U.S. Patent No.'s 6,373,577; 6,289,286; 5,322,798; 5,341,215; 6,268,125 are contemplated in the methods of the invention.

5

[00219] Briefly, SPR based assays involve immobilizing a member of a binding pair on a surface, and monitoring its interaction with the other member of the binding pair in solution in real time. SPR is based on measuring the change in refractive index of the solvent near the surface that occurs upon complex formation or dissociation. The surface onto which the immobilization occur is the sensor chip, which is at the heart of the SPR technology; it consists of a glass surface coated with a thin layer of gold and forms the basis for a range of specialized surfaces designed to optimize the binding of a molecule to the surface. A variety of sensor chips are commercially available especially from the companies listed *supra*, all of which may be used in the methods of the invention.

10
15 Examples of sensor chips include those available from BIAcore AB, Inc., *e.g.*, Sensor Chip CM5, SA, NTA, and HPA. A molecule of the invention may be immobilized onto the surface of a sensor chip using any of the immobilization methods and chemistries known in the art, including but not limited to, direct covalent coupling via amine groups, direct covalent coupling via sulfhydryl groups, biotin attachment to avidin coated surface, aldehyde coupling to carbohydrate groups, and attachment through the histidine tag with NTA chips.

20
25 [00220] In some embodiments, the kinetic parameters of the binding of molecules of the invention comprising variant Fc regions, *e.g.*, immunoglobulins comprising variant Fc region, to an FcγR may be determined using a BIAcore instrument (*e.g.*, BIAcore instrument 1000, BIAcore Inc., Piscataway, NJ). Any FcγR can be used to assess the interaction with the molecules of the invention comprising variant Fc regions. In a specific embodiment the FcγR is FcγRIIIA, preferably a soluble monomeric FcγRIIIA. For example, in one embodiment, the soluble monomeric FcγRIIIA is the extracellular region of FcγRIIIA joined to the linker-AVITAG sequence (*see*, U.S. Provisional Application No. 60/439,498, filed on January 9, 2003 (Attorney Docket No. 11183-004-888) and U.S. Provisional Application No. 60/456,041 filed on March 19, 2003.

30
In another specific embodiment, the FcγR is FcγRIIB, preferably a soluble dimeric FcγRIIB. For example in one embodiment, the soluble dimeric FcγRIIB protein is prepared in accordance with the methodology described

in U.S. Provisional application No. 60/439,709 filed on January 13, 2003.

[00221] An exemplary assay for determining the kinetic parameters of a molecule comprising a variant Fc region, wherein the molecule is the 4-4-20 antibody, to an FcγR using a BIAcore instrument comprises the following: BSA-FITC is immobilized on one of the four flow cells of a sensor chip surface, preferably through amine coupling chemistry such that about 5000 response units (RU) of BSA-FITC is immobilized on the surface. Once a suitable surface is prepared, 4-4-20 antibodies carrying the Fc mutations are passed over the surface, preferably by one minute injections of a 20 μg/mL solution at a 5 μL/mL flow rate. The level of 4-4-20 antibodies bound to the surface ranges between 400 and 700 RU. Next, dilution series of the receptor (FcγRIIA and FcγRIIB-Fc fusion protein) in HBS-P buffer (20mM HEPES, 150 mM NaCl, 3mM EDTA, pH 7.5) are injected onto the surface at 100 μL/min. Antibody regeneration between different receptor dilutions is carried out preferably by single 5 second injections of 100mM NaHCO₃ pH 9.4; 3M NaCl. Any regeneration technique known in the art is contemplated in the method of the invention.

[00222] Once an entire data set is collected, the resulting binding curves are globally fitted using computer algorithms supplied by the SPR instrument manufacturer, e.g., BIAcore, Inc. (Piscataway, NJ). These algorithms calculate both the K_{on} and K_{off} , from which the apparent equilibrium binding constant, K_d is deduced as the ratio of the two rate constants (i.e., K_{off}/K_{on}). More detailed treatments of how the individual rate constants are derived can be found in the BIAevaluation Software Handbook (BIAcore, Inc., Piscataway, NJ). The analysis of the generated data may be done using any method known in the art. For a review of the various methods of interpretation of the kinetic data generated see Myszkka, 1997, *Current Opinion in Biotechnology* 8: 50-7; Fisher et al., 1994, *Current Opinion in Biotechnology* 5: 389-95; O'Shannessy, 1994, *Current Opinion in Biotechnology*, 5:65-71; Chaiken et al., 1992, *Analytical Biochemistry*, 201: 197-210; Morton et al., 1995, *Analytical Biochemistry* 227: 176-85; O'Shannessy et al., 1996, *Analytical Biochemistry* 236: 275-83.

[00223] In preferred embodiments, the kinetic parameters determined using an SPR analysis, e.g., BIAcore, may be used as a predictive measure of how a molecule of the invention will function in a functional assay, e.g., ADCC. An exemplary method for predicting the efficacy of a molecule of the invention based on kinetic parameters obtained from an SPR analysis may comprise the following: determining the K_{off} values for binding

of a molecule of the invention to FcγRIIIA and FcγRIIB; plotting (1) $K_{\text{off}}(\text{wt})/K_{\text{off}}(\text{mut})$ for FcγRIIIA; (2) $K_{\text{off}}(\text{mut})/K_{\text{off}}(\text{wt})$ for FcγRIIB against the ADCC data. Numbers higher than one show a decreased dissociation rate for FcγRIIIA and an increased dissociation rate for FcγRIIB relative to wild tyoe; and possess and enhanced ADCC function.

5

5.3 METHODS OF RECOMBINANTLY PRODUCING MOLECULES OF THE INVENTION

5.3.1 POLYNUCLEOTIDES ENCODING MOLECULES OF THE INVENTION

10 [00224] The present invention also includes polynucleotides that encode the molecules, including the polypeptides and antibodies, of the invention identified by the methods of the invention. The polynucleotides encoding the molecules of the invention may be obtained, and the nucleotide sequence of the polynucleotides determined, by any method known in the art.

15 [00225] Once the nucleotide sequence of the molecules (*e.g.*, antibodies) that are identified by the methods of the invention is determined, the nucleotide sequence may be manipulated using methods well known in the art, *e.g.*, recombinant DNA techniques, site directed mutagenesis, PCR, *etc.* (*see*, for example, the techniques described in Sambrook *et al.*, 2001, Molecular Cloning, A Laboratory Manual, 3rd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY; and Ausubel *et al.*, eds., 1998, Current Protocols in Molecular Biology, John Wiley & Sons, NY),

to generate, for example, antibodies having a different amino acid sequence, for example by generating amino acid substitutions, deletions, and/or insertions.

25 [00226] In a specific embodiment, when the nucleic acids encode antibodies, one or more of the CDRs are inserted within framework regions using routine recombinant DNA techniques. The framework regions may be naturally occurring or consensus framework regions, and preferably human framework regions (*see, e.g.*, Chothia *et al.*, 1998, *J. Mol. Biol.* 278: 457-479 for a listing of human framework regions).

30 [00227] In another embodiment, human libraries or any other libraries available in the art, can be screened by standard techniques known in the art, to clone the nucleic acids encoding the molecules of the invention.

5.3.2 RECOMBINANT EXPRESSION OF MOLECULES OF THE INVENTION

[00228] Once a nucleic acid sequence encoding molecules of the invention (*i.e.*, antibodies) has been obtained, the vector for the production of the molecules may be produced by recombinant DNA technology using techniques well known in the art. Methods which are well known to those skilled in the art can be used to construct expression vectors containing the coding sequences for the molecules of the invention and appropriate transcriptional and translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. (See, for example, the techniques described in Sambrook *et al.*, 1990, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY and Ausubel *et al.* eds., 1998, Current Protocols in Molecular Biology, John Wiley & Sons, NY).

[00229] An expression vector comprising the nucleotide sequence of a molecule identified by the methods of the invention (*i.e.*, an antibody) can be transferred to a host cell by conventional techniques (*e.g.*, electroporation, liposomal transfection, and calcium phosphate precipitation) and the transfected cells are then cultured by conventional techniques to produce the molecules of the invention. In specific embodiments, the expression of the molecules of the invention is regulated by a constitutive, an inducible or a tissue, specific promoter.

[00230] The host cells used to express the molecules identified by the methods of the invention may be either bacterial cells such as *Escherichia coli*, or, preferably, eukaryotic cells, especially for the expression of whole recombinant immunoglobulin molecule. In particular, mammalian cells such as Chinese hamster ovary cells (CHO), in conjunction with a vector such as the major intermediate early gene promoter element from human cytomegalovirus is an effective expression system for immunoglobulins (Foecking *et al.*, 1998, *Gene* 45:101; Cockett *et al.*, 1990, *Bio/Technology* 8:2).

[00231] A variety of host-expression vector systems may be utilized to express the molecules identified by the methods of the invention. Such host-expression systems represent vehicles by which the coding sequences of the molecules of the invention may be produced and subsequently purified, but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, express the molecules of the invention *in situ*. These include, but are not limited to, microorganisms such as bacteria (*e.g.*, *E. coli* and *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing coding sequences for the molecules

identified by the methods of the invention; yeast (*e.g.*, *Saccharomyces Pichia*) transformed with recombinant yeast expression vectors containing sequences encoding the molecules identified by the methods of the invention; insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing the sequences encoding the molecules identified by the methods of the invention; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus (CaMV) and tobacco mosaic virus (TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing sequences encoding the molecules identified by the methods of the invention; or mammalian cell systems (*e.g.*, COS, CHO, BHK, 293, 293T, 3T3 cells, lymphotic cells (*see* U.S. 5,807,715), Per C.6 cells (human retinal cells developed by Crucell) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the adenovirus late promoter; the vaccinia virus 7.5K promoter).

[00232] In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the molecule being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of pharmaceutical compositions of an antibody, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the *E. coli* expression vector pUR278 (Ruther *et al.*, 1983, *EMBO J.* 2:1791), in which the antibody coding sequence may be ligated individually into the vector in frame with the *lac Z* coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, 1985, *Nucleic Acids Res.* 13:3101-3109; Van Heeke & Schuster, 1989, *J. Biol. Chem.* 24:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption and binding to a matrix glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

[00233] In an insect system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. The antibody coding sequence may be cloned individually into non-essential regions (*e.g.*, the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (*e.g.*, the polyhedrin promoter).

[00234] In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the antibody coding sequence of interest may be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the immunoglobulin molecule in infected hosts (*e.g.*, see Logan & Shenk, 1984, *Proc. Natl. Acad. Sci. USA* 81:355-359). Specific initiation signals may also be required for efficient translation of inserted antibody coding sequences. These signals include the ATG initiation codon and adjacent sequences. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, *etc.* (see Bittner *et al.*, 1987, *Methods in Enzymol.* 153:51-544).

[00235] In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (*e.g.*, glycosylation) and processing (*e.g.*, cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO, VERO, BHK, HeLa, COS, MDCK, 293, 293T, 3T3, WI38, BT483, Hs578T, HTB2, BT20 and T47D, CRL7030 and Hs578Bst.

[00236] For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express an antibody of the invention may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (*e.g.*, promoter, enhancer, sequences, transcription terminators, polyadenylation sites, *etc.*), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media,

and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which
 5 express the antibodies of the invention. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that interact directly or indirectly with the antibodies of the invention.

[00237] A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler *et al.*, 1977, *Cell* 11: 223), hypoxanthine-
 10 guanine phosphoribosyltransferase (Szybalska & Szybalski, 1992, *Proc. Natl. Acad. Sci. USA* 48: 202), and adenine phosphoribosyltransferase (Lowy *et al.*, 1980, *Cell* 22: 817) genes can be employed in tk-, hgprt- or aprt- cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler *et al.*, 1980, *Proc. Natl. Acad. Sci. USA* 77:357; O'Hare
 15 *et al.*, 1981, *Proc. Natl. Acad. Sci. USA* 78: 1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, *Proc. Natl. Acad. Sci. USA* 78: 2072); neo, which confers resistance to the aminoglycoside G-418 *Clinical Pharmacy* 12: 488-505; Wu and Wu, 1991, 3:87-95; Tolstoshev, 1993, *Ann. Rev. Pharmacol. Toxicol.* 32:573-596; Mulligan, 1993, *Science* 260:926-932; and Morgan and Anderson, 1993, *Ann. Rev.*
 20 *Biochem.* 62:191-217; May, 1993, *TIB TECH* 11(5):155-215). Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel *et al.* (eds.), 1993, *Current Protocols in Molecular Biology*, John Wiley & Sons, NY; Kriegler, 1990, *Gene Transfer and Expression, A Laboratory Manual*, Stockton Press, NY; and in Chapters 12 and 13, Dracopoli *et al.* (eds), 1994, *Current Protocols in Human Genetics*,
 25 John Wiley & Sons, NY.; Colberre-Garapin *et al.*, 1981, *J. Mol. Biol.* 150:1; and hygromycin (Santerre *et al.*, 1984, *Gene* 30:147).

[00238] The expression levels of an antibody of the invention can be increased by vector amplification (for a review, see Bebbington and Hentschel, The use of vectors based on gene amplification for the expression of cloned genes in mammalian cells in DNA
 30 cloning, Vol. 3 (Academic Press, New York, 1987). When a marker in the vector system expressing an antibody is amplifiable, increase in the level of inhibitor present in culture of host cell will increase the number of copies of the marker gene. Since the amplified region is associated with the nucleotide sequence of the antibody, production of the antibody will also increase (Crouse *et al.*, 1983, *Mol. Cell. Biol.* 3:257).

- [00239] The host cell may be co-transfected with two expression vectors of the invention, the first vector encoding a heavy chain derived polypeptide and the second vector encoding a light chain derived polypeptide. The two vectors may contain identical selectable markers which enable equal expression of heavy and light chain polypeptides.
- 5 Alternatively, a single vector may be used which encodes both heavy and light chain polypeptides. In such situations, the light chain should be placed before the heavy chain to avoid an excess of toxic free heavy chain (Proudfoot, 1986, *Nature* 322:52; Kohler, 1980, *Proc. Natl. Acad. Sci. USA* 77:2197). The coding sequences for the heavy and light chains may comprise cDNA or genomic DNA.
- 10 [00240] Once a molecule of the invention (*i.e.*, antibodies) has been recombinantly expressed, it may be purified by any method known in the art for purification of polypeptides or antibodies, for example, by chromatography (*e.g.*, ion exchange, affinity, particularly by affinity for the specific antigen after Protein A, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique
- 15 for the purification of polypeptides or antibodies.

5.4 PROPHYLACTIC AND THERAPEUTIC METHODS

- [00241] The present invention encompasses administering one or more of the molecules of the invention (*e.g.*, antibodies) to an animal, preferably a mammal, and most preferably a human, for preventing, treating, or ameliorating one or more symptoms
- 20 associated with a disease, disorder, or infection. The molecules of the invention are particularly useful for the treatment or prevention of a disease or disorder where an enhanced efficacy of effector cell function (*e.g.*, ADCC) mediated by Fc γ R is desired. The methods and compositions of the invention are particularly useful for the treatment or prevention of primary or metastatic neoplastic disease (*i.e.*, cancer), and infectious diseases.
- 25 Molecules of the invention may be provided in pharmaceutically acceptable compositions as known in the art or as described herein. As detailed below, the molecules of the invention can be used in methods of treating or preventing cancer (particularly in passive immunotherapy), autoimmune disease, inflammatory disorders or infectious diseases.
- [00242] The molecules of the invention may also be advantageously utilized in
- 30 combination with other therapeutic agents known in the art for the treatment or prevention of a cancer, autoimmune disease, inflammatory disorders or infectious diseases. In a specific embodiment, molecules of the invention may be used in combination with monoclonal or chimeric antibodies, lymphokines, or hematopoietic growth factors (such as, *e.g.*, IL-2, IL-3 and IL-7), which, for example, serve to increase the number or activity of

effector cells which interact with the molecules and, increase immune response. The molecules of the invention may also be advantageously utilized in combination with one or more drugs used to treat a disease, disorder, or infection such as, for example anti-cancer agents, anti-inflammatory agents or anti-viral agents, *e.g.*, as detailed in sections 5.4.1.2 and 5.4.2.1 below.

5.4.1 CANCERS

[00243] The invention encompasses methods and composition for treatment or prevention of cancer or metastasis in a subject comprising administering to the subject a therapeutically effective amount of one or more molecules comprising a variant Fc region.

10 [00244] Molecules of the invention (*i.e.*, polypeptides, antibodies) comprising variant Fc regions can be used to prevent, inhibit or reduce the growth of primary tumors or metastasis of cancerous cells. In one embodiment, the molecule of the invention comprises a variant Fc that binds Fc γ RIIIA and/or Fc γ RIIA with a greater affinity than a comparable polypeptide comprising a wild type Fc region binds Fc γ RIIIA and/or Fc γ RIIA, and/or said

15 variant Fc region has an enhanced effector function, *e.g.*, ADCC, CDC, phagocytosis, opsonization, *etc.* Such molecules can be used alone to treat or prevent cancer. In another embodiment, the molecule of the invention comprises a variant Fc region that binds Fc γ RIIIA and/or Fc γ RIIA with a greater affinity than a comparable polypeptide comprising a wild type Fc region binds Fc γ RIIIA and/or Fc γ RIIA, and further binds Fc γ RIIB with a

20 lower affinity than a comparable polypeptide comprising a wild-type Fc region binds Fc γ RIIB, and/or said variant Fc region has an enhanced effector function, *e.g.*, ADCC, CDC, phagocytosis, opsonization, *etc.* Such molecules can also be used alone to treat or prevent cancer.

[00245] In some embodiments, the invention encompasses methods and compositions

25 for the treatment or prevention of cancer in a subject with Fc γ R polymorphisms such as those homozygous for the Fc γ RIIIA-158V or Fc γ RIIIA-158F alleles. In some embodiments, the invention encompasses engineering therapeutic antibodies, *e.g.*, tumor specific monoclonal antibodies in accordance with the methods of the invention such that the engineered antibodies have enhanced efficacy in patients homozygous for the low affinity

30 allele of Fc γ RIIIA (158F). In other embodiments, the invention encompasses engineering therapeutic antibodies, *e.g.*, tumor specific monoclonal antibodies in accordance with the methods of the invention such that the engineered antibodies have enhanced efficacy in patients homozygous for the high affinity allele of Fc γ RIIIA (158V).

[00246] In some embodiments, the engineered antibodies of the invention are particularly effective in treating and/or preventing non-Hodgkin's lymphoma (NHL). The engineered antibodies of the invention are therapeutically more effective than current therapeutic regimens for NHL, including but not limited to chemotherapy, and immunotherapy using anti-CD20 mAb, Rituximab. The efficacy of anti-CD20 monoclonal antibodies however depends on the FcγR polymorphism of the subject (Carton *et al.*, 2002 *Blood*, 99: 754-8; Weng *et al.*, 2003 *J Clin Oncol*.21(21):3940-7).

These receptors are expressed on the surface of the effector cells and mediate ADCC. High affinity alleles, of the low affinity activating receptors, improve the effector cells' ability to mediate ADCC. The methods of the invention allow engineering anti-CD20 antibodies harboring Fc mutations to enhance their affinity to FcγR on effector cells via their altered Fc domains. The engineered antibodies of the invention provide better immunotherapy reagents for patients regardless of their FcγR polymorphism.

