Title of the Invention:

Fc Region-Containing Polypeptides That Exhibit Improved Effector Function Due to Alterations Of The Extent Of Fucosylation, And Methods For Their Use

1. Cross-Reference to Related Applications

[0001] This Application claims priority from United States Patent Applications Serial Nos. 61/249,510 (filed on October 7, 2009), which application is herein incorporated by reference in its entirety. This Application additionally incorporates by rerference in their entireties United States Patent Applications Serial Nos. 11/952,568 (filed on December 7, 2007) and 60/869,254 (filed on December 6, 2006).

2. FIELD OF THE INVENTION

[0002] The present invention relates to Fc region-containing polypeptides that exhibit improved effector function due to alterations of the extent of fucosylation, and to methods of using such polypeptides for treating or preventing cancer and other diseases. The Fc region-containing polypeptides of the present invention are preferably immunoglobulins (*e.g.*, antibodies), in which the Fc region comprises at least one amino acid substitution relative to the corresponding amino acid sequence of a wild type Fc region, and which is sufficient to attenuate post-translational fucosylation and mediate improved binding to an activating Fc receptor and reduced binding to an inhibitory Fc receptor.

[0003] The methods of the invention are particularly useful in preventing, treating, or ameliorating one or more symptoms associated with a disease, disorder, or infection where an enhanced efficacy of effector cell function (*e.g.*, ADCC) mediated by FcγR is desired, *e.g.*, cancer, infectious disease. The methods of the invention are also of use in enhancing the therapeutic efficacy of therapeutic antibodies the effect of which is mediated by ADCC. Conversely, the methods of the invention are particularly useful in preventing, treating, or ameliorating one or more symptoms associated with a disease or disorder in which decreased efficacy of effector cell function mediated by FcγR is desired, *e.g.*, inflammation, etc. The methods of the invention are thus also of use in enhancing the therapeutic efficacy of therapeutic antibodies which attenuate inflammatory processes.

3. BACKGROUND OF THE INVENTION

3.1 Fc RECEPTORS AND THEIR ROLES IN THE IMMUNE SYSTEM

[0004] The interaction of antibody-antigen complexes with cells of the immune 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 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.

[0005] The Fc receptors, members of the immunoglobulin gene superfamily of proteins, are surface glycoproteins that can bind the Fc portion of immunoglobulin 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 FcγR, for IgE as FεR, and for IgA as FcαR. 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): 161-168l; 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 well known in the art, see, *e.g.*, Immunobiology: The Immune System in Health and Disease, 4th ed. 1999, Elsevier Science Ltd/Garland Publishing, New York, which is hereby incorporated by reference in its entirety.

Fcγ Receptors

[0006] 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. There

are three known FcγRs, designated FcγRI(CD64), FcγRII(CD32), and FcγRIII(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.

FcγRII(CD32)

[0007] FcγRII 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 FcγR, present on all hematopoietic cells, including monocytes, macrophages, B cells, NK cells, neutrophils, mast cells, and platelets. FcγRII has only two immunoglobulin-like regions in its immunoglobulin binding chain and hence a much lower affinity for IgG than FcγRI. There are three human FcγRII genes (FcγRII-A, FcγRII-B, FcγRII-C), all of which bind IgG in aggregates or immune complexes.

[0008] Distinct differences within the cytoplasmic domains of FcγRII-A and FcγRII-B create two functionally heterogenous responses to receptor ligation. The fundamental difference is that the A isoform initiates intracellular signaling leading to cell activation such as phagocytosis and respiratory burst, whereas the B isoform initiates inhibitory signals, *e.g.*, inhibiting B-cell activation.

Signaling through FcγRs

[0009] 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, FcγRIIIA, whereas ITIM-containing complexes only include FcγRIIB.

[0010] 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.*, PI_3K). Cellular activation leads to release of proinflammatory mediators.

[0011] 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 inosital 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.

3.2 DISEASES OF RELEVANCE

3.2.1 CANCER

[0012] 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.

[0013] More than 1.2 million Americans develop cancer each year. Cancer is the second leading case 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.

[0014] 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

[0015] 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.

[0016] 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 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 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.

[0017] Despite the availability of a variety of chemotherapeutic agents, 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 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 multidrug resistance. Thus, because of drug resistance, many cancers prove refractory to standard chemotherapeutic treatment protocols.

[0018] 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 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.

3.2.2 INFLAMMATORY DISEASES AND AUTOIMMUNE DISEASES

[0019] 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.

[0020] 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.

[0021] 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.

[0022] 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.

[0023] 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.

[0024] 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 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.

[0025] The typical course of the disease is one of persistent but fluctuating joint 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 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).

[0026] 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 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 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.

3.2.3 INFECTIOUS DISEASES

[0027] 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.

[0028] 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.

[0029] 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).

[0030] 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). 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.

4. SUMMARY OF THE INVENTION

[0031] The invention relates to methods of treating or preventing cancer and other diseases, disorders and infections using 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 modification(s) alter (relative to a wild-type Fc region) the Ratio of Affinities of the variant Fc region to an activating FcγR (such as FcγRIIA or FcγRIIIA) relative to an inhibiting FcγR (such as FcγRIIB):

$$\text{Ratio of Affinities} = \frac{\text{Wild-Type to Variant Change in Affinity to Fc}\gamma\text{R}_{\text{Activating}}}{\text{Wild-Type to Variant Change in Affinity to Fc}\gamma\text{R}_{\text{Inhibiting}}}$$

[0032] Of particular interest are Ratios of Affinities in which either FcγRIIIA or FcγRIIA is the $Fc\gamma R_{Activating}$ and FcγRIIB is the $Fc\gamma R_{Inhibiting}$. Where an Fc variant has a Ratio of Affinities greater than 1, the methods of the invention have particular use in providing a therapeutic or prophylactic treatment of a disease, disorder, or infection, or the amelioration of a symptom thereof, where an enhanced efficacy of effector cell function (*e.g.*, ADCC) mediated by FcγR is desired, *e.g.*, cancer or infectious disease. Such an increased Ratio of Affinities may result from the Fc region of the molecule having (relative to a wild type Fc) an increase in affinity to an $Fc\gamma R_{Activating}$ (e.g., FcγRIIIA or FcγRIIA) coupled with either an unchanged affinity to an $Fc\gamma R_{Inhibiting}$ (e.g., FcγRIIB) or a decrease in affinity to such $Fc\gamma R_{Inhibiting}$. Alternatively, an increased Ratio of Affinities may result from the Fc region of such molecule exhibiting an increase in affinity to both an $Fc\gamma R_{Activating}$ and an $Fc\gamma R_{Inhibiting}$ (relative to a wild-type Fc), provided that the increase in affinity to the $Fc\gamma R_{Activating}$ exceeds the increase in affinity to the $Fc\gamma R_{Inhibiting}$, or may result from the Fc region of such molecule exhibiting a decreased affinity to both the $Fc\gamma R_{Activating}$ and an $Fc\gamma R_{Inhibiting}$ (relative to a wild-type Fc), provided that the decrease in affinity to the $Fc\gamma R_{Activating}$ is less than the decrease in affinity to the $Fc\gamma R_{Inhibiting}$, or may result from an unchanged affinity to an $Fc\gamma R_{Activating}$ coupled with a decrease in affinity to an $Fc\gamma R_{Inhibiting}$.

[0033] Where an Fv variant has a Ratio of Affinities less than 1, the methods of the invention have particular use in providing a therapeutic or prophylactic treatment of a disease or disorder, or the amelioration of a symptom thereof, where a decreased efficacy of effector cell function mediated by FcγR is desired, *e.g.*, autoimmune or inflammatory disorders. Such a decreased Ratio of Affinities may result from the Fc

region of the molecule having (relative to a wild type Fc) a decrease in affinity to an $Fc\gamma R_{Activating}$ (e.g., FcγRIIIA or FcγRIIA) coupled with either an unchanged affinity to an $Fc\gamma R_{Inhibiting}$ (e.g., FcγRIIB) or an increase in affinity to such $Fc\gamma R_{Inhibiting}$. Alternatively, a decreased Ratio of Affinities may result from the Fc region of such molecule exhibiting a decrease in affinity to both an $Fc\gamma R_{Activating}$ and an $Fc\gamma R_{Inhibiting}$ (relative to a wild-type Fc), provided that the decrease in affinity to the $Fc\gamma R_{Activating}$ exceeds the decrease in affinity to the $Fc\gamma R_{Inhibiting}$, or may result from the Fc region of such molecule exhibiting an increased affinity to both an $Fc\gamma R_{Activating}$ and an $Fc\gamma R_{Inhibiting}$ (relative to a wild-type Fc), provided that the increase in affinity to the $Fc\gamma R_{Activating}$ is less than the increase in affinity to the $Fc\gamma R_{Inhibiting}$, or may result from an unchanged affinity to an $Fc\gamma R_{Activating}$ coupled with an increase in affinity to an $Fc\gamma R_{Inhibiting}$.

[0034] Current approaches to optimize the Fc region function (*e.g.*, antibody-dependent cell mediated cytotoxicity (ADCC), complement dependent cytotoxicity (CDC) activity) in therapeutic monoclonal antibodies and soluble polypeptides fused to Fc regions have focused on a limited number of single amino acid changes based on structural analysis and/or computer aided designs. Alternative approaches in engineering Fc regions have focused on the glycosylation of the Fc region to optimize Fc region function. The validity of using an Fc variant's Ratio of Affinities to assess its therapeutic potential has been suggested with respect to Fc variants whose sequences were derived using computer algorithms to search sequence-structure space (Lazar, G.A. *et al.* Proc. Natl. Acad. Sci. (USA) 103:4005-4010 (2006)). This approach identified four Fc variants: (1) S239D; (2) I322E; (3) S239D and I322E; and (4) S239D, I332E and A330L, all of which bound FcγRIIIa as well as FcγRIIb with greater affinity than wild-type (Lazar, G.A. *et al.* Proc. Natl. Acad. Sci. (USA) 103:4005-4010 (2006). In contrast, the present invention is based, in part, on selecting desired variant Fc-containing molecules that exhibit an altered Ratio of Affinities for FcγRIII and FcγRII, from an unbiased library of Fc variants. This approach enabled the identification of a larger universe of desired Fc variants, as well as variants having Ratios of Affinities far in excess of those reported by Lazar, G.A. *et al.* (Proc. Natl. Acad. Sci. (USA) 103:4005-4010 (2006)). The present invention encompasses methods for engineering Fc regions and identification and screening of novel Fc variants outside the expected regions identified by structural studies. Expected regions

as used herein refer to those regions that based on structural and/or biochemical studies are in contact with an Fc ligand.

[0035] The therapeutic or prophylactic molecules that are used in accordance with the methods of the invention thus comprise variant Fc regions comprising one or more amino acid modifications that exhibit an altered Ratio of Affinities, especially wherein the $Fc\gamma R_{Activating}$ is either FcγRIIA or FcγRIIIA and the $Fc\gamma R_{Inhibiting}$ is FcγRIIB. In a preferred embodiment, the molecules of the invention further specifically bind FcγRIIB (via the Fc region) with a lower affinity than a comparable molecule (*i.e.*, having the same amino acid sequence as the molecule of the invention except for the one or more amino acid modifications in the Fc region) comprising the wild-type Fc region binds FcγRIIB. 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 FcγRIIIA and/or FcγRIIA and enhance the affinity of the variant Fc region for FcγRIIB 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 FcγRIIIA and/or FcγRIIA but do not alter the affinity of the variant Fc regions for FcγRIIB relative to a comparable molecule with a wild type Fc region. A preferred embodiment is a variant Fc region that has enhanced affinity for FcγRIIIA and FcγRIIA but reduced affinity for FcγRIIB relative to a comparable molecule with a wild type Fc region.

[0036] The Fc variants of the present invention may be combined with other Fc modifications, including but not limited to modifications that alter effector function. The invention encompasses combining an Fc variant of the invention with other Fc modifications to provide additive, synergistic, or novel properties in antibodies or Fc fusions. Preferably, the Fc variants of the invention enhance the phenotype of the modification with which they are combined. For example, if an Fc variant of the invention is combined with a mutant known to bind FcγRIIIA with a higher affinity than a comparable molecule comprising a wild type Fc region; the combination with a mutant of the invention results in a greater fold enhancement in FcγRIIIA affinity.

[0037] In one embodiment, the Fc variants of the present invention may be combined with other known Fc variants such as those disclosed in Duncan et al, 1988, Nature 332:563-564; Lund et al., 1991, J. Immunol 147:2657-2662; Lund et al, 1992, Mol

Immunol 29:53-59; Alegre et al, 1994, Transplantation 57:1537-1543; Hutchins et al. , 1995, Proc Natl. Acad Sci U S A 92:11980-11984; Jefferis et al, 1995, Immunol Lett. 44:111-117; Lund et al., 1995, Faseb J 9:115-119; Jefferis et al, 1996, Immunol Lett 54:101-104; Lund et al, 1996, J Immunol 157:49634969; Armour et al, 1999, Eur J Immunol 29:2613-2624; Idusogie et al, 2000, J Immunol 164:41784184; Reddy et al, 2000, J Immunol 164:1925-1933; Xu et al., 2000, Cell Immunol 200:16-26; Idusogie et al, 2001, J Immunol 166:2571-2575; Shields et al., 2001, J Biol Chem 276:6591-6604; Shields et al, 2002, J Biol Chem 277:26733-26740; Jefferis et al, 2002, Immunol Lett 82:57-65; Presta et al., 2002, Biochem Soc Trans 30:487-490); Lazar , G.A. et al. Proc. Natl. Acad. Sci. (USA) 103:4005-4010 (2006); US 5,624,821; US 5,885,573; US 6,194,551; PCT WO 00/42072; PCT WO 99/58572; PCT WO 04/063351; U.S. Patent Application Publication 2005/0037000; and U.S. Patent Application Publication 2005/0064514; each of which is incorporated herein by reference in its entirety. In certain embodiments, the Fc variants of the present invention may be combined with one or more of the Fc variants, *i.e.*, amino acid modifications relative to a wild-type Fc region, presented in tables 4, 5, 9, and 10, *infra*.

[0038] 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 comprising variant Fc regions, one chain contains the wild-type Fc region and the other chain comprises one or more modifications. Methods of engineering heterodimeric Fc containing molecules are known in the art and encompassed within the invention.

[0039] 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 an $Fc\gamma R_{Activating}$ but does not bind an $Fc\gamma R_{Inhibiting}$ or binds an $Fc\gamma R_{Inhibiting}$ 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 an alternative 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 binds an

$Fc\gamma R_{Inhibiting}$, does not bind an $Fc\gamma R_{Activating}$ or binds an an $Fc\gamma R_{Activating}$ with 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 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 FcγR, wherein said FcγR is FcγIIIA. 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 FcγR, wherein said FcγR is FcγRIIA. 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 FcγR, wherein said FcγR is FcγRIIB.

[0040] The affinities and binding properties of the molecules of the invention for an FcγR are initially determined using *in vitro* assays (biochemical or immunological based assays) known in the art for determining Fc-FcγR interactions, *i.e.*, specific binding of an Fc region to an FcγR including but not limited to ELISA assay, surface plasmon resonance assay, immunoprecipitation assays (*See* Section 6.2). Preferably, the binding properties of the molecules of the invention are also characterized by in vitro functional assays for determining one or more FcγR mediator effector cell functions (*See* Section 6.2.2). 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.

[0041] 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 and exhibits a Ratio of Affinities greater than 1, provided that said variant Fc region does not solely have a substitution at any one of positions 329, 331, or 332, or does not include, or is not solely a substitution of, any one of: (1) alanine at any of positions 256, 290, 298, 312, 333, 334, 359, 360, 326, or 430; (2) a lysine at position 330; (3) a threonine at position 339; (4) a methionine at position 320; (5) a serine at position 326; (6) an asparagine at position 326; (7) an

aspartic acid at position 326; (8) a glutamic acid at position 326; (9) a glutamine at position 334; (10) a glutamic acid at position 334; (11) a methionine at position 334; (12) a histidine at position 334; (13) a valine at position 334; (14) a leucine at position 334; (15) a lysine at position 335, or (16) solelely a glutamic acid at position 332; (17) solely a glutamic acid at position 332 and an aspartic acid at position 239; (18) solely a glutamic acid at position 332, an aspartic acid at position 239, and a leucine at position 330.

[0042] The invention particularly concerns a molecule having such a variant Fc region, wherein said variant Fc region is additionally characterized in possessing at least one amino acid modification relative to a wild-type Fc region at position 234, 235, 243, 247, 255, 270, 284, 292, 300, 305, 316, 370, 392, 396, 416, 419 and/or 421.

[0043] The invention further concerns such molecules wherein the variant Fc regions are additionally characterized in possessing substitutions at at least the two positions: (a) 235 and 243; (b) 243 and 292; (c) 243 and 300; (d) 243 and 305; (e) 243 and 396; (f) 247 and 270; (g) 247 and 421; (h) 255 and 270; (i) 255 and 396; (j) 270 and 316; (k) 270 and 396; (l) 270 and 416; (m) 270 and 421; (n) 292 and 300; (o) 292 and 305; (p) 292 and 396; (q) 300 and 396; (r) 305 and 396; (s) 316 and 416; (t) 392 and 270; (u) 392 and 396; (v) 419 and 270; or (w) 419 and 396.

[0044] The invention further concerns such molecules wherein the variant Fc regions are additionally characterized in possessing substitutions at at least the three positions: ((a) 243, 247 and 421; (b) 243, 292 and 300; (c) 243, 292 and 305; (d) 243, 292 and 396; (e) 243 300 and 396; (f) 243, 305 and 396; (g) 247, 270 and 421; (h) 255, 270 and 396; (i) 270, 316 and 416; (j) 270, 392 and 396; (k) 270, 396 and 419; (l) 292 300 and 396; or (m) 292, 305 and 396.

[0045] The invention further concerns the embodiments of such antibodies wherein the at least one modification in the Fc domain comprises a substitution of L234 or a substitution of L235, or substitutions of both L234 and L235, and particularly, wherein the substitution of L234 is L234F and said substitution at L235 is L235V.

[0046] The invention additionally concerns the above-described methods wherein the variant Fc region possesses at least amino acid modifications relative to a wild-type Fc region at one or more of positions: 243, 292, 300 or 396 (*i.e.*, at position 243, 292, 300 or 396; or at both position 243 and at position 292; or at both position 243 and at position 300; or at both position 243 and at position 396; or at both position 292 and at position 300; or at both position 292 and at position 396; or at both position 300 and at

position 396; or at position 243, at position 292 and at position 300; or at position 243, at position 292 and at position 396; or at position 292, at position 300 and at position 396; or at position 243, at position 292, at position 300 and at position 396). The invention particularly concerns concerns the embodiment of the above-described methods wherein such variant Fc region possess the modifications: L234F, L235I, F243L, R292P and/or Y300L.

[0047] The invention further concerns such molecules wherein the variant Fc regions exhibit a Ratio of Affinities greater than 1, and wherein such variant Fc regions have at least any of the following substitutions: (a) F243L; (b) D270E; (c) R292G; or (d) R292P.

[0048] The invention further concerns such molecules wherein the variant Fc regions exhibit a Ratio of Affinities greater than 1, and wherein such variant Fc regions have at least any of the following pairs of substitutions: (a) F243L and R292P; (b) F243L and Y300L; (c) F243L and P396L; (d) D270E and P396L; (e) R292P and Y300L; (f) R292P and V305I; (g) R292P and P396L; (h) Y300L and P396L; and (i) P396L and Q419H.

[0049] The invention further concerns such molecules wherein the variant Fc regions exhibit a Ratio of Affinities greater than 1, and wherein such variant Fc regions have at least any of the following trios of substitutions: (a) F243L, P247L and N421K; (b) F243L, R292P andY300L; (c) F243L, R292P and Y300L; (d) F243L, R292P and V305I; (e) F243L, R292P and P396L; (f) F243L, Y300L and P396L; (g) P247L, D270E and N421K; (h) R255L, D270E and P396L; (i) D270E, G316D and R416G; (j) D270E, K392T and P396L; (k) D270E, P396L and Q419H; (l) V284M, R292L and K370N or (m) R292P, Y300L and P396L.

[0050] The invention further concerns such molecules wherein the variant Fc regions exhibit a Ratio of Affinities greater than 1, and wherein such variant Fc regions have at least any of the following tetrads of substitutions: (a) L234F, F243L, R292P and Y300L; (b) L235I, F243L, R292P and Y300L; (c) L235Q, F243L, R292P and Y300L; (d) F243L, R292P, Y300L, and P396L; (e) F243L, P247L, D270E and N421K; (f) F243L, R255L, D270E and P396L; (g) F243L, D270E, G316D and R416G; (h) F243L, D270E, K392T and P396L; (i) F243L, D270E, P396L and Q419H; (j) F243L, R292P, V305I and P396L; (k) D270E, G316D, P396L and R416G; (l) P247L, D270E, Y300L and N421K; (m) R255L, D270E, R292G and P396L; or (n) R255L, D270E, Y300L and P396L.

[0051] The invention further concerns such molecules wherein the variant Fc regions exhibit a Ratio of Affinities greater than 1, and wherein such variant Fc regions have at least any of the following pentads of substitutions: (a) L235V, F243L, R292P, Y300L and P396L; (b) L235P, F243L, R292P, Y300L and P396L; (c) F243L, R292P, V305I, Y300L and P396L; or (d) F243L, R292P, Y300L, V305I and P396L.

[0052] The invention further concerns such molecules wherein the variant Fc regions exhibit a Ratio of Affinities less than 1, and wherein such variant Fc regions have at least any of the following substitutions: (a) P396L or (b) Y300L.

[0053] The invention further concerns such molecules wherein the variant Fc regions exhibit a Ratio of Affinities less than 1, and wherein such variant Fc regions have at least any of the following pairs of substitutions: (a) F243L and P396L; (b) P247L and N421K; (c) R255L and P396L; (d) R292P and V305I; (e) K392T and P396L; or (f) P396L and Q419H.

[0054] The invention further concerns such molecules wherein the variant Fc regions exhibit a Ratio of Affinities less than 1, and wherein such variant Fc regions have at least the following three substitutions: F243L, R292P and V305I.

[0055] 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.

[0056] 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, which is incorporated herein by reference in its entirety). 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.

[0057] 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 (via its Fc region) 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 (via its Fc region) with an altered affinity relative to a molecule comprising a wild-type Fc region, provided 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 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 at position 335; or a methionine at position 301.

[0058] 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 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, 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 (via its Fc region) 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 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 relative to a wild-type Fc region, such that said molecule binds an FcγR (via its Fc region) 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.

[0059] In a specific embodiment, the invention encompasses a molecule comprising a variant Fc region, wherein said variant Fc region does not include a substitution or does not solely have a substitution at any of positions 330, 243, 247, 298, 241, 240, 244, 263, 262, 235, 269, or 328 and does not have a leucine at position 243, an asparagine at position 298, a leucine at position 241, and isoleucine or an alanine at position 240, a histidine at position 244, a valine at position 330, or an isoleucine at position 328.

[0060] 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 FcγRIIIA and/or FcγRIIA 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 more amino acid modifications (*e.g.*, substitutions), which modifications increase the affinity of the variant Fc region for FcγRIIIA and/or FcγRIIA 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 FcγRIIIA and/or FcγRIIA 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.

[0061] The invention encompasses molecules with altered affinities for the activating and/or inhibitory Fcγ receptors. In particular, the invention contemplates molecules with variant Fc regions, having one or more amino acid modifications, which modifications increase the affinity of the variant Fc region for FcγRIIB but decrease the affinity of the variant Fc region for FcγRIIIA and/or FcγRIIA, 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 decrease the affinity of the variant Fc region for FcγRIIB and also decrease the affinity of the variant Fc regions for FcγRIIIA and/or FcγRIIA relative to a comparable molecule with a wild-type Fc region. In yet 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 FcγRIIB and also increase the affinity of the variant Fc regions for FcγRIIIA and/or FcγRIIA 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 FcγRIIIA and/or FcγRIIA but do not alter the affinity of the variant Fc region for FcγRIIB 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 increase the affinity of the variant Fc region for FcγRIIIA and/or FcγRIIA but reduce the affinity of the variant Fc region for FcγRIIB relative to a comparable molecule with a wild-type Fc region.

[0062] 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 FcγRIIIA and decrease the affinity of the variant Fc region for FcγRIIB, relative to a comparable molecule comprising a wild-type Fc region which binds FcγRIIIA and FcγRIIB 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.

[0063] 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 FcγRIIA and decrease the affinity of the variant Fc region for FcγRIIB, relative to a comparable molecule comprising a wild-type Fc region which binds FcγRIIA and FcγRIIB with wild-type affinity. In a certain embodiment, the one or more amino acid modifications is not a substitution with arginine at position 320.