[00247] An exemplary method for determining the efficacy of the engineered anti-CD20 antibodies in a subject may include the following: Plasmids harboring chimeric anti-HER2/neu heavy chain genes with Fc mutations that show substantially increased killing in ADCC can be used as a backbone to transfer in the variable domain from the Rituximab heavy chain gene. The variable region from the anti-HER2/neu Fc variant is replaced with the variable region from Rituximab. Plasmids containing wild type Fc domains or a D265A mutation to abrogate FcR binding, or the anti-CD20 Fc variants are transiently cotransfected with the Rituximab light chain gene into 293H cells, conditioned media and the antibody is purified over a protein G column using routine methods.

[00248] Anti-CD20 mAbs harboring the Fc variants are tested by ADCC using a cultured B cell line to determine the ability of the Fc mutations to enhance ADCC. Standard ADCC is performed using methods disclosed herein. Lymphocytes are harvested from peripheral blood using a Ficoll-Paque gradient (Pharmacia). Target Daudi cells, a B-cell line expressing CD20, are loaded with Europium (PerkinElmer) and incubated with effectors for 4 hrs at 37°C. Released Europium is detected using a fluorescent plate reader (Wallac). The resulting ADCC data indicates the efficacy of the Fc variants to trigger NK cell mediated cytotoxicity and establish which anti-CD20 Fc variants can be tested with both patient samples and elutriated monocytes. Fc variants showing the greatest potential for enhancing the efficacy of the anti-CD20 antibody are then tested in an ADCC assay using PBMCs from patients. PBMC from healthy donors are used as effector cells. *In vitro* ADCC assays using anti-CD20 variants and Rituximab are performed in primary

lymphoma cells from patients with follicular lymphoma. The specific FcγR polymorphism of the donors is determined and cataloged using methods known in the art. ADCC assay is performed by effector cells from patients with different FcγRIIIA and FcγRIIA genotypes.

[00249] According to an aspect of the invention, molecules (*e.g.*, antibodies) of the invention comprising variant Fc regions enhance the efficacy of cancer immunotherapy by increasing the potency of the antibody effector function relative to a molecule containing the wild-type Fc region, *e.g.*, ADCC, CDC, phagocytosis, opsonization, *etc.* In a specific embodiment, antibody dependent cellular toxicity and/or phagocytosis of tumor cells is enhanced using the molecules of the invention with variant Fc regions. Molecules of the invention may enhance the efficacy of immunotherapy cancer treatment by enhancing at least one antibody-mediated effector function. In one particular embodiment, a molecule of the invention comprising a variant Fc region enhances the efficacy of immunotherapy treatment by enhancing the complement dependent cascade. In another embodiment of the invention, the molecule of the invention comprising a variant Fc region enhances the efficacy of immunotherapy treatment by enhancing the phagocytosis and/or opsonization of the targeted tumor cells. In another embodiment of the invention, the molecule of the invention comprising a variant Fc region enhances the efficacy of treatment by enhancing antibody-dependent cell-mediated cytotoxicity (“ADCC”) in destruction of the targeted tumor cells.

[00250] The invention further contemplates engineering therapeutic antibodies (*e.g.*, tumor specific monoclonal antibodies) for enhancing the therapeutic efficacy of the therapeutic antibody, for example, by enhancing the effector function of the therapeutic antibody (*e.g.*, ADCC). Preferably the therapeutic antibody is a cytotoxic and/or opsonizing antibody. It will be appreciated by one of skill in the art, that once molecules of the invention with desired binding properties (*e.g.*, molecules with variant Fc regions with at least one amino acid modification, which modification enhances the affinity of the variant Fc region for FcγRIIIA and/or FcγRIIA relative to a comparable molecule, comprising a wild-type Fc region) have been identified (*See* Section 5.2 and Table 5) according to the methods of the invention, therapeutic antibodies may be engineered using standard recombinant DNA techniques and any known mutagenesis techniques, as described in Section 5.2.2 to produce engineered therapeutic carrying the identified mutation sites with the desired binding properties. Any of the therapeutic antibodies listed in Table 6 that have demonstrated therapeutic utility in cancer treatment, may be engineered according to the methods of the invention, for example, by modifying the Fc region to have an enhanced affinity for FcγRIIIA and/or FcγRIIA compared to a therapeutic antibody having a wild-

type Fc region, and used for the treatment and or prevention of a cancer characterized by a cancer antigen.

- [00251] The invention also encompasses engineering any other polypeptide comprising an Fc region which has therapeutic utility, including but not limited to
- 5 ENBREL, according to the methods of the invention, in order to enhance the therapeutic efficacy of such polypeptides, for example, by enhancing the effector function of the polypeptide comprising an Fc region.

TABLE 6. THERAPEUTIC ANTIBODIES THAT CAN BE ENGINEERED ACCORDING TO THE METHODS OF THE INVENTION

Company	Product	Disease	Target
Abgenix	ABX-EGF	Cancer	EGF receptor
AltaRex	OvaRex	ovarian cancer	tumor antigen CA125
	BravaRex	metastatic cancers	tumor antigen MUC1
Antisoma	Theragyn (pemtumomabytrrium-90)	ovarian cancer	PEM antigen
	Therex	breast cancer	PEM antigen
Boehringer Ingelheim	Blvatuzumab	head & neck cancer	CD44
Centocor/J&J	Panorex	Colorectal cancer	17-1A
	ReoPro	PTCA	gp IIIb/IIIa
	ReoPro	Acute MI	gp IIIb/IIIa
	ReoPro	Ischemic stroke	gp IIIb/IIIa
Corixa	Bexocar	NHL	CD20
CRC Technology	MAB, idiotypic 105AD7	colorectal cancer vaccine	gp72
Crucell	Anti-EpCAM	cancer	Ep-CAM
Cytoclonal	MAB, lung cancer	non-small cell lung cancer	NA
Genentech	Herceptin	metastatic breast cancer	HER-2
	Herceptin	early stage breast cancer	HER-2
	Rituxan	Relapsed/refractory low-grade or follicular NHL	CD20
	Rituxan	intermediate &	CD20

DEMANDES OU BREVETS VOLUMINEUX

**LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVETS
COMPREND PLUS D'UN TOME.**

CECI EST LE TOME __1__ DE __2__

NOTE: Pour les tomes additionels, veuillez contacter le Bureau Canadien des Brevets.

JUMBO APPLICATIONS / PATENTS

**THIS SECTION OF THE APPLICATION / PATENT CONTAINS MORE
THAN ONE VOLUME.**

THIS IS VOLUME __1__ OF __2__

NOTE: For additional volumes please contact the Canadian Patent Office.

The embodiments of the present invention for which an exclusive property or privilege is claimed are defined as follows:

1. An antibody which comprises an antigen binding region and a variant Fc region, or a fragment thereof which comprises said variant Fc region, wherein said variant Fc region:

(A) differs from a wild-type Fc region by comprising an amino acid substitution of leucine or histidine at position 396, according to the EU index as in Kabat, relative to said wild-type Fc region; and

(B) binds an FcγR with an increased affinity relative to said wild-type Fc region.

2. The antibody or fragment of claim 1, wherein said variant Fc region differs from said wild-type Fc region in comprising additional amino acid modification(s) of said wild-type Fc region selected from the group consisting of: 210M; 217S; 227S; 240A; 242F; 244H; 246T; 247S; 248M; 250A; 250S; 255I; 255L; 258D; 268D; 268N; 303I; 305L; 323I; 326I; 334N; 358P; 370E; 375C; 379M; 384K; 392T; 400F; 410H; 419H; 419L; and 427A.

3. The antibody or fragment of claim 1, wherein said variant Fc region differs from said wild-type Fc region in comprising additional amino acid modification(s) of said wild-type Fc region selected from the group consisting of:

(1) 221E; 270E; 308A, 311H and 402D;

(2) 319F and 352L;

(3) 288R; 307A; and 344E;

(4) 210M and 261N;

and

(5) 243L; 305I; 378D; and 404S.

4. The antibody or fragment of any one of claims 1-3, wherein said FcγR is FcγRIIIA.

5. The antibody or fragment of claim 4, wherein said variant Fc region of said antibody or fragment additionally has decreased affinity for FcγRIIB relative to said wild-type Fc region.

6. The antibody or fragment of claim 5, wherein said variant Fc region comprises amino acid modifications of said wild-type Fc region selected from the group consisting of:

(1) 221E; 270E; 308A; 311H; 396L and 402D;

(2) 243L; 305I; 376D; 404S; and 396L;

- (3) 255I and 396L;
- (4) 370E and 396L.
- (5) 392T and 396L;
- and
- (6) 410H and 396L.

7. The antibody or fragment of any one of claims 1-6 wherein said wild-type Fc region is an Fc region of a human IgG Fc region.
8. The antibody or fragment of claim 7, wherein said human IgG Fc region is a human IgG1, IgG2, IgG3, or IgG4 Fc region.
9. The antibody or fragment of claim 7, wherein said variant Fc region of said antibody specifically binds FcγRIIIA with at least two times greater affinity than the wild-type Fc region.
10. The antibody or fragment of any one of claims 1-9, wherein said antigen binding region binds a cancer antigen.
11. The antibody or fragment of claim 10, wherein said antibody or fragment binds a cancer antigen and mediates antibody dependent cell mediated cytotoxicity 2-fold more effectively than an antibody comprising said wild-type Fc region.
12. The antibody or fragment of claim 10, wherein said cancer antigen is HER-2/neu, MAGE-1, MAGE-3, BAGE, GAGE-1, GAGE-2, MUM-1, CDK4, MUC-1, N-acetylglucosaminyltransferase, p15, beta-catenin, human papillomavirus-E6, or human papillomavirus-E7.
13. The antibody or fragment of any one of claims 1-5 and 7-12, wherein said amino acid substitution at position 396 is a substitution of leucine.
14. An isolated nucleic acid comprising a nucleotide sequence encoding an antibody which comprises an antigen binding region and a variant Fc region, wherein said variant Fc region:
 - (A) differs from a wild-type Fc region by comprising an amino acid substitution of leucine or histidine at position 396, according to the EU index as in Kabat, relative to said wild-type Fc region; and

- (B) binds an FcγR with an increased affinity relative to said wild-type Fc region.
15. An isolated vector comprising the nucleic acid of claim 14.
 16. The vector of claim 15 which is an expression vector.
 17. An isolated host cell comprising the nucleic acid of claim 14.
 18. A method for recombinantly producing an antibody, said method comprising: (i) culturing in a medium a host cell comprising the nucleic acid of claim 14, under conditions suitable for the expression of said antibody; and (ii) recovering said antibody from said medium; wherein said produced antibody comprises an antigen binding region and a variant Fc region, wherein said variant Fc region:
 - (A) differs from a wild-type Fc region by comprising an amino acid substitution of leucine or histidine at position 396, according to the EU index as in Kabat, relative to said wild-type Fc region; and
 - (B) binds an FcγR with an increased affinity relative to said wild-type Fc region.
 19. An isolated nucleic acid comprising a nucleotide sequence encoding a heavy chain of an antibody, wherein said antibody comprises an antigen binding region and a variant Fc region, wherein said variant Fc region:
 - (A) differs from a wild-type Fc region by comprising an amino acid substitution of leucine or histidine at position 396, according to the EU index as in Kabat, relative to said wild-type Fc region; and
 - (B) binds an FcγR with an increased affinity relative to said wild-type Fc region.
 20. An isolated vector comprising the nucleic acid of claim 19.
 21. The vector of claim 20 which is an expression vector.
 22. An isolated host cell comprising the nucleic acid of claim 19.
 23. A method for recombinantly producing an antibody, said method comprising: (i) culturing in a medium a host cell comprising the nucleic acid of claim 19, and culturing in a medium a host cell comprising a nucleic acid molecule encoding a light chain of said antibody, said culturing being under conditions suitable for the expression of said light and heavy chains of said antibody; and (ii) permitting said expressed light and heavy chains to

associate with one another to thereby produce said antibody; wherein said produced antibody comprises an antigen binding region and a variant Fc region, wherein said variant Fc region:

- (A) differs from a wild-type Fc region by comprising an amino acid substitution of leucine or histidine at position 396, according to the EU index as in Kabat, relative to said wild-type Fc region; and
- (B) binds an FcγR with an increased affinity relative to said wild-type Fc region.

24. The nucleic acid molecule of claim 14, wherein said variant Fc region differs from said wild-type Fc region in comprising additional amino acid modification(s) of said wild-type Fc region selected from the group consisting of: 210M; 217S; 227S; 240A; 242F; 244H; 246T; 247S; 248M; 250A; 250S; 255I; 255L; 258D; 268D; 268N; 303I; 305L; 323I; 326I; 334N; 358P; 370E; 375C; 379M; 384K; 392T; 400F; 410H; 419H; 419L; and 427A.

25. The nucleic acid molecule of claim 14, wherein said variant Fc region differs from said wild-type Fc region in comprising additional amino acid modification(s) of said wild-type Fc region selected from the group consisting of:

- (1) 221E; 270E; 308A, 311H and 402D;
- (2) 319F and 352L;
- (3) 288R; 307A; and 344E;
- (4) 210M and 261N;
- and
- (5) 243L; 305I; 378D; and 404S.

26. The nucleic acid molecule of any one of claims 14, 24 and 25, wherein said FcγR is FcγRIIIA.

27. The nucleic acid molecule of claim 26, wherein said variant Fc region of said antibody additionally has decreased affinity for FcγRIIB relative to said wild-type Fc region.

28. The nucleic acid molecule of claim 14, wherein said variant Fc region comprises amino acid modifications of said wild-type Fc region selected from the group consisting of:

- (1) 221E; 270E; 308A; 311H; 396L and 402D;
- (2) 243L; 305I; 376D; 404S; and 396L;
- (3) 255I and 396L;
- (4) 370E and 396L.
- (5) 392T and 396L;

and

(6) 410H and 396L.

29. The nucleic acid molecule of claim 14, wherein said wild-type Fc region is an Fc region of a human IgG Fc region.

30. The nucleic acid molecule of claim 29, wherein said human IgG Fc region is a human IgG1, IgG2, IgG3, or IgG4 Fc region.

31. The nucleic acid molecule of any one of claims 14, 24 and 25, wherein said variant Fc region of said antibody is a human IgG1, IgG2, IgG3, or IgG4 Fc region that specifically binds FcγRIIIA with at least two times greater affinity than the wild-type Fc region.

32. The nucleic acid molecule of any one of claims 14, 24 and 25, wherein said antigen binding region binds a cancer antigen, wherein said cancer antigen is: 17-1A, adult erythrocyte primary endoderm I antigen, alpha fetoprotein, an envelope antigen of an RNA tumor virus, bladder tumor oncofetal antigen, Burkitt's lymphoma antigen-38.13, CA125, CD3, CD18, CD19, human B-lymphoma antigen-CD20, CD22, CD33, CD44, CD52, CEA, CO17-1A, CTA-1, CTLA-4, epidermal growth factor receptor, Ep-CAM, fetal erythrocyte I antigen, fibrosarcoma antigen, ganglioside GD2, ganglioside GD3, ganglioside GM2, ganglioside GM3, GICA 19-9, gp IIb/IIIa, gp72, HER-2/neu, high molecular weight melanoma antigen, HLA-DR antigen, human leukemia T cell antigen-Gp37, human lung carcinoma antigen L20, human lung carcinoma antigen L6, human milk fat globule antigen, IgE, KS 1/4 pan-carcinoma antigen, LEA, lung adenocarcinoma F3 antigen, malignant human lymphocyte antigen-APO-1, melanoma antigen gp75, melanoma-associated antigen p97, neoglycoprotein, nuC242, polymorphic epithelial mucin antigen, prostate specific antigen, prostate specific membrane antigen, prostatic acid phosphatase, SK-1 antigen, TAG-72, T-antigen, tumor antigen CA125, tumor antigen MUC1, tumor-specific transplantation type of cell-surface antigen, vascular endothelial growth factor, vascular endothelial growth factor-receptor, or $\alpha v\beta 3$.

33. The nucleic acid molecule of claim 32, wherein said antibody binds a cancer antigen and mediates antibody dependent cell mediated cytotoxicity 2-fold more effectively than an antibody comprising said wild-type Fc region.

34. The nucleic acid molecule of claim 32, wherein said cancer antigen is HER-2/neu, CA125, prostate specific antigen, prostate specific membrane antigen, CEA, or human B-lymphoma antigen-CD20.

35. The nucleic acid molecule of any one of claims 14, 26, 27, 29 and 30, wherein said amino acid substitution at position 396 is a substitution of leucine.

36. The nucleic acid molecule of claim 19, wherein said variant Fc region differs from said wild-type Fc region in comprising additional amino acid modification(s) of said wild-type Fc region selected from the group consisting of: 210M; 217S; 227S; 240A; 242F; 244H; 246T; 247S; 248M; 250A; 250S; 255I; 255L; 258D; 268D; 268N; 303I; 305L; 323I; 326I; 334N; 358P; 370E; 375C; 379M; 384K; 392T; 400F; 410H; 419H; 419L; and 427A.

37. The nucleic acid molecule of claim 19, wherein said variant Fc region differs from said wild-type Fc region in comprising additional amino acid modification(s) of said wild-type Fc region selected from the group consisting of:

- (1) 221E; 270E; 308A, 311H and 402D;
- (2) 319F and 352L;
- (3) 288R; 307A; and 344E;
- (4) 210M and 261N;
- and
- (5) 243L; 305I; 378D; and 404S.

38. The nucleic acid molecule of any one of claims 19, 36 and 37, wherein said FcγR is FcγRIIIA.

39. The nucleic acid molecule of claim 38, wherein said variant Fc region of said antibody additionally has decreased affinity for FcγRIIB relative to said wild-type Fc region.

40. The nucleic acid molecule of claim 19, wherein said variant Fc region comprises amino acid modifications of said wild-type Fc region selected from the group consisting of:

- (1) 221E; 270E; 308A; 311H; 396L and 402D;
- (2) 243L; 305I; 376D; 404S; and 396L;
- (3) 255I and 396L;
- (4) 370E and 396L;
- (5) 392T and 396L;
- and

(6) 410H and 396L.

41. The nucleic acid molecule of claim 19, wherein said wild-type Fc region is an Fc region of a human IgG Fc region.

42. The nucleic acid molecule of claim 41, wherein said human IgG Fc region is a human IgG1, IgG2, IgG3, or IgG4 Fc region.

43. The nucleic acid molecule of any one of claims 19, 36 and 37, wherein said variant Fc region of said antibody is a human IgG1, IgG2, IgG3, or IgG4 Fc region that specifically binds FcγRIIIA with at least two times greater affinity than the wild-type Fc region.

44. The nucleic acid molecule of any one of claims 19, 38, 39 and 41-43, wherein said amino acid substitution at position 396 is a substitution of leucine.

45. The method of claim 18, wherein said variant Fc region differs from said wild-type Fc region in comprising additional amino acid modification(s) of said wild-type Fc region selected from the group consisting of: 210M; 217S; 227S; 240A; 242F; 244H; 246T; 247S; 248M; 250A; 250S; 255I; 255L; 258D; 268D; 268N; 303I; 305L; 323I; 326I; 334N; 358P; 370E; 375C; 379M; 384K; 392T; 400F; 410H; 419H; 419L; and 427A.