[0064] 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); 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); or a substitution at position 243 with leucine, at position 292 with proline, at position 300 with leucine, at position 305 with isoleucine, and at position 396 with leucine (MgFc88); or a substitution at position 243 with leucine, at position 292 with proline, at position 300 with leucine, and at position 396 with leucine (MgFc88A); or a substitution at position 243 with leucine, at position 292 with proline, and at position 300 with leucine (MgFc155); or a substitution at position 243 with leucine, at position 292 with proline, and at position 300 with leucine; or a substitution at position 243 with leucine, at position 292 with proline, and at position 396 with leucine; or a substitution at position 243 with leucine, and at position 292 with proline; or a substitution at position 243 with leucine; or a substitution at position 273 with phenylalanine; or a substitution at position 247 with leucine, at position 270 with glutamic acid, and at position 421 with lysine. In a related embodiment, the variant Fc region further comprises one or more amino acid modifications disclosed in tables 4, 5, 9, and 10 *infra*.

[0065] In certain embodiments, the invention encompasses methods for screening and identifying therapeutic and/or prophylactic molecules comprising variant Fc regions with altered FcγR affinities (*e.g.*, enhanced FcγRIIIA affinity) using yeast surface display technology (for review *see* Boder and Wittrup, 2000, *Methods in Enzymology*, 328: 430-444, which is incorporated herein by reference in its entirety). 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 described herein (*see, e.g.,* U.S Patent application Publications 2005/0037000 and 2005/0064514, and International Patent Application Publication WO 04/063351, each of which is hereby incorporated by reference in its entirety). Yeast display offers the advantage of utilizing actual binding to a desired receptor to identify variant Fc regions that have enhanced binding to that receptor.

[0066] In certain embodiments, the invention encompasses methods for screening and identifying therapeutic and/or prophylactic molecules comprising variant Fc regions with altered FcγR affinities (*e.g.*, enhanced FcγRIIIA affinity) using yeast display technology known in the art or 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 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 one or more FcγR mediated effector cell function such as those described herein in Section 6.2.2. 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 or known in the art 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.

[0067] In preferred embodiments, the invention encompasses methods for screening and characterizing FcγR-Fc interaction using biochemical assays developed by the inventors and disclosed in U.S Patent application Publications 2005/0037000 and 2005/0064514, and International Patent Application Publication WO 04/063351, each of which is hereby incorporated by reference in its entirety. The disclosed assays allow detection and quantitation of the FcγR-Fc interaction, despite the inherently weak affinity of the receptor for its ligand, *e.g.*, in the micromolar range for FcγRIIB and FcγRIIIA. The method involves the formation of an FcγR complex (*e.g.*, FcγRIIIA, FcγRIIB) that has an improved avidity for an Fc region, relative to an uncomplexed FcγR.

[0068] 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.

[0069] In preferred embodiments, molecules of the invention (*e.g.*, immunoglogulins or fragments thereof) comprising the variant Fc regions are further characterized in an

animal model for interaction with an FcγR or in an animal model of disease state. Preferred animal models for use in the methods of the invention are, for example, transgenic mice expressing human FcγRs, *e.g.*, any mouse model described in U.S. Patent No. 5,877,397, and 6,676,927 which are incorporated herein by reference in their entirety. Transgenic mice for use in the methods of the invention include, but are not limited to, knockout FcγRIIIA mice carrying human FcγRIIIA; knockout FcγRIIIA mice carrying human FcγRIIA; knockout FcγRIIIAmice carrying human FcγRIIB and human FcγRIIIA; knockout FcγRIIIA mice carrying human FcγRIIB and human FcγRIIA; knockout FcγRIIIA and FcγRIIA mice carrying human FcγRIIIA and FcγRIIA; and knockout FcγRIIIA, FcγRIIA and FcγRIIB mice carrying human FcγRIIIA, FcγRIIA and FcγRIIB. The mouse strain used for knockout studies may be any suitable inbred strain (*e.g.*, B6) as determined routinely in the art. In preferred embodiments, the mouse strain is that of a nude genotype, *i.e.*, immune compromised, to allow xenograft studies (*e.g.*, cancer models). Such nude strains include, but are not limited to FoxN1 and N/N. In other embodiments the mice carrying one or more human FcγRs further comprise one or more additional genetic mutations including one or more knockouts, *e.g.* RAG1 -/-.

[0070] In a specific embodiment, the invention provides modified immunoglobulins comprising a variant Fc region with an enhanced affinity for FcγRIIIA and/or FcγRIIA. Such immunoglobulins include IgG molecules that naturally contain FcγR binding regions (*e.g.*, FcγRIIIA and/or FcγRIIB binding regions), or immunoglobulin derivatives that have been engineered to contain an FcγR binding region (*e.g.*, FcγRIIIA and/or FcγRIIB 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 FcγR binding region (*e.g.*, a FcγRIIIA and/or FcγRIIB 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 FcγR binding region.

[0071] In certain embodiment, the invention encompasses immunoglobulins comprising a variant Fc region with an enhanced affinity for FcγRIIIA and/or FcγRIIA 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 FcγRIIIA and/or FcγRIIA 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.

[0072] 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 FcγR activating receptor and/or an FcγR 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 FcγRIIIA and/or FcγRIIA. 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 FcγRIIIA and/or FcγRIIA and further decreases the affinity of the Fc region for FcγRIIB. 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.

[0073] In a specific embodiment, the invention encompasses engineering a monoclonal antibody specific for Her2/neu protooncogene (amino acid sequence **SEQ ID NO:31**) (*e.g.*, 4D5 antibody as disclosed in Carter *et al.*, 1992, *Proc. Natl. Acad. Sci. USA* 89:4285-9; U.S. Patent No. 5,677,171; or International Patent Application Publication WO 01/00245, each of which is hereby incorporated by references in its entirety) by 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. In another specific embodiment, modification of the humanized Her2/neu monoclonal antibody may also further decrease the affinity of the Fc region

for FcγRIIB. 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. In a certain embodiment, the 4D5 antibody is chimeric. In another embodiment, the 4D5 antibody is humanized. In a specific embodiment, the 4D5 antibody to be engineered in accordance with the methods of the invention comprises a heavy chain having the amino acid sequence **SEQ ID NO:32**. In another specific embodiment, the 4D5 antibody to be engineered in accordance with the methods of the invention comprises a light chain having the amino acid sequence **SEQ ID NO:33**. In still other embodiments, the 4D5 antibody to be engineered in accordance with the methods of the invention is humanized and comprises a heavy chain having the amino sequence **SEQ ID NO:34**. In further embodiments, the 4D5 antibody to engineered in accordance with the methods of the invention is humanized and comprises a light chain having the amino sequence **SEQ ID NO:35**.

[0074] In a specific embodiment, the antibodies of the invention bind Her2/neu. The anti-Her2/neu antibodies of the invention may have a heavy chain variable region comprising the amino acid sequence of CDR1 (**SEQ ID NO:36**) and/or CDR2 (**SEQ ID NO:37**) and/or CDR3 (**SEQ ID NO:38**) and/or a light chain variable region comprising the amino acid sequence of CDR1 (**SEQ ID NO:39**) and/or a CDR2 (**SEQ ID NO:40**) and/or CDR3 (**SEQ ID NO:41**).

[0075] In a specific embodiment, the invention encompasses a 4D5 antibody (*e.g.*, chimeric, humanized) comprising a variant Fc region having a substitution at position 243 with leucine, at position 292 with proline, at position 300 with leucine, at position 305 with isoleucine, and at position 396 with leucine. In another specific embodiment, the invention encompasses a 4D5 antibody having a leucine at position 243, a proline at position 292, a leucine at position 300, an isoleucine at position 305, and a leucine at position 396. In other embodiments, the invention encompasses a 4D5 antibody comprising a variant Fc region having a substitution at position 243 with leucine, at position 292 with proline, and at position 300 with leucine. In other embodiments, the invention encompasses a 4D5 antibody having a leucine at position 243, a proline at position 292, and a leucine at position 300. In other embodiments, the invention encompasses a 4D5 antibody comprising a variant Fc region having a substitution at position 247 with leucine, at position 270 with glutamic acid, and at position 421 with

lysine. In another embodiment, the invention encompasses a 4D5 antibody having a leucine at position 247, a glutamic acid at position 292, and a lysine at position 421.

[0076] In another specific embodiment, the invention encompasses engineering an anti-CD20 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 FcγRIIIA and/or FcγRIIA. In a related embodiment, the anti-CD20 antibody is mouse human chimeric anti-CD20 monoclonal antibody, 2H7 Further nonlimiting examples of anti-CD20 antibodies that can be used in the methods of the invention are disclosed in U.S. Patent Application No.: 11/271,140, filed November 10, 2005, hereby incorporated by reference in its entirety. In another specific embodiment, modification of the anti-CD20 monoclonal antibody, 2H7 may also further decrease the affinity of the Fc region for FcγRIIB. 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.

[0077] In a specific embodiment, the invention encompasses a 2H7 antibody (*e.g.*, chimeric, humanized) comprising a variant Fc region having a substitution at position 243 with leucine, at position 292 with proline, at position 300 with leucine, at position 305 with isoleucine, and at position 396 with leucine. In another specific embodiment, the invention encompasses a 2H7 antibody having a leucine at position 243, a proline at position 292, a leucine at position 300, an isoleucine at position 305, and a leucine at position 396. In other embodiments, the invention encompasses a 4D5 antibody comprising a variant Fc region having a substitution at position 243 with leucine, at position 292 with proline, and at position 300 with leucine. In other embodiments, the invention encompasses a 2H7 antibody having a leucine at position 243, a proline at position 292, and a leucine at position 300. In other embodiments, the invention encompasses a 2H7 antibody comprising a variant Fc region having a substitution at position 247 with leucine, at position 270 with glutamic acid, and at position 421 with lysine. In another embodiment, the invention encompasses a 2H7 antibody having a leucine at position 247, a glutamic acid at position 292, and a lysine at position 421.

[0078] In another specific embodiment, the invention encompasses engineering an anti-FcγRIIB antibody, in particular an anti-FcγRIIB antibody that specifically binds human FcγRIIB, more particularly native human FcγRIIB, by 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. Non-limiting examples of representative anti-FcγRIIB antibodies are disclosed in U.S. Provisional Application No. 60/403,266 filed on August 12, 2002; U.S. Application No. 10/643,857 filed on August 14, 2003; and U.S Patent Application Publication Numbers: 2004-0185045; 2005-0260213; and 2006-0013810, all of which are hereby incorporated by reference in their entireties. Examples of anti-FcγRIIB antibodies that may be engineered in accordance with the methods of the invention are the monoclonal antibodies produced by clone 2B6, 3H7, 8B5.4.3, 1D5, 2E1, 2H9, 2D11, 8B5 and 1F2 having ATCC accession numbers PTA-4591, PTA-4592, PTA-7610, PTA-5958, PTA-5961, PTA-5962, PTA-5960, PTA-7610 and PTA-5959, respectively (deposited at ATCC, 10801 University Boulevard, Manassas, VA 02209-2011, all of which are incorporated herein by reference), or chimeric, humanized or other engineered versions thereof.

[0079] In a specific embodiment, the invention encompasses engineering a humanized antibody comprising the heavy chain variable domain and/or light chain variable domain of 2B6, 3H7 or 8B5.3.4. In another specific embodiment, the invention encompasses engineering a humanized antibody comprising the CDRs of 2B6, 3H7 or 8B5.3.4. In a specific embodiment, the invention encompasses engineering a humanized antibody comprising the heavy chain variable domain having the amino acid sequence of SEQ ID NO: 1, **SEQ ID NO:2** or **SEQ ID NO:3** and the light chain variable domain having the amino acid sequence of SEQ ID NO: 4, **SEQ ID NO:5**, **SEQ ID NO:6**, **SEQ ID NO:7** or SEQ ID NO: 8. In a specific embodiment, the invention encompasses engineering a humanized antibody comprising the heavy chain variable domain having the amino acid sequence of **SEQ ID NO:9** and the light chain variable domain having the amino acid sequence of **SEQ ID NO:10**.

[0080] In a specific embodiment, the invention encompasses engineering a humanized 2B6 antibody comprising a heavy chain having the amino acid sequence **SEQ ID NO:42**. In another specific embodiment, the invention encompasses engineering a humanized 2B6 antibody comprising a heavy chain having the amino acid sequence **SEQ ID NO:29**. In still other embodiments, the invention encompasses engineering a humanized 2B6 antibody comprising a light chain having the amino acid sequence **SEQ ID NO:30**. In a preferred embodiment, the invention encompasses engineering a humanized 2B6 antibody comprising a heavy chain containing the amino

acid sequence **SEQ ID NO:29** and a light chain containing the sequence **SEQ ID NO:30**. In a specific aspect of the invention, the invention encompasses the use of plasmid pMGx0675, which includes the nucleotide sequences **SEQ ID NO:43** and **SEQ ID NO:44** that encode the heavy chain amino acid sequence **SEQ ID NO:29** and the light chain amino acid sequence **SEQ ID NO:30**, respectively. Plasmid pMGx0675 been deposited with the American Type Culture Collection (10801 University Blvd., Manassas, VA. 20110-2209) on May 23, 2006 under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedures, and assigned accession number PTA 7609, and is incorporated herein by reference.

[0081] In specific embodiments, the invention encompasses engineering antibodies, preferably humanized, that bind the extracellular domain of native human FcγRIIB. The humanized anti- FcγRIIB antibodies encompassed by the invention may have a heavy chain variable region comprising the amino acid sequence of CDR1 (**SEQ ID NO:15**, **SEQ ID NO:16**, an amino acid sequence corresponding to amino acids 31-35 as set forth in **SEQ ID NO:2**, or an amino acid sequence corresponding to amino acids 31-35 as set forth in **SEQ ID NO:3**) and/or CDR2 (**SEQ ID NO:17**, **SEQ ID NO:18**, an amino acid sequence corresponding to amino acids 50-66 as set forth in **SEQ ID NO:2**, or an amino acid sequence corresponding to amino acids 50-66 as set forth in **SEQ ID NO:3**) and/or CDR3 (**SEQ ID NO:19**, **SEQ ID NO:20**, an amino acid sequence corresponding to amino acids 100-111 as set forth in **SEQ ID NO:2**, or an amino acid sequence corresponding to amino acids 100-111 as set forth in **SEQ ID NO:3**) and/or a light chain variable region comprising the amino acid sequence of CDR1 (**SEQ ID NO:21**, **SEQ ID NO:22**, or an amino acid sequence corresponding to amino acids 24-34 as set forth in **SEQ ID NO:8**) and/or a CDR2 (**SEQ ID NO:23**, **SEQ ID NO:24**, **SEQ ID NO:25**, **SEQ ID NO:26**, or an amino acid sequence corresponding to amino acids 50-56 as set forth in **SEQ ID NO:62**) and/or CDR3 (**SEQ ID NO:27**, **SEQ ID NO:28**, or an amino acid sequence corresponding to amino acids 90-98 as set forth in **SEQ ID NO:8**).

[0082] In a specific embodiment, the invention encompasses a 2B6 antibody comprising a variant Fc region having a substitution at position 243 with leucine, at position 292 with proline, at position 300 with leucine, at position 305 with isoleucine, and at position 396 with leucine. In another specific embodiment, the invention encompasses a 2B6 antibody having a leucine at position 243, a proline at position 292,

a leucine at position 300, an isoleucine at position 305, and a leucine at position 396. In other embodiments, the invention encompasses a 2B6 antibody comprising a variant Fc region having a substitution at position 243 with leucine, at position 292 with proline, and at position 300 with leucine. In other embodiments, the invention encompasses a 2B6 antibody having a leucine at position 243, a proline at position 292, and a leucine at position 300. In other embodiments, the invention encompasses a 2B6 antibody comprising a variant Fc region having a substitution at position 247 with leucine, at position 270 with glutamic acid, and at position 421 with lysine. In another embodiment, the invention encompasses a 2B6 antibody having a leucine at position 247, a glutamic acid at position 292, and a lysine at position 421.

[0083] In a specific embodiment, modification of the anti-FcγRIIB antibody may also decrease the affinity of the Fc region for FcγRIIB relative to the wild-type antibody. 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 anti-FcγRIIB 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) ; or a substitution at position 243 with leucine, at position 292 with proline, at position 300 with leucine, at position 305 with isoleucine, and at position 396 with leucine (MgFc88); or a substitution at position 243 with leucine, at position 292 with proline, at position 300 with leucine, and at position 396 with leucine

(MgFc88A); or a substitution at position 234 with leucine, at position 292 with proline, and at position 300 with leucine (MgFc155); or a substitution at position 243 with leucine, at position 292 with proline, and at position 300 with leucine; or a substitution at position 243 with leucine, at position 292 with proline, and at position 396 with leucine; or a substitution at position 243 with leucine, and at position 292 with proline; or a substitution at position 243 with leucine; or a substitution at position 273 with phenylalanine; or a substitution at position 247 with leucine, at position 270 with glutamic acid, and at position 421 with lysine. In a related embodiment, the variant Fc region further comprises one or more amino acid modifications disclosed in tables 4, 5, 9, and 10, *infra*.

[0084] In a specific embodiments, the invention encompasses an antibody that binds to CD79a or CD79b (*e.g.*, chimeric, humanized) comprising a variant Fc region and use of the antibodies for treatment of cancer. In specific embodiments, the antibody that binds to CD79b or CD79b has a substitution at position 243 with leucine, at position 292 with proline, at position 300 with leucine, at position 305 with isoleucine, and at position 396 with leucine. In another specific embodiment, the invention encompasses an antibody that binds to CD79a or CD79b having a leucine at position 243, a proline at position 292, a leucine at position 300, an isoleucine at position 305, and a leucine at position 396. In other embodiments, the invention an antibody that binds to CD79a or CD79b receptor comprising a variant Fc region having a substitution at position 243 with leucine, at position 292 with proline, and at position 300 with leucine. In other embodiments, the invention encompasses an antibody that binds to CD79a or CD79b having a leucine at position 243, a proline at position 292, and a leucine at position 300. In other embodiments, the invention encompasses an antibody that binds to CD79a or CD79b comprising a variant Fc region having a substitution at position 247 with leucine, at position 270 with glutamic acid, and at position 421 with lysine. In another embodiment, the invention encompasses an antibody that binds to CD79a or CD79b having a leucine at position 247, a glutamic acid at position 292, and a lysine at position 421.

[0085] In a specific embodiments, the invention encompasses an antibody that binds to ErbB1 (*e.g.*, chimeric, humanized) comprising a variant Fc region and use of the antibodies for treatment of cancer. In specific embodiments, the antibody that binds to ErbB1 has a substitution at position 243 with leucine, at position 292 with proline, at position 300 with leucine, at position 305 with isoleucine, and at position 396 with

leucine. In another specific embodiment, the invention encompasses an antibody that binds to ErbB1 having a leucine at position 243, a proline at position 292, a leucine at position 300, an isoleucine at position 305, and a leucine at position 396. In other embodiments, the invention an antibody that binds to ErbB1 receptor comprising a variant Fc region having a substitution at position 243 with leucine, at position 292 with proline, and at position 300 with leucine. In other embodiments, the invention encompasses an antibody that binds to ErbB1 having a leucine at position 243, a proline at position 292, and a leucine at position 300. In other embodiments, the invention encompasses an antibody that binds to ErbB1 comprising a variant Fc region having a substitution at position 247 with leucine, at position 270 with glutamic acid, and at position 421 with lysine. In another embodiment, the invention encompasses an antibody that binds to ErbB1 having a leucine at position 247, a glutamic acid at position 292, and a lysine at position 421.

[0086] In a specific embodiments, the invention encompasses an antibody that binds to A33, CD5, CD11c, CD19, CD22, CD23, CD27, CD40, CD45, CD103, CTLA4, ErbB3, ErbB4, VEGF receptor, TNF-a receptor, TNF-ß receptor, or TNF-γ receptor (*e.g.*, chimeric, humanized) comprising a variant Fc region and use of the antibodies for treatment of cancer. In specific embodiments, the antibody that binds to A33, CD5, CD11c, CD19, CD22, CD23, CD27, CD40, CD45, CD103, CTLA4, ErbB3, ErbB4, VEGF receptor, TNF-a receptor, TNF-ß receptor, or TNF-γ receptor has a substitution at position 243 with leucine, at position 292 with proline, at position 300 with leucine, at position 305 with isoleucine, and at position 396 with leucine. In another specific embodiment, the invention encompasses an antibody that binds to A33, CD5, CD11c, CD19, CD22, CD23, CD27, CD40, CD45, CD103, CTLA4, ErbB3, ErbB4, VEGF receptor, TNF-a receptor, TNF-ß receptor, or TNF-γ receptor having a leucine at position 243, a proline at position 292, a leucine at position 300, an isoleucine at position 305, and a leucine at position 396. In other embodiments, the invention an antibody that binds to A33, CD5, CD11c, CD19, CD22, CD23, CD27, CD40, CD45, CD103, CTLA4, ErbB3, ErbB4, VEGF receptor, TNF-a receptor, TNF-ß receptor, or TNF-γ receptor comprising a variant Fc region having a substitution at position 243 with leucine, at position 292 with proline, and at position 300 with leucine. In other embodiments, the invention encompasses an antibody that binds to A33, CD5, CD11c, CD19, CD22, CD23, CD27, CD40, CD45, CD103, CTLA4, ErbB3, ErbB4, VEGF receptor, TNF-a receptor, TNF-ß receptor, or TNF-γ receptor having a leucine at

position 243, a proline at position 292, and a leucine at position 300. In other embodiments, the invention encompasses an antibody that binds to A33, CD5, CD11c, CD19, CD22, CD23, CD27, CD40, CD45, CD103, CTLA4, ErbB3, ErbB4, VEGF receptor, TNF-a receptor, TNF-ß receptor, or TNF-γ receptor comprising a variant Fc region having a substitution at position 247 with leucine, at position 270 with glutamic acid, and at position 421 with lysine. In another embodiment, the invention encompasses an antibody that binds to A33, CD5, CD11c, CD19, CD22, CD23, CD27, CD40, CD45, CD103, CTLA4, ErbB3, ErbB4, VEGF receptor, TNF-a receptor, TNF-ß receptor, or TNF-γ receptor having a leucine at position 247, a glutamic acid at position 292, and a lysine at position 421.

[0087] 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 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.

[0088] The invention further provides methods for the production of the molecules 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 suitable for the expression of said molecule; and (ii) recovery of said molecule from said medium.

[0089] 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 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.

[0090] In one embodiment, the invention encompasses a method of 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 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 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 FcγRIIIA via its Fc region with a greater affinity than the therapeutic antibody comprising the wild-type Fc region binds FcγRIIIA, 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 FcγRIIIA via its Fc region with a greater affinity than a therapeutic antibody comprising the wild-type Fc region binds FcγRIIIA, and said therapeutic antibody further specifically binds FcγRIIB with a lower affinity than a therapeutic antibody comprising the wild-type Fc region binds FcγRIIB, 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.

[0091] 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 FcγRIIB via its Fc region with a greater affinity than a comparable molecule comprising the wild type Fc region, and said molecule further specifically binds FcγRIIIA via its Fc region 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.

[0092] 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, protozae, and viruses.

[0093] 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.

[0094] 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.

[0095] 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.

[0095a] In one embodiment, there is provided a method of attenuating the post-translational fucosylation of a molecule comprising a modified Fc region, comprising:

(A) modifying an IgG Fc region to comprise an amino acid substitution relative to a wild-type Fc region to yield a modified Fc region;

(B) expressing a candidate molecule comprising said modified Fc region in a host cell capable of mediating normal glycosylation;

(C) assessing a ratio of high mannose oligosaccharide glycosylation to complex oligosaccharide glycosylation exhibited by said modified Fc region;

(D) identifying said candidate molecule comprising said modified Fc region as a suitable molecule if said modified Fc region exhibits said ratio that is greater than 0.2; and

(E) expressing said suitable molecule possessing said modified Fc region in said host cell.

4.1 DEFINITIONS

[0096] 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 (also referred to as "Cγ2" domain) usually extends from amino acid 231 to amino acid 338. The CH3 domain of a human IgG Fc region usually extends from amino acids 342 to 447. 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.

[0097] 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), expressly incorporated herein by references. The "EU index as in Kabat" refers to the numbering of the human IgG1 EU antibody.

[0098] 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 bonds in the same positions.

[0099] 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.*, IgG_1, IgG_2, IgG_3, IgG_4, IgA_1 and IgA_2) or subclass.