46. The method of claim 18, wherein said variant Fc region differs from said wild-type Fc region in comprising additional amino acid modification(s) of said wild-type Fc region selected from the group consisting of:

(1) 221E; 270E; 308A, 311H and 402D;

(2) 319F and 352L;

(3) 288R; 307A; and 344E;

(4) 210M and 261N;

and

(5) 243L; 305I; 378D; and 404S.

47. The method of claim 18, wherein said amino acid substitution at position 396 is a substitution of leucine.

48. The method of claim 23, wherein said variant Fc region differs from said wild-type Fc region in comprising additional amino acid modification(s) of said wild-type Fc region selected from the group consisting of: 210M; 217S; 227S; 240A; 242F; 244H; 246T; 247S;

248M; 250A; 250S; 255I; 255L; 258D; 268D; 268N; 303I; 305L; 323I; 326I; 334N; 358P; 370E; 375C; 379M; 384K; 392T; 400F; 410H; 419H; 419L; and 427A.

49. The method of claim 23, wherein said variant Fc region differs from said wild-type Fc region in comprising additional amino acid modification(s) of said wild-type Fc region selected from the group consisting of:

- (1) 221E; 270E; 308A, 311H and 402D;
- (2) 319F and 352L;
- (3) 288R; 307A; and 344E;
- (4) 210M and 261N;
- and
- (5) 243L; 305I; 378D; and 404S.

50. The method of claim 23, wherein said amino acid substitution at position 396 is a substitution of leucine.

51. Use of the antibody or fragment thereof of any one of claims 1-13 in the manufacture of a medicament for treating cancer characterized by a cancer antigen in a patient in need thereof, wherein said antigen-binding region binds said cancer antigen.

52. The use of claim 51, wherein said cancer antigen is a breast, ovarian, prostate, cervical, vaginal, vulvar, uterine, lung, colorectal, gastric, kidney, adrenal, thyroid, pituitary, eye, colon, brain, esophageal, stomach, rectal, liver, gallbladder, testicular, penal, oral, pharynx, skin, bladder, leukemia, lymphoma, sarcoma, or pancreatic carcinoma antigen.

53. The use of claim 51, wherein said medicament is for use in combination with one or more—additional cancer therapies selected from the group consisting of chemotherapy, immunotherapy, radiation therapy, hormonal therapy, and surgery.

54. The use of claim 51, wherein said cancer antigen is Her2/neu, epidermal growth factor receptor, tumor antigen CA125, PEM, CD44, gp IIIb/IIIa, human B-lymphoma antigen-CD20, gp72, Ep-CAM, VEGF, CD18, IgE, mimics ganglioside GD3, VEGF-receptor, nuC242, CD22, CEA, TAG-72, AFP, CTLA-4, $\alpha\beta_3$, CD52, HLA-DR 10 beta, DNA-associated proteins, CD3, CD33, HLA-DR antigen, SK-1, KS 1/4 pan-carcinoma antigen, prostatic acid phosphate, prostate specific antigen, melanoma-associated antigen p97, melanoma antigen gp75, HMW-MAA, prostate specific membrane antigen, polymorphic epithelial mucin antigen, high molecular weight melanoma antigen, human milk

fat globule antigen, TAG-72, 17-1A, GICA 19-9, CTA-1, LEA, Burkitt's lymphoma antigen-38.13, CD19, ganglioside GD2, ganglioside GD3, ganglioside GM2, ganglioside GM3, tumor-specific transplantation type of cell-surface antigen, T-antigen DNA tumor virus, envelope antigen of RNA tumor virus, alpha-fetoprotein, human lung carcinoma antigen L6, human lung carcinoma antigen L20, fibrosarcoma antigen, human leukemia T cell antigen-Gp37, vascular endothelial growth factor, vascular endothelial growth factor-receptor, neoglycoprotein, sphingolipids, EGFR, HER2, malignant human lymphocyte antigen-APO-1, fetal erythrocyte I antigen, I(Ma), M18, M39, CO17-1A, SSEA-1, VEP8, VEP9, Myl, VIM-D5, D₁56-22, TRA-1-85, C14, lung adenocarcinoma F3 antigen, bladder tumor oncofetal antigen, AH6, Y hapten, Le^y, TL5, E₁ series, FC10.2, CO-514, NS-10, CO-43, G49, GICA 19-9, T₅A₇, R₂₄, 4.2, G_{D3}, D1.1, OFA-1, G_{M2}, OFA-2, G_{D2}, M1:22:25:8, SSEA-3, SSEA-4, T-antigen, adult erythrocyte primary endoderm I antigen, or tumor antigen MUC-1.

55. The use of claim 54, wherein said cancer antigen is Her2/neu.

56. A pharmaceutical composition comprising a therapeutically effective amount of the antibody of claim 10, and a pharmaceutically acceptable carrier.

57. The pharmaceutical composition of claim 56, wherein composition further comprises an additional anti-cancer agent selected from the group consisting of a chemotherapeutic agent, a radiation therapeutic agent, a hormonal therapeutic agent, and an immunotherapeutic agent.