[00100] As used herein, the term "variant Fc region" is intended to denote an Fc region that has been modified, by substitution, insertion or deletion of one or more amino acid residues relative to the Fc region of the unmodified molecule (i.e., the "wild-type" immunoglobulin). The present invention relates to molecules that possess Fc regions having an altered Ratio of Affinities for an FcγR that activates a cellular effector function (i.e., "an $Fc\gamma R_{Activating}$," such as FcγRIIA or FcγRIIIA) relative to an FcγR that inhibits a cellular effector function (i.e., "an $Fc\gamma R_{Inhibiting}$," such as FcγRIIB):

$$\text{Ratio of Affinities} = \frac{\text{Wild-Type to Variant Change in Affinity to } Fc\gamma R_{Activating}}{\text{Wild-Type to Variant Change in Affinity to } Fc\gamma R_{Inhibiting}}$$

[00101] Thus, for any particular molecule having a variant Fc region, the molecule's Ratio of Affinities is determined by calculating the difference in affinity of the variant Fc region of the molecule to an $Fc\gamma R_{Activating}$ (for example, to FcγRIIA or to FcγRIIIA), relative to the affinity of a wild-type immunoglobulin to such $Fc\gamma R_{Activating}$ (e.g., $Affinity_{Fc\gamma RIIIA}$ of the variant Fc region - $Affinity_{Fc\gamma RIIIA}$ of the wild-type immunoglobulin), and dividing such difference by the difference in affinity to an $Fc\gamma R_{Inhibiting}$ (for example, FcγRIIB) of the variant Fc region of the molecule, relative to the affinity of a wild-type immunoglobulin to such $Fc\gamma R_{Inhibiting}$ (e.g., $Affinity_{Fc\gamma RIIB}$ of the variant Fc region - $Affinity_{Fc\gamma RIIB}$ of the wild-type immunoglobulin). An increased Ratio of Affinities may result from the Fc region of the molecule having (relative to a wild type Fc) an increase in affinity to an $Fc\gamma R_{Activating}$ (for example, to FcγRIIA or to FcγRIIIA) coupled with either an unchanged affinity to an $Fc\gamma R_{Inhibiting}$ (for example, FcγRIIB) or a decrease in affinity to such $Fc\gamma R_{Inhibiting}$. Alternatively, an increased Ratio of Affinities may result from the Fc region of such molecule exhibiting an increase in affinity to both an $Fc\gamma R_{Activating}$ and an $Fc\gamma R_{Inhibiting}$ (relative to a wild-type Fc), provided that the increase in affinity to the $Fc\gamma R_{Activating}$ exceeds the increase in affinity to the $Fc\gamma R_{Inhibiting}$ (i.e., binding to the $Fc\gamma R_{Activating}$ is "greatly" increased compared to binding to the $Fc\gamma R_{Inhibiting}$, which is merely increased), or may result from

the Fc region of such molecule exhibiting a decreased affinity to both an $Fc\gamma R_{Activating}$ and an $Fc\gamma R_{Inhibiting}$ (relative to a wild-type Fc), provided that the decrease in affinity to the $Fc\gamma R_{Activating}$ is less than the decrease in affinity to an $Fc\gamma R_{Inhibiting}$ (i.e., binding to the $Fc\gamma R_{Inhibiting}$ is "greatly" decreased compared to binding to the $Fc\gamma R_{Activating}$, which is merely decreased), or may result from an unchanged affinity to an $Fc\gamma R_{Activating}$ coupled with a decrease in affinity to an $Fc\gamma R_{Inhibiting}$. A decreased Ratio of Affinities may result from the Fc region of the molecule having (relative to a wild type Fc) a decrease in affinity to an $Fc\gamma R_{Activating}$ coupled with either an unchanged affinity to an $Fc\gamma R_{Inhibiting}$ or an increase in affinity to an $Fc\gamma R_{Inhibiting}$. Alternatively, a decreased Ratio of Affinities may result from the Fc region of such molecule exhibiting a decrease in affinity to both an $Fc\gamma R_{Activating}$ and an $Fc\gamma R_{Inhibiting}$ (relative to a wild-type Fc), provided that the decrease in affinity to the $Fc\gamma R_{Activating}$ exceeds the decrease in affinity to the $Fc\gamma R_{Inhibiting}$ (i.e., binding to the $Fc\gamma R_{Activating}$ is "greatly" decreased compared to binding to the $Fc\gamma R_{Inhibiting}$, which is merely decreased), or may result from the Fc region of such molecule exhibiting an increased affinity to both an $Fc\gamma R_{Activating}$ and an $Fc\gamma R_{Inhibiting}$ (relative to a wild-type Fc), provided that the increase in affinity to the $Fc\gamma R_{Activating}$ is less than the increase in affinity to the an $Fc\gamma R_{Inhibiting}$ (i.e., binding to the $Fc\gamma R_{Inhibiting}$ is "greatly" increased compared to binding to he $Fc\gamma R_{Activating}$, which is merely increased), or may result from an unchanged affinity to an $Fc\gamma R_{Activating}$ coupled with an increase in affinity to an $Fc\gamma R_{Inhibiting}$.

[00102] 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.

[00103] 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.

[00104] 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.

[00105] 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.

[00106] 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. Immunomodatory agents include, but are not limited to, small molecules, peptides, polypeptides, fusion proteins, antibodies, inorganic molecules, mimetic agents, and organic molecules.

[00107] 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.

[00108] 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 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.

[00109] 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.

[00110] 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.

[00111] 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, 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 prophylactic agent alone, or in combination with other agents, that provides a prophylactic benefit in the prevention of disease.

[00112] 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 as result of the administration of a prophylactic or therapeutic agent.

[00113] 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 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 to a subject with a disorder.

[00114] "Effector function" as used herein is meant a biochemical event that results from the interaction of an antibody Fc region with an Fc receptor or ligand. Effector functions include but are not limited to antibody dependent cell mediated cytotoxicity (ADCC), antibody dependent cell mediated phagocytosis (ADCP), and complement

dependent cytotoxicity (CDC). Effector functions include both those that operate after the binding of an antigen and those that operate independent of antigen binding.

[00115] "Effector cell" as used herein is meant a cell of the immune system that expresses one or more Fc receptors and mediates one or more effector functions. Effector cells include but are not limited to monocytes, macrophages, neutrophils, dendritic cells, eosinophils, mast cells, platelets, B cells, large granular lymphocytes, Langerhans' cells, natural killer (NK) cells, and may be from any organism including but not limited to humans, mice, rats, rabbits, and monkeys.

[00116] "Fc ligand" as used herein is meant a molecule, preferably a polypeptide, from any organism that binds to the Fc region of an antibody to form an Fc-ligand complex. Fc ligands include but are not limited to FcγRs, FcγRs, FcγRs, FcRn, C1q, C3, staphylococcal protein A, streptococcal protein G, and viral FcγR. Fc ligands may include undiscovered molecules that bind Fc.

5. BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 DECISION TREE FOR SELECTION OF Fc MUTANTS

[00117] An exemplary protocol for selecting Fc mutants.

FIG. 2 SCHEMATIC OF SEQUENCE OF 8B5.3.4 VARIABLE HEAVY CHAIN DOMAIN

[00118] Depiction of the of the 8B5.3.4 VH nucleotide and amino acid sequence (SEQ ID NOS:12 and 9, respectively).

FIG. 3 SCHEMATIC OF SEQUENCE OF 8B5.3.4 VARIABLE LIGHT CHAIN DOMAIN

[00119] Depiction of the 8B5.3.4 VL nucleotide and amino acid sequence (SEQ ID NOS:11 and 10, respectively).

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

[00120] 6 μL of antibody at a concentration of approximately 20 μg/mL was injected at 5μL/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 immobilized sensor ship is shown. The marker was set on wild-type captured antibody response.

FIG. 5 SENSOGRAM OF REAL TIME BINDING OF FcγRIIIA TO CH 4-4-20 ANTIBODIES CARRYING VARIANT Fc REGIONS

[00121] Binding of FcγRIIIA 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.

[00122] Mutants used were as follows: Mut 6 (S219V), Mut 10 (P396L, A330S, K288N); Mut 18 (K326E); Mut 14 (K334E, K288N); Mut 11 (R255L, F243L); Mut 16 (F372Y); Mut 19 (K334N, K246I).

FIGS. 6 A-H ANALYSIS OF KINETIC PARAMETERS OF FcγRIIIA BINDING TO ANTIBODIES CARRYING VARIANT Fc REGIONS

[00123] Kinetic parameters for FcγRIIIA 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. 7 SENSOGRAM OF REAL TIME BINDING OF FcγRIIB-Fc FUSION PROTEINS TO ANTIBODIES CARRYING VARIANT Fc REGIONS

[00124] Binding of FcγRIIB-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.

FIGs. 8 A-C ANALYSIS OF KINETIC PARAMETERS FcγRIIB-Fc FUSION PROTEINS TO ANTIBODIES CARRYING VARIANT Fc REGIONS

[00125] Kinetic parameters for FcγRIIB-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.

[00126] Mutants used were as follows: Mut 6 (S219V), Mut 10 (P396L, A330S, K288N); Mut 18 (K326E); Mut 14 (K334E, K288N); Mut 11 (R255L, F243L); Mut 16 (F372Y); Mut 19 (K334N, K246I).

FIG. 9 RATIOS OF K_{off} (WT)/K_{off} (MUT) FOR FcγRIIIA-Fc PLOTTED AGAINST ADCC DATA

[00127] Numbers higher than one show a decreased dissociation rate for FcγRIIIA binding and increased dissociation rate for FcγRIIB-Fc binding relative to wild-type. Mutants in the box have lower off rate for FcγRIIIA binding and higher off rate for FcγRIIB-Fc binding.

FIG. 10 COMPETITION WITH UNLABELED FcγRIIIA

[00128] A kinetic screen was implemented to identify Fc region mutants with improved K_{off} rates for binding FcγRIIIA. A library of Fc region variants containing P396L mutation was incubated with 0.1 μM biotinylated FcγRIIIA-Linker-Avitag for one hour and then washed. Subsequently 0.8 uM unlabeled FcγRIIIA was incubatd with the labeled yeast for different time points. Yeast was spun down and unlabeled FcγRIIIA was removed, Receptor bound yease was stained with SA (streptavidin):PE (phycoerythrin) for FACS analysis.

FIGS. 11 A-C FACS ANALYSIS BASED ON THE KINETIC SCREEN

[00129] Based on the calculated K_{off} from the data presented in FIG. 22, a one minute time point selection was chosen. A 10-fold excess of library was incubated with 0.1 μM biotinylated FcγRIIIA-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 nonselcted 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 FcγRIIIA /PE and goat anti-human Fc/FITC.

FIGS. 12 A-B SELECTION BASED ON SOLID PHASE DEPLETION OF FcγRIIB Fc BINDERS

[00130] A. The P396L library was screened based on FcγRIIB depletion and FcγRIIIA selection using magnetic beads. The FcγRIIB 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 FcγRIIIA. Subsequently cells were selected twice by FACS using 0.1 μM biotinylated FcγRIIIA linker-avitag. Yeast cells were analyzed for both FcγRIIIA and FcγRIIB binding after each sort and compared to wild type binding.

B. Fc Mutants were selected from the FcγRIIB depleted yeast population using biotinylated FcγRIIIA 158F linker avitag monomer as a ligand. The sort gate was set to select the top 0.25% FcγRIIIA 158F binders. The resulting enriched population was analyzed by FACS for binding to the different FcγRIIIA (158F and 158V), FcγRIIIB and FcγRIIA (131R).

FIG. 13 RELATIVE RATES OF SKBR3 TARGET CELL LYSIS MEDIATED BY CHIMERIC 4D5 HARBORING FC MUTANTS

[00131] Relative rates of lysis was calculated for each Fc mutant tested. Lysis rates 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%.

FIG. 14 RELATIVE RATES OF DAUDI CELL LYSIS MEDIATED BY CHIMERIC 2H7 HARBORING FC MUTANTS

[00132] Relative rates 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 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%.

FIG. 15, Panels A-E Fc RECEPTOR PROFILES VIA FACS UPON CYTOKINE TREATMENT OF MONOCYTES.

[00133] Cytokine treatment of monocytes increases low affinity Fc receptor expression. Elutriated monocytes were cultured using specific cytokines in serum free media. Fc receptor profiles were assayed using FACS.

FIG. 16 IMPROVED TUMOR CELL KILLING USING FC MUTANTS IN MACROPHAGE-DERIVED MONOCYTES BASED ADCC.

[00134] Ch4D5 MAb concentration over 2 logs was tested using effector:target ratio of 35:1. Percent lysis was calculated as in FIG. 30.

FIG. 17 COMPLEMENT DEPENDENT CYTOTOXICITY ASSAY FLOW CHART.

[00135] The flow chart summarizes the CDC assays used.

FIG. 18 COMPLEMENT DEPENDENT CYTOTOXICITY ACTIVITY

[00136] Fc mutants that show enhanced binding to FcγRIIIA also showed improved complement activity. Anti-CD20 ChMAb over 3 orders of magnitude was titrated. Percent lysis was calculated as in as in FIG. 30.

FIG. 19 C1q BINDING TO 2B6 ANTIBODY

[00137] **A.** The diagram depicts the BIAcore format for analysis of 2B6 binding to the first component of the complement cascade.

[00138] **B.** Sensogram of real time binding of 2B6 antibody carrying variant Fc regions to C1q.

FIGS. 20 A-D C1q BINDING TO 2B6 MUTANT ANTIBODY.

[00139] Sensogram of real time binding of 2B6 mutants to C1q (3.25nM). Mutants depicted at MgFc51 (Q419H, P396L); MgFc51/60 in Panel A; MgFc55 and MgFc55/60 (Panel B), MgFc59 and MgFc59/60 (Panel C); and MgFc31/60 (Panel D).

FIGS. 21 A-D Fc VARIANTS WITH DECREASED BINDING TO FcγRIIB

[00140] Binding of FcR to ch4D5 antibodies to compare effect of D270E (60) on R255L, P396L double mutant (MgFc55). K_D was analyzed at different concentrations of FcR; 400nM CD16A 158V; 800nM CD16A 158F; 200nM CD32B; 200nM CD32A 131H. Analysis was performed using separate K_D using Biacore 3000 software.

FIGS. 22 A-D KINETIC CHARACTERISTICS OF 4D5 MUTANTS SELECTED FROM FcγRIIB DEPLETIONS/FcγRIIAH131 SELECTION

[00141] Binding of FcR to ch4D5 antibodies carrying different Fc mutations selected by CD32B depletion and CD32A H131 screening strategy. K_D was analyzed at different concentrations of FcR; 400nM CD16A 158V; 800nM CD16A 158F; 200nM CD32B; 200nM CD32A 131H. Analysis was performed using separate K_D using Biacore 3000 software.

FIG. 23. PLOT OF MDM ADCC DATA AGAINST THE K_{OFF} DETERMINED FOR CD32A 131H BINDING AS DETERMINED BY BIACORE.

[00142] The mutants are as follows: MgFc 25 (E333A, K334A, S298A); MgFc68 (D270E); MgFc38 (K392T, P396L); MgFc55 (R255L, P396L); MgFc31 (P247L, N421K); MgFc59(K370E, P396L).

FIGS. 24 A-B. ADCC ACTIVITY OF MUTANTS IN A HER2/NEU CHIMERIC MONOCLONAL ANTIBODY

[00143] Chimeric HER2/neu monoclonal antibodies containing mutant Fc regions were assessed, in duplicate, for their ADCC activity and compared to the ADCC activity of the wild type, chimeric Her2/neu antibody. The mutants analyzed are as follows: MGFc88 (F243L, R292P, Y300L, V305I, P396L), MGFc88A (F243L, R292P, Y300L, P396L), MGFc155 (F243L, R292P, Y300L).

FIGS. 25 A-B. ESTIMATED TUMOR WEIGHT IN MICE TREATED WITH WILD-TYPE OR Fc MUTANT h2B6

[00144] Balb/c nude mice were inoculated subcutaneously with Daudi cells and administered 25 μg, 2.5 μg or 0.25 μg weekly doses of either wild-type h2B6 (**A**) or h2B6 harboring Fc mutant MGFc 0088 (F243L, R292P, Y300L, V305I, P396L) (**B**). Mice administered buffer alone were used as control. Tumor wieght was calculated based on the estimated volume of the subcutaneous tumor according to the formula $(width^2 \times length)/2$.

FIGS. 26 A-B. SURVIVAL IN TUMOR BEARING MICE TREATED WITH WILD-TYPE OR Fc MUTANT h2B6

[00145] Nude mice were inoculated with Daudi cells and administered 25 μg, 2.5 μg or 0.25 μg weekly doses of either wild-type h2B6 (**A**) or h2B6 harboring Fc mutant MGFc 0088 (F243L, R292P, Y300L, V305I, P396L) (**B**). Mice administered buffer alone were used as control.

FIG. 27. ESTIMATED TUMOR WEIGHT IN HUMAN Fc RECEPTOR–EXPRESSING TRANSGENIC MICE TREATED WITH WILD-TYPE OR Fc-OPTIMIZED h2B6 0088

[00146] mCD16-/- huCD16A+ RAG1-/- C57Bl/6 mice were inoculated subcutaneously with Raji cells and after two weeks were intraperitoneally administered six weekly doses of either buffer alone (PBS), or 250 μg, 25 μg or 2.5 μg of wild-type h2B6 (Rituxan) or h2B6 comprising mutant FcMG0088 (F243L, R292P, Y300L,

V305I P396L) (MGA321). Tumor weight was calculated based on the estimated volume of the subcutaneous tumor according to the formula (width^2 X length)/2. Lines correspond to tumor weight, over time, for each individual mouse tested.

FIGS. 28 A–B. ESTIMATED TUMOR WEIGHT IN HUMAN Fc RECEPTOR–EXPRESSING TRANSGENIC MICE TREATED WITH WILD-TYPE OR Fc-OPTIMIZED h2B6 0088

[00147] mCD16-/- huCD16A+ RAG1-/- C57BI/6 mice were inoculated subcutaneously with Raji cells and after three weeks were intraperitoneally administered five weekly doses of 250 μg, 25 μg or 2.5 μg of wild-type h2B6 (Rituxan "Rituximab") (**A**) or h2B6 comprising mutant FcMG0088 (F243L, R292P, Y300L, V305I P396L) (h2B6 0088 "MGA321") (**B**). Tumor weight was calculated based on the estimated volume of the subcutaneous tumor according to the formula (width^2 X length)/2.

FIG. 29. SURVIVAL IN TUMOR-BEARING HUMAN Fc RECEPTOR–EXPRESSING TRANSGENIC MICE TREATED WITH WILD-TYPE OR Fc-OPTIMIZED h2B6 31/60

[00148] mCD16-/- huCD16A+ nude (FoxN1) mice were intraperitoneally inoculated with EL4-CD32B cells and intraperitoneally administered on days 0, 1, 2, 3 and 6 either wild-type h2B6 1.3 or h2B6 1.3 comprising mutant 31/60 (P247L, D270E, N421K) (h2B6 1.3 3160). Mice administered buffer alone (PBS) were used as control.

FIG. 30. SURVIVAL IN TUMOR-BEARING HUMAN Fc RECEPTOR–EXPRESSING TRANSGENIC MICE TREATED WITH WILD-TYPE OR Fc-OPTIMIZED h2B6 31/60

[00149] mCD16-/- huCD16A+ nude (FoxN1) mice were intraperitoneally inoculated with EL4-CD32B cells and intraperitoneally administered on days 0–3 and 6 doses of 10 μg/g body weight of either h2B6 comprising mutant 31/60 (P247L, D270E, N421K) (h2B6 3160) or wild-type h2B6.

FIGS. 31 A–B. SURVIVAL IN TUMOR-BEARING HUMAN Fc RECEPTOR–EXPRESSING TRANSGENIC MICE TREATED WITH WILD-TYPE OR Fc-OPTIMIZED h2B6 0088

[00150] (**A**) mCD16-/- huCD16A+ nude (FoxN1 or N/N) mice were intraperitoneally inoculated with EL4-CD32B cells and intraperitoneally administered on days 0–3 with doses of either h2B6 3.5 N297Q (negative control), h2B6 3.5 comprising mutant FcMG0088 (F243L, R292P, Y300L, V305I P396L) (h2B6 3.5 0088) or wild-type h2B6

3.5. Mice administered buffer alone (PBS) were used as control. (**B**) Tumor-bearing mice as in **A** were intraperitoneally administered on days 0–3 and 6 doses of 4 μg/g body weight of either PBS, h2B6 comprising mutant FcMG0088 (F243L, R292P, Y300L, V305I P396L) (h2B6 0088) or wild-type h2B6.

FIG. 32. SURVIVAL IN TUMOR-BEARING HUMAN Fc RECEPTOR–EXPRESSING TRANSGENIC MICE TREATED WITH WILD-TYPE OR Fc-OPTIMIZED h2B6 0088

[00151] mCD16-/- huCD16A+ hCD32A+ nude (FoxN1 or N/N) mice were intraperitoneally inoculated with EL4-CD32B cells and intraperitoneally administered on days 0–3 of either h2B6 3.5 N297Q (negative control), h2B6 comprising mutant FcMG0088 (F243L, R292P, Y300L, V305I P396L) (h2B6 3.5 0088) or wild-type h2B6 3.5. Mice administered buffer alone (PBS) were used as control.

FIGS. 33 A–C. SURVIVAL IN TUMOR-BEARING TRANSGENIC MICE TREATED WITH WILD-TYPE OR Fc-OPTIMIZED h2B6 0088

[00152] Nude (FoxN1) mice were intraperitoneally inoculated with EL4-CD32B cells and intraperitoneally administered on days 0–3 of either h2B6 3.5 N297Q (negative control), h2B6 comprising mutant FcMG0088 (F243L, R292P, Y300L, V305I P396L) (h2B6 3.5 88) or wild-type h2B6 3.5. Mice administered buffer alone (PBS) were used as control. The transgenic mice strains examined were (**A**) mCD16-/- huCD16A+, (**B**) mCD16-/- huCD16A+ hCD32A+ and (**C**) mCD16-/- hCD32A+.

FIG. 34. SURVIVAL IN TUMOR-BEARING HUMAN Fc RECEPTOR–EXPRESSING TRANSGENIC MICE TREATED AT VARYING INTERVALS WITH WILD-TYPE OR Fc-OPTIMIZED h2B6 0088

[00153] mCD16-/- huCD16A+ nude (FoxN1 or N/N) mice were intraperitoneally inoculated with EL4-CD32B cells and intraperitoneally treated with h2B6 comprising mutant FcMG0088 (F243L, R292P, Y300L, V305I, P396L) (MGA321) at the indicated time intervals.

FIGS. 35 A–B. ESTIMATED TUMOR WEIGHT IN HUMAN Fc RECEPTOR–EXPRESSING TRANSGENIC MICE TREATED WITH WILD-TYPE OR Fc-OPTIMIZED ch4D5 0088

[00154] Nude (FoxN1) mice with the transgenic genotype mCD16-/- hCD16A+ (**A**) or mCD16-/- hCD16A+ hCD32A+ (**B**) were inoculated subcutaneously with mSCOV3 cells and, starting on day 0, were intraperitoneally administered eight weekly doses of

either ch4D5 N297Q (negative control) or ch4D5 comprising mutant FcMG0088 (F243L, R292P, Y300L, V305I, P396L) (ch4D5 0088). Mice administered buffer alone (PBS) were used as control. Tumor weight was calculated based on the estimated volume of the subcutaneous tumor according to the formula (width^2 X length)/2.

FIGS. 36 A–B. SURVIVAL IN TUMOR-BEARING HUMAN Fc RECEPTOR–EXPRESSING TRANSGENIC MICE TREATED WITH WILD-TYPE OR Fc-OPTIMIZED ch4D5 0088

[00155] mCD16-/- huCD16A+ nude (N/N) mice were intraperitoneally inoculated with mSKOV3 cells and, starting on day 0, were intraperitoneally administered six weekly doses of either 100 μg (**A**) or 1 μg (**B**) of either wild-type ch4D5, ch4D5 N297Q (negative control) or ch4D5 comprising mutant FcMG0088 (F243L, R292P, Y300L, V305I P396L) (ch4D5 0088). Mice administered buffer alone (PBS) were used as control.

FIGS. 37 A–B. SURVIVAL IN TUMOR-BEARING HUMAN Fc RECEPTOR–EXPRESSING TRANSGENIC MICE TREATED WITH WILD-TYPE OR Fc-OPTIMIZED VARIANTS OF ch4D5

[00156] mCD16-/- huCD16A+ nude (N/N) mice were intraperitoneally inoculated with mSKOV3 cells and, starting on day 0, were intraperitoneally administered eight weekly doses of either 100 μg (**A**) or 10 μg (**B**) of either wild-type ch4D5, ch4D5 N297Q (negative control), ch4D5 comprising mutant FcMG0088 (F243L, R292P, Y300L, V305I P396L) (ch4D5 0088), ch4D5 mutant MGFc0155 (F243L, R292P, Y300L) (ch4D5 0155) or ch4D5 mutant MCFc3160 (P247L, D270E, N421K) ("ch4D5 3160"). Mice administered buffer alone (PBS) were used as control.