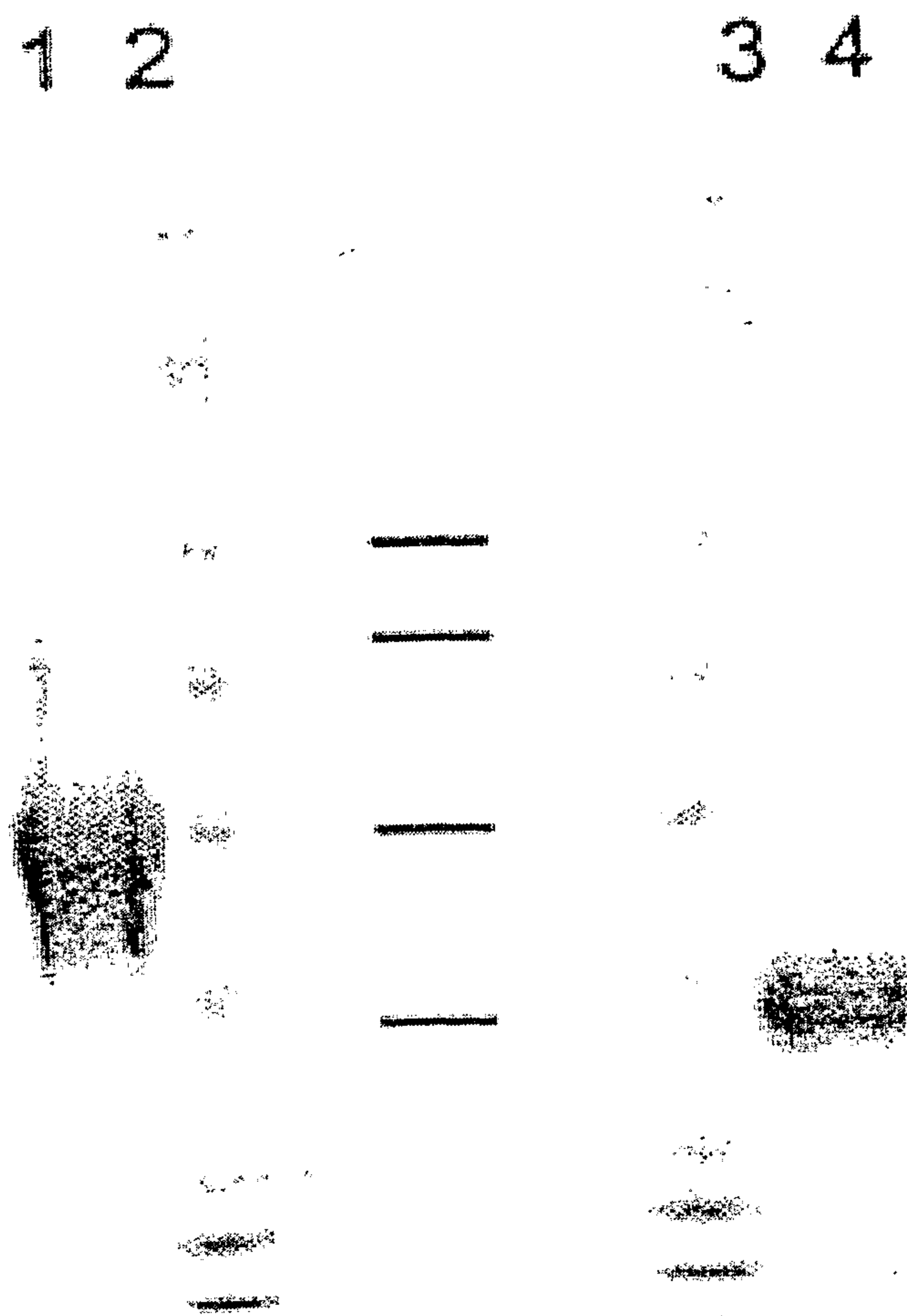


FIG. 1

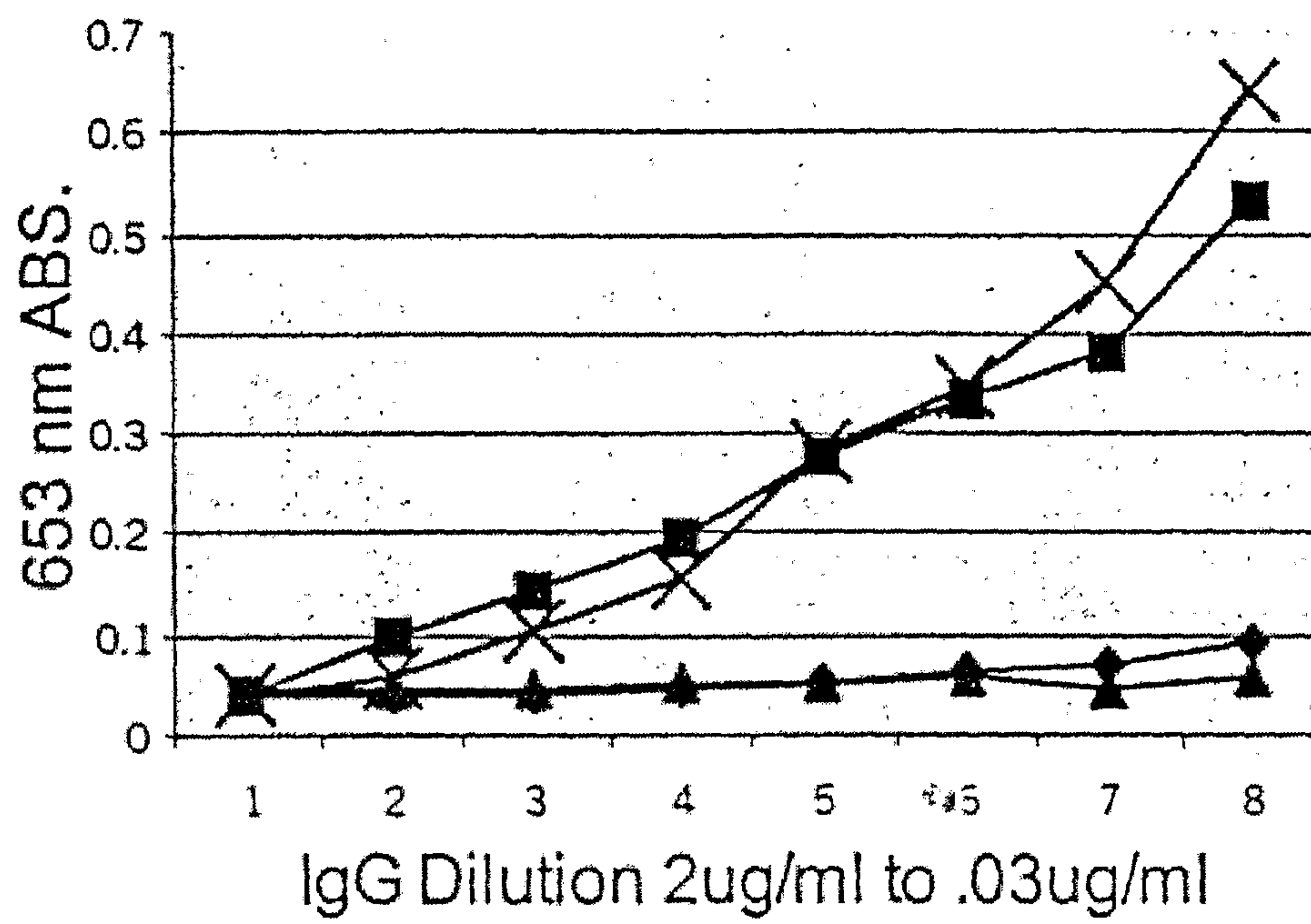


FIG. 2

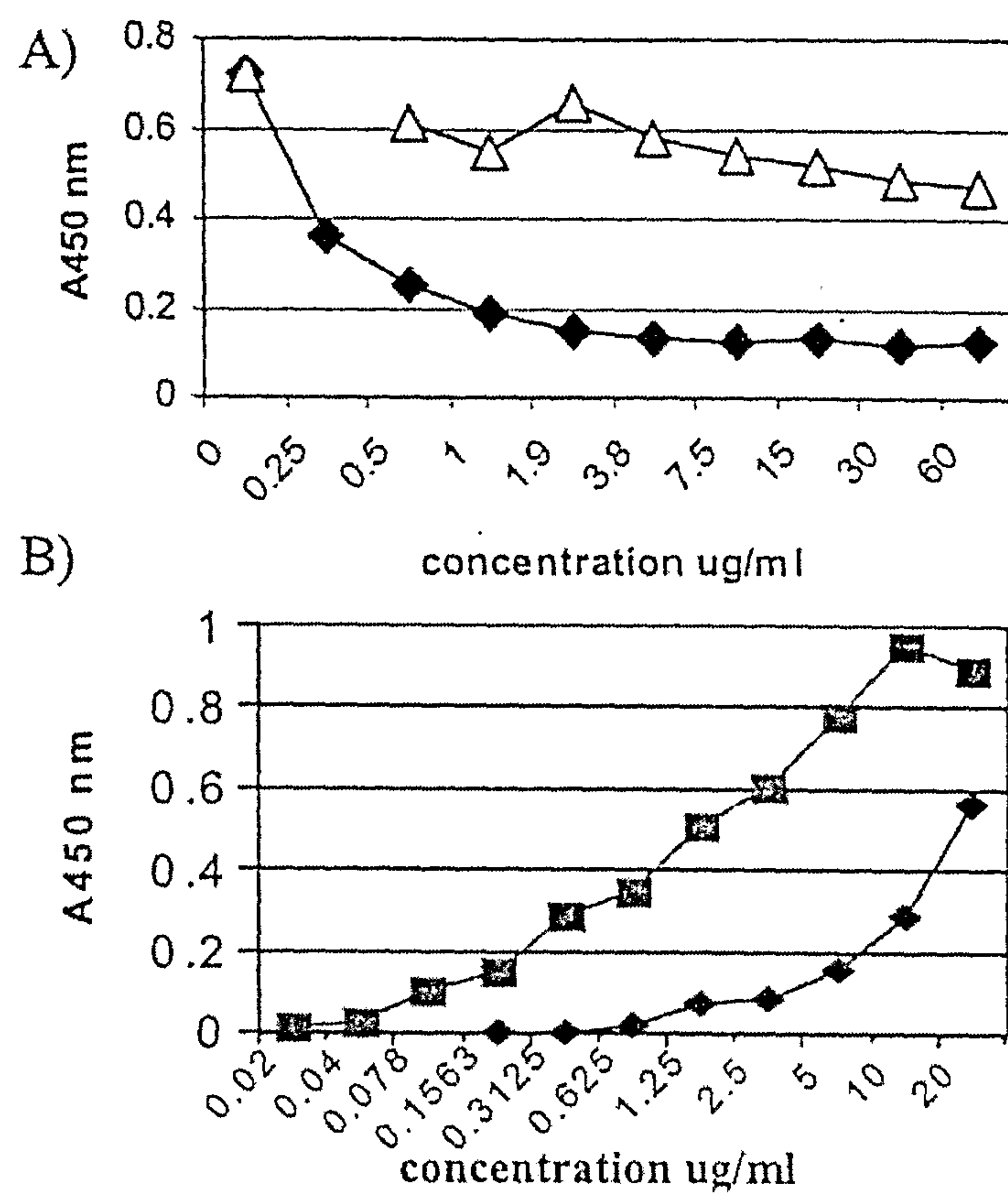


FIG. 3

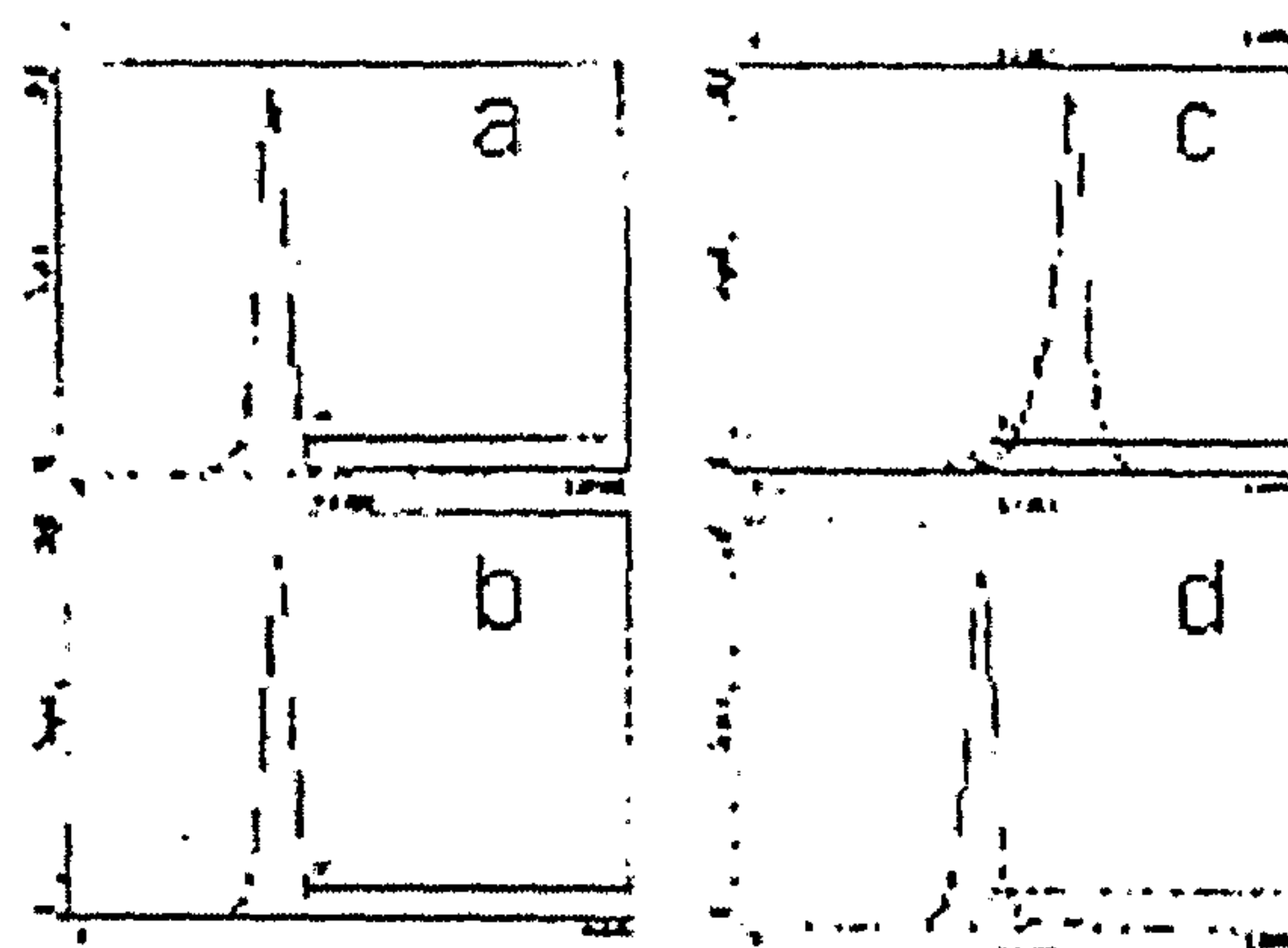
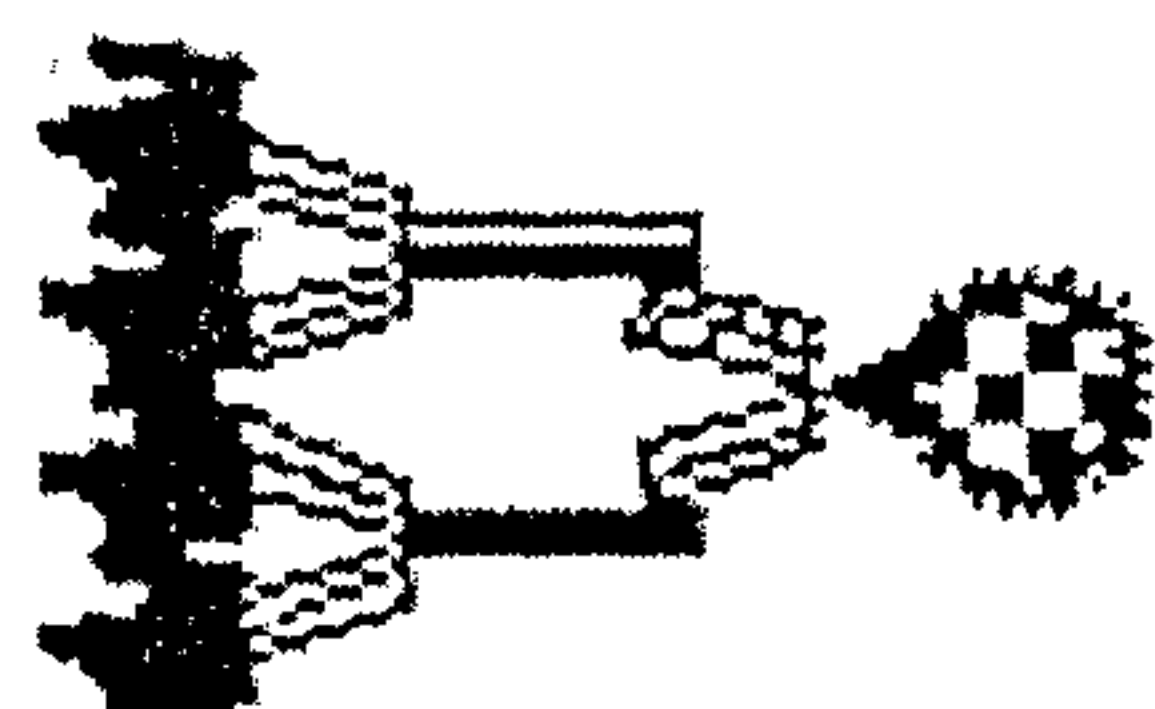