FIGS. 38 A–B. SURVIVAL IN TUMOR-BEARING HUMAN Fc RECEPTOR–EXPRESSING TRANSGENIC MICE TREATED WITH WILD-TYPE OR Fc-OPTIMIZED ch4D5 0088

[00157] Nude (N/N) mice with the transgenic genotype mCD16-/- hCD16A+ (**A**) or mCD16-/- hCD16A+ hCD32A+ (**B**) were intraperitoneally inoculated with mSKOV3 cells and, starting on day 0, intraperitoneally administered eight weekly doses of either ch4D5 comprising mutant FcMG0088 (F243L, R292P, Y300L, V305I P396L) (ch4D5 0088) or ch4D5 N297Q (negative control). Mice administered buffer alone (PBS) were used as control.

FIG. 39 A-D. Fc OPTIMIZATION ENHANCES TUMOR-CELL DEPLETION IN VIVO.

[00158] (A-B) Enhanced reduction of tumor burden by Fc-engineered hu2B6 treatment of Daudi cell subcutaneous xenografts in Balb/c FoxN1 (nu/nu) mice (6-8 mice/group). Statistical significance between curves was determined by a student T test; WT vs MG12, P=0.431; WT vs MG4, P = 0.002; MG4 vs MG12, P = 0.002. (C-D) Kaplan-Meier survival plots of mFcγRIII-/- human CD16A+ FoxN1 mice injected intra-peritoneally with CD32B-EL4 cells (10 mice/group) and treated with either WT-Fc hu2B6 or the indicated Fc-engineered forms of hu2B6 (C, 10 μg/g; D, 4 μg/g). Data was analyzed for significance using log-rank analysis.

FIG. 40. GLYCOSYLATION PATTERNS OF ANTIBODIES HAVING ALTERED FC REGIONS.

[00159] (Panels A-D) show the results of investigations into the glycosylation patterns of the antibodies of the present invention (plotted is detected signal in lumens vs. chromatographic elution time in minutes). In all panels, ■ is GlcNAc; ● is galactose; ○ is mannose and △ is fucose. Panel A shows shows the assignment of N-linked oligosaccharides as determined using an antibody reference panel. Panels B and C show the results on two preparations of antibody ch45D4-FcMT2. Panel D is a digest control. Arrows indicate the assignments of glycosylation patterns to peaks; dashed arrows indicate the expected positions of the glycosylation patterns of Panel A.

FIG. 41. $GlcNAc_2Man_9$ ("Man9") GLYCOSYLATION STRUCTURE.

[00160] The $GlcNAc_2Man_9$ ("Man9") glycosylation structure. Cleavage of mannose units results in the formation of $GlcNAc_2Man_8$ ("Man8"), $GlcNAc_2Man_7$ ("Man7"), $GlcNAc_2Man_6$ ("Man6") and $GlcNAc_2Man_5$ ("Man5") structures.

FIG. 42. $GlcNAc_2Man_5$ ("Man5") GLYCOSYLATION STRUCTURE.

[00161] The $GlcNAc_2Man_5$ ("Man5") structure is processed by the successive action of glycosyltransferases to generate "G0F," "G1F" and "G2F" oligosaccharides.

FIG. 43. GLYCOSYLATION STRUCTURE AND FcγR BINDING OF FC TETRA VARIANT: F243X, R292P, Y300L, P396L

[00162] The Figure shows the effect of altering the identity of the residue substituted at position F243 of a tetra variant (F243X, R292P, Y300L, P396L) on the observed

glycosylation profile of the variant and on the relative binding ($K_{dissociation}$ wild type / $K_{dissociation}$ variant) of the Fc variant to the Fc receptors, CD16A, CD32A and CD32B.

FIG. 44. GLYCOSYLATION STRUCTURE AND FcγR BINDING OF FC TETRA VARIANT: F243L, R292X, Y300L, P396L

[00163] The Figure shows the effect of altering the identity of the residue substituted at position R292 of a tetra variant (F243L, R292X, Y300L, P396L) on the observed glycosylation profile of the variant and on the relative binding ($K_{dissociation}$ wild type / $K_{dissociation}$ variant) of the Fc variant to the Fc receptors, CD16A, CD32A and CD32B.

FIG. 45. EFFECT OF PROGRESSIVE ALTERATIONS OF RESIDUES R292, Y300 AND P396 OF AN F243C Fc VARIANT

[00164] The Figure shows the effect of progressively altering the identity of the residue substituted at position R292, Y300 and P396 of an F243C Fc variant on the observed glycosylation profile of the variant and on the relative binding ($K_{dissociation}$ wild type / $K_{dissociation}$ variant) of the Fc variant to the Fc receptors, CD16A, CD32A and CD32B.

FIG. 46. EFFECT OF PROGRESSIVE ALTERATIONS OF RESIDUES R292, Y300 AND P396 OF AN F243C Fc VARIANT

[00165] The Figure shows the effect of progressively altering the identity of the residue substituted at position R292, Y300 and P396 of an F243L Fc variant on the observed glycosylation profile of the variant and on the relative binding ($K_{dissociation}$ wild type / $K_{dissociation}$ variant) of the Fc variant to the Fc receptors, CD16A, CD32A and CD32B.

FIG. 47. EFFECT OF PROGRESSIVE ALTERATIONS OF RESIDUES R292, Y300 AND P396 OF AN F243R Fc VARIANT

[00166] The Figure shows the effect of progressively altering the identity of the residue substituted at position R292, Y300 and P396 of an F243R Fc variant on the observed glycosylation profile of the variant and on the relative binding ($K_{dissociation}$ wild type / $K_{dissociation}$ variant) of the Fc variant to the Fc receptors, CD16A, CD32A and CD32B.

FIG. 48. EFFECT OF PROGRESSIVE ALTERATIONS OF RESIDUES R292, Y300 AND P396 OF AN F243V Fc VARIANT

[00167] The Figure shows the effect of progressively altering the identity of the residue substituted at position R292, Y300 and P396 of an F243V Fc variant on the observed glycosylation profile of the variant and on the relative binding ($K_{dissociation}$ wild type / $K_{dissociation}$ variant) of the Fc variant to the Fc receptors, CD16A, CD32A and CD32B.

6. DETAILED DESCRIPTION

[00168] The present invention relates to methods of treatment of cancer or other diseases using 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 FcγR. Enhancing the ability of immunoglobulins to mediate antibody-dependent cell-mediated cytotoxicity (ADCC), complment-dependent cytotoxicity (CDC) or antibody-dependent cell-mediated phagocytosis (ADCP) provides an approach for enhancing the therapeutic activity of immunoglobulins against cancers and infectious diseases.

[00169] The invention thus encompasses therapeutic antibodies in the treatment or prevention of a disease or disorder, or the amelioration of a symptom thereof, where an enhanced efficacy of effector cell function (*e.g.*, ADCC) mediated by FcγR is desired, *e.g.*, cancer or infectious disease, or where a modulation of effector cell function mediated by FcγR is desired, *e.g.*, autoimmune or inflammitory disorders. In some embodiments, the invention encompasses the use of molecules comprising Fc regions with amino acid modifications including but not limited to any of the modifications disclosed in U.S Patent Application Publications 2005/0037000 and 2005/0064514; and International Patent Application Publication WO 04/063351. Each of the above mentioned applications is incorporated herein by reference in its entirety.

[00170] The polypeptides of the present invention may have variant Fc domains. Modification of the Fc domain normally leads to an altered phenotype, for example altered serum half-life, altered stability, altered susceptibility to cellular enzymes or altered effector function. It may be desirable to modify the antibody of the invention with respect to effector function, so as to enhance the effectiveness of the antibody in treating cancer, for example. Reduction or elimination of effector function is desirable in certain cases, for example in the case of antibodies whose mechanism of action

involves blocking or antagonism, but not killing of the cells bearing a target antigen. Increased effector function is generally desirable when directed to undesirable cells, such as tumor and foreign cells, where the FcγRs are expressed at low levels, for example, tumor specific B cells with low levels of FcγRIIB (e.g., non-Hodgkins lymphoma, CLL, and Burkitt's lymphoma). In said embodiments, molecules of the invention with conferred or altered effector function activity are useful for the treatment and/or prevention of a disease, disorder or infection where an enhanced efficacy of effector function activity is desired.

[00171] In certain embodiments, the molecules of the invention comprise one or more modifications to the amino acids of the Fc domain, which reduce the affinity and avidity of the Fc region and, thus, the molecule of the invention, for one or more FcγR receptors. In other embodiments, the molecules of the invention comprise one or more modifications to the amino acids of the Fc region, which increase the affinity and avidity of the Fc region and, thus, the molecule of the invention, for one or more FcγR receptors. In other embodiments, the molecules comprise a variant Fc domain wherein said variant confers or mediates increased ADCC activity and/or an increased binding to FcγRIIA, relative to a molecule comprising no Fc domain or comprising a wild-type Fc domain. In alternate embodiments, the molecules comprise a variant Fc domain wherein said variant confers or mediates decreased ADCC activity (or other effector function) and/or an increased binding to FcγRIIB, relative to a molecule comprising no Fc domain or comprising a wild-type Fc domain.

[00172] In some embodiments, the invention encompasses molecules comprising a variant Fc region, which variant Fc region does not show a detectable binding to any FcγR, relative to a comparable molecule comprising the wild-type Fc region. In other embodiments, the invention encompasses molecules comprising a variant Fc region, which variant Fc region only binds a single FcγR, preferably one of FcγRIIA, FcγRIIB, or FcγRIIIA.

[00173] The polypeptides of the present invention may comprise altered affinities for an activating and/or inhibitory Fcγ receptor. In one embodiment, the antibody or polypeptide comprises a variant Fc region that has increased affinity for FcγRIIB and decreased affinity for FcγRIIIA and/or FcγRIIA, relative to a comparable molecule with a wild-type Fc region. In another embodiment, the polypeptides of the present invention comprise a variant Fc region, which has decreased affinity for FcγRIIB and increased affinity for FcγRIIIA and/or FcγRIIA, relative to a comparable molecule with

a wild-type Fc region. In yet another embodiment, the polypeptides of the present invention comprise a variant Fc region that has decreased affinity for FcγRIIB and decreased affinity for FcγRIIIA and/or FcγRIIA, relative to a comparable molecule with a wild-type Fc region. In still another embodiment, the polypeptides of the present invention comprise a variant Fc region, which has unchanged affinity for FcγRIIB and decreased (or increased) affinity for FcγRIIIA and/or FcγRIIA, relative to a comparable molecule with a wild-type Fc region.

[00174] In certain embodiments, the invention encompasses immunoglobulins comprising a variant Fc region with an altered affinity for FcγRIIIA and/or FcγRIIA such that the immunoglobulin has an enhanced effector function, e.g., antibody dependent cell mediated cytotoxicity. Non-limiting examples of effector cell functions include antibody-dependent cell mediated cytotoxicity (ADCC), antibody-dependent phagocytosis, phagocytosis, opsonization, opsonophagocytosis, cell binding, rosetting, C1q binding, and complement dependent cell mediated cytotoxicity.

[00175] In a preferred embodiment, the alteration in affinity or effector function is at least 2-fold, preferably at least 4-fold, at least 5-fold, at least 6-fold, at least 7-fold, at least 8-fold, at least 9-fold, at least 10-fold, at least 50-fold, or at least 100-fold, relative to a comparable molecule comprising a wild-type Fc region. In other embodiments of the invention, the variant Fc region immunospecifically binds one or more FcRs with at least 65%, preferably at least 70%, 75%, 80%, 85%, 90%, 95%, 100%, 125%, 150%, 175%, 200%, 225%, or 250% greater affinity relative to a molecule comprising a wild-type Fc region. Such measurements can be in vivo or in vitro assays, and in a preferred embodiment are in vitro assays such as ELISA or surface plasmon resonance assays.

[00176] In different embodiments, the molecules comprise a variant Fc domain wherein said variant agonizes at least one activity of an FcγR receptor, or antagonizes at least one activity of an FcγR receptor. In a preferred embodiment, the molecules comprise a variant that agonizes (or antagonizes) one or more activities of FcγRIIB, for example, B cell receptor-mediated signaling, activation of B cells, B cell proliferation, antibody production, intracellular calcium influx of B cells, cell cycle progression, FcγRIIB-mediated inhibition of FcεRI signaling, phosphorylation of FcγRIIB, SHIP recruitment, SHIP phosphorylation and association with Shc, or activity of one or more downstream molecules (e.g., MAP kinase, JNK, p38, or Akt) in the FcγRIIB signal transduction pathway. In another embodiment, the molecules comprise a variant that agonizes (or antagonizes) one or more activities of FcεRI, for example, mast cell

activation, calcium mobilization, degranulation, cytokine production, or serotonin release.

[00177] In certain embodiments, the molecules comprise an Fc domain comprising domains or regions from two or more IgG isotypes (e.g., IgG1, IgG2, IgG3 and IgG4). The various IgG isotypes exhibit differing physical and functional properties including serum half-life, complement fixation, FcγR binding affinities and effector function activities (e.g. ADCC, CDC, etc.) due to differences in the amino acid sequences of their hinge and/or Fc domains, for example as described in Flesch and Neppert (1999) J. Clin. Lab. Anal. 14:141-156; Chappel *et al.* (1993) J. Biol. Chem. 33:25124-25131; Chappel *et al.* (1991) Proc. Natl. Acad. Sci. (U.S.A.) 88:9036-9040; Brüggemann *et al.* (1987) J. Exp. Med 166:1351-1361. This type of variant Fc domain may be used alone, or in combination with an amino acid modification, to affect Fc-mediated effector function and/or binding activity. In combination, the amino acid modification and IgG hinge/Fc region may display similar functionality (e.g., increased affinity for FcγRIIA) and may act additively or, more preferably, synergistically to modify the effector functionality in the molecule of the invention, relative to a molecule of the invention comprising a wild-type Fc region. In other embodiments, the amino acid modification and IgG Fc region may display opposite functionality (e.g., increased and decreased affinity for FcγRIIA, respectively) and may act to selectively temper or reduce a specific functionality in the molecule of the invention, relative to a molecule of the invention not comprising an Fc region or comprising a wild-type Fc region of the same isotype.

[00178] In specific embodiments, the invention encompasses therapeutic antibodies that immunospecifically bind FcγRIIB via their variable domains with greater affinity than FcγRIIA, *e.g.*, antibodies derived from mouse monoclonal antibody produced by clone 2B6 or 3H7, having ATCC accession numbers PTA-4591 and PTA-4592, respectively. In other embodiments, the anti-FcγRIIB antibodies of the invention are derived from a mouse monoclonal antibody produced by clone 1D5, 2E1, 2H9, 2D11, or 1F2, having ATCC Accession numbers, PTA-5958, PTA-5961, PTA-5962, PTA-5960, and PTA-5959, respectively. Hybridomas producing antibodies 2B6 and 3H7 have been deposited with the American Type Culture Collection (10801 University Blvd., Manassas, VA. 20110-2209) on August 13, 2002 under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedures, and assigned accession numbers PTA-4591 (for

hybridoma producing 2B6) and PTA-4592 (for hybridoma producing 3H7), respectively, and are incorporated herein by reference. Hybridomas producing 1D5, 2E1, 2H9, 2D11, and 1F2 were deposited under the provisions of the Budapest Treaty with the American Type Culture Collection (10801 University Blvd., Manassas, VA. 20110-2209) on May 7, 2004, and assigned accession numbers PTA-5958, PTA-5961, PTA-5962, PTA-5960, and PTA-5959, respectively and are incorporated herein by reference. In preferred embodiments, the anti-FcγRIIB antibodies of the invention (*e.g.*, 2B6) comprise a variant Fc region, wherein said variant Fc region has a substitution at portion at position 243 with leucine, at position 292 with proline, at position 300 with leucine, at position 305 with isoleucine, and at position 396 with leucine. In another embodiment, the invention encompasses a 2B6 antibody having a leucine at position 243, a proline at position 292, a leucine at position 300, an isoleucine at position 305, and a leucine at position 396. In other embodiments, the invention encompasses a 2B6 antibody comprising a variant Fc region having a substitution at position 243 with leucine, at position 292 with proline, and at position 300 with leucine. In other embodiments, the invention encompasses a 2B6 antibody having a leucine at position 243, a proline at position 292, and a leucine at position 300. In other embodiments, the invention encompasses a 2B6 antibody comprising a variant Fc region having a substitution at position 247 with leucine, at position 270 with glutamic acid, and at position 421 with lysine. In another embodiment, the invention encompasses a 2B6 antibody having a leucine at position 247, a glutamic acid at position 292, and a lysine at position 421.

[00179] In a specific embodiment, the invention encompasses therapeutic antibodies that bind the Her2/neu protooncogene (amino acid sequence **SEQ ID NO:31**) (*e.g.*, Ab4D5 antibody as disclosed in Carter *et al.*, 1992, *Proc. Natl. Acad. Sci. USA* 89:4285-9; U.S. Patent No. 5,677,171; or International Patent Application Publication WO 01/00245, each of which is hereby incorporated by references in its entirety). In a certain embodiment, the 4D5 antibody is chimeric. In another embodiment, the 4D5 antibody is humanized. In a specific embodiment, the 4D5 antibody is engineered to comprise a variant 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 FcγRIIIA and/or FcγRIIA and/or decreases the affinity of the Fc region for FcγRIIB and/or modulates the effector function of the antibody relative to a comparable antibody comprising a wild-type Fc region. In certain embodiments, the 4D5 antibody

of the invention comprises a variant Fc region having a substitution at position 243 with leucine, at position 292 with proline, at position 300 with leucine, at position 305 with isoleucine, and at position 396 with leucine. In other embodiments, the invention encompasses a 4D5 antibody having a leucine at position 243, a proline at position 292, a leucine at position 300, an isoleucine at position 305, and a leucine at position 396. In yet another embodiment, the invention encompasses a 4D5 antibody comprising a variant Fc region having a substitution at position 243 with leucine, at position 292 with proline, and at position 300 with leucine. In still other embodiments, the invention encompasses a 4D5 antibody having a leucine at position 243, a proline at position 292, and a leucine at position 300. In other embodiments, the invention encompasses a 4D5 antibody comprising a variant Fc region having a substitution at position 247 with leucine, at position 270 with glutamic acid, and at position 421 with lysine. In another embodiment, the invention encompasses a 4D5 antibody having a leucine at position 247, a glutamic acid at position 292, and a lysine at position 421.

[00180] 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 FcγR (*e.g.*, does not bind FcγRIIA, FcγRIIB, or FcγRIIIA, as determined by, for example, an ELISA assay), relative to a comparable molecule comprising the wild-type Fc region.

[00181] 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 FcγR, wherein said FcγR is FcγIIIA. 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 FcγR, wherein said FcγR is FcγRIIA. 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 FcγR, wherein said FcγR is FcγRIIB. 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.

[00182] In certain embodiments, the molecules comprise a variant Fc region, having one or more amino acid modifications in one or more regions, which modification(s) alter (relative to a wild-type Fc region) the Ratio of Affinities of the variant Fc region to an activating FcγR (such as FcγRIIA or FcγRIIIA) relative to an inhibiting FcγR (such as FcγRIIB):

$$\text{Ratio of Affinities} = \frac{\text{Wild-Type to Variant Change in Affinity to Fc}\gamma\text{R}_{\text{Activating}}}{\text{Wild-Type to Variant Change in Affinity to Fc}\gamma\text{R}_{\text{Inhibiting}}}$$

[00183] Where an Fc variant has a Ratio of Affinities greater than 1, the methods of the invention have particular use in providing a therapeutic or prophylactic treatment of a disease, disorder, or infection, or the amelioration of a symptom thereof, where an enhanced efficacy of effector cell function (e.g., ADCC) mediated by FcγR is desired, e.g., cancer or infectious disease. Where an Fc variant has a Ratio of Affinities less than 1, the methods of the invention have particular use in providing a therapeutic or prophylactic treatment of a disease or disorder, or the amelioration of a symptom thereof, where a decreased efficacy of effector cell function mediated by FcγR is desired, e.g., autoimmune or inflammatory disorders. **Table 1** lists exemplary single, double, triple, quadruple and quintuple mutations by whether their Ratio of Affinities is greater than or less than 1. Specific binding data for various mutations is listed in **Table 2**, and more information concerning these mutations may be found in the Antibody Engineering Technology Art.

Table 1: Exemplary Single and Multiple Mutations Listed by Ratio of Affinities

Ratio	Single	Double	Triple	Quadruple	Quintuple
> 1	F243L D270E R292G R292P	F243L & R292P F243L & Y300L F243L & P396L D270E & P396L R292P & Y300L R292P & V305I R292P & P396L Y300L & P396L	F243L, P247L & N421K F243L, R292P & Y300L F243L, R292P & V305I F243L, R292P & P396L F243L, Y300L & P396L P247L, D270E & N421K R255L, D270E & P396L D270E, G316D & R416G	L234F, F243L, R292P & Y300L L235I, F243L, R292P & Y300L L235Q, F243L, R292P & Y300L F243L, P247L, D270E & N421K F243L, R255L, D270E & P396L F243L, D270E, G316D & R416G F243L, D270E, K392T & P396L F243L, D270E, P396L & Q419H	L235V, F243L, R292P, Y300L & P396L L235P, F243L, R292P, Y300L & P396L F243L, R292P, V305I, Y300L & P396L

Table 1: Exemplary Single and Multiple Mutations Listed by Ratio of Affinities					
Ratio	**Single**	**Double**	**Triple**	**Quadruple**	**Quintuple**
		P396L & Q419H	D270E, K392T & P396L D270E, P396L & Q419H V284M, R292L & K370N R292P, Y300L & P396L	F243L, R292P, Y300L, & P396L F243L, R292P, V305I & P396L P247L, D270E, Y300L & N421K R255L, D270E, R292G & P396L R255L, D270E, Y300L & P396L D270E, G316D, P396L & R416G	
< 1	Y300L P396L	F243L & P396L P247L & N421K R255L & P396L R292P & V305I K392T & P396L P396L & Q419H	F243L, R292P & V305I		

Table 2: Detailed Binding Information for Exemplary Fc Variants					
Fc sequence	**CD16A V158**	**CD16A F158**	**CD32B**	**Ratio of Affinities CD16A/CD32B**	
				V158	**F158**
Ratio of Affinities > 1					
Class I: Increased Binding to CD16; Decreased Binding to CD32B					
F243L	4.79	3.44	0.84	5.70	4.10
F243L P247L D270E N421K	2.30	3.45	0.32	7.19	10.78
F243L P247L N421K	1.89	1.71	0.17	11.12	10.06
F243L R255L D270E P396L	1.75	1.64	0.38	4.61	4.32
F243L D270E G316D R416G	1.50	1.34	0.20	7.50	6.70
F243L D270E K392T P396L	3.16	2.44	0.44	7.18	5.55
F243L D270E P396L Q419H	1.46	1.15	0.26	5.62	4.42
F243L R292P	4.73		0.12	39.4	
F243L R292P	4	1.67	0.16	25	10.44
F243L R292P P300L	6.69	2.3	0.32	20.9	7.19
F243L R292P V305I	2.56	1.43	ND	>25	>25
F243L R292P V305I P396L	5.37	2.53	0.40	13.43	6.33
P247L D270E N421K	1.89	2.46	0.58	3.26	4.24
R255L D270E R292G P396L	1.39	1.30	0.65	2.14	2.00
R255L D270E Y300L P396L	1.52	1.74	0.87	1.75	2.00
R255L D270E P396L	1.34	1.65	0.87	1.54	1.90
D270E	1.25	1.48	0.39	3.21	3.79
D270E G316D R416G	2.18	2.49	0.78	2.79	3.19
D270E K392T P396L	1.81	2.28	0.79	2.29	2.89
D270E P396L	1.38	1.65	0.89	1.55	1.85
D270E P396L G316D R416G	1.22		1.07	1.14	

Table 2: Detailed Binding Information for Exemplary Fc Variants					
Fc sequence	CD16A V158	CD16A F158	CD32B	Ratio of Affinities CD16A/CD32B	
				V158	F158
D270E P396L Q419H	1.64	2.00	0.68	2.41	2.94
V284M R292P K370N	1.14	1.37	0.37	3.1	3.7
R292G	1.54		0.25	6.2	
R292P	2.90		0.25	11.60	
R292P V305I	1.32	1.28	0.37	3.6	3.46
Class II: Decreased Binding to CD16; Greatly Decreased Binding to CD32B					
R292P		0.64	0.25		2.56
R292P F243L		0.6	0.12		5.00
Class III: Increased Binding to CD16; Unchanged Binding to CD32B					
F243I R292P Y300L V305I P396L	10.9	3.12	1.05	10.4	2.97
F243L R292P Y300L P396L	10.06	5.62	1.07	9.40	5.25
R292P V305I P396L	1.85	1.90	0.92	2.01	2.07
Class IV: Greatly Increased Binding to CD16; Increased Binding to CD32B					
F243L R292P Y300L V305I P396L	10.06	8.25	1.38	7.29	5.98
D270E G316D P396L R416G	1.22		1.07	1.14	
Ratio of Affinities < 1					
Class V: Unchanged Binding to CD16; Increased Binding to CD32B					
R255L P396L	1.09		2.22	0.49	
Y300L	1.01		1.18		0.99
Class VI: Increased Binding to CD16; Greatly Increased Binding to CD32B					
F243L P396L	1.49	1.60	2.22	0.67	0.72
P247L N421K	1.29	1.73	2.00	0.65	0.87
R255L P396L		1.39	2.22	0.49	0.63
R292P V305I	1.59	2.11	2.67	0.60	0.79
K392T P396L	1.49	1.81	2.35	0.63	0.77
P396L	1.27	1.73	2.58	0.49	0.67
P396L Q419H	1.19	1.19	1.33	0.89	0.89
Class VII: Decreased Binding to CD16; Increased / Unchanged Binding to CD32B					
D270E G316D P396L R416G		0.94	1.07		0.88

[00184] In other embodiments, the molecules comprise a variant Fc region having one or more amino acid substitutions, which substitutions alter (relative to a wild-type Fc region) the binding of the variant Fc region, e.g., enhance the binding to an activating FcγR (such as FcγRIIA or FcγRIIIA) and/or reduce the binding to an inhibiting FcγR (such as FcγRIIB). Various Fc mutations having one or more amino acid changes were engineered and analyzed by surface plasmon resonance for k_{off}, as shown in **Table 3**. Dissociation rate constants for binding the various FcγR were determined by BIAcore analysis and directly compared with those for the wild-type Fc, with the ratio (x = WT k_{off}/mutant k_{off}) indicated in the right-hand columns of **Table 3** with respect to each FcγR tested.

Table 3: Comparison Of k_{off} Of Fc Mutants to Wild-Type Fc

M	Amino Acid Change(s)								CD16A^V	CD16A^F	CD32A^H	CD32B
One Amino Acid												
1	F243L								4.8	3.4	0.6	0.8
2		D270E							1.3	1.5	2.2	0.4
3			R292P						2.4	1.6	0.7	0.3
4				S298N					nd	nd	nt	0.2
5					Y300L				1.0	1.2	2.9	1.2
6						V305I			0.9	0.6	1.3	1.2
7							A330V		0.6	1.2	0.4	0.3
8								P396L	1.3	1.7	1.6	2.6
Two Amino Acids												
9	F243L							P396L	2.2	2.0	1.5	1.6
10	F243L		R292P						4.0	1.7	0.5	0.2
11			R292P			V305I			1.3	1.3	0.8	0.4
Three Amino Acids												
12	F243L		R292P		Y300L				7.4	4.6	1.0	0.6
13	F243L		R292P			V305I			2.6	1.4	0.2	0.1
14	F243L		R292P					P396L	6.3	3.4	1.4	0.4
15			R292P			V305I		P396L	1.9	1.9	1.5	0.9
Four Amino Acids												
16	F243L		R292P		Y300L			P396L	10.1	5.6	1.7	1.1
17	F243L		R292P			V305I		P396L	4.0	2.3	0.8	0.4
Five Amino Acids												
18	F243L		R292P		Y300L	V305I		P396L	10.1	8.3	3.2	1.4

Abbreviations: M, Mutant Number; nd, no detectable binding; nt, not tested. Values with ≥ 80% difference (≥ 0.8 fold) from wild-type in either direction are in bold. Shading denotes Fc mutants identified directly by yeast display; all other mutants were constructed by site-directed mutagenesis.

[00185] There is also extensive guidance in the Antibody Engineering Technology Art concerning desirable modifications. Exemplary modifications that may be desirable in certain circumstances are listed below:

[00186] In a specific embodiment, in variant Fc regions, any amino acid modifications (e.g., substitutions) at any of positions 235, 240, 241, 243, 244, 247, 262, 263, 269, 298, 328, or 330 and preferably one or more of the following residues: A240, I240, L241, L243, H244, N298, I328 or V330. In a different specific embodiment, in variant Fc regions, any amino acid modifications (e.g., substitutions) 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 preferably one or more of the following residues: H280, Q280, Y280, G290, S290, T290, Y290, N294, K295, P296, D298, N298, P298, V298, I300 or L300.

[00187] In a preferred embodiment, in variant Fc regions that bind an FcγR with an altered affinity, any amino acid modifications (e.g., substitutions) 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 or 439. Preferably, the variant Fc region has any of the following residues: A256, N268, Q272, D286, Q286, S286, A290, S290, A298, M301, A312, E320, M320, Q320, R320, E322, A326, D326, E326, N326, S326, K330, T339, A333, A334, E334, H334, L334, M334, Q334, V334, K335, Q335, A359, A360 or A430.

[00188] In a different embodiment, in variant Fc regions that bind an FcγR (via its Fc region) with a reduced affinity, any amino acid modifications (e.g., substitutions) 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.

[0001] In a different embodiment, in variant Fc regions that bind an FcγR (via its Fc region) with an enhanced affinity, any amino acid modifications (e.g., substitutions) 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. In a different embodiment, in variant Fc regions that binds FcγRIIA with an enhanced affinity, any of the following residues: A255, A256, A258, A267, A268, N268, A272, Q272, A276, A280, A283, A285, A286, D286, Q286, S286, A290, S290, M301, E320, M320, Q320, R320, E322, A326, D326, E326, S326, K330, A331, Q335, A337 or A430.

[00189] In other embodiments, the invention encompasses the use of any Fc variant known in the art, such as those disclosed in Jefferis *et al.* (2002) Immunol Lett 82:57-65; Presta *et al.* (2002) Biochem Soc Trans 30:487-90; Idusogie *et al.* (2001) J Immunol 166:2571-75; Shields *et al.* (2001) J Biol Chem 276:6591-6604; Idusogie *et al.* (2000) J Immunol 164:4178-84; Reddy *et al.* (2000) J Immunol 164:1925-33; Xu *et al.* (2000) Cell Immunol 200:16-26; Armour *et al.* (1999) Eur J Immunol 29:2613-24; Jefferis *et al.* (1996) Immunol Lett 54:101-04; Hinton et al; 2004, J. Biol. Chem. 279(8): 6213-6; Lund *et al.* (1996) J Immunol 157:4963-69; Hutchins *et al.* (1995) Proc. Natl. Acad. Sci. (U.S.A.) 92:11980-84; Jefferis *et al.* (1995) Immunol Lett. 44:111-17; Lund *et al.* (1995) FASEB J 9:115-19; Alegre *et al.* (1994) Transplantation 57:1537-43; Lund *et al.* (1992) Mol Immunol 29:53-59; Lund *et al.* (1991) J. Immunol 147:2657-62; Duncan *et al.* (1988) Nature 332:563-64; US Patent Nos. 5,624,821; 5,885,573; 6,194,551; 7,276,586; and 7,317,091; and PCT Publications WO 00/42072 and PCT WO 99/58572.

[00190] Preferred variants include one or more modifications at any of positions: 228, 230, 231, 232, 233, 234, 235, 239, 240, 241, 243, 244, 245, 247, 262, 263, 264, 265, 266, 271, 273, 275, 281, 284, 291, 296, 297, 298, 299, 302, 304, 305, 313, 323, 325, 326, 328, 330 or 332.

[00191] Particularly preferred variants include one or more modifications selected from groups A-AI:

A. 228E, 228K, 228Y or 228G;

B. 230A, 230E, 230Y or 230G;

C. 231E, 231K, 231Y, 231P or 231G;

D. 232E, 232K, 232Y, 232G;

E. 233D;

F. 234I or 234F;

G. 235D, 235Q, 235P, 235I or 235V;

H. 239D, 239E, 239N or 239Q;

I. 240A, 240I, 240M or 240T;

J. 243R, 243, 243Y, 243L, 243Q, 243W, 243H or 243I;

K. 244H;

L. 245A;

M. 247G, 247V or 247L;

N. 262A, 262E, 262I, 262T, 262E or 262F;

O. 263A, 263I, 263M or 263T;

P. 264F, 264E, 264R, 264I, 264A, 264T or 264W;

Q. 265F, 265Y, 265H, 265I, 265L, 265T, 265V, 265N or 265Q;

R. 266A, 266I, 266M or 266T;

S. 271D, 271E, 271N, 271Q, 271K, 271R, 271S, 271T, 271H, 271A, 271V, 271L, 271I, 271F, 271M, 271Y, 271W or 271G;

T. 273I;

U. 275L or 275W;

V. 281D, 281K, 281Y or 281P;

W. 284E, 284N, 284T, 284L, 284Y or284M;

X. 291D, 291E, 291Q, 291T, 291H, 291I or 291G;

Y. 299A, 299D, 299E, 299F, 299G, 299H, 299I, 299K, 299L, 299M, 299N, 299P, 299Q, 299R, 299S, 299V, 299W or 299Y;

Z. 302I;

AA. 304D, 304N, 304T, 304H or 304L

AB. 305I;

AC. 313F;

AD. 323I;

AE. 325A, 325D, 325E, 325G, 325H, 325I, 325L, 325K, 325R, 325S, 325F, 325M, 325T, 325V, 325Y, 325W or 325P;

AF. 328D, 328Q, 328K, 328R, 328S, 328T, 328V, 328I, 328Y, 328W, 328P, 328G, 328A, 328E, 328F, 328H, 328M or 328N;

AG. 330L, 330Y, 330I or 330V;

AH. 332A, 332D, 332E, 332H, 332N, 332Q, 332T, 332K, 332R, 332S, 332V, 332L, 332F, 332M, 332W, 332P, 332G or 332Y; and

AI. 336E, 336K or 336Y.

[00192] Still more particularly preferred variants include one or more modifications selected from Groups 1-105 of **Table 4**:

Table 4

Group	Variant	Group	Variant
1	A330L / I332E	54	S239D / D265L / N297D / I332E
2	D265F / N297E / I332E	55	S239D / D265T / N297D / I332E
3	D265Y / N297D / I332E	56	S239D / D265V / N297D / I332E
4	D265Y / N297D / T299L / I332E	57	S239D / D265Y / N297D / I332E
5	F241E / F243Q / V262T / V264F	58	S239D / I332D
6	F241E / F243Q / V262T / V264E / I332E	59	S239D / I332E
7	F241E / F243R / V262E / V264R	60	S239D / I332E / A330I
8	F241E / F243R / V262E / V264R / I332E	61	S239D / I332N
9	F241E / F243Y / V262T / V264R	62	S239D / I332Q
10	F241E / F243Y / V262T / V264R / I332E	63	S239D / N297D / I332E
11	F241L / F243L / V262I / V264I	64	S239D / N297D / I332E / A330Y
12	F241L / V262I	65	S239D / N297D / I332E / A330Y / F241S / F243H / V262T / V264T
13	F241R / F243Q / V262T / V264R	66	S239D / N297D / I332E / K326E
14	F241R / F243Q / V262T / V264R / I332E	67	S239D / N297D / I332E / L235D
15	F241W / F243W / V262A / V264A	68	S239D / S298A / I332E
16	F241Y / F243Y / V262T / V264T	69	S239D / V264I / A330L / I332E
17	F241Y / F243Y / V262T / V264T / N297D / I332E	70	S239D / V264I / I332E
18	F243L / V262I / V264W	71	S239D / V264I / S298A / I332E
19	P243L / V264I	72	S239E / D265N
20	L328D / I332E	73	S239E / D265Q
21	L328E / I332E	74	S239E / I332D
22	L328H / I332E	75	S239E / I332E
23	L328I / I332E	76	S239E / I332N
24	L328M / I332E	77	S239E / I332Q
25	L328N / I332E	78	S239E / N297D / I332E
26	L328Q / I332E	79	S239E / V264I / A330Y / I332 E
27	L328T / I332E	80	S239E / V264I / I332 E

28	L328V / I332E	81	S239E / V264I / S298A / A330Y / I332E
29	N297D / A330Y / I332E	82	S239N / A330L / I332E
30	N297D / I332E	83	S239N / A330Y / I332E
31	N297D / I332E / S239D / A330L	84	S239N / I332D
32	N297D / S298A / A330Y / I 332E	85	S239N / I332E
33	N297D / T299L / I332E	86	S239N / I332N
34	N297D / T299F / I332E / N297D / T299H / I332E	87	S239N / I332Q
35	N297D / T299I / I332E	88	S239N1S298A / I332E
36	N297D / T299L / I332E	89	S239Q / I332D
37	N297D / T299V / I332E	90	S239Q / I332E
38	N297E / I332E	91	S239Q / I332N
39	N297S / I332E	92	S239Q / I332Q
40	P230A / E233D / I332E	93	S239Q / V264I / I332E
41	P244H / P245A / P247V	94	S298A / I332E
42	S239D / A330L / I332E	95	V264E / N297D / I332E
43	S239D / A330Y / I332E	96	V264I / A330L / I332E
44	S239D / A330Y / I332E / K326E	97	V264I / A330Y / I332E
45	S239D / A330Y / I332E / K326T	98	V264I / I332E
46	S239D / A330Y / I332E / L234I	99	V264I / S298A / I332E
47	S239D / A330Y / I332E / L235D	100	Y296D / N297D / I332E
48	S239D / A330Y / I332E / V240I	101	Y296E / N297D / I332 E
49	S239D / A330Y / I332E / V264T	102	Y296H / N297D / I332E
50	S239D / A330Y / I332E / V266I	103	Y296N / N297D / I332E
51	S239D / D265F / N297D / I332E	104	Y296Q / N297I / I332E
52	S239D / D265H / N297D / I332E	105	Y296T / N297D / I332E.
53	S239D / D265I / N297D / I332E		

[00193] Effector function can be modified by techniques such as those described in the Antibody Engineering Technology Art, or by other means. For example, cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region, resulting in the generation of a homodimeric antibody that may have improved internalization capability and/or increased complement-mediated cell killing and ADCC. See Caron *et al.* (1992) J. Exp Med. 176:1191-1195; and B. Shopes (1992) J. Immunol. 148:2918-2922. Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff *et al.* (1993) Cancer Research 53:2560-2565. Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. Stevenson *et al.* (1989) Anti-Cancer Drug Design 3:219-230.

[00194] The affinities and binding properties of the molecules of the invention for an FcγR are initially determined using *in vitro* assays (biochemical or immunological based assays) known in the art for determining Fc-FcγR interactions, *i.e.*, specific binding of an Fc region to an FcγR including but not limited to ELISA assay, surface plasmon resonance assay, immunoprecipitation assays (*See* Section 6.2.2). Preferably,

the binding properties of the molecules of the invention are also characterized by in vitro functional assays for determining one or more FcγR mediator effector cell functions (*See* Section 6.2.2). 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.

[00195] 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.

[00196] In a particular embodiment, the invention encompasses amino acid modification in the CH1 domain of the Fc region.

[00197] In particularly preferred embodiments, the invention encompasses molecules comprising a variant Fc region wherein said variant has an increased binding to FcγRIIA (CD32A) and/or an increased ADCC activity, as measured using methods known to one skilled in the art and exemplified herein. The ADCC assays used in accordance with the methods of the invention may be NK dependent or macrophage dependent.

[00198] The Fc variants of the present invention may be combined with other known Fc modifications including but not limited to modifications which alter effector function and modification which alter FcγR binding affinity. In a particular embodiment, an Fc variant of the invention comprising a first amino acid modification in the CH3 domain, CH2 domain or the hinge region may be combined with a second Fc modification such that the second Fc modification is not in the same domain as the

first so that the first Fc modification confers an additive, synergistic or novel property on the second Fc modification. In some embodiments, the Fc variants of the invention do not have any amino acid modification in the CH2 domain.

[00199] 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 which is incorporated herein by reference in its entirety). 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.

[00200] The FcγR interacting domain maps to the lower hinge region and select sites within the CH2 and CH3 domains of the IgG heavy chain. Amino acid residues flanking the actual contact positions and amino acid residues in the CH3 domain play a role in IgG/FcγR interactions as indicated by mutagenesis studies and studies using small peptide inhibitors, respectively (Sondermann *et al.*, 2000 *Nature*, 406: 267-273; Diesenhofer et al., 1981, Biochemistry, 20: 2361-2370; Shields et al., 2001, J. Biol. Chem. 276: 6591-6604; each of which is incorporated herein by reference in its entirety). Direct contact as used herein refers to those amino acids that are within at least 1 A, at least 2, or at least 3 angstroms of each other or within 1 Å, 1.2 Å, 1.5 Å, 1.7 Å or 2 Å Van Der Waals radius.

[00201] 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 via the Fc region with an altered affinity relative to a molecule comprising a wild-type Fc region, provided that said variant Fc region does not have or is 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 via the Fc region with an altered affinity relative to a molecule comprising a wild-type Fc region, provided that said variant Fc region does not have or is 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 at position 335; or a methionine at position 301.

[00202] In a specific embodiment, the invention encompasses a molecule comprising a variant Fc region, wherein said variant Fc region does not have or is 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, 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, 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 via the Fc region with a reduced affinity relative to a molecule comprising a wild-type Fc region provided that said variant Fc region does not have or is 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, 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 via the Fc region with an enhanced affinity relative to a molecule comprising a wild-type Fc region provided that said variant Fc region does not have or is 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.

[00203] 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 substitution at any of positions 330, 243, 247, 298, 241, 240, 244, 263, 262, 235, 269, or 328 and does not have a leucine at position 243, an asparagine at position 298, a leucine at position 241, and isoleucine or an alanine at position 240, a histidine at position 244, a valine at position 330, or an isoleucine at position 328.

[00204] 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); 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) ; or a substitution at position 243 with leucine, at position 292 with proline, at position 300 with leucine, at position 305 with isoleucine, and at position 396 with leucine (MgFc88); or a substitution at position 243 with leucine, at position 292 with proline, at position 300 with leucine, and at position 396 with leucine (MgFc88A); or a substitution at position 234 with leucine, at position 292 with proline, and at position 300 with leucine (MgFc155); or a substitution at position 243 with leucine, at position 292 with proline, and at position 300 with leucine; or a substitution at position 243 with leucine, at position 292 with proline, and at position 396 with leucine; or a substitution at position 243 with leucine, and at position 292 with proline; or a substitution at position 243 with leucine; or a substitution at position 273 with phenylalanine; or a substitution at position 247 with leucine, at position 270 with glutamic acid, and at position 421 with lysine.

[00205] In one specific embodiment, the invention encompasses a molecule comprising a variant Fc region wherein said variant Fc region comprises a substitution at position 396 with leucine, at position 270 with glutamic acid and at position 243 with leucine. In another specific embodiment the molecule further comprises one or more amino acid modification such as those disclosed herein.

[00206] 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: 119, 125, 132, 133, 141, 142, 147, 149, 162, 166, 185, 192, 202, 205, 210, 214, 217, 219, 215, 216, 217, 218, 219, 221, 222, 223, 224, 225, 227, 288, 229, 231, 232, 233, 234, 235, 240, 241, 242, 243, 244, 246, 247, 248, 250, 251, 252, 253, 254, 255, 256, 258, 261, 262, 263, 268, 269, 270, 272, 273, 274, 275, 276, 279, 280, 281, 282, 284, 287, 288, 289, 290, 291, 292, 293, 295, 298, 300, 301, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 315, 316, 317, 318, 319, 320, 323, 326, 327, 328, 330, 333, 334, 335, 337, 339, 340, 343, 344, 345, 347, 348, 352, 353, 354, 355, 358, 359, 360, 361, 362, 365, 366, 367, 369, 370, 371, 372, 375, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 392, 393, 394, 395, 396, 397, 398, 399, 400, 401, 402, 404, 406, 407, 408, 409, 410, 411, 412, 414, 415, 416417, 419, 420, 421, 422, 423, 424, 427, 428, 431, 433, 435, 436, 438, 440, 441, 442, 443, 446, 447. Preferably such mutations result in molecules that have an altered affinity for an FcγR

and/or have an altered effector cell mediated function as determined using methods disclosed and exemplified herein and known to one skilled in the art.

[00207] 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, 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, which is incorporated herein by reference in its entirety.

[00208] Glycosylation is a co-translational / post-translational modification that results in the attachment of a glucosylated high mannose oligosaccharide (GlcNAc2Man9Glu3), which is subsequently processed, first to a $GlcNAc_2Man_9$ ("Man9") structure (**Figure 41**), and then successively to produce a $GlcNAc_2Man_8$ ("Man8"), a $GlcNAc_2Man_7$ ("Man7"), a $GlcNAc_2Man_6$ ("Man6") and ultimately, a $GlcNAc_2Man_5$ ("Man5") structure. The $GlcNAc_2Man_5$ ("Man5") structure is then processed by the successive action of glycosyltransferases to generate a "G0F," "G1F" and "G2F" oligosaccharide that exhibits a complex diantennary structure (**Figure 42**) (Jefferis, R. (2005) "*Glycosylation of Recombinant Antibody Therapeutics*," Biotechnol. Prog. 21:11-16; Kornfeld, R. *et al.* (1985) "*Assembly Of Asparagine-Linked Oligosaccharides*," Ann. Rev. Biochem. 54:631-664).

[00209] 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, which is incorporated herein by reference in its entirety. 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.

[00210] 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; all of which are incorporated herein by reference in their entirety. 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 position 297 (*e.g.*, from asparagine to a residue without an available amine group, *e.g.*, glutamine) and/or positions adjacent to 297. In a specific embodiment, the invention encompasses modifying position 296 so that position 296 and not position 297 is glycosylated.

[00211] Fucosylated carbohydrate structures are involved in a variety of biological and pathological processes in eukaryotic organisms including tissue development, angiogenesis, fertilization, cell adhesion, inflammation, and tumor metastasis (Ma, B. *et al.* (Epub 2006 Sep 14) "*Fucosylation In Prokaryotes And Eukaryotes*," Glycobiology. 16(12):158R-184R). Therapeutic antibodies fully lacking the core fucose of the Fc oligosaccharides have been found to exhibit much higher ADCC in humans than their fucosylated counterparts (Iida, S. *et al.* (2009) "*Two Mechanisms Of The Enhanced Antibody-Dependent Cellular Cytotoxicity (ADCC) Efficacy Of Non-Fucosylated Therapeutic Antibodies In Human Blood*," BMC Cancer. 18:9:58). However, the production of such antibodies has previously required the enzymatic removal of fucose residues (for example, using N-glycosidase F) that had been added to the antibodies by a glycosylase.

[00212] One aspect of the present invention relates to the recognition that variations in the Fc region of an antibody can interfere with the cellular glycosylation mechanism and thereby yield antibodies that exhibit a decreased extent of glycosylation (and in particular, of fucosylation).

[00213] The invention thus provides a means for making antibodies having a decreased extent of glycosylation (and in particular, of fucosylation) by substituting one, or more two, three, four, five or more of the native Fc residues to form a variant Fc region. Without intending to be bound by any theory of mechanism of action, it is believed that suitable substitutions yield a polypeptide having a variant Fc region that is either less fucosylated than a native Fc region, or is not fucosylated at all, and that such polypeptides, due to their altered (or absent) extent of glycosylation (and in particular, of fucosylation) exhibit improved effector function, relative to polypeptides having a native Fc region. Polynucleotides encoding polypeptides that comprise such variant Fc regions can therefore be introduced into host cells (*e.g.*, CHO cells, yeast, etc.), including host cells that are capable of mediating normal glycosylation (and in particular, normal fucosylation), but will be expressed as polypeptides that are less post-translationally modified by glycosylase (or fucosylase) or are not post-translationally modified by such enzymes, relative to polypeptides comprising native Fc regions and thereby exhibit improved effector function (*e.g.*, improved binding to the activating receptors (*e.g.*, CD16A, CD32A) and reduced binding to CD32B. Such improvement of effector function may be measured using an off-rate analysis. Such analysis provides the best indicator for potential improvement of in vivo FcγR binding

activity because as a kinetic variable it directly reflects the Fc-FcγR interaction independent of antibody concentration.

[00214] As used herein, such decreased extent of glycosylation (and in particular, of fucosylation) is preferably less than 80%, more preferably less than 60%, still more preferably less than 40%, and most preferably less than 20% of the extent of glycosylation (and in particular, of fucosylation) exhibited by such antibody on the absence of such variation in its Fc region. In a more preferred embodiment, such variations in the Fc region of the antibody will substantially eliminate or fully eliminate the extent of glycosylation (and in particular, of fucosylation) exhibited by such antibody. The ability of such Fc variants to decrease the extent of glycosylation (and in particular, of fucosylation) is a general characteristic and is exemplified herein with respect to anti-Her2/neu antibodies.