FIG. 4

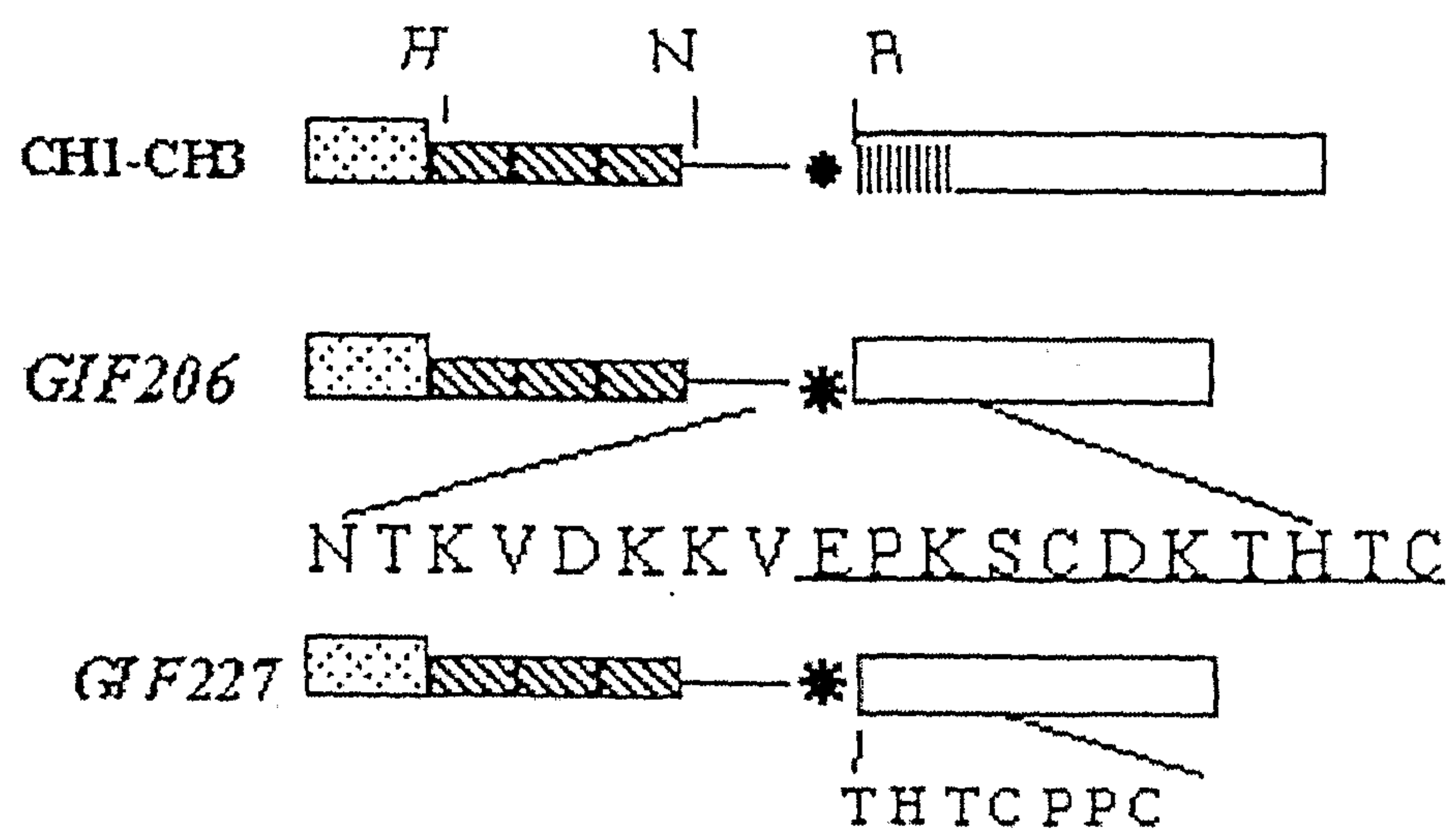


FIG. 5

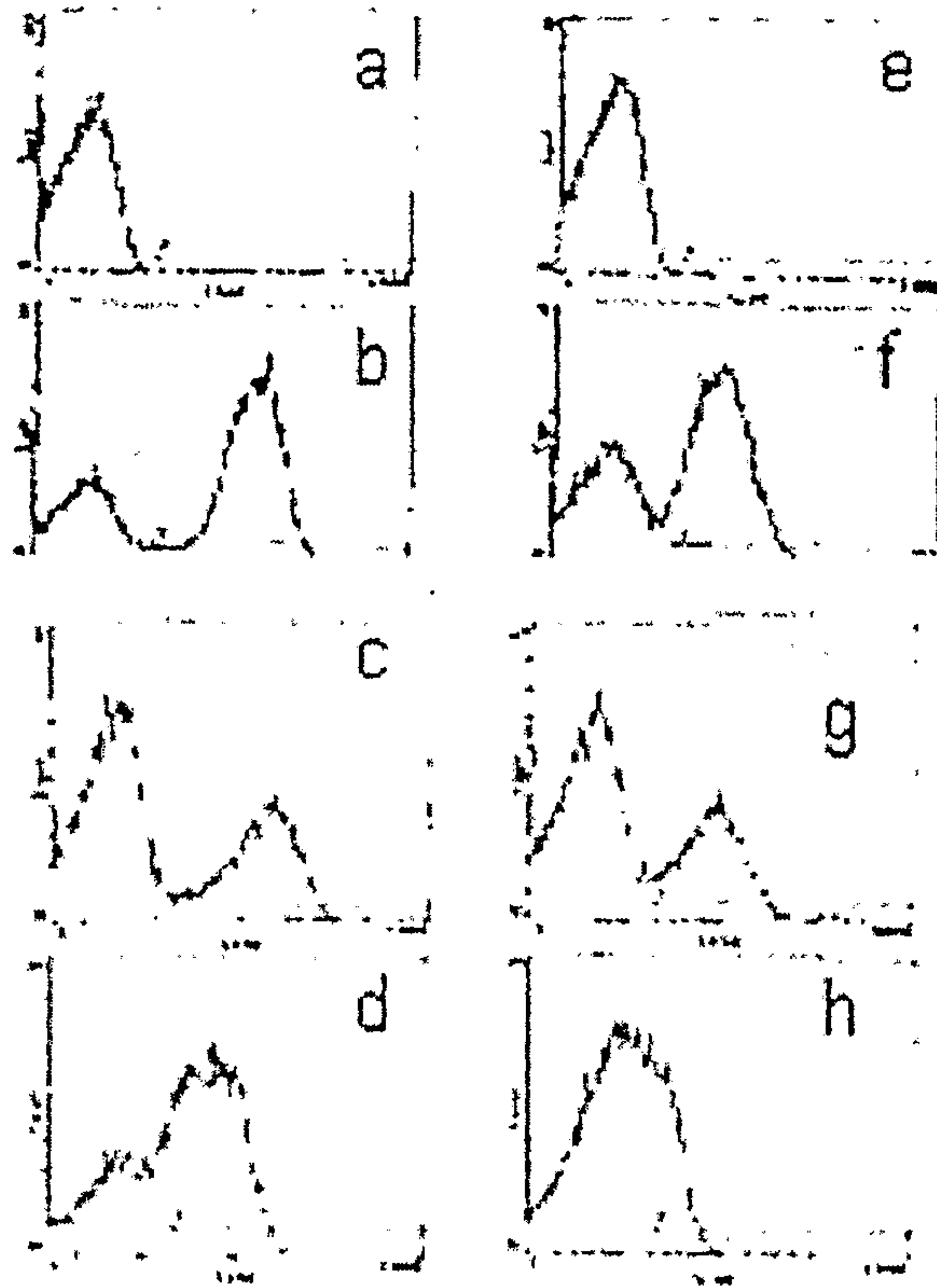


FIG. 6

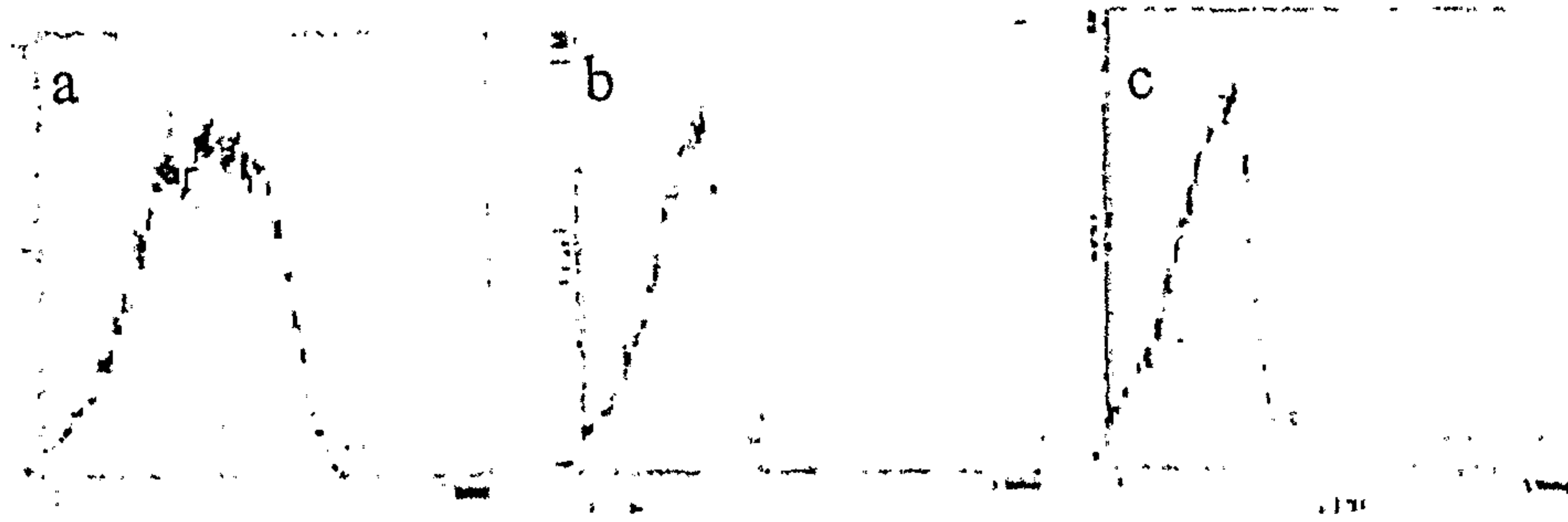


FIG. 7

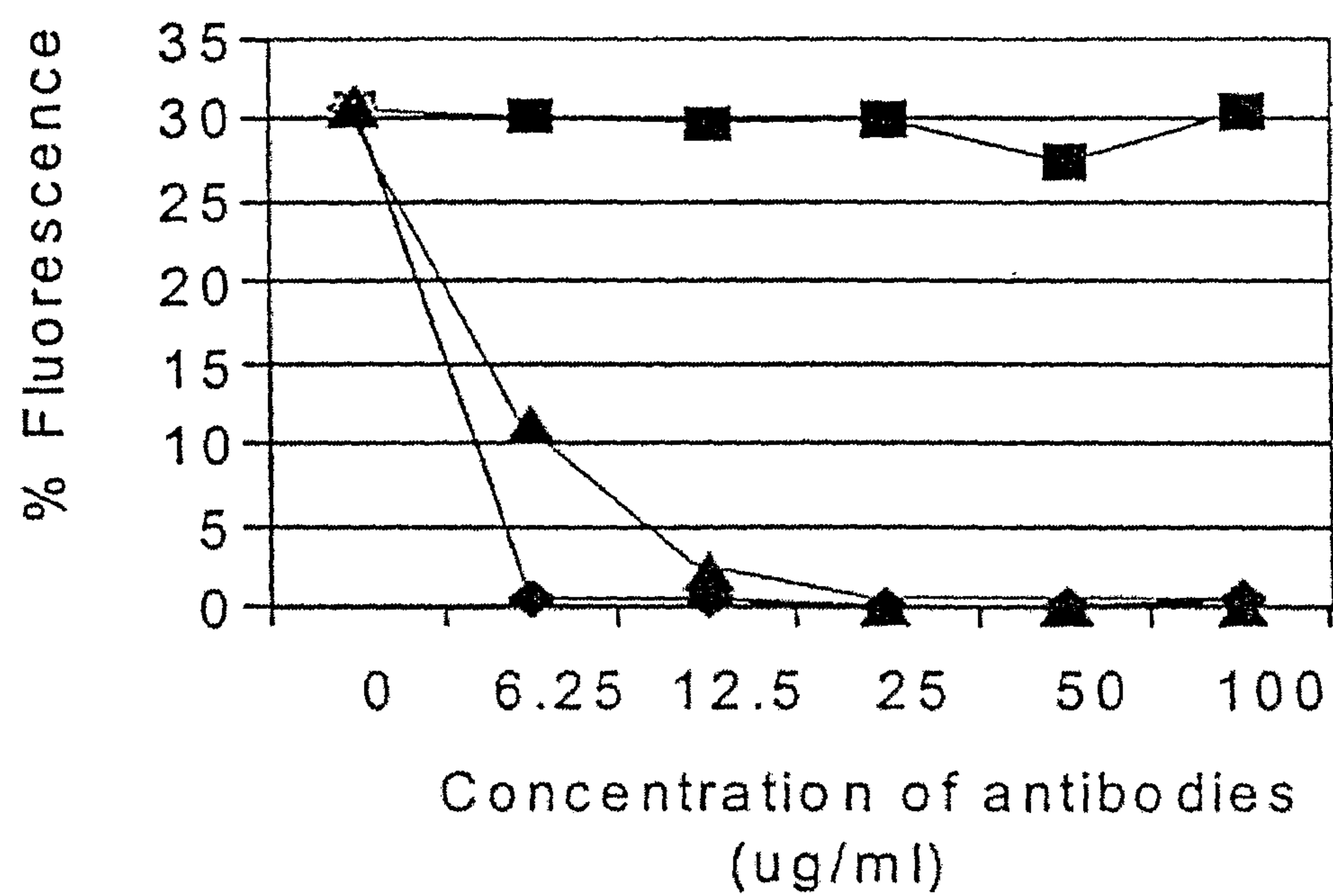


FIG. 8

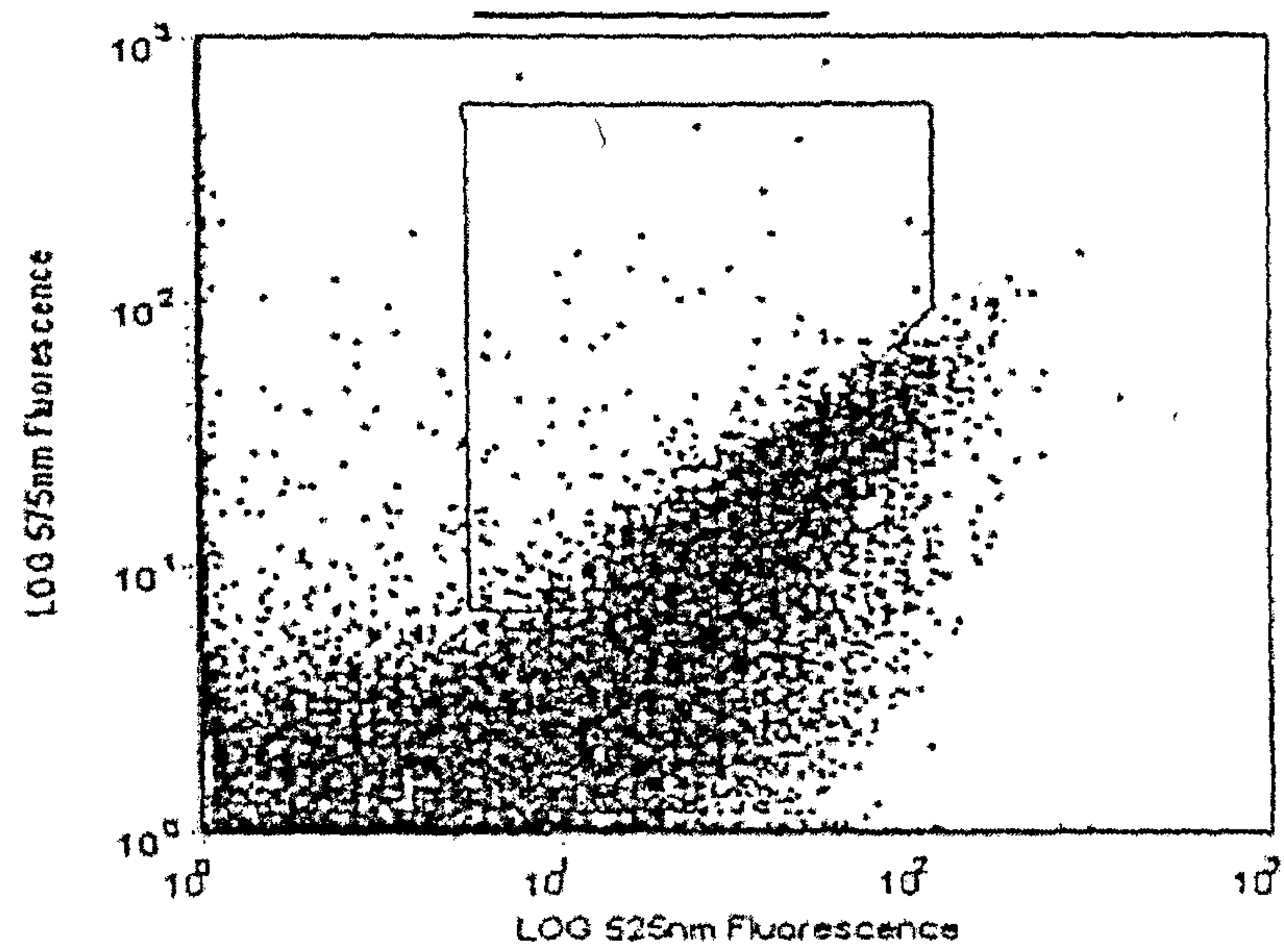


FIG. 9

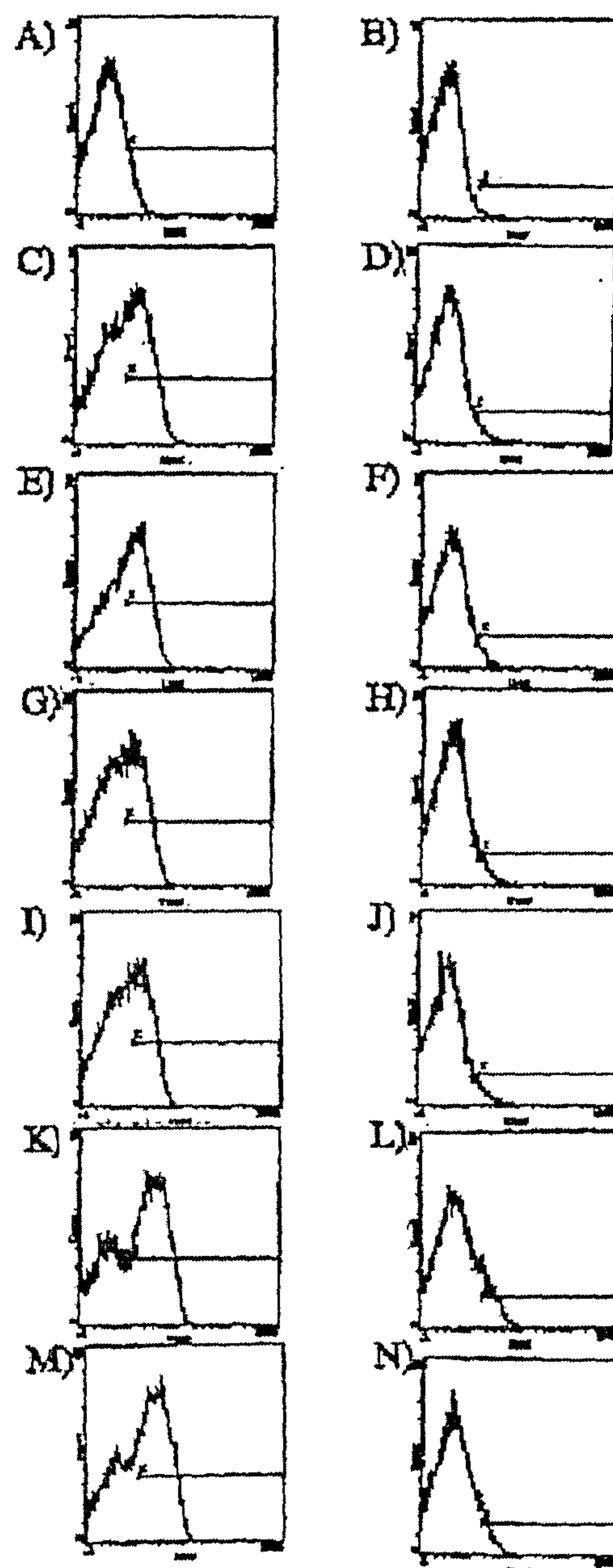


FIG. 10

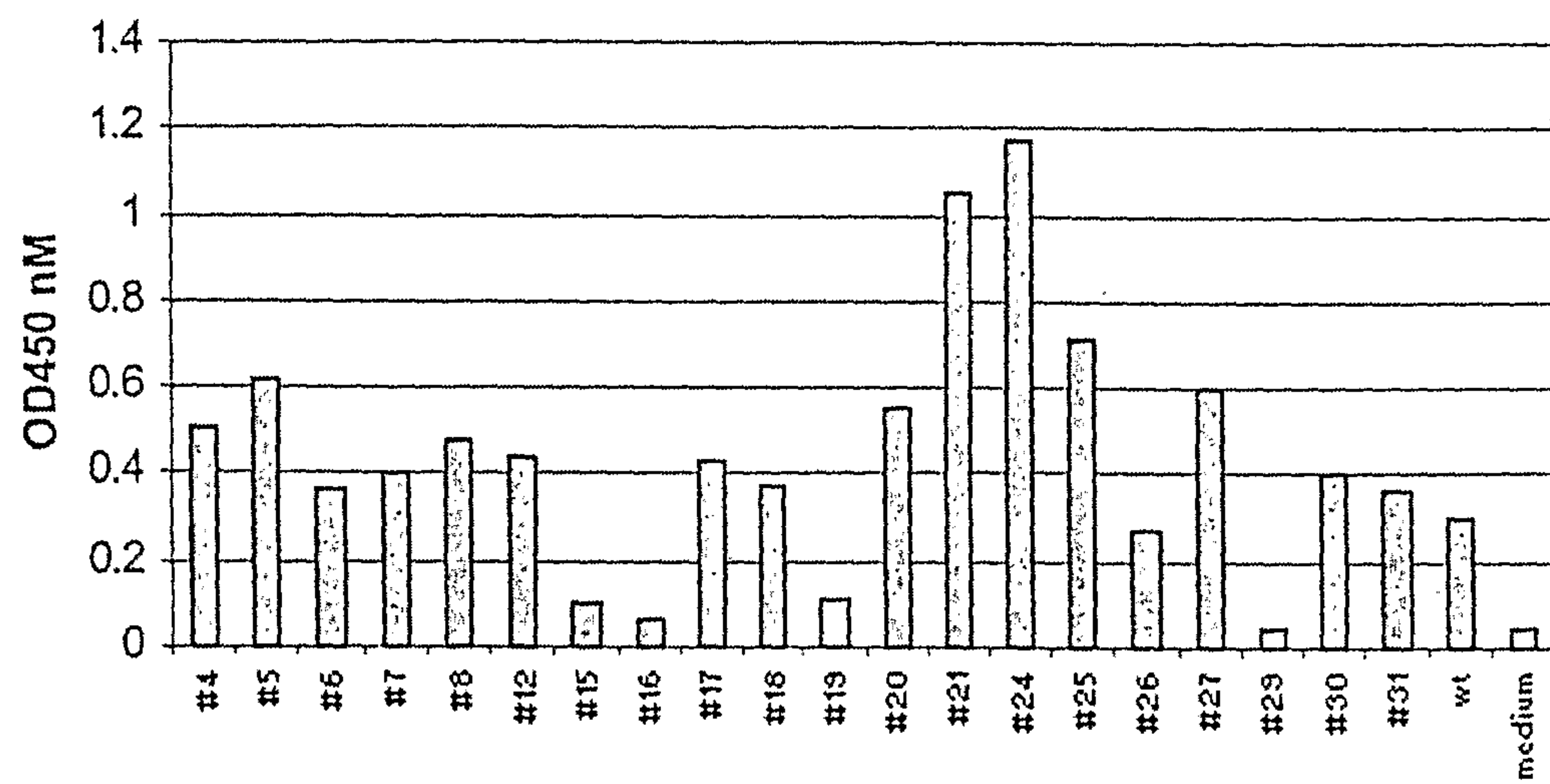