The invention particularly concerns polypeptides (*e.g.*, antibodies) that possess variant Fc regions that result in an enhanced ratio of high manose oligosaccharide glycosylation to complex oligosaccharide glycosylation. As used herein, such a ratio denotes a comparsison of the extent to which the glycosylation exhibited by an Fc region is a complex oligosaccharide of either G0F, G1F or G2F (see, **Figure 42**) relative to the extent to which the glycosylation exhibited by an Fc region is Man5, Man6, Man7, Man8 or Man9 (see, **Figure 41**). The ratio of high manose oligosaccharide glycosylation to complex oligosaccharide glycosylation is therefore: Σ (% Man5 + % Man6 + % Man7 + % Man8 + % Man9) : Σ (% G0F + % G1F + % G2F). Preferably, the enhanced ratio of high manose oligosaccharide glycosylation to complex oligosaccharide glycosylation will be greater than about 0.2. More preferably, this ratio will be greater than about 0.5, still more preferably this ratio will be greater than about 1.0, greater than about 1.5, greater than about 2.0, greater than about 2.5, greater than about 3.0, greater than about 3.5, greater than about 4.0, greater than about 4.5, greater than about 5.0, greater than about 5.5, or greater than 6.0. Most preferably, the upper ranges of such ratio will be less than about 10, more preferably, less than about 9.5, less than about 9, less than about 8.5, less than about 8, less than about 7.5, less than about 7, less than about 6.5, less than about 6, or less than about 5.5. Specific contemplated ranges of such ratio include greater than about 0.2 but less than about 5.5; greater than about 0.5 but less than about 5.5; greater than about 1.0 but less than about 5.5; greater than about 2.0 but less than about 5.5; greater than about 3.0 but less than

about 5.5; greater than about 4.0 but less than about 5.5; and greater than about 5.0 but less than about 5.5.

[00215] Preferably, Fc variants exhibiting a decreased extent of glycosylation (and in particular, of fucosylation) comprise an amino acid substitution of either or both of positions L234 and/or L235. More preferably, such Fc variants will further comprise at least one additional amino acid substitution at any or all of positions: F243, R292, Y300, V305, and/or P396. Thus, preferred Fc variants exhibiting a decreased extent of glycosylation (and in particular, of fucosylation) comprise amino acid substitutions at either or both of positions L234 and/or L235, and also at position F243; position R292; position Y300; position V305; position P396; positions F243 and R292; positions F243 and Y300; positions F243 and V305; positions F243 and P396; positions R292 and Y300; positions R292 and V305; positions R292 and P396; positions Y300 and V305; positions Y300 and P396; positions V305 and P396; positions F243, R292 and Y300; positions F243, R292 and V305; positions F243, R292 and P396; positions F243, Y300 and V305; positions F243, Y300 and P396; positions F243, V305 and P396; positions R292, Y300 and V305, positions R292, Y300 and P396; positions R292, V305 and P396; positions Y300, V305 and P396; positions F243, R292, Y300 and V305; positions F243, R292, Y300 and P396; positions F243, R292, V305 and P396; positions F243, Y300, V305 and P396; positions R292, Y300, V305 and P396; or positions F243, R292, Y300, V305 and P396. The L234 substitutions L234F and/or the L235 substitution L235V are particularly preferred.

6.1 POLYPEPTIDES AND ANTIBODIES WITH VARIANT Fc REGIONS

[00216] It will be appreciated by one skilled in the art that aside from amino acid substitutions, the present invention contemplates other modifications of the Fc region amino acid sequence in order to generate an Fc region variant with one or more altered properties, *e.g.*, altered effector function. The invention contemplates deletion of one or more amino acid residues of the Fc region in order to reduce binding to an FcγR. Preferably, no more than 5, no more than 10, no more than 20, no more than 30, no more than 50 Fc region residues will be deleted according to this embodiment of the invention. The Fc region herein comprising one or more amino acid deletions will preferably retain at least about 80%, and preferably at least about 90%, and most preferably at least about 95%, of the wild type Fc region. In some embodiments, one or

more properties of the molecules are maintained such as for example, non-immunogenicity, FcγRIIIA binding, FcγRIIA binding, or a combination of these properties.

[00217] In alternate embodiments, the invention encompasses amino acid insertion to generate the Fc region variants, which variants have altered properties including altered effector function. In one specific embodiment, the invention encompasses introducing at least one amino acid residue, for example one to two amino acid residues and preferably no more than 10 amino acid residues adjacent to one or more of the Fc region positions identified herein. In alternate embodiments, the invention further encompasses introducing at least one amino acid residue, for example one to two amino acid residues and preferably no more than 10 amino acid residues adjacent to one or more of the Fc region positions known in the art as impacting FcγR interaction and/or binding.

[00218] The invention further encompasses incorporation of unnatural amino acids to generate the Fc variants of the invention. Such methods are known to those skilled in the art such as those using the natural biosynthetic machinery to allow incorporation of unnatural amino acids into proteins, *see*, *e.g.*, Wang et al., 2002 Chem. Comm. 1: 1-11; Wang et al., 2001, Science, 292: 498-500; van Hest et al., 2001. Chem. Comm. 19: 1897-1904, each of which is incorporated herein by reference in its entirety. Alternative strategies focus on the enzymes responsible for the biosynthesis of amino acyl-tRNA, *see*, e.g., Tang et al., 2001, J. Am. Chem. 123(44): 11089-11090; Kiick et al., 2001, FEBS Lett. 505(3): 465; each of which is incorporated herein by reference in its entirety.

[00219] The affinities and binding properties of the Fc variants, or fragments thereof, of use in the invention are intially determined using a yeast-display system, preferably combined with *in vitro* assays (biochemical or immunological based assays) known in the art for determining Fc-FcγR interactions, *i.e.*, specific binding of an Fc region to an FcγR including but not limited to ELISA assay, surface plasmon resonance assay, immunoprecipitation assays (*See* Section 6.2.1). In certain embodiments, candidate Fc variants identified using the yeast display system are further incorporated into an antibody or fragment thereof for testing in said *in vitro* assay. Preferably, the binding properties of the molecules of the invention are also characterized by in vitro functional assays for determining one or more FcγR mediator effector cell functions (*See* Section

6.2.5). Such methods have previously been disclosed by the inventors, see, *e.g.*, U.S Patent Application Publications 2005/0037000 and 2005/0064514, and International Patent Application Publication WO 04/063351, each of which is hereby incorporated by reference in its entirety, and have been used to identify and characterize novel Fc mutations based on binding characteristics to FcγRIIIA and FcγRIIB, see, *e.g.*, **Table 5**.

TABLE 5
Fc MUTATIONS IDENTIFIED USING YEAST DISPLAY AND ELISA ASSAY

Clone #	Mutation sites	Domain	IIIA binding	IIB binding
4	A339V, Q347H	CH2, CH3	+	+
5	L251P, S415I	CH2, CH3	+	+
8	V185M, K218N, R292L, D399E	CH1,hinge,CH2, CH3	no change	-
12	K290E, L142P	CH1,CH2	+	not tested
16	A141V, H268L, K288E, P291S	CH1,CH2	-	not tested
19	L133M, P150Y, K205E, S383N, N384K	CH1,CH2,CH3	-	not tested
21	P396L	CH3	•	•+
25	P396H	CH3	•••	••
6	K392R	CH3	no change	no change
15	R301C, M252L, S192T	CH1,CH2	-	not tested
17	N315I	CH2	no change	not tested
18	S132I	CH1	no change	not tested
26	A162V	CH1	no change	not tested
27	V348M, K334N, F275I, Y202M, K147T	CH1,Ch2	+	+
29	H310Y, T289A, G337E	CH2	-	not tested
30	S119F, G371S, Y407N, E258D	CH1,CH2,CH3	+	no change
31	K409R, S166N	CH1,CH3	no change	not tested
20	S408I, V215I, V125I	CH1,Hinge,CH3	+	no change
24	G385E, P247H	CH2, CH3	•••	+
16	V379M	CH3	••	no change
17	S219Y	Hinge	•	-
18	V282M	CH2	•	-
31	F275I, K334N, V348M	CH2	+	no change
35	D401V	CH3	+	no change
37	V280L, P395S	CH2	+	-

TABLE 5
Fc MUTATIONS IDENTIFIED USING YEAST DISPLAY AND ELISA ASSAY

Clone #	Mutation sites	Domain	IIIA binding	IIB binding
40	K222N	Hinge	•	no change
41	K246T, Y319F	CH2	•	no change
42	F243I, V379L	CH2,CH3	•+	-
43	K334E	CH2	•+	-
44	K246T, P396H	CH2,CH3	•	••+
45	H268D, E318D	CH2	•+	•••••
49	K288N, A330S, P396L	CH2,CH3	•••••	•••
50	F243L, R255L, E318K	CH2	•	-
53	K334E, T359N, T366S	CH2,CH3	•	no change
54	I377F	CH3	•+	+
57	K334I	CH2	•	no change
58	P244H, L358M, V379M, N384K, V397M	CH2,CH3	•+	•+
59	K334E, T359N, T366S (independent isolate)	CH2,CH3	•+	no change
61	I377F (independent isolate)	CH3	•••	••+
62	P247L	CH2	••	••+
64	P217S, A378V, S408R	Hinge, CH3	••	••••+
65	P247L, I253N, K334N	CH2	•••	••+
66	K288M, K334E	CH2	•••	-
67	K334E, E380D	CH2,CH3	•+	-
68	P247L (independent isolate)	CH2	+	••••
69	T256S, V305I, K334E, N390S	CH2,CH3	•+	no change
70	K326E	CH2	•+	••+
71	F372Y	CH3	+	•••••+
72	K326E (independent isolate)	CH2	+	••
74	K334E, T359N, T366S (independent isolate)	CH2,CH3	••	no change
75	K334E (independent isolate)	CH2	••+	no change
76	P396L (independent isolate)	CH3	•+	no change
78	K326E (independent isolate)	CH2	••	•••+
79	K246I, K334N	CH2	•	••••
80	K334E (independent isolate)	CH2	•	no change
81	T335N, K370E, A378, T394M, S424L	CH2,CH3	•	no change

TABLE 5
Fc MUTATIONS IDENTIFIED USING YEAST DISPLAY AND ELISA ASSAY

Clone #	Mutation sites	Domain	IIIA binding	IIB binding
82	K320E, K326E	CH2	•	•
84	H224L	Hinge	•	•••••
87	S375C, P396L	CH3	•+	••••+
89	E233D, K334E	CH2	•+	**no change**
91	K334E (independent isolate)	CH2	•	**no change**
92	K334E (independent isolate)	CH2	•	**no change**
94	K334E, T359N, T366S, Q386R	CH2	•	**no change**

relative to comparable molecule with wild-type Fc region, • ≡ 1-fold increase in affinity; + ≡ up to 50% increase in affinity; and - ≡ 1-fold decrease in affinity

[00220] In most preferred embodiments, the molecules of the invention have similar binding properties in in vivo models (such as those disclosed herein and/or known in the art) 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 representative flow chart of the screening and characterization of molecules of the invention is described in FIG. 1.

[00221] The invention encompasses molecules comprising a variant Fc region that binds with a greater affinity to one or more FcγRs. Such molecules preferably mediate effector function more effectively as discussed *infra.* In other embodiments, the invention encompasses molecules comprising a variant Fc region that bind with a weaker affinity to one or more FcγRs. Reduction or elimination of effector function is desirable in certain cases for example in the case of antibodies whose mechanism of action involves blocking or antagonism but not killing of the cells bearing a target antigen. Reduction or elimination of effector function would be desirable in cases of autoimmune disease where one would block FcγR activating receptors in effector cells (This type of function would be present in the host cells). In general increased effector function would be directed to tumor and foreign cells.

[00222] The Fc variants of the present invention may be combined with other Fc modifications, including but not limited to modifications that alter effector function. The invention encompasses combining an Fc variant of the invention with other Fc modifications to provide additive, synergistic, or novel properties in antibodies or Fc

fusions. Preferably the Fc variants of the invention enhance the phenotype of the modification with which they are combined. For example, if an Fc variant of the invention is combined with a mutant known to bind FcγRIIIA with a higher affinity than a comparable molecule comprising a wild type Fc region; the combination with a mutant of the invention results in a greater fold enhancement in FcγRIIIA affinity.

[00223] In one embodiment, the Fc variants of the present invention may be combined with other known Fc variants such as those disclosed in Duncan et al, 1988, Nature 332:563-564; Lund et al., 1991, J. Immunol 147:2657-2662; Lund et al, 1992, Mol Immunol 29:53-59; Alegre et al, 1994, Transplantation 57:1537-1543; Hutchins et al. , 1995, Proc Natl. Acad Sci U S A 92:11980-11984; Jefferis et al, 1995, Immunol Lett. 44:111-117; Lund et al., 1995, Faseb J 9:115-119; Jefferis et al, 1996, Immunol Lett 54:101-104; Lund et al, 1996, J Immunol 157:49634969; Armour et aL, 1999, Eur J Immunol 29:2613-2624; Idusogie et al, 2000, J Immunol 164:41784184; Reddy et al, 2000, J Immunol 164:1925-1933; Xu et al., 2000, Cell Immunol 200:16-26; Idusogie et al, 2001, J Immunol 166:2571-2575; Shields et al., 2001, J Biol Chem 276:6591-6604; Jefferis et al, 2002, Immunol Lett 82:57-65; Presta et al., 2002, Biochem Soc Trans 30:487-490); US 5,624,821; US 5,885,573; US 6,194,551; PCT WO 00/42072; PCT WO 99/58572; each of which is incorporated herein by reference in its entirety.

[00224] In some embodiments, the Fc variants of the present invention are incorporated into an antibody or Fc fusion that comprises one or more engineered glycoforms, *i.e.*, a carbohydrate composition that is covalently attached to a molecule comprising an Fc region, wherein said carbohydrate composition differs chemically from that of a parent molecule comprising an Fc region. Engineered glycoforms may be useful for a variety of purposes, including but not limited to enhancing or reducing effector function. Engineered glycoforms may be generated by any method known to one skilled in the art, for example by using engineered or variant expression strains, by co-expression with one or more enzymes, for example DI N-acetylglucosaminyltransferase III (GnTI11), by expressing a molecule comprising an Fc region in various organisms or cell lines from various organisms, or by modifying carbohydrate(s) after the molecule comprising Fc region has been expressed. Methods for generating engineered glycoforms are known in the art, and include but are not limited to those described in Umana et al, 1999. Nat. Biotechnol 17:176-180; Davies et al., 20017 Biotechnol Bioeng 74:288-294; Shields et al, 2002, J Biol Chem 277:26733-

26740; Shinkawa et aL, 2003, J Biol Chem 278:3466-3473) US 6,602,684; USSN 10/277,370; USSN 10/113,929; PCT WO 00/61739A1; PCT WO 01/292246A1; PCT WO 02/311140A1; PCT WO 02/30954A1; Potillegent™ technology (Biowa, Inc. Princeton, NJ); GlycoMAb™ glycosylation engineering technology (GLYCART biotechnology AG, Zurich, Switzerland); each of which is incorporated herein by reference in its entirety. See, e.g., WO 00061739; EA01229125; US 20030115614; Okazaki et al., 2004, JMB, 336: 1239-49 each of which is incorporated herein by reference in its entirety.

[00225] The Fc variants of the present invention may be optimized for a variety of properties. Properties that may be optimized include but are not limited to enhanced or reduced affinity for an FcγR, enhanced or reduced effector function. In a preferred embodiment, the Fc variants of the present invention are optimized to possess enhanced affinity for a human activating FcγR, preferably FcγR, FcγRIIA, FcγRIIc, FcγRIIIA, and FcγRIIIB, most preferably FcγRIIIA. In an alternate preferred embodiment, the Fc variants are optimized to possess reduced affinity for the human inhibitory receptor FcγRIIB. These preferred embodiments are anticipated to provide antibodies and Fc fusions with enhanced therapeutic properties in humans, for example enhanced effector function and greater anti-cancer potency as described and exemplified herein. These preferred embodiments are anticipated to provide antibodies and Fc fusions with enhanced tumor elimination in mouse xenograft tumor models.

[00226] In an alternate embodiment the Fc variants of the present invention are optimized to have reduced affinity for a human FcγR, including but not limited to FcγRI, FcγRIIA, FcγRIIB, FcγRIIc, FcγRIIIA, and FcγRIIIB. These embodiments are anticipated to provide antibodies and Fc fusions with enhanced therapeutic properties in humans, for example reduced effector function and reduced toxicity.

[00227] In alternate embodiments the Fc variants of the present invention possess enhanced or reduced affinity for FcγRs from non-human organisms, including but not limited to mice, rats, rabbits, and monkeys. Fc variants that are optimized for binding to a non-human FcγR may find use in experimentation. For example, mouse models are available for a variety of diseases that enable testing of properties such as efficacy, toxicity, and pharmacokinetics for a given drug candidate. As is known in the art, cancer cells can be grafted or injected into mice to mimic a human cancer, a process referred to as xenografting. Testing of antibodies or Fc fusions that comprise Fc

variants that are optimized for one or more mouse FcγRs, may provide valuable information with regard to the efficacy of the antibody or Fc fusion, its mechanism of action, and the like. In certain embodiments, molecules of the invention comprising a variant human Fc region are tested in transgenic mice expressing one or more human Fcγ receptors (*e.g.*, FcγRIIIA, FcγRIIA, FcγRIIB).

[00228] While it is preferred to alter binding to an FcγR, the instant invention further contemplates Fc variants with altered binding affinity to the neonatal receptor (FcRn). Although not intending to be bound by a particular mechanism of action, Fc region variants with improved affinity for FcRn are anticipated to have longer serum half-lives, and such molecules will have useful applications in methods of treating mammals where long half-life of the administered polypeptide is desired, *e.g.*, to treat a chronic disease or disorder. Although not intending to be bound by a particular mechanism of action, Fc region variants with decreased FcRn binding affinity, on the contrary, are expected to have shorter half-lives, and such molecules may, for example, be administered to a mammal where a shortened circulation time may be advantageous, *e.g.*, for in vivo diagnostic imaging or for polypeptides which have toxic side effects when left circulating in the blood stream for extended periods. Fc region variants with decreased FcRn binding affinity are anticipated to be less likely to cross the placenta, and thus may be utilized in the treatment of diseases or disorders in pregnant women.

[00229] In other embodiments, these variants may be combined with other known Fc modifications with altered FcRn affinity such as those disclosed in International Publication Nos. WO 98/23289; and WO 97/34631; and U.S. Patent No. 6,277,375, each of which is incorporated herein by reference in its entirety.

[00230] The invention encompasses any other method known in the art for generating antibodies having an increased half-life *in vivo*, for example, by introducing one or more amino acid modifications (*i.e.*, substitutions, insertions or deletions) into an IgG constant domain, or FcRn binding fragment thereof (preferably a Fc or hinge-Fc domain fragment). See, *e.g.*, International Publication Nos. WO 98/23289; and WO 97/34631; and U.S. Patent No. 6,277,375, each of which is incorporated herein by reference in its entirety to be used in combination with the Fc variants of the invention. Further, antibodies of the invention can be conjugated to albumin in order to make the antibody or antibody fragment more stable *in vivo* or have a longer half-life *in vivo*. The techniques well-known in the art, see, *e.g.*, International Publication Nos. WO

93/15199, WO 93/15200, and WO 01/77137, and European Patent No. EP 413,622, all of which are incorporated herein by reference in their entirety.

[00231] The variant(s) described herein may be subjected to further modifications, often times depending on the intended use of the variant. Such modifications may involve further alteration of the amino acid sequence (substitution, insertion and/or deletion of amino acid residues), fusion to heterologous polypeptide(s) and/or covalent modifications. Such further modifications may be made prior to, simultaneously with, or following, the amino acid modification(s) disclosed herein which results in altered properties such as an alteration of Fc receptor binding and/or ADCC activity.

[00232] Alternatively or additionally, the invention encompasses combining the amino acid modifications disclosed herein with one or more further amino acid modifications that alter C1q binding and/or complement dependent cytoxicity function of the Fc region as determined in vitro and/or in vivo. Preferably, the starting molecule of particular interest herein is usually one that binds to C1q and displays complement dependent cytotoxicity (CDC). The further amino acid substitutions described herein will generally serve to alter the ability of the starting molecule to bind to C1q and/or modify its complement dependent cytotoxicity function, *e.g.*, to reduce and preferably abolish these effector functions. In other embodiments molecules comprising substitutions at one or more of the described positions with improved C1q binding and/or complement dependent cytotoxicity (CDC) function are contemplated herein. For example, the starting molecule may be unable to bind C1q and/or mediate CDC and may be modified according to the teachings herein such that it acquires these further effector functions. Moreover, molecules with preexisting C1q binding activity, optionally further having the ability to mediate CDC may be modified such that one or both of these activities are altered, *e.g.*, enhanced. In some embodiments, the invention encompasses variant Fc regions with altered CDC activity without any alteration in C1q binding. In yet other embodiments, the invention encompasses variant Fc regions with altered CDC activity and altered C1q binding.

[00233] To generate an Fc region with altered C1q binding and/or complement dependent cytotoxicity (CDC) function, the amino acid positions to be modified are generally selected from positions 270, 322, 326, 327, 329, 331, 333, and 334, where 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, National Institutes of Health, Bethesda, Md. (199). These amino acid modifications

may be combined with one or more Fc modifications disclosed herein to provide a synergistic or additive effect on C1q binding and/or CDC activity. In other embodiments, the invention encompasses Fc variants with altered C1q binding and/or complement dependent cytotoxicity (CDC) function comprising an amino acid substitution at position 396 with leucine and at position 255 with leucine; or an amino acid substitution at position 396 with leucine and at position 419 with histidine; an amino acid substitution at position 396 with leucine and at position 370 with glutamic acid; an amino acid substitution at position 396 with leucine and at position 240 with alanine; an amino acid substitution at position 396 with leucine and at position 392 with threonine; an amino acid substitution at position 247 with leucine and at position 421 with lysine. The invention encompasses any known modification of the Fc region which alters C1q binding and/or complement dependent cytotoxicity (CDC) function such as those disclosed in Idusogie *et al.*, 2001, *J. Immunol.* 166(4) 2571-5; Idusogie *et al.*, *J. Immunol.* 2000 164(8): 4178-4184; each of which is incorporated herein by reference in its entirety.

[00234] As disclosed above, the invention encompasses an Fc region with altered effector function, *e.g.*, modified C1q binding and/or FcR binding and thereby altered CDC activity and/or ADCC activity. In specific embodiments, the invention encompasses variant Fc regions with improved C1q binding and improved FcγRIII binding; *e.g.* having both improved ADCC activity and improved CDC activity. In alternative embodiments, the invention encompasses a variant Fc region with reduced CDC activity and/or reduced ADCC activity. In other embodiments, one may increase only one of these activities, and optionally also reduce the other activity, *e.g.* to generate an Fc region variant with improved ADCC activity, but reduced CDC activity and vice versa.

6.1.1 MUTANTS WITH ENHANCED ALTERED AFFINITIES FOR FcγRIIIA and/or FcγRIIA

[00235] 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 FcγR. 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

FcγRIIIA and/or FcγRIIA 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 FcγRIIIA and/or FcγRIIA 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 FcγRIIIA and/or FcγRIIA 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 FcγRIIIA and/or FcγRIIA 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 modifications 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 modifications do 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.

[00236] 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 via it Fc region with a greater affinity than a comparable molecule comprising the wild-type Fc region binds FcγRIIA, 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 position322; 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 FcγRIIA 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 FcγRIIA 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 FcγRIIA 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

[00237] 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 FcγRIIIA and/or FcγRIIA 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.

[00238] In a specific embodiment, the one or more amino acid modifications which increase the affinity of the variant Fc region for one or more activating FcγRs 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 threonineand 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 positon 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 243 with leucine, at position 292 with proline, at position 300 with leucine, at position 305 with isoleucine, and at position 396 with leucine; or a substitution at position 243 with leucine, at position 292 with proline, at position 300 with leucine, and at position 396 with leucine; or a substitution at position 234 with leucine, at position 292 with proline, and at position 300 with leucine; or a substitution at position 234 with leucine, at position 292 with proline, and at position 396 with leucine; or a substitution at position 234 with leucine, at position 292 with proline, and at position 305 with isoleucine; or a substitution at position 234 with

leucine and at position 292 with proline; or a substitution at position 234 with leucine; or a substitution at position 247 with leucine, at position 270 with glutamic acid, and at position 421 with lysine. Examples of other amino acid substitutions that result in an enhanced affinity for FcγRIIIA in vitro are disclosed below and summarized in **Table 3.**

[00239] 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.

[00240] 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 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 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 FcγRIIIA 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 FcγRIIIA 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 FcγRIIIA 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 FcγRIIIA 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 377 with phenylalaine. 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 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 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 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 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 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 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 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 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 224 with leucine.

[00241] 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 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 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 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 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 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 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 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 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 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 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 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 FcγRIIIA 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 FcγRIIIA 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 FcγRIIIA 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 FcγRIIIA 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. 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 243 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 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

292 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 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 300 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 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 305 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 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 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 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 273 with phenylalanine.

[00242] 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 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 FcγRIIIA 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.

[00243] In some embodiments, the molecules of the invention have an altered affintiy for FcγRIIIA and/or FcγRIIA as determined using *in vitro* assays (biochemical or immunological based assays) known in the art for determining Fc-FcγR interactions, *i.e.*, specific binding of an Fc region to an FcγR including but not limited to ELISA assay, surface plasmon resonance assay, immunoprecipitation assays (*See* Section 6.2.5.1). Preferably, the binding properties of these molecules with altered affinities for activating FcγR receptors are also correlated to their activity as determined by in vitro functional assays for determining one or more FcγR mediator effector cell functions (*See* Section 6.2.7), *e.g.*, molecules with variant Fc regions with enhanced affinity for FcγRIIIA 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.*, FcγRIIIA 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 FcγR binding in in vitro based assays but do exhibit the desired phenotype *in vivo*.