FIG. 11

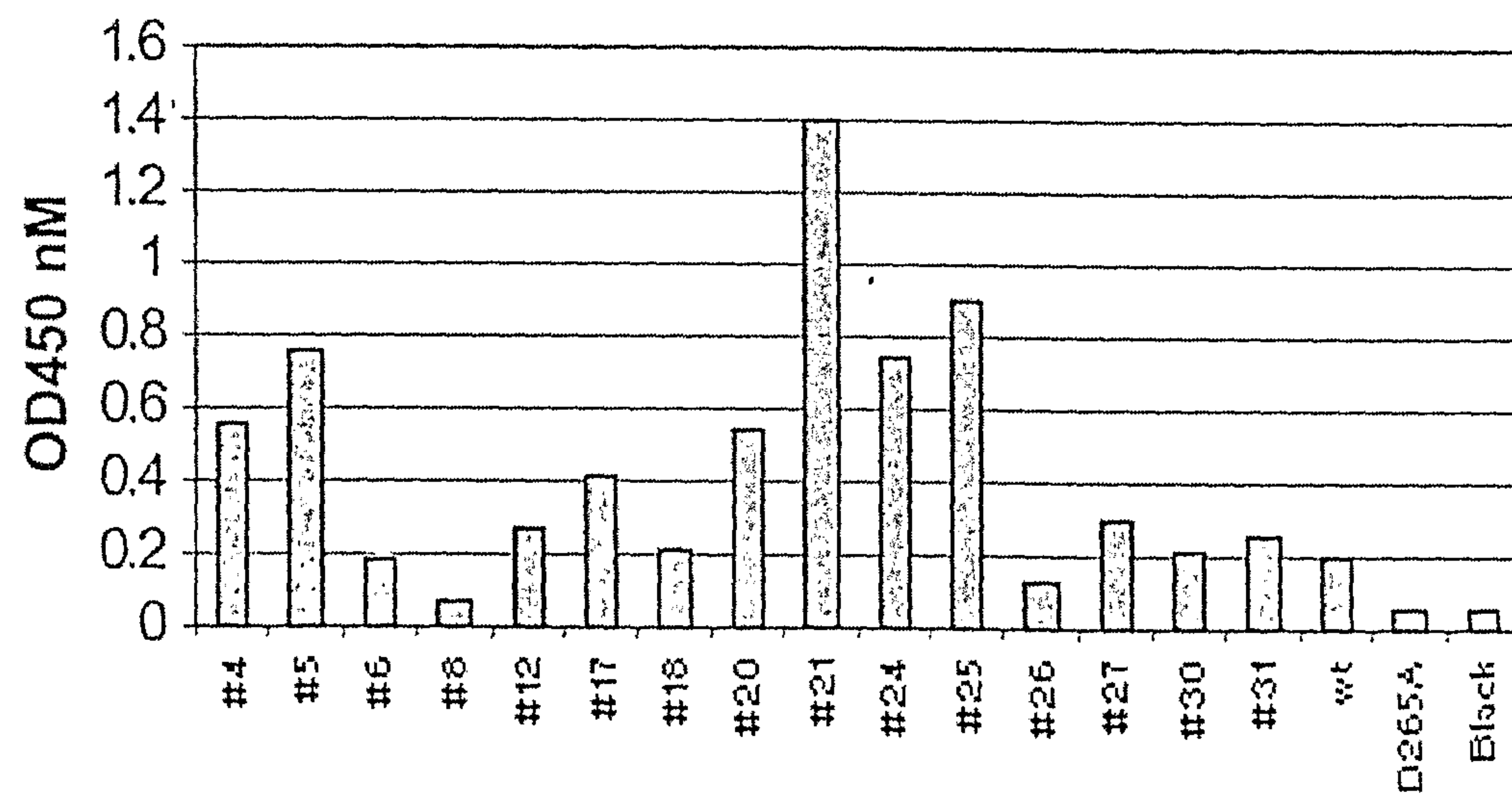


FIG. 12

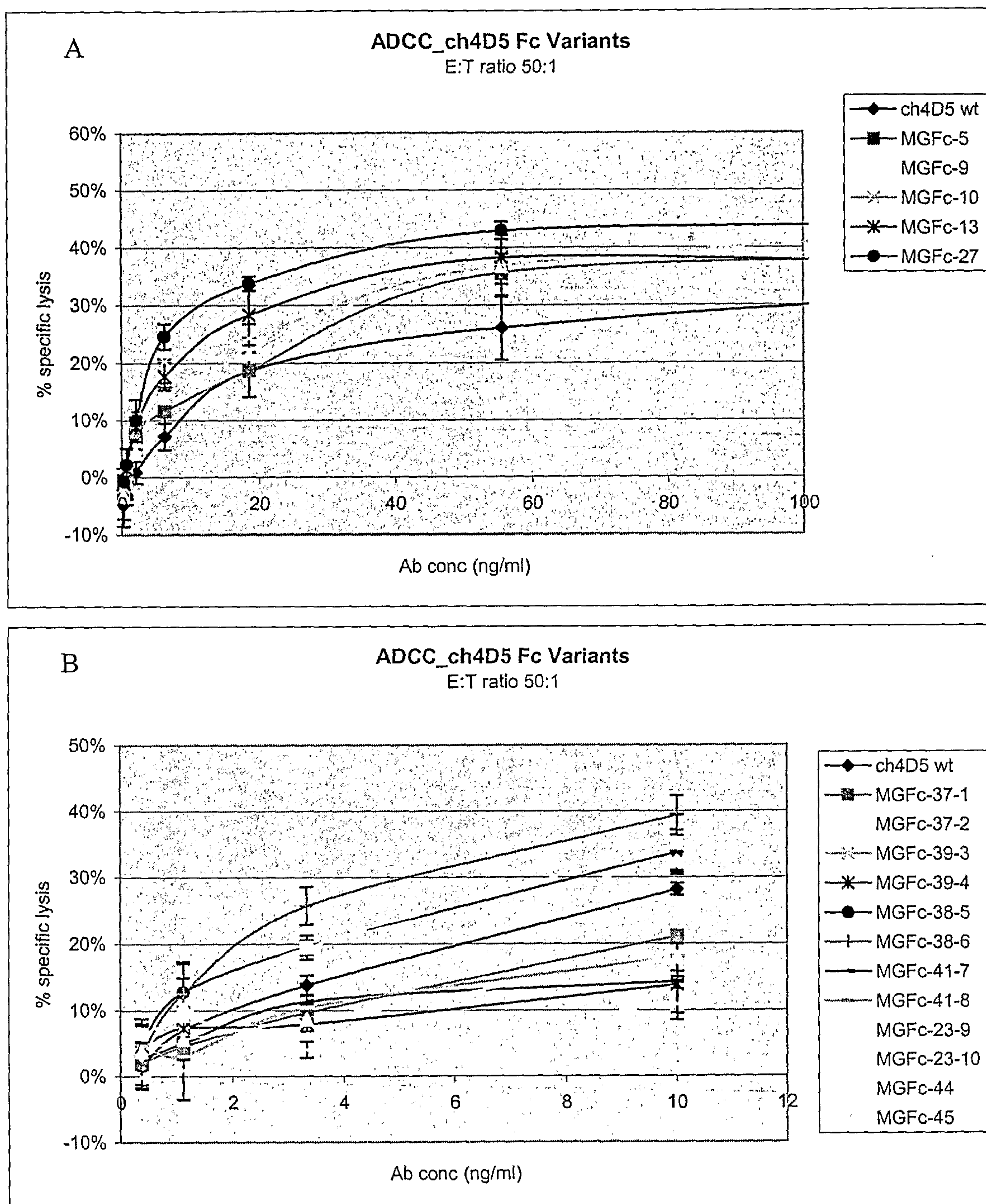


FIG. 13

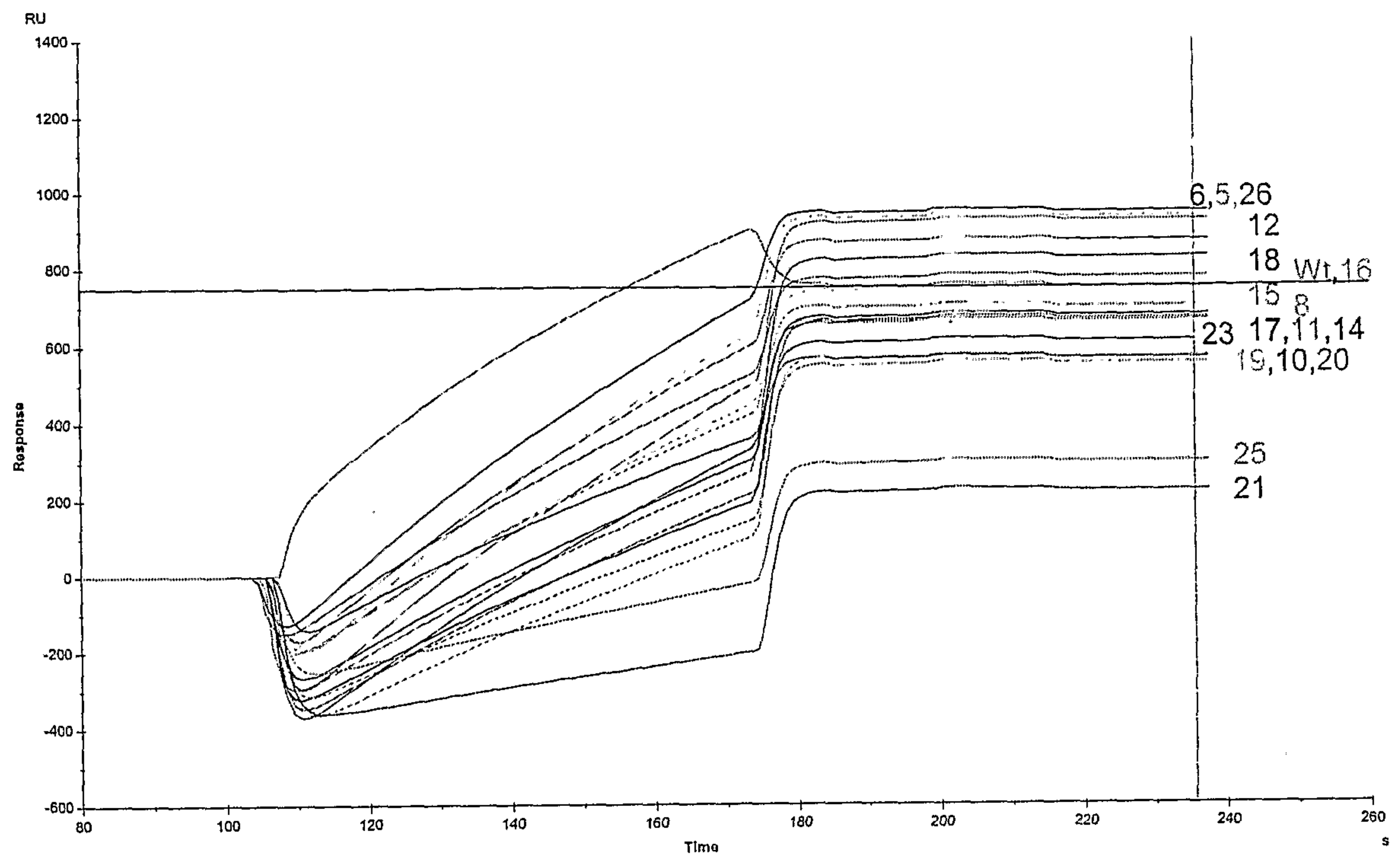


FIG. 14

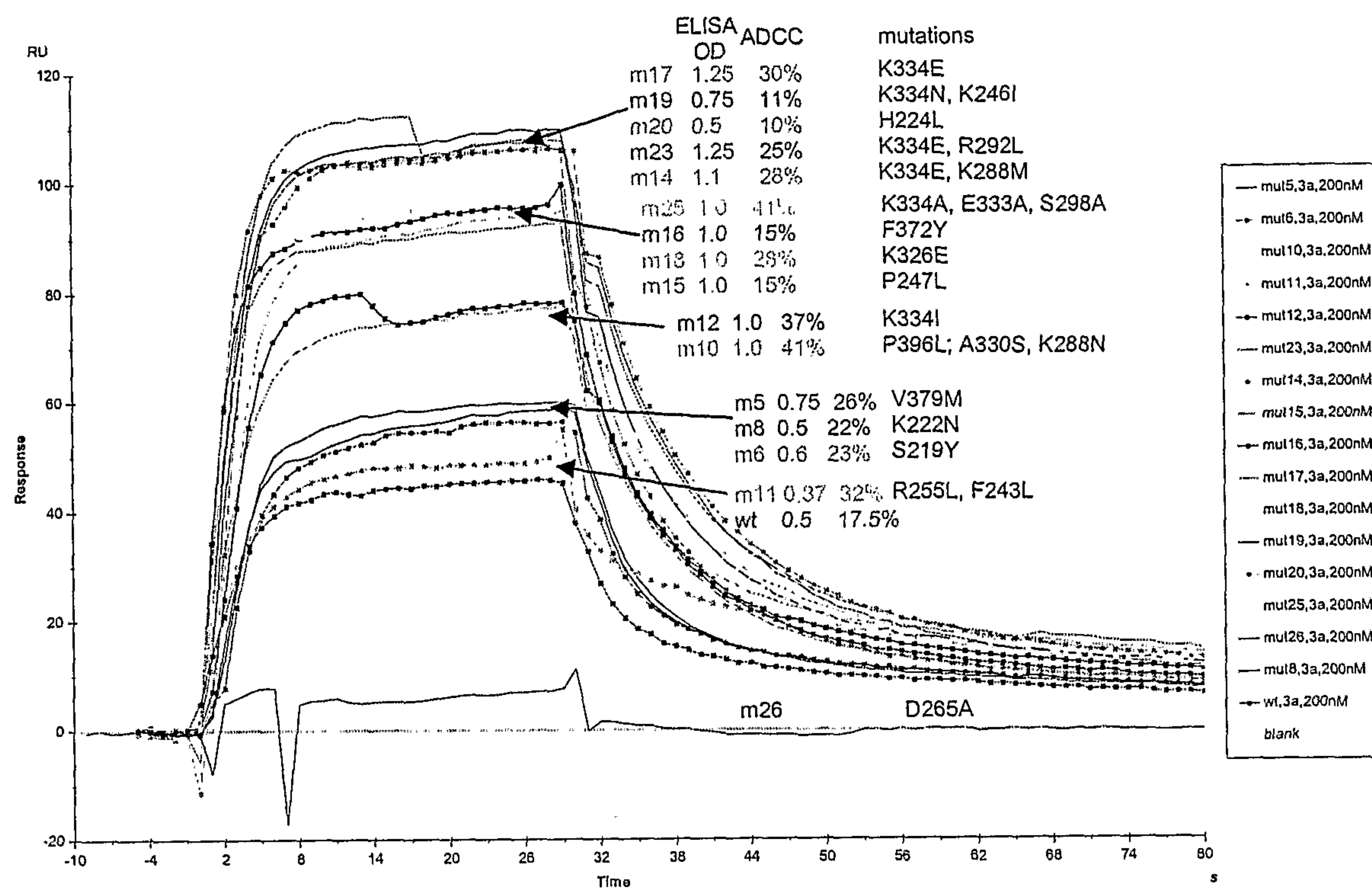


FIG. 15

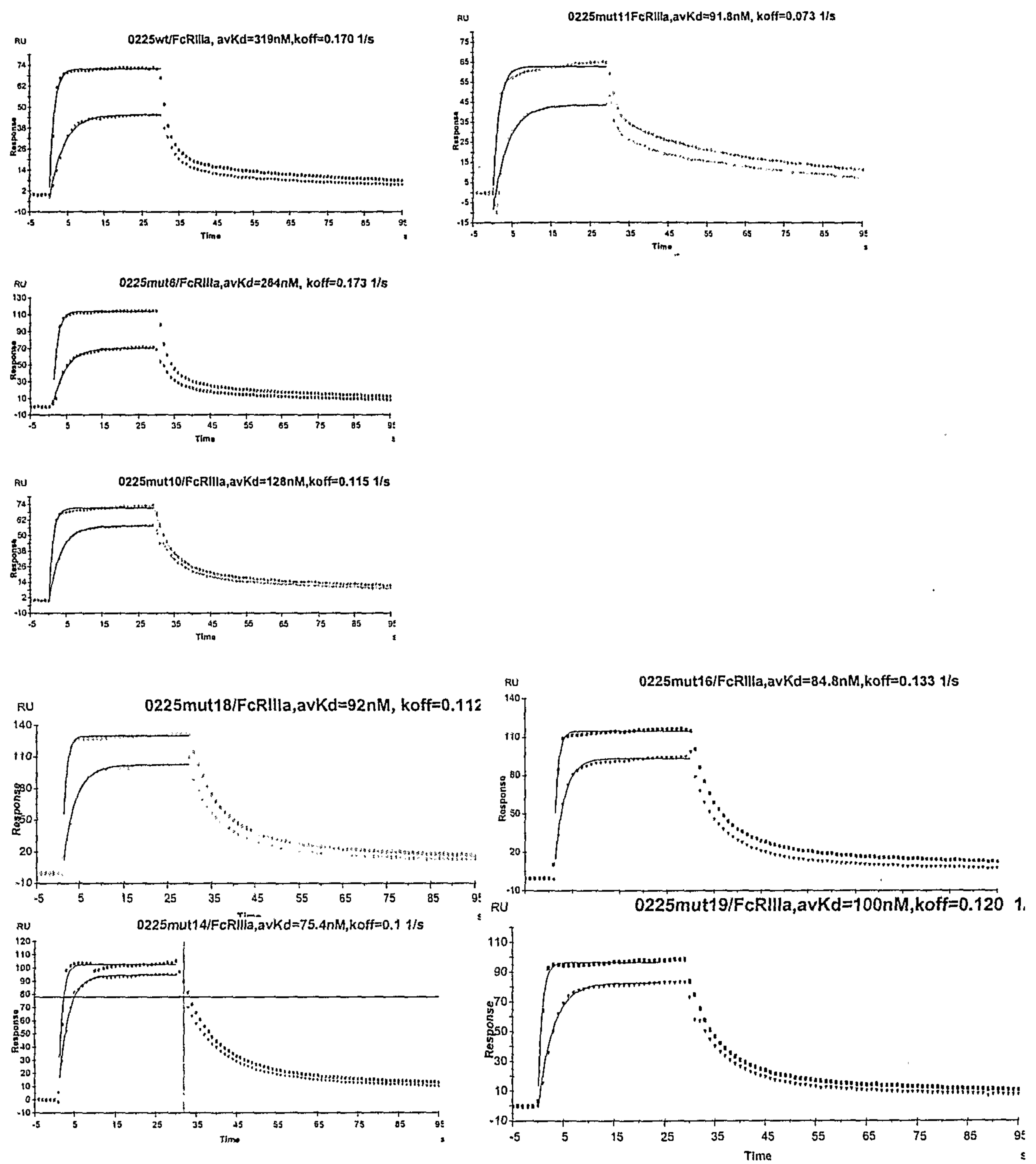


FIG. 16

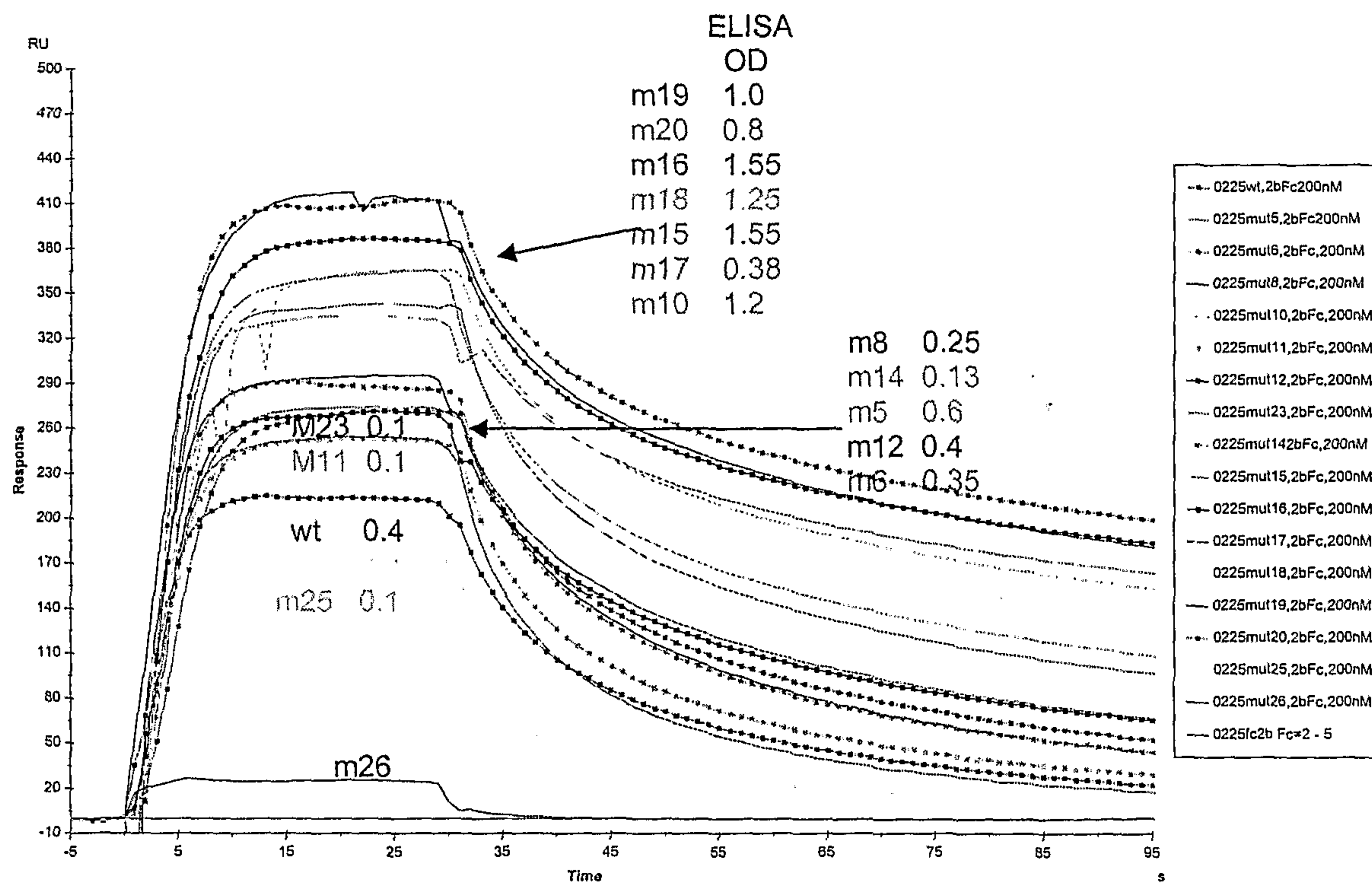


FIG. 17