6.1.2 MUTANTS WITH ENHANCED AFFINITY FOR FcγRIIIA AND REDUCED OR NO AFFINITY FOR FcγRIIB

[00244] 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 FcγRIIIA and decreases the affinity of the variant Fc region for FcγRIIB, relative to a comparable molecule comprising a wild-type Fc region which binds FcγRIIIA and FcγRIIB 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 FcγRIIIA 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 leat 400% and decreases the affinity of the variant Fc region for FcγRIIB 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 leat 400%.

[00245] In a specific embodiment, the molecule of the invention comprising a variant Fc region with an enhanced affinity for FcγRIIIA and a lowered affinity or no affinity for FcγRIIB, as determined based on an ELISA assay and/or an ADCC based assay using ch-4-4-20 antibody, or a surface plasmon resonance assay using a chimeric 4D5 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, as 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; or a substitution at position 243 with

leucine, at position 292 with proline, at position 305 with isoleucine, and at position 396 with leucine; or a substitution at position 243 leucine, at position 292 with proline, and at position 305 with isoleucine; or a substitution at position 292 with proline, at position 305 with isoleucine, and at position 396 with leucine; or a substitution at position 243 with leucine, and at position 292 with proline; or a substitution at position 292 with proline; or a substitution at position 243 with leucine, at position 292 with proline, and at position 396 with leucine; or a substitution at position 243 with leucine, at position 292 with proline, at position 300 with leucine; or a substitution at position 243 with leucine.

[00246] In a specific embodiment, the molecule of the invention comprising a variant Fc region with an enhanced affinity for FcγRIIIA and a lowered affinity or no affinity for FcγRIIB 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. In yet another specific embodiment, the molecule of the invention comprising a variant Fc region with an enhanced affinity for FcγRIIIA and a lowered affinity or no affinity for FcγRIIB as determined based on an ELISA assay and/or an ADCC based assay using ch-4-4-20 antibody, or a surface plasmon resonance assay using a chimeric 4D5 antibody, carrying the variant Fc region comprises a substitution at position 243 with leucine; at position 292 with proline; and at position 300 with leucine.

[00247] 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 FcγRIIB 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 FcγRIIB 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 FcγRIIB 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 FcγRIIB 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 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 FcγRIIB 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 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 FcγRIIB with about a 5 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 wild-type Fc region, such that said polypeptide specifically binds FcγRIIB 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.

6.1.3 MUTANTS WITH ENHANCED AFFINITY TO FcγRIIIA AND FcγRIIB

[00248] 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 FcγRIIIA and FcγRIIB 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 leat 400% and decreases the affinity of the variant Fc region for FcγRIIB 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 leat 400%. In a specific embodiment, the molecule of the invention comprising a variant Fc region with an enhanced affinity for FcγRIIIA and an enhanced affinity for FcγRIIB (as determined based on an ELISA assay and/or an ADCC based assay using ch-4-4-20 antibody, or a surface plasmon resonance assay using a chimeric 4D5 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 positon 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 postion 320 with glutamic acid and at position 326 with glutamic acid; or a substitution at positoion 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 substition 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 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 with isoleucine; or a substitution at position 247 with leucine, at position 313 with arginne, 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 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 substitution at position 231 with valine, at position 386 with histidine, and at position 412 with methionine; or a substitution at position 215 with proline, at position 274 with asparagine, at position 287 with glycine, at position 334 with asparagine, at positon 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 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 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 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 woth 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; or a substitution at position 243 with leucine, at position 292 with proline, at position 300 with leucine, at position 305 with isoleucine, and at position 396 with leucine; or a substitution at position 243 with leucine, at position 292 with proline, at position 300 with leucine, and at position 396 with leucine; or a substitution at position 243 with leucine, and at position 396 with leucine; or at position 292 with proline, and at position 305 with isoleucine.

6.1.4 MUTANTS THAT DO NOT BIND ANY FcγR

[00249] 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 emobodiment, 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 postion 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.

6.1.5 MUTANTS WITH ALTERED FcγR-MEDIATED EFFECTOR FUNCTIONS

[00250] The invention encompasses immunoglobulin comprising Fc variants with altered effector functions. In some embodiments, immunoglobulins comprising Fc variants mediate effector function more effectively in the presence of effector cells as determined using assays known in the art and exemplified herein. In other embodiments, immunoglobulins comprising Fc variants mediate effector function less effectively in the presence of effector cells as determined using assays known in the art and exemplified herein. In specific embodiments, the Fc variants of the invention may be combined with other known Fc modifications that alter effector function, such that

the combination has an additive, synergistic effect. The Fc variants of the invention have altered effector function in vitro and/or in vivo.

[00251] 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 6.2.6.

[00252] In a specific embodiment, the immunoglobulins of the invention comprising a variant Fc region with enhanced affinity for FcγRIIIA and/or FcγRIIA 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 FcγRIIIA and/or FcγRIIA 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 10^4-fold, at least 10^5-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 FcγRIIIA and/or FcγRIIA have altered C1q binding activity. In some embodiments, the immunoglobulins of the invention with enhanced affinity for FcγRIIIA and/or FcγRIIA 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 10^4-fold, at least 10^5-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 FcγRIIIA and/or FcγRIIA have altered complement dependent cytotoxicity. In yet another specific embodiment, the immunoglobulins of the invention with enhanced affinity for FcγRIIIA and/or FcγRIIA 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 FcγRIIIA and/or FcγRIIA 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 10^4-fold, at least 10^5-fold higher

complement dependent cytotoxicity than an immunoglobulin comprising a wild-type Fc region.

[00253] In certain embodiments, the Fc variants of the invention may be combined with or comprise any of the Fc variants previously identified by the inventors to modulate effector function as disclosed in U.S Patent Application Publications 2005/0037000 and 2005/0064514, and International Patent Application Publication WO 04/063351, each of which is hereby incorporated by reference in its entirety. Examples of such Fc variants previously identified by the authors are provided in **Tables 6 and 7** *infra*.

[00254] In other embodiments, immunoglobulins of the invention with enhanced affinity for FcγRIIIA and/or FcγRIIA 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 FcγRIIIA and/or FcγRIIA 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.

Table 6
Summary of ADCC Activity of Mutants in ch4D5

	Fc Variant		ADCC			
Label	Ref	Amino Acid Variation	1 μg/ml		0.5 μg/ml	
			% Specific Lysis	Normalized	% Specific Lysis	Normalized
MGFc-27	2C4	G316D, A378V, D399E	33%	2.24	22%	3.60
MGFc-31	3B9	P247L, N421K	30%	2.05	17%	2.90
MGFc-10	1E1	K288N, A330S, P396L	24%	1.66	10%	1.67
MGFc-28	2C5	N315I, V379M, T394M	20%	1.37	10%	1.69
MGFc-29	3D11	F243I, V379L, G420V	20%	1.35	7%	1.17
CH4-4-20 (P54008)			15%	1.00	6	1.00
MGFc-35	3D2	R255Q, K326E	11%	0.79	3%	0.53
MGFc-36	3D3	K218R, G281D, G385R	10%	0.67	5%	0.78
MGFc-30	3A8	F275Y	9%	0.64	2%	0.37
MGFc-32	3C8	D280E, S354F, A431D,L441I	9%	0.62	4%	0.75
MGFc-33	3C9	K317N, F423Δ	3%	0.18	-1%	-0.22
MGFc-34	3B10	F421L, E258G	-1%	-0.08	-4%	-0.71
MGFc-26		D265A	1%	0.08	-3%	-0.45

6.2

Table 7: SUMMARY OF MUTANTS

Fc Variant	Amino Acid changes	FcR3A, K_D/K_{off}	FcR2B, K_D/K_{off}	ELISA IIIA binding	ELISA IIB binding	Phagocytosis (mutant/WT)	4-4-20 ADCC (mutant/wt)	Anti-HER2 ADCC (mutant/wt)
Wt	**none**	**198/0.170**	**94/.094**	1	1	1	1	1
MGFc 5	V379M	160/0.167	70/0.10	2X	N/C	0.86	2.09	1.77
MGFc 9	P243I, V379L	99.7/0.105	120/0.113	1.5X	reduced	?	2.25	2.04
MGFc 10	K288N, A330S, P396L	128/0.115	33.4/0.050	5X	3X	1.2	2.96	2.50
MGFc 11	F243L, R255L	90/0.075	74.7/0.09	1x	reduced	0.8	2.38	1.00
MGFc13	K334E, T359N, T366S	55.20.128	72/0.11	1.5X	N/C	[	1.57	3.67
MGFc 14	K288M, K334E	75.4/0.1	95.6/0.089	3X	reduced	[	1.74	
MGFc 23	K334E, R292L	70.2/0.105	108/0.107			[	2.09	1.6
MGFc 27	G316D, A378V, D399E	72/0.117	46/0.06	1.5X	14X	1.4	3.60	6.88
MGFc 28	N315I, A379M, D399E			1X	9X	1.37	1.69	1.00
MGFc 29	P243I, V379L, G420V	108/0.082	93.4/.101	2.5X	7X	0.93	1.17	1.00
MGFc 31	P247L, N421K	62/0.108	66/0.065	3X	N/C	1.35	2.90	1.00
MGFc 37	K248M	154/0.175	100/0.091	1.4X	reduced	0.98	3.83	0.67
MGFc 38	K392T, P396L	84/0.104	50/0.041	4.5X	2.5X	1.4	3.07	2.50
MGFc 39	E293V, Q295E, A327T	195/0.198	86/0.074	1.4X	reduced	1.5	4.29	0.50
MGFc 40	K248M	180/0.186	110/0.09	1.4X	reduced	1.14	4.03	
MGFc 41	H268N, P396L	178/0.159	46.6/0.036	2.2X	4.5X	1.96	2.24	0.67
MGFc 43	Y319F, P352L, P396L	125/0.139	55.7/0.041	3.5X	2X	1.58	1.09	

[00255] 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 FcγRIIIA and/or FcγRIIA 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 asapragine 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; or a substitution at position 243 with leucine, at position 292 with proline, at position 300 with leucine, at position 305 with isoleucine, and at position 396 with leucine; or a substitution at position 243 with leucine, at position 292 with proline, at position 300 with leucine, and at position 396 with leucine; or a substitution at position 243 with leucine, at position 292 with proline, and at position 300 with leucine.

[00256] 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 8**.

TABLE 8. AMINO ACID MODIFICATION WHICH INCREASE ADCC

E333A, K334A	T250S, P396L
R292L, K334E	P247S, P396L
V379M	K290E, V369A, T393A, P396L
S219Y	K210N, K222I, K320M, P396L
V282M	L410H, P396L
K222N	Q419L,P396L
F243I,V379L	V427A, P396L
F243L,R255L,E318K	P217S, V305I, I309L, N390H, P396L
K334I	E258D, P396L
K334E,T359N,T366S	N384K, P396L
K288M, K334E	V323I, P396L
K288N, A330S,P396L	K246N, Q419R, P396L
K326E	V273I, K326E, L328I, P396L
G316D,A378V,D399E	K326I, S408N, P396L
N315I,V379M,T394M	K334N, P396L
F243I,V379L,G420V	V379M, P396L
E293V,Q295E,A327T	P227S, P396L
Y319F,P352L,P396L	P217S, P396L
K392T,P396L	K261N, K210M, P396L
K248M	Q419H, P396L
H268N,P396L	K370E, P396L
K290T, N390I, P396L	L242F, P396L
K326I, P396L	F243L, V305I, A378D, F404S, P396L
H268D, P396L	R255L, P396L
K210M, P396L	V240A, P396L
L358P, P396L	P217A, T359A, P396L
K288R, T307A, K344E,P396L	P244H, P396L
D270E, G316D, R416G	V215I, K290V, P396L
P247L, N421K	F275L, Q362H, N384K, P396L
P247L, N421K, D270E	V305L, P396L
Q419H, P396L, D270E	S400F, P396L
K370E, P396L, D270E	V303I, P396L
R255L, P396L, D270E	F243L, R292P, Y300L, V305I, P396L
V240A, P396L, D270E	F243L, R292P, Y300L, P396L
K392T, P396L, D270E	F243L, R292P, Y300L

[00257] Alternatively or additionally, it may be useful to combine the above amino acid modifications or any other amino acid modifications disclosed herein with one or more further amino acid modifications that alter C1q binding and/or complement dependent cytoxicity function of the Fc region. The starting molecule of particular interest herein is usually one that binds to C1q and displays complement dependent cytotoxicity (CDC). The further amino acid substitutions described herein will generally serve to alter the ability of the starting molecule to bind to C1q and/or modify

its complement dependent cytotoxicity function, *e.g.*, to reduce and preferably abolish these effector functions. However, molecules comprising substitutions at one or more of the described positions with improved C1q binding and/or complement dependent cytotoxicity (CDC) function are contemplated herein. For example, the starting molecule may be unable to bind C1q and/or mediate CDC and may be modified according to the teachings herein such that it acquires these further effector functions. Moreover, molecules with preexisting C1q binding activity, optionally further having the ability to mediate CDC may be modified such that one or both of these activities are enhanced.

[00258] As disclosed above, one can design an Fc region with altered effector function, *e.g.*, by modifying C1q binding and/or FcR binding and thereby changing CDC activity and/or ADCC activity. For example, one can generate a variant Fc region with improved C1q binding and improved FcγRIII binding; *e.g.*, having both improved ADCC activity and improved CDC activity. Alternatively, where one desires that effector function be reduced or ablated, one may engineer a variant Fc region with reduced CDC activity and/or reduced ADCC activity. In other embodiments, one may increase only one of these activities, and optionally also reduce the other activity, *e.g.*, to generate an Fc region variant with improved ADCC activity, but reduced CDC activity and vice versa.

[00259] 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 9**. **Table 9** summarizes the various mutants that were 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 μg/mL - 1.0 μg/mL.

TABLE 9: MUTATIONS IDENTIFIED IN THE Fc REGION				
Mutations	**Domain**	**Binding to FcγRIIIA (ELISA)**	**Binding to FcγRIIB (ELISA)**	**4-4-20 ADCC (Relative Lysis (Mut/Wt)**
pYD-CH1 library FACS screen with A 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**
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** **N/C means no change; N/B means no binding; NT means not tested**				
V379M	CH3	↑2x	N/C	**1.47**
S219Y	Hinge	↑1x	↓ or N/B	**1.28**
V282M	CH2	↑1x	↓ or N/B	**1.25; 1**
F275I, K334N,V348M	CH2	↑0.5x	N/C	

TABLE 9: MUTATIONS IDENTIFIED IN THE Fc REGION				
Mutations	**Domain**	**Binding to FcγRIIIA (ELISA)**	**Binding to FcγRIIB (ELISA)**	**4-4-20 ADCC (Relative Lysis (Mut/Wt)**
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**
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 FcγIIIA 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	
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	

TABLE 9: MUTATIONS IDENTIFIED IN THE Fc REGION				
Mutations	**Domain**	**Binding to FcγRIIIA (ELISA)**	**Binding to FcγRIIB (ELISA)**	**4-4-20 ADCC (Relative Lysis (Mut/Wt)**
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	
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				
K334E,E380D	CH2,CH3	N/C	↑4.5x	
T366N	CH3	N/C	↑ 5x	
P244A,K326I,C367R,S375I,K447T	CH2,CH3	N/C	↑ 3x	

TABLE 9: MUTATIONS IDENTIFIED IN THE Fc REGION				
Mutations	**Domain**	**Binding to FcγRIIIA (ELISA)**	**Binding to FcγRIIB (ELISA)**	**4-4-20 ADCC (Relative Lysis (Mut/Wt)**
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	
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
K326I, 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

TABLE 9: MUTATIONS IDENTIFIED IN THE Fc REGION				
Mutations	**Domain**	**Binding to FcγRIIIA (ELISA)**	**Binding to FcγRIIB (ELISA)**	**4-4-20 ADCC (Relative Lysis (Mut/Wt)**
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: O.8 μM, 1' with cold 8 μM FcγRIIIA: P396L Library				
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 FcγRIIIA 158V solid phase selection: Naïve Library				
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

TABLE 9: MUTATIONS IDENTIFIED IN THE Fc REGION				
Mutations	**Domain**	**Binding to FcγRIIIA (ELISA)**	**Binding to FcγRIIB (ELISA)**	**4-4-20 ADCC (Relative Lysis (Mut/Wt)**
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	
FcγRIIB depletion FcgRIIA 131H solid phase selection: Naïve Library				
R292P, V305I	CH2,CH2	NT	NT	
D270E, G316D, R416G	CH2,CH3	NT	NT	
V284M, R292L, K370N	CH2,CH3	NT	NT	
R292P, V305I, F243L	CH2	NT	NT	

[00260] 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 FcγRIIIA and/or FcγRIIA. Such immunoglobulins include IgG molecules that naturally contain FcγR binding regions (*e.g.*, FcγRIIIA and/or FcγRIIB binding region), or immunoglobulin derivatives that have been engineered to contain an FcγR binding region (*e.g.*, FcγRIIIA and/or FcγRIIB 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 FcγR binding region (*e.g.*, a FcγRIIIA and/or FcγRIIB 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 FcγR binding region.

[00261] 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 FcγR 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 acid. 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.

[00262] 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.

[00263] 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.*

[00264] The antibodies of the present invention may be monospecific, bispecific, trispecific or of greater multispecificity. Multispecific antibodies may be specific for 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.

[00265] Multispecific antibodies have binding specificities for at least two different antigens. While such molecules normally will only bind two antigens (i.e. bispecific antibodies, BsAbs), antibodies with additional specificities such as trispecific antibodies are encompassed by the instant invention. Examples of BsAbs include without limitation those with one arm directed against a tumor cell antigen and the other arm directed against a cytotoxic molecule.

[00266] Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different

specificities (Millstein et al., Nature, 305:537-539 (1983); which is incorporated herein by reference in its entirety). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., EMBO J., 10:3655-3659 (1991).

[00267] According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when, the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

[00268] In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., Methods in Enzymology, 121:210 (1986). According to another approach described in WO96/27011, a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the CH3 domain of an antibody constant domain. In this method, one or more small amino

acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

[00269] Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

[00270] Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. See, *e.g.*, Tutt et al. J. Immunol. 147: 60 (1991), which is incorporated herein by reference.

[00271] 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 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 tunicamycin, etc. Additionally, the derivative may contain one or more non-classical amino acids.

[00272] 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 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, which are incorporated herein by reference in their entireties. Humanized antibodies are antibody 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 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, which are incorporated herein by reference in their entireties. 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, 1994), and chain shuffling (U.S. Patent No. 5,565,332), all of which are hereby incorporated by reference in their entireties. Humanized antibodies may be generated using any of the methods disclosed in U.S. Patent Nos. 5,693,762 (Protein Design Labs), 5,693,761, (Protein Design Labs) 5,585,089 (Protein Design Labs), 6,180,370 (Protein Design Labs), and U.S. Publication Nos. 20040049014, 200300229208, each of which is incorporated herein by reference in its entirety.

[00273] 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 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, each of which is incorporated herein by reference in its entirety.

[00274] Human antibodies can also be produced using transgenic mice which are 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 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, which are incorporated by reference herein in their entireties. In addition, companies such as Abgenix, Inc. (Freemont, CA), Medarex (NJ) and Genpharm (San Jose, CA) can be engaged to provide human antibodies directed against a selected antigen using technology similar to that described above.

[00275] 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*, 12:899-903, 1988).

[00276] 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 FcγRIIIA and/or FcγRIIA. 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 at least one amino acid residue, which modification increases the affinity of the Fc region for FcγRIIIA and/or FcγRIIA and further decreases the affinity of the Fc region for FcγRIIB. 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.

[00277] 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 rgion for FcγRIIIA and/or FcγRIIA. In another specific embodiment, modification of the humanized

Her2/neu monoclonal antibody may also further decrease the affinity of the Fc region for FcγRIIB. 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.

[00278] 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 rgion for FcγRIIIA and/or FcγRIIA. In another specific embodiment, modification of the anti-CD20 monoclonal antibody, 2H7 may also further decrease the affinity of the Fc region for FcγRIIB. 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.

[00279] In another specific embodiment, the invention encompasses engineering an antibody that binds A33, CD5, CD11c, CD19, CD20, CD22, CD23, CD27, CD40, CD45, CD79a, CD79b, CD103, CTLA4, ErbB1, ErbB3, ErbB4, VEGF receptor, TNF-a receptor, TNF-ß receptor, or TNF-γ receptor (particularly a humanized or chimerized form of the antibody) by modification (*e.g.*, substitution, insertion, deletion) of at least one amino acid residue which modification increases the affinity of the Fc rgion for FcγRIIIA and/or FcγRIIA. In another specific embodiment, modification of the antibody that binds A33, CD5, CD11c, CD19, CD20, CD22, CD23, CD27, CD40, CD45, CD79a, CD79b, CD103, CTLA4, ErbB1, ErbB3, ErbB4, VEGF receptor, TNF-a receptor, TNF-ß receptor, or TNF-γ receptor may also further decrease the affinity of the Fc region for FcγRIIB. In yet another specific embodiment, the antibody that binds A33, CD5, CD11c, CD19, CD20, CD22, CD23, CD27, CD40, CD45, CD79a, CD79b, CD103, CTLA4, ErbB1, ErbB3, ErbB4, VEGF receptor, TNF-a receptor, TNF-ß receptor, or TNF-γ receptor may further have an enhanced effector function as determined by standard assays known in the art and disclosed and exemplified herein.

[00280] In certain embodiments, the invention encompasses engineering an antibody (or chimeric, humanized or other engineered versions thereof), comprising the heavy chain variable domain and/or light chain variable domain of the monoclonal antibody produced by clone 2B6, 3H7, 8B5.4.3, 1D5, 2E1, 2H9, 2D11 or 1F2 having ATCC accession numbers PTA-4591, PTA-4592, PTA-7610, PTA-5958, PTA-5961, PTA-

5962, PTA-5960, and PTA-5959, respectively (deposited at ATCC, 10801 University Boulevard, Manassas, VA 02209-2011, all of which are incorporated herein by reference). In a specific embodiment, the invention encompasses engineering a humanized antibody comprising the heavy chain variable and/or light chain variable domains of 2B6, 3H7 or 8B5.3.4. In another specific embodiment, the invention encompasses engineering a humanized antibody comprising the CDRs of 2B6, 3H7 or 8B5.3.4. In another specific embodiment, the invention encompasses engineering a humanized antibody comprising the heavy chain variable domain having the amino acid sequence of SEQ ID NO: 1, **SEQ ID NO:2** or **SEQ ID NO:3** and the light chain variable domain having the amino acid sequence of SEQ ID NO: 4, **SEQ ID NO:5**, **SEQ ID NO:6**, **SEQ ID NO:7** or SEQ ID NO: 8. In another specific embodiment, the invention encompasses engineering an anti-FcγRIIB antibody comprising the heavy chain variable domain having the amino acid sequence of **SEQ ID NO:13** and the light chain variable domain having the amino acid sequence of **SEQ ID NO:14**. In another specific embodiment, the invention encompasses engineering a humanized anti-FcγRIIB antibody comprising the heavy chain variable domain having the amino acid sequence of **SEQ ID NO:3** and the light chain variable domain having the amino acid sequence of **SEQ ID NO:8**. In another specific embodiment, the invention encompasses engineering a humanized anti-FcγRIIB antibody comprising the heavy chain variable domain having the amino acid sequence of **SEQ ID NO:9** and the light chain variable domain having the amino acid sequence of **SEQ ID NO:10**.

[00281] In another specific embodiment, the invention encompasses engineering an anti-FcγRIIB 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, U.S. Application No. 10/643,857 filed on August 14, 2003, U.S. Provisional Application No. 60/562,804 filed on April 16, 2004, U.S. Provisional Application No. 60/582,044 filed on June 21, 2004, U.S. Provisional Application No. 60/582,045 filed on June 21, 2004, U.S. Provisional Application No. 60/636,663 filed on December 15, 2004 and U.S. Application Serial No. 10/524,134 filed February 11, 2005 by modification (*e.g.*, substitution, insertion, deletion) of at least one amino acid residue which modification increases the affinity of the Fc rgion for FcγRIIIA and/or FcγRIIA. In another specific embodiment, the invention encompasses engineering a humanized anti-FcγRIIB antibody including but not limited to any of the antibodies disclosed in U.S. Provisional

Application No. 60/569,882 filed on May 10, 2004, U.S. Provisional Application No. 60/582, 043 filed on June 21, 2004 and U.S. Application No. 11/126,978, filed on May 10, 2005 by modification (*e.g.*, substitution, insertion, deletion) of at least one amino acid residue which modification increases the affinity of the Fc rgion for FcγRIIIA and/or FcγRIIA. Each of the above mentioned applications is incorporated herein by reference in its entirety. Examples of anti-FcγRIIB antibodies, which may or may not be humanized, 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 ,1D5 monoclonal antibody having ATCC accession number PTA-5958, 1F2 monoclonal antibody having ATCC accession number PTA-5959, 2D11 monoclonal antibody having ATCC accession number PTA-5960, 2E1 monoclonal antibody having ATCC accession number PTA-5961, 8B5.3.4 having ATCC accession number PTA-7610, and 2H9 monoclonal antibody having ATCC accession number PTA-5962 (all deposited at 10801 University Boulevard, Manassas, VA 02209-2011), which are incorporated herein by reference. 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.