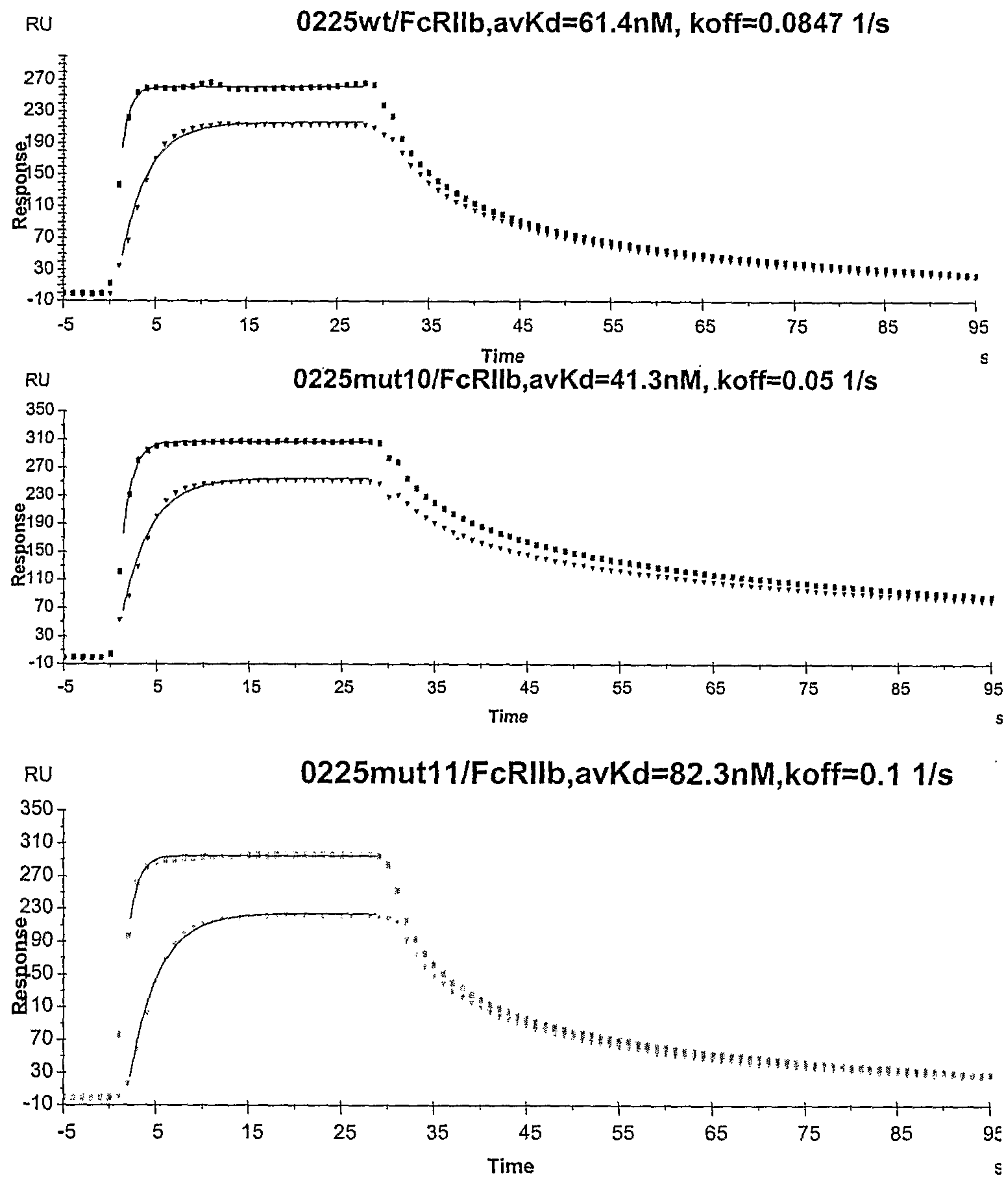


FIG. 18

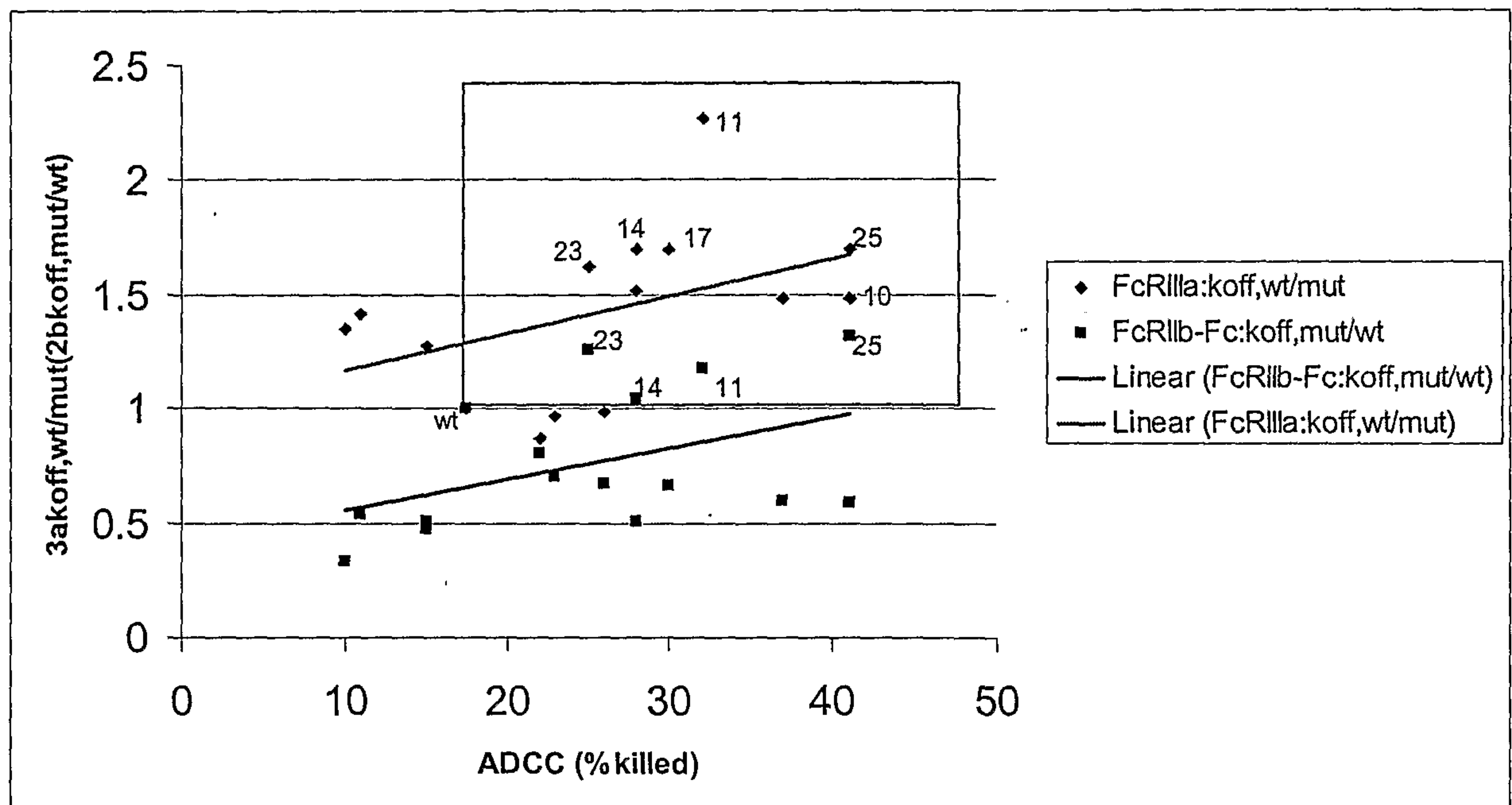


FIG. 19

FACS analysis of FcR3A competition

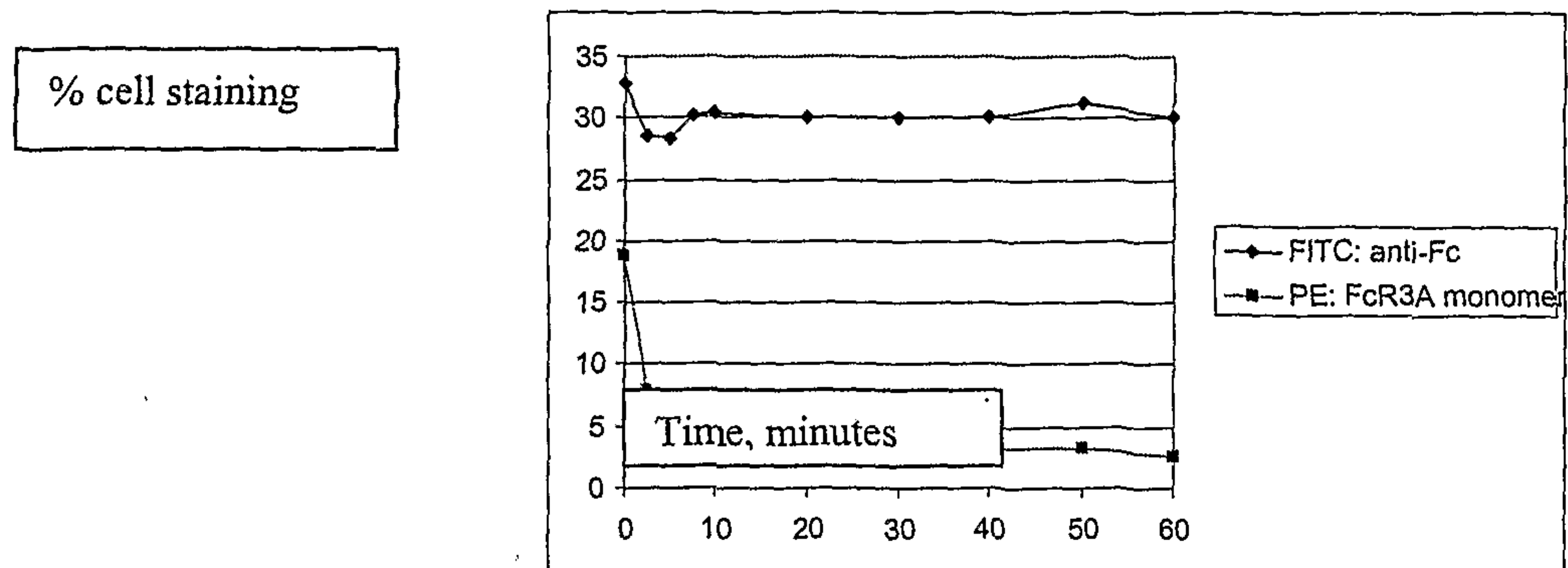


FIG. 20

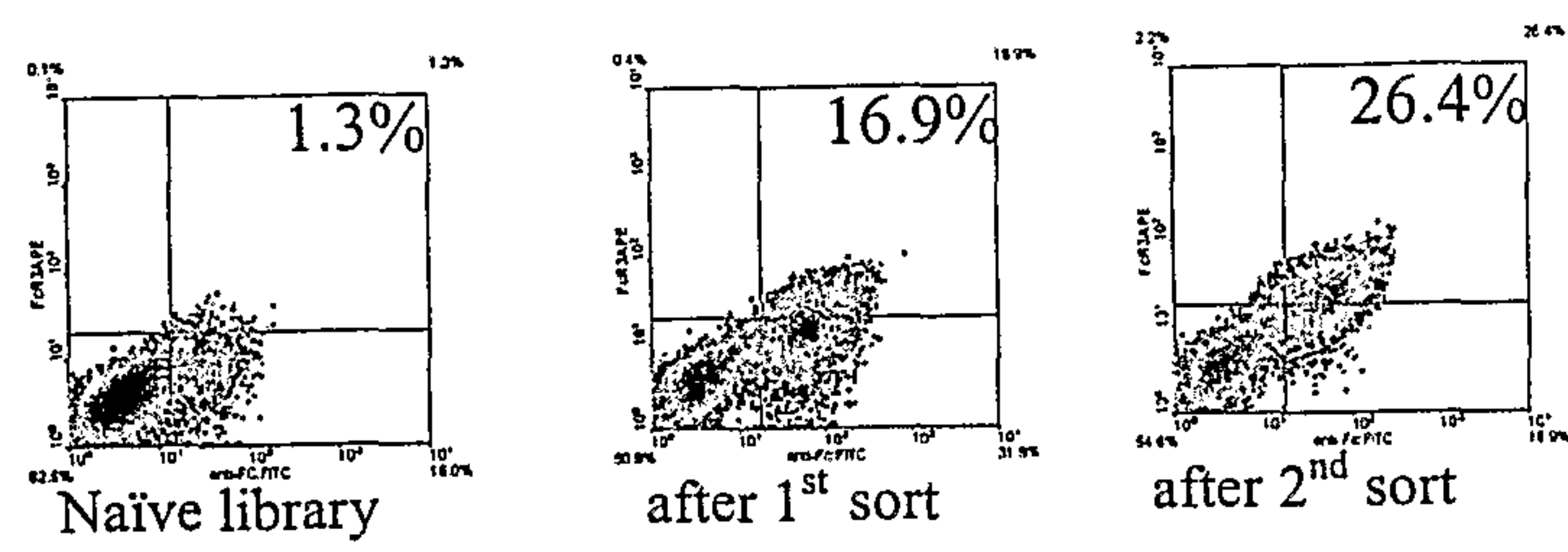
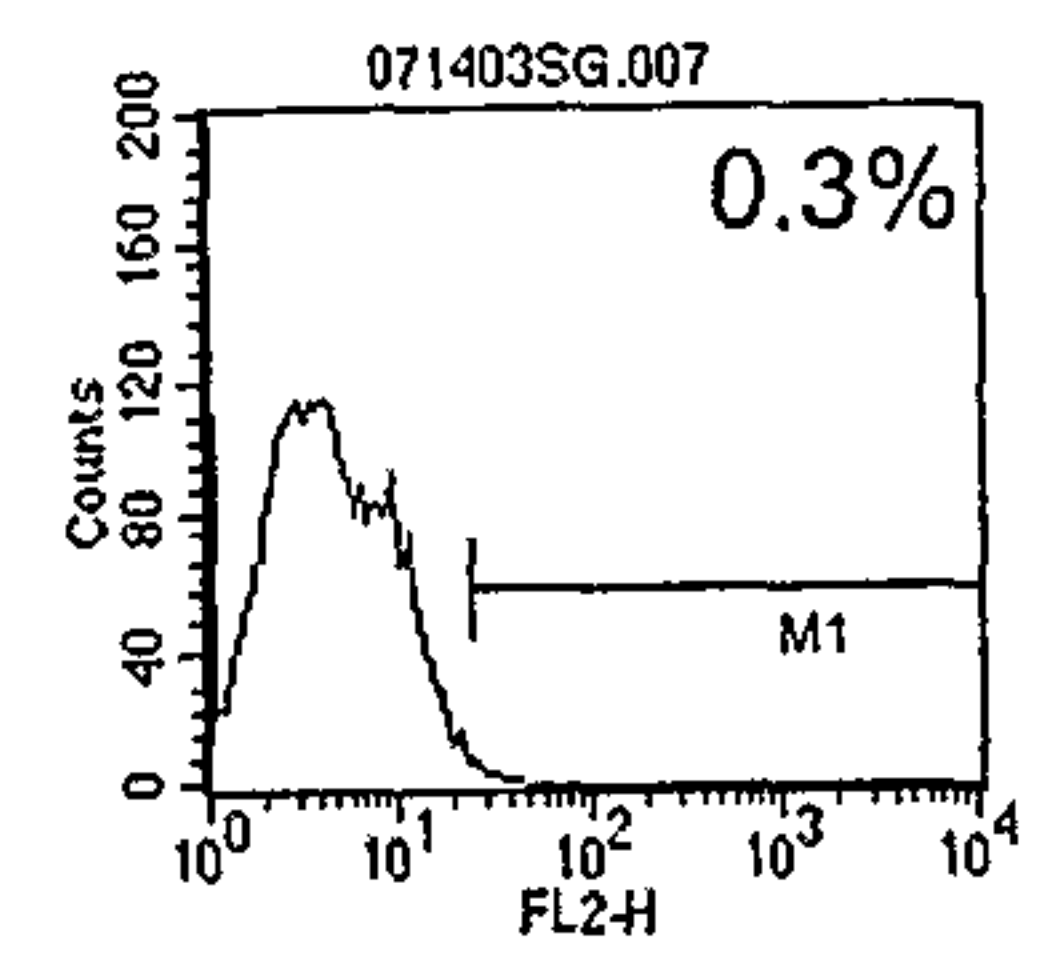
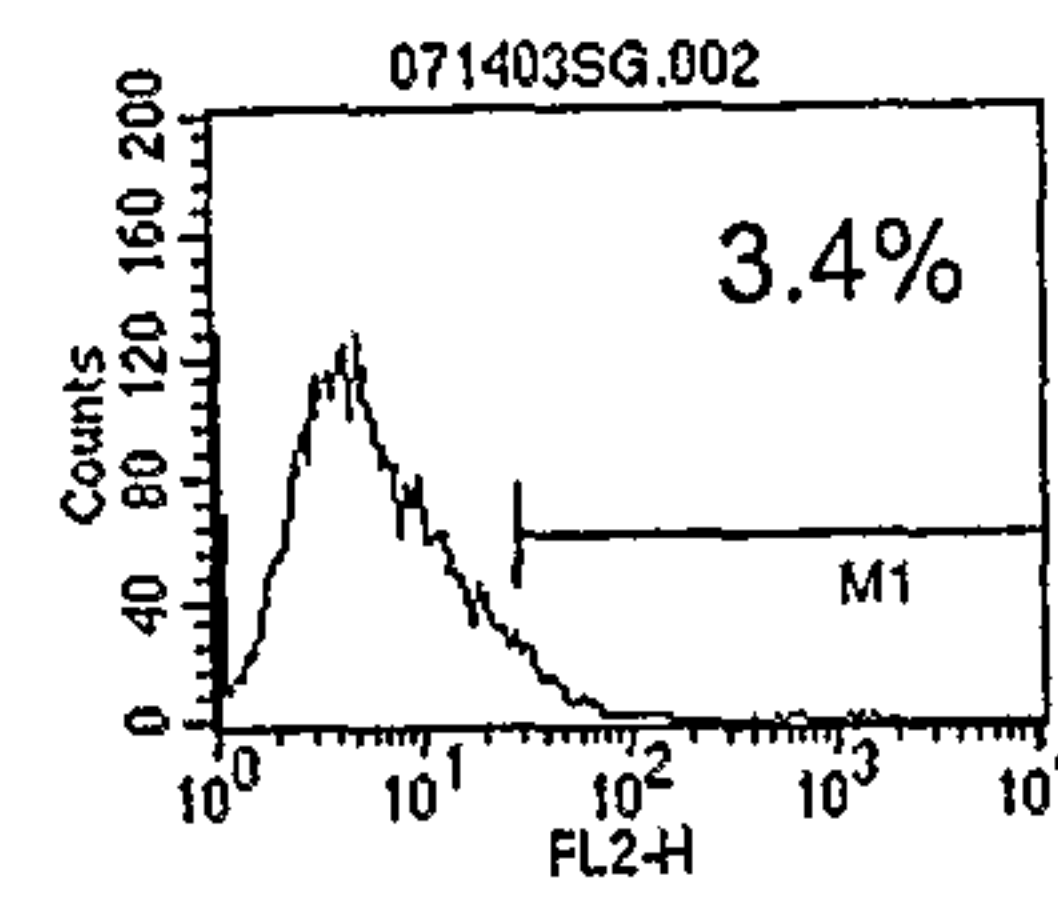
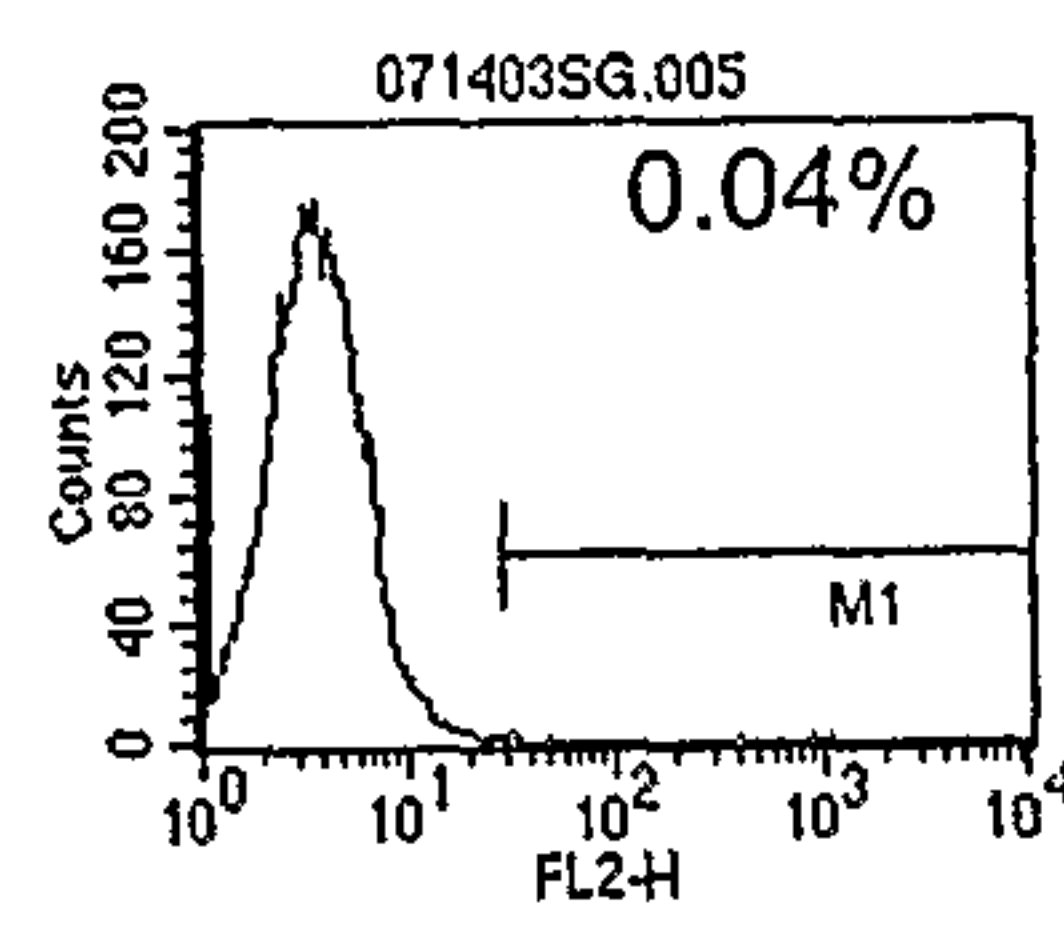


FIG. 21

3aLAVI(V158) P051



2bFc S-biot

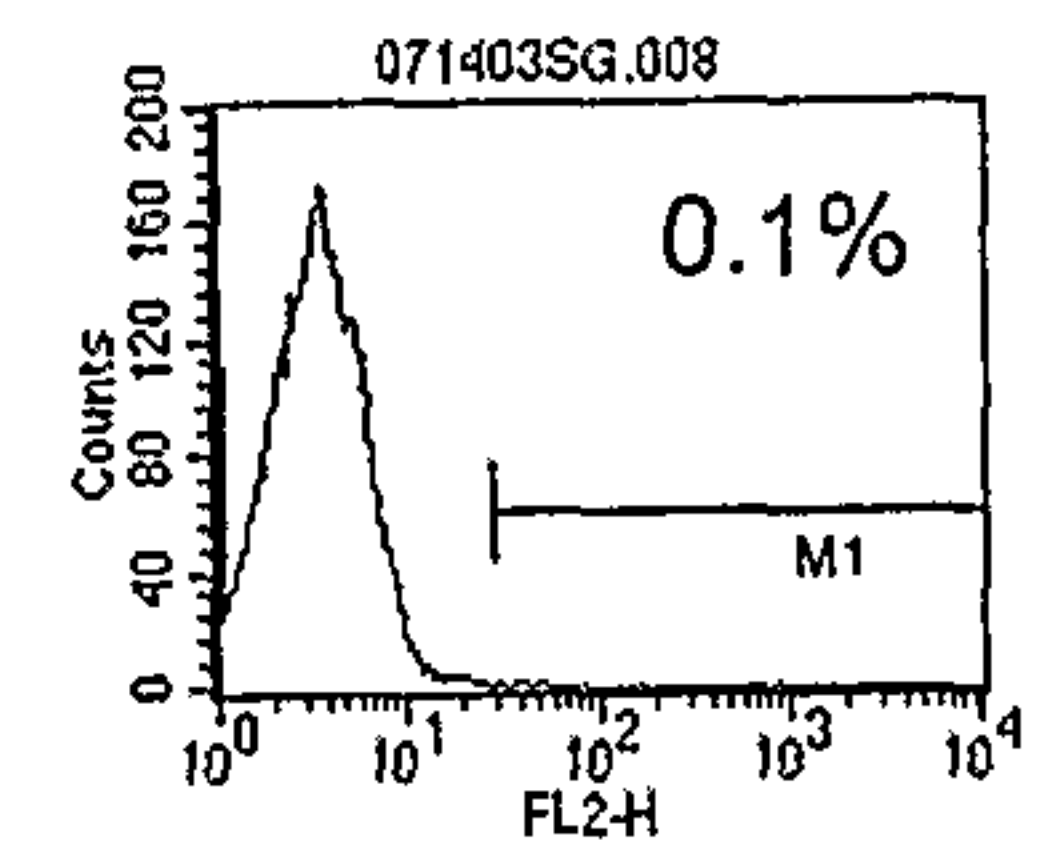
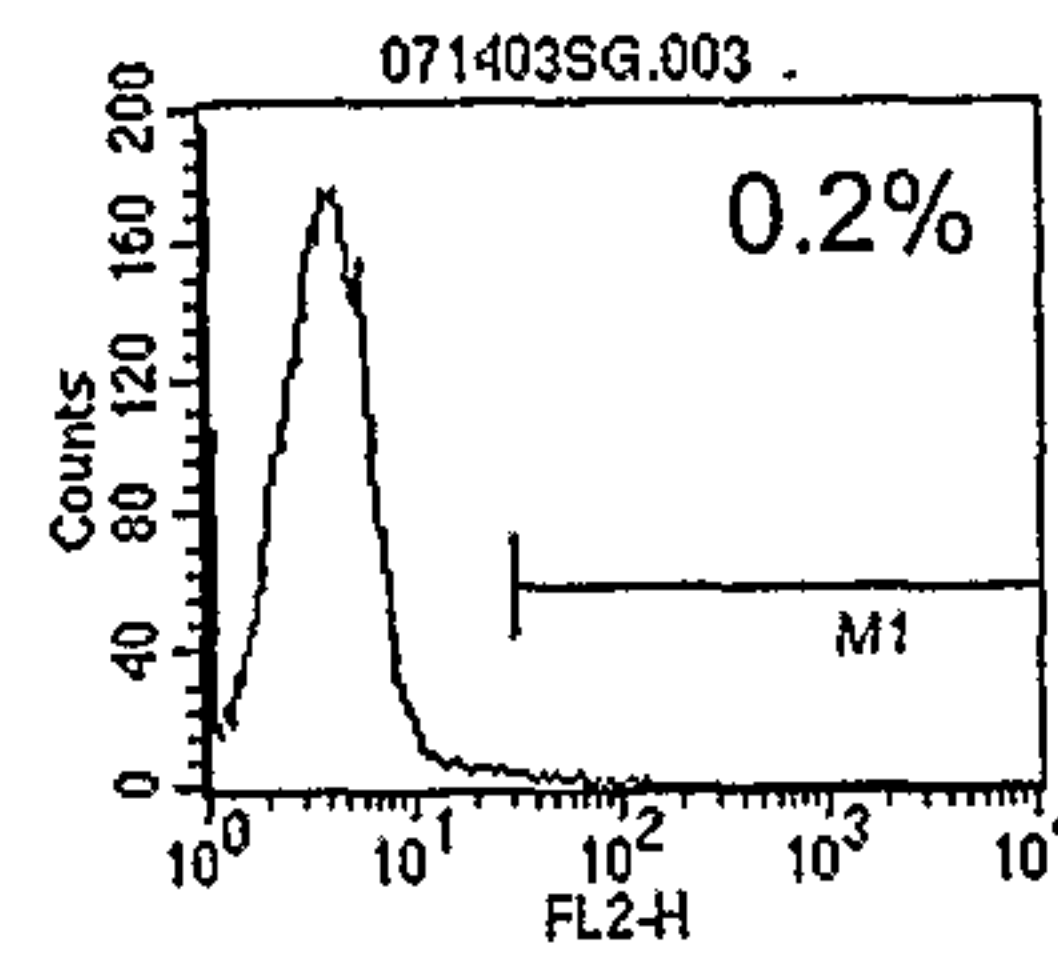
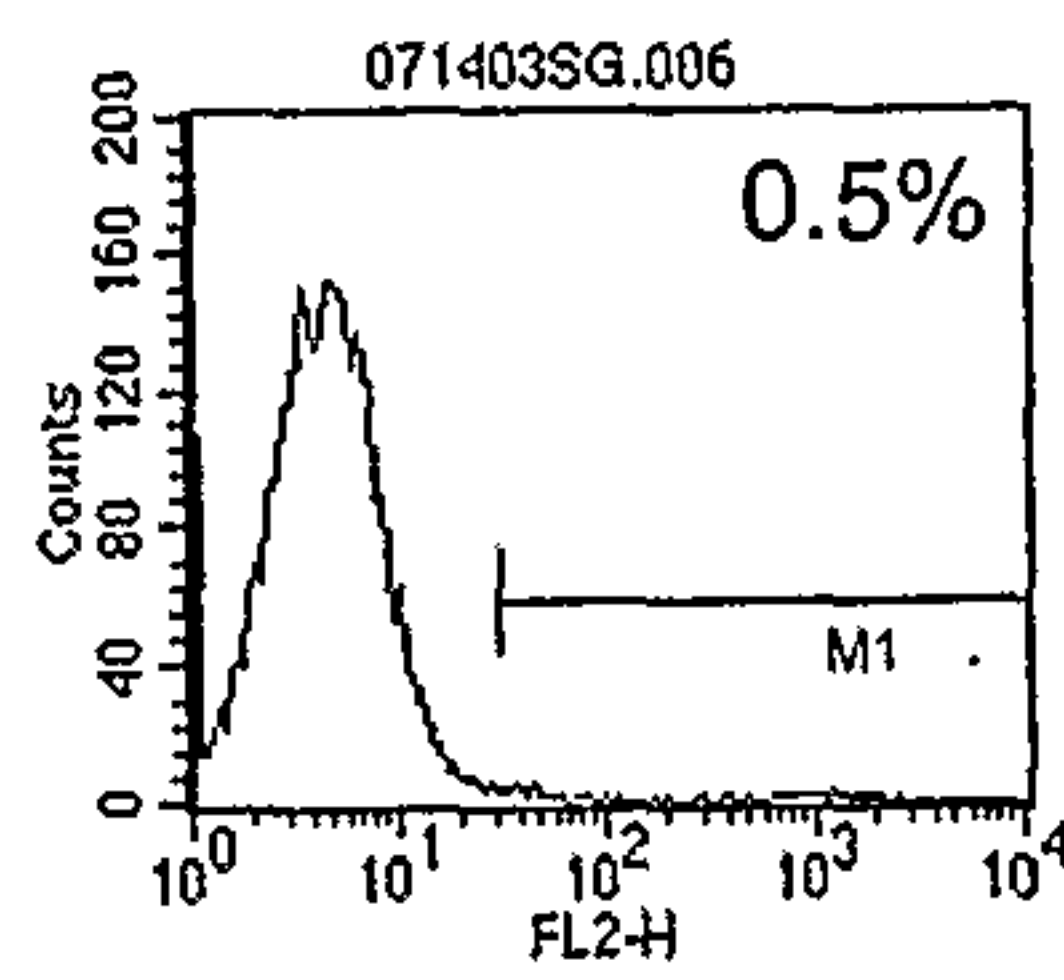
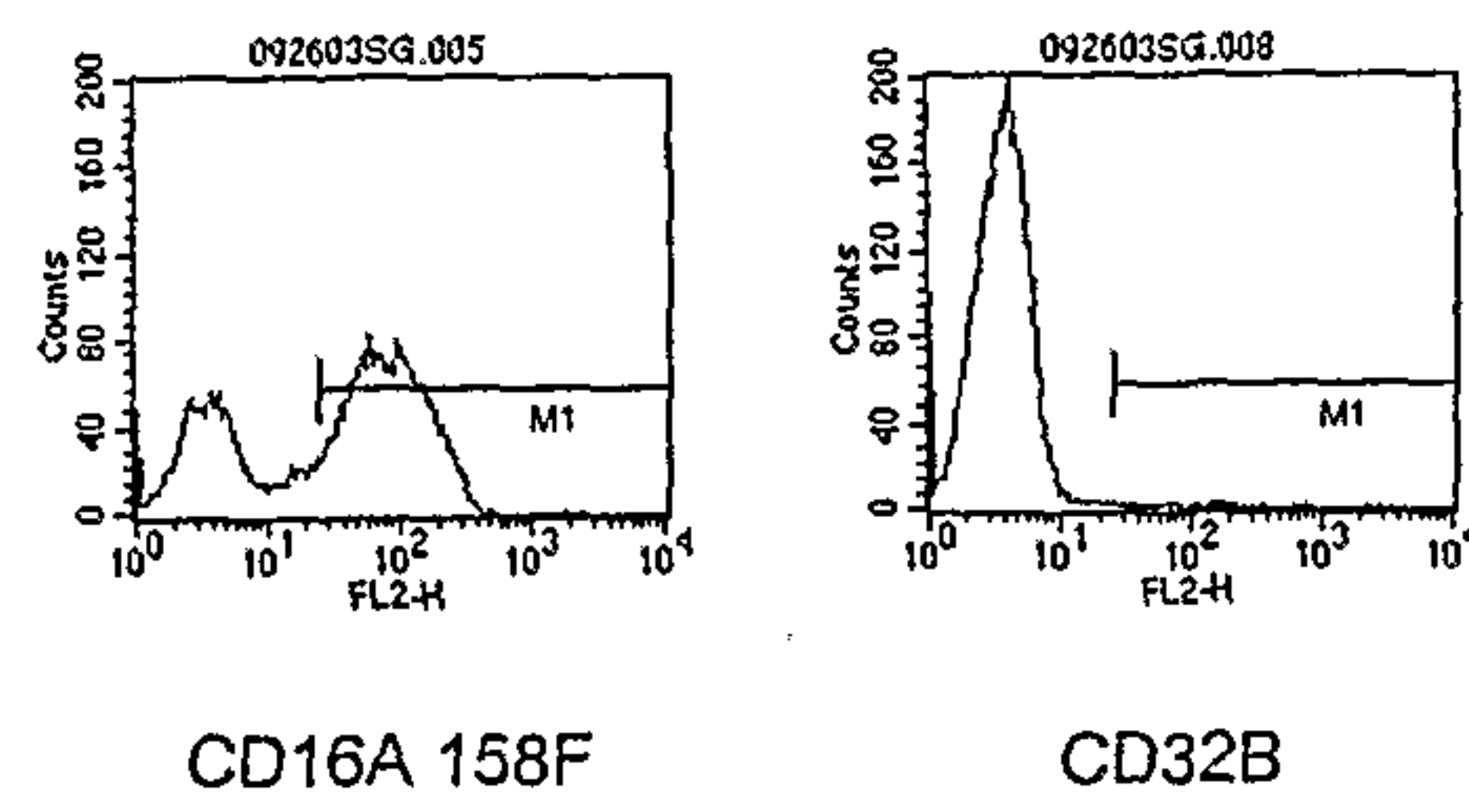
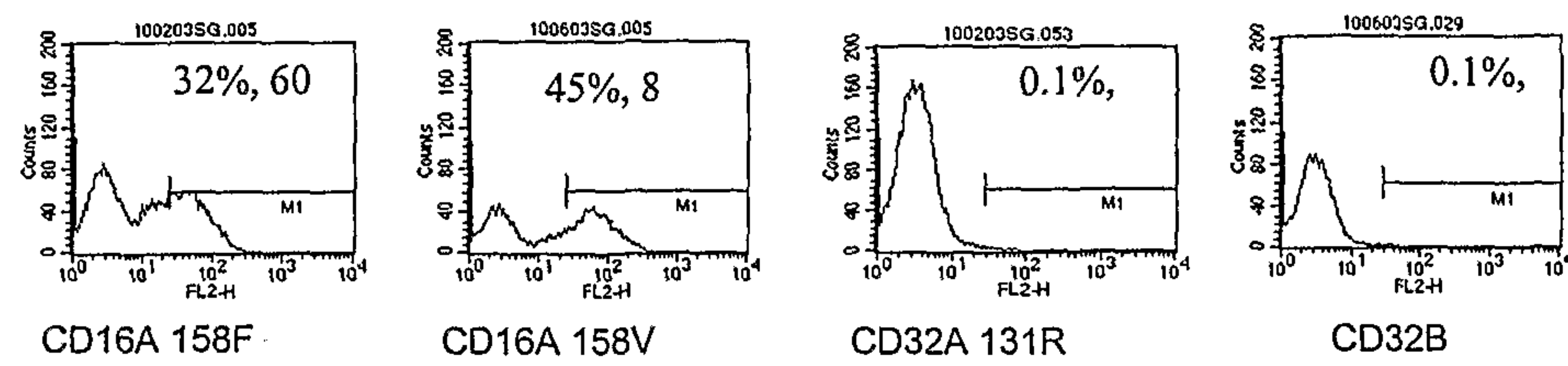


FIG. 22

A.**B.****FIG. 23**

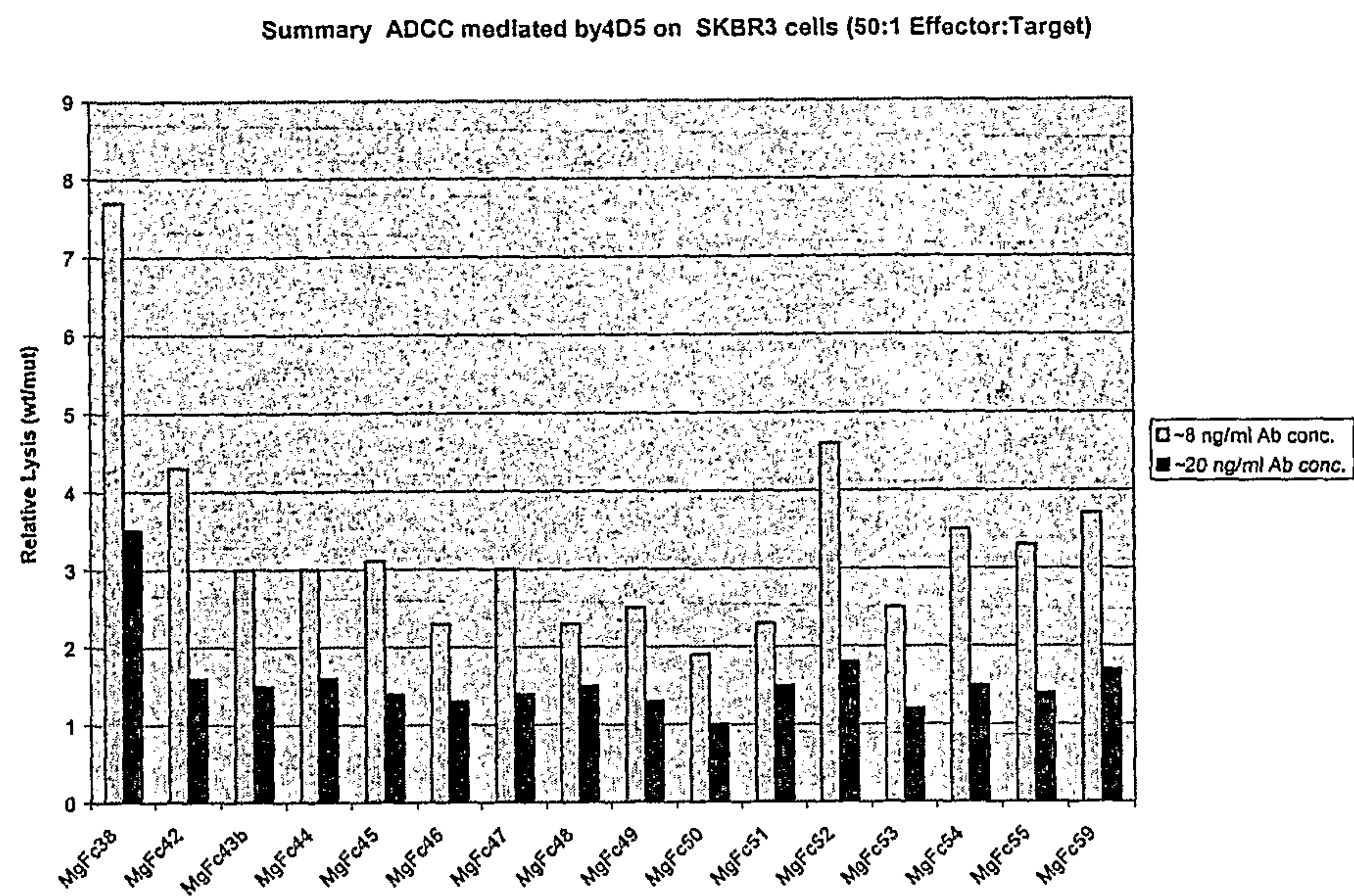


FIG. 24

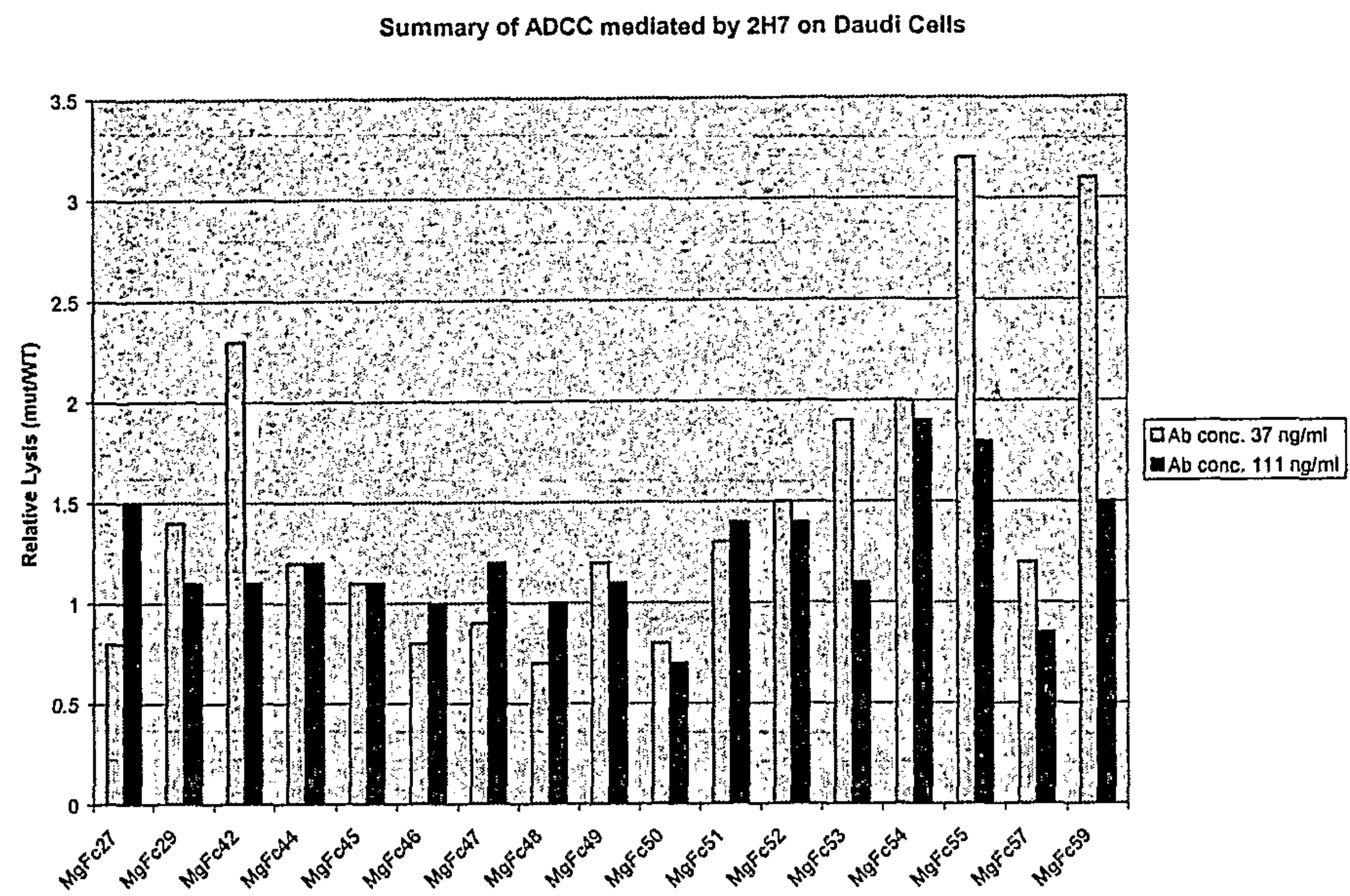


FIG. 25