[00282] In a specific embodiment, the invention encompasses engineering an anti-FcγRIIB antibody according to methods of the present invention that comprises one or more complementarily determining regions (CDRs), preferably all 6 CDRs, of the antibody produced by clone 2B6, 3H7, or 8B5.3.4 with ATCC accession numbers PTA-4591, PTA-4592, and PTA-7610, respectively (*e.g.*, the heavy chain CDR3). In a specific embodiment, an anti-FcγRIIB antibody engineered according to methods of the invention comprises one or more complementarily determining regions (CDRs), preferably all 6 CDRs, of the antibody produced by clone 1D5, 2E1, 2H9, 2D11, and 1F2 having ATCC Accession numbers, PTA-5958, PTA-5961, PTA-5962, PTA-5960, and PTA-5959, respectively (*e.g.*, the heavy chain CDR3). In another embodiment, an anti-FcγRIIB antibody engineered according to methods of the invention binds to the same epitope as the mouse monoclonal antibody produced from clone 2B6, 3H7, or 8B5.3.4 with ATCC accession numbers PTA-4591, PTA-4592, and PTA-7610,

respectively and/or competes with the mouse monoclonal antibody produced from clone 2B6, 3H7, or 8B5.3.4 with ATCC accession numbers PTA-4591, PTA-4592, and PTA-7610, respectively as determined, *e.g.*, in an ELISA assay or other appropriate competitive immunoassay, and also binds FcγRIIB with a greater affinity than said antibody or a fragment thereof binds FcγRIIA. In another embodiment, an anti-FcγRIIB antibody engineered according to methods of the invention binds to the same epitope as the mouse monoclonal antibody produced from clone 1D5, 2E1, 2H9, 2D11, and 1F2 having ATCC Accession numbers, PTA-5958, PTA-5961, PTA-5962, PTA-5960, and PTA-5959, respectively, and/or competes with the mouse monoclonal antibody produced from clone 1D5, 2E1, 2H9, 2D11, and 1F2 having ATCC Accession numbers, PTA-5958, PTA-5961, PTA-5962, PTA-5960, and PTA-5959, respectively, as determined, *e.g.*, in an ELISA assay or other appropriate competitive immunoassay, and also binds FcγRIIB, via its variable region, with a greater affinity than said antibody or a fragment thereof binds FcγRIIA.

[00283] The present invention also encompasses engineering an anti-FcγRIIB antibody comprising a heavy chain variable domain and/or light chain variable domain amino acid sequence that is at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% identical to the amino acid sequence of the heavy chain variable domain and/or light chain variable domain of the mouse monoclonal antibody produced by clone 2B6, 3H7, 8B5.3.4, 1D5, 2E1, 2H9, 2D11, or 1F2 having ATCC accession numbers PTA-4591, PTA-4592, PTA-7610, PTA-5958, PTA-5961, PTA-5962, PTA-5960, and PTA-5959, respectively. The present invention further encompasses the engineering of anti-FcγRIIB antibodies comprising an amino acid sequence of one or more CDRs that is at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% identical to the amino acid sequence of one or more CDRs of the mouse monoclonal antibody produced by clone 2B6, 3H7, 8B5.3.4, 1D5, 2E1, 2H9, 2D11, or 1F2 having ATCC accession numbers PTA-4591, PTA-4592, PTA-7610, PTA-5958, PTA-5961, PTA-5962, PTA-5960, and PTA-5959, respectively. The determination of percent identity of two amino acid sequences can be determined by any method known to one skilled in the art, including BLAST protein searches.

[00284] The present invention also encompasses the the engineering of one or more anti-FcγRIIB antibodies comprising one or more variable domains encoded by a

nucleotide sequence that hybridizes to the nucleotide sequence of one or more variable domains of a mouse monoclonal antibody produced by clone 2B6, 3H7, 8B5.3.4, 1D5, 2E1, 2H9, 2D11, or 1F2 having ATCC accession numbers PTA-4591, PTA-4592, PTA-7610, PTA-5958, PTA-5961, PTA-5962, PTA-5960, and PTA-5959, respectively, under stringent conditions. In a preferred embodiment, the invention encompasses engineering one or more anti-FcγRIIB antibodies comprising a variable light chain and/or variable heavy chain domain encoded by a nucleotide sequence that hybridizes under stringent conditions to the nucleotide sequence of the variable light chain and/or variable heavy chain domain of the mouse monoclonal antibody produced by clone 2B6, 3H7, 8B5.3.4, 1D5, 2E1, 2H9, 2D11, or 1F2 having ATCC accession numbers PTA-4591, PTA-4592, PTA-7610, PTA-5958, PTA-5961, PTA-5962, PTA-5960, and PTA-5959, respectively, under stringent conditions. In another preferred embodiment, the invention provides engineering anti-FcγRIIB antibodies comprising one or more CDRs encoded by a nucleotide sequence that hybridizes under stringent conditions to the nucleotide sequence of one or more CDRs of the mouse monoclonal antibody produced by clone 2B6, 3H7, 8B5.3.4, 1D5, 2E1, 2H9, 2D11, or 1F2 having ATCC accession numbers PTA-4591, PTA-4592, PTA-7610, PTA-5958, PTA-5961, PTA-5962, PTA-5960, and PTA-5959, respectively. Stringent hybridization conditions include, but are not limited to, hybridization to filter-bound DNA in 6X sodium chloride/sodium citrate (SSC) at about 45°C followed by one or more washes in 0.2X SSC/0.1% SDS at about 50-65°C, highly stringent conditions such as hybridization to filter-bound DNA in 6X SSC at about 45°C followed by one or more washes in 0.1X SSC/0.2% SDS at about 60°C, or any other stringent hybridization conditions known to those skilled in the art (see, for example, Ausubel, F.M. *et al.*, eds. 1989 Current Protocols in Molecular Biology, vol. 1, Green Publishing Associates, Inc. and John Wiley and Sons, Inc., NY at pages 6.3.1 to 6.3.6 and 2.10.3, incorporated herein by reference).

[00285] In a preferred embodiment, the engineered antibodies of the invention are humanized by any method known in the art or described herein and/or comprise the CDR regions of a humanized FcγRIIB specific antibody or humanized CD20 specific antibody, whrein said CDRs are derived from a murine antibody specific for FcγRIIB or CD20, respectively. In some embodiments, the humanized antibodies described herein comprise alterations, including but not limited to amino acid deletions,

insertions, modifications, of the acceptor antibody, *i.e.,* human, heavy and/or light chain variable domain framework regions that are necessary for retaining binding specificity of the donor monoclonal antibody. In some embodiments, the framework regions of the humanized antibodies described herein do not necessarily consist of the precise amino acid sequence of the framework region of a natural occurring human antibody variable region, but contains various alterations, including but not limited to amino acid deletions, insertions, modifications that alter the property of the humanized antibody, for example, improve the binding properties of a humanized antibody variable region specific for the same target as the murine FcγRIIB or CD20 specific antibody. In most preferred embodiments, a minimal number of alterations are made to the framework region in order to avoid large-scale introductions of non-human framework residues and to ensure minimal immunogenicity of the humanized antibody of the invention in humans. The donor monoclonal antibody is preferably a monoclonal antibody produced by clones 2B6, 3H7, 8B5.3.4, 1D5, 2E1, 2H9, 2D11, or 1F2 (having ATCC accession numbers PTA-4591, PTA-4592, PTA-7610, PTA-5958, PTA-5961, PTA-5962, PTA-5960, and PTA-5959, respectively) which bind FcγRIIB, or the monoclonal antibody is a CD20 antibody, such as rituximab or 2H7.

[00286] In a specific embodiment, the invention encompasses engineering a CDR-grafted antibody that comprises a heavy chain variable region domain comprising framework residues of the recipient antibody and residues from the donor monoclonal antibody, which specifically binds FcγRIIB, *e.g.,* monoclonal antibody produced from clones 2B6, 3H7, 8B5.3.4, 1D5, 2E1, 2H9, 2D11, or 1F2 having ATCC accession numbers PTA-4591, PTA-4592, PTA-7610, PTA-5958, PTA-5961, PTA-5962, PTA-5960, and PTA-5959, respectively. In another specific embodiment, the invention encompasses engineering a CDR-grafted antibody that comprises a light chain variable region domain comprising framework residues of the recipient antibody and residues from the donor monoclonal antibody, which specifically binds FcγRIIB, *e.g.,* monoclonal antibody produced from clones 2B6, 3H7, 8B5.3.4, 1D5, 2E1, 2H9, 2D11, or 1F2.

[00287] Preferably the FcγRIIB humanized antibodies bind the extracellular domain of native human FcγRIIB. The humanized anti- FcγRIIB antibodies of the combinations can have a heavy chain variable region comprising the amino acid sequence of CDR1 (SEQ ID NO: 15 or SEQ ID NO: 16) and/or CDR2 (**SEQ ID**

NO:17 or **SEQ ID NO:18**) and/or CDR3 (SEQ ID NO: 19 or **SEQ ID NO:20**) and/or a light chain variable region comprising the amino acid sequence of CDR1 (**SEQ ID NO:21** or **SEQ ID NO:22**) and/or a CDR2 (**SEQ ID NO:23**, **SEQ ID NO:24**, **SEQ ID NO:25**, or **SEQ ID NO:26**) and/or CDR3 (**SEQ ID NO:27** or **SEQ ID NO:28**).

[00288] In a specific embodiment, the invention encompasses the engineering of a humanized anti-FcγRIIB antibody with the heavy chain variable domain having the amino acid sequence of **SEQ ID NO:1**, **SEQ ID NO:2**, or **SEQ ID NO:3** and a light chain variable domain having the amino acid sequence of **SEQ ID NO:4**, **SEQ ID NO:5**, **SEQ ID NO:6**; **SEQ ID NO:7** or **SEQ ID NO:8**.

[00289] In one specific embodiment, the invention encompasses engineering a humanized anti-FcγRIIB antibody, wherein the VH region of the FcγRIIB antibody consists of the FR segments from the human germline VH segment VH1-18 (Matsuda *et al.*, 1998, J. Exp. Med. 188:2151062) and JH6 (Ravetch *et al.*, 1981, Cell 27(3 Pt. 2): 583-91), and one or more CDR regions of a 2B6 VH, having the amino acid sequence of SED ID NO: 1, **SEQ ID NO:17**, or **SEQ ID NO:19**. In one embodiment, the 2B6 VH has the amino acid sequence of **SEQ ID NO:1**, **SEQ ID NO:3**, or **SEQ ID NO:29**. In another specific embodiment, the humanized anti-FcγRIIB antibody further comprises a VL region, which consists of the FR segments of the human germline VL segment VK-A26 (Lautner-Rieske *et al.*, 1992, Eur. J. Immunol. 22:1023-1029) and JK4 (Hieter *et al.*, 1982, J. Biol. Chem. 257:1516-22), and one or more CDR regions of a 2B6VL, having the amino acid sequence of **SEQ ID NO:21**, **SEQ ID NO:23**, **SEQ ID NO:24**, **SEQ ID NO:25**, and **SEQ ID NO:27**. In one embodiment, the 2B6 VL has the amino acid sequence of **SEQ ID NO:4**, **SEQ ID NO:5**, **SEQ ID NO:6**; **SEQ ID NO:7**, **SEQ ID NO:8**, or **SEQ ID NO:30**, and optionally in combination with one of the above-referenced 2B6 VH.

[00290] In some embodiments, the anti-FcγRIIB antibody angineered in accordance with the methods of the invention has a VH chain and/or a VH domain comprising the amino acid sequence (H2B6VH-3) (**SEQ ID NO:3**):

QVQLVQSGAEVKKPGASVKVSCKASGYTFTNYWIHWVRQAPGQGLEWIGVIDPSDTYP
NYNKKFKGRVTMTVDTSTSTAYMELRSLRSDDTAVYYCARNGDSDYYSGMDYWGQGTT
VTVSS

[00291] In some embodiments, the anti-FcγRIIB antibody angineered in accordance with the methods of the invention has a VL chain and/or VL domain comprising the amino acid sequence (H2B6VL-5) (**SEQ ID NO:8**):

EIVLTQSPDFQSVTPKEKVTFTCRTSQSIGTNIHWYQQKPDQSPKLLIKEVSESISGV
PSRFSGSGSGTDFTLTINSLEAEDAATYYCQQSNTWPFTFGGGTKVEIK

[00292] In some embodiments, the anti-FcγRIIB antibody angineered in accordance with the methods of the invention has a VH chain and/or VH domain comprising the amino acid sequence (H2B6VH-3):

QVQLVQSGAEVKKPGASVKVSCKASGYTFTNYWIHWVRQAPGQGLEWIGVIDPSDTYP
NYNKKFKGRVTMTVDTSTSTAYMELRSLRSDDTAVYYCARNGDSDYYSGMDYWGQGTT
VTVSS (**SEQ ID NO:3**), and a VL chain and/or VL domain comprising the amino acid sequence (H2B6VL-5) (**SEQ ID NO:8**):

EIVLTQSPDFQSVTPKEKVTFTCRTSQSIGTNIHWYQQKPDQSPKLLIKEVSESISGV
PSRFSGSGSGTDFTLTINSLEAEDAATYYCQQSNTWPFTFGGGTKVEIK

[00293] In some embodiments, the anti-FcγRIIB antibody angineered in accordance with the methods of the invention has a VH domain and/or VH chain comprising the amino acid sequence (8B5.3.4 VH, see FIG. 2):

EVKLEESGGGLVQPGGSMKLSCEASGFTFSDAWMDWVRQSPEKGLEWVAEIRNKAKNH
ATYYAESVIGRFTISRDDSKSSVYLQMNSLRAEDTGIYYCGALGLDYWGQGTTLTVSS
(**SEQ ID NO:9**).

[00294] In some embodiments, the anti-FcγRIIB antibody angineered in accordance with the methods of the invention has a VL domain and/or VL chain comprising the amino acid sequence (8B5.3.4 VL, see FIG. 3) (**SEQ ID NO:10**):

DIQMTQSPSSLLAALGERVSLTCRASQEISGYLSWLQQKPDGTIKRLIYAASTLDSGV
PKRFSGSESGSDYSLTISSLESEDFADYYCLQYFSYPLTFGAGTKLELK

[00295] In some embodiments, the anti-FcγRIIB antibody angineered in accordance with the methods of the invention has a VH domain and/or VH chain comprising the amino acid sequence (8B5.3.4 VH) (**SEQ ID NO:9**, see FIG. 2):

EVKLEESGGGLVQPGGSMKLSCEASGFTFSDAWMDWVRQSPEKGLEWVAEIRNKAKNH
ATYYAESVIGRFTISRDDSKSSVYLQMNSLRAEDTGIYYCGALGLDYWGQGTTLTVSS,
and a VL domain and/or VL chain comprising the amino acid sequence (8B5.3.4 VL, see FIG 3) (**SEQ ID NO:10**):

DIQMTQSPSSLLAALGERVSLTCRASQEISGYLSWLQQKPDGTIKRLIYAASTLDSGV
PKRFSGSESGSDYSLTISSLESEDFADYYCLQYFSYPLTFGAGTKLELK

[00296] In another specific embodiment, the anti-FcγRIIB antibody angineered in accordance with the methods of the invention is a humanized 3H7 antibody, wherein the FcγRIIB VH region consists of the FR segments from a human germline VH segment and the CDR regions of the 3H7 VH, having the amino acid sequence of SED ID NO: 37. In another specific embodiment, the humanized 3H7 antibody further comprises a VL region, which consists of the FR segments of a human germline VL segment and the CDR regions of 3H7VL, having the amino acid sequence of **SEQ ID NO:7**.

[00297] In particular, the invention encompasses the engineering of an anti-FcγRIIB antibody wherein the antibody immunospecifically binds to an extracellular domain of native human FcγRIIB, said FcγRIIB antibody comprising (or alternatively, consisting of) CDR sequences of 2B6, 3H7, or 8B5.3.4 in any of the following combinations: a VH CDR1 and a VL CDR1; a VH CDR1 and a VL CDR2; a VH CDR1 and a VL CDR3; a VH CDR2 and a VL CDR1; VH CDR2 and VL CDR2; a VH CDR2 and a VL CDR3; a VH CDR3 and a VH CDR1; a VH CDR3 and a VL CDR2; a VH CDR3 and a VL CDR3; a VH1 CDR1, a VH CDR2 and a VL CDR1; a VH CDR1, a VH CDR2 and a VL CDR2; a VH CDR1, a VH CDR2 and a VL CDR3; a VH CDR2, a VH CDR3 and a VL CDR1, a VH CDR2, a VH CDR3 and a VL CDR2; a VH CDR2, a VH CDR2 and a VL CDR3; a VH CDR1, a VL CDR1 and a VL CDR2; a VH CDR1, a VL CDR1 and a VL CDR3; a VH CDR2, a VL CDR1 and a VL CDR2; a VH CDR2, a VL CDR1 and a VL CDR3; a VH CDR3, a VL CDR1 and a VL CDR2; a VH CDR3, a VL CDR1 and a VL CDR3; a VH CDR1, a VH CDR2, a VH CDR3 and a VL CDR1; a VH CDR1, a VH CDR2, a VH CDR3 and a VL CDR2; a VH CDR1, a VH CDR2, a VH CDR3 and a VL CDR3; a VH CDR1, a VH CDR2, a VL CDR1 and a VL CDR2; a VH CDR1, a VH CDR2, a VL CDR1 and a VL CDR3; a VH CDR1, a VH CDR3, a VL CDR1 and a VL CDR2; a VH CDR1, a VH CDR3, a VL CDR1 and a VL CDR3; a VH CDR2, a VH CDR3, a VL CDR1 and a VL CDR2; a VH CDR2, a VH CDR3, a VL CDR1 and a VL CDR3; a VH CDR2, a VH CDR3, a VL CDR2 and a VL CDR3; a VH CDR1, a VH CDR2, a VH CDR3, a VL CDR1 and a VL CDR2; a VH CDR1, a VH CDR2, a VH CDR3, a VL CDR1 and a VL CDR3; a VH CDR1, a VH CDR2, a VL CDR1, a VL CDR2, and a VL CDR3; a VH CDR1, a VH CDR3, a VL CDR1, a VL CDR2, and a VL CDR3; a VH CDR2, a VH CDR3, a VL CDR1, a VL CDR2, and a VL CDR3; or any combination thereof of the VH CDRs and VL CDRs disclosed herein.

[00298] In a specific embodiment, the anti-FcγRIIB 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 positon 392 with threonine and at position 396 with leucine (MgFc38); or a substitution at position 221 with glutamic acid, at positon 270 with glutamic acid, at positon 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); or a substitution at position 243 with leucine, at position 292 with proline, at position 300 with leucine, at position 305 with isoleucine, and at position 396 with leucine (MgFc88); or a substitution at position 243 with leucine, at position 292 with proline, at position 300 with leucine, and at position 396 with leucine (MgFc88A); or a substitution at position 234 with leucine, at position 292 with proline, and at position 300 with leucine (MgFc155); or a substitution at position 243 with leucine, at position 292 with proline, and at position 300 with leucine; or a substitution at position 243 with leucine, at position 292 with proline, and at position 396 with leucine; or a substitution at position 243 with leucine, and at position 292 with proline; or a substitution at position 243 with leucine; or a substitution at position 273 with phenylalanine.

6.1.6 POLYPEPTIDE AND ANTIBODY CONJUGATES

[00299] 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.

[00300] 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 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 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 (*e.g.*, growth hormone ("GH"); proteases, or ribonucleases.

[00301] 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 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.*, *Biotechniques*, 17(4):754-761, 1994).

[00302] 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*, 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 (each of these patents and publications are hereby incorporated by reference in its entirety). Molecules of the invention comprising variant Fc regions, or 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.

[00303] 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 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 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 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, 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), lanthanium (^{140}La), lutetium (^{177}Lu), manganese (^{54}Mn), molybdenum (^{99}Mo), palladium (^{103}Pd), phosphorous (^{32}P), praseodymium (^{142}Pr), promethium (^{149}Pm), rhenium (^{186}Re, ^{188}Re), rhodium (^{105}Rh), ruthemium (^{97}Ru), samarium (^{153}Sm), scandium (^{47}Sc), selenium (^{75}Se), strontium (^{85}Sr), sulfur (^{35}S), technetium (^{99}Tc), thallium (^{201}Ti), tin (^{113}Sn, ^{117}Sn), tritium (^{3}H), 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.

[00304] 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 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, 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, 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).

[00305] 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 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 each of which is incorporated herein by reference in their entireties.

[00306] Techniques for conjugating such therapeutic moieties to antibodies are well known; *see, e.g.*, Arnon *et al.*, "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in *Monoclonal Antibodies And Cancer Therapy*, Reisfeld *et al.* (eds.), 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 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.

[00307] 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 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.

6.2 SCREENING OF MOLECULES WITH VARIANT Fc REGIONS FOR ENHANCED FcγRIII BINDING AND CHARACTERIZATION OF SAME

[00308] In preferred 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 in combination with one or more biochemical based assays, preferably in a high throughput manner, as described herein or as disclosed in U.S Patent Application Publications 2005/0037000 and 2005/0064514, and International Patent Application Publication WO 04/063351, each of which is hereby incorporated by reference in its entirety. 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 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 one or more FcγR mediated effector cell functions such as those described herein in Section 6.2.7. 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.

[00309] 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), which is incorporated herein by reference in its entirety. 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.

[00310] 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. 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 phosphate (Tailor *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 (Estin *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, $D_1$56-22 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 , E_1 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_5A_7 found in myeloid cells, R_{24} 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).

[00311] The invention particularly concerns the embodiment in which the binding of the Fc variant to an FcγR activates a cellular effector which targets cells that array a cancer antigen such as A33 (a colorectal carcinoma antigen; Almqvist, Y. 2006, *Nucl Med Biol.* Nov;33(8):991-998); B1 (Egloff, A.M. *et al.* 2006, *Cancer Res.* 66(1):6-9); BAGE (Bodey, B. 2002 *Expert Opin Biol Ther.* 2(6):577-84); beta-catenin (Prange W. *et al.* 2003 *J Pathol.* 201(2):250-9); CA125 (Bast, R.C. Jr. *et al.* 2005 *Int J Gynecol Cancer* 15 Suppl 3:274-81); CD5 (Calin, G.A. *et al.* 2006 *Semin Oncol.* 33(2):167-73; CD19 (Troussard, X. *et al.* 1998 *Hematol Cell Ther.* 40(4):139-48); CD20 (Thomas, D.A. *et al.* 2006 Hematol Oncol Clin North Am. 20(5):1125-36); CD22 (Kreitman, R.J. 2006 AAPS J. 18;8(3):E532-51); CD23 (Rosati, S. *et al.* 2005 *Curr Top Microbiol Immunol.* 5;294:91-107); CD25 (Troussard, X. *et al.* 1998 *Hematol Cell Ther.* 40(4):139-48); CD27 (Bataille, R. 2006 *Haematologica* 91(9):1234-40); CD28 (Bataille, R. 2006 *Haematologica* 91(9):1234-40); CD36 (Ge, Y. 2005 *Lab Hematol.* 11(1):31-7); CD40/CD154 (Messmer, D. *et al.* 2005 *Ann N Y Acad Sci.* 1062:51-60); CD45 (Jurcic, J.G. 2005 *Curr Oncol Rep.* 7(5):339-46); CD56 (Bataille, R. 2006 *Haematologica* 91(9):1234-40); CD79a/CD79b (Troussard, X. *et al.* 1998 *Hematol Cell Ther.* 40(4):139-48; Chu, P.G. *et al.* 2001 Appl Immunohistochem Mol Morphol. 9(2):97-106); CD103 (Troussard, X. *et al.* 1998 *Hematol Cell Ther.* 40(4):139-48); CDK4 (Lee, Y.M. *et al.* 2006 *Cell Cycle* 5(18):2110-4); CEA (carcinoembryonic antigen; Mathelin, C. 2006 *Gynecol Obstet Fertil.* 34(7-8):638-46; Tellez-Avila, F.I. *et al.* 2005 *Rev Invest Clin.* 57(6):814-9); CTLA4 (Peggs, K.S. *et al.* 2006 *Curr Opin Immunol.* 18(2):206-13); EGF-R (epidermal growth factor receptor; Adenis, A. *et al.* 2003 *Bull Cancer.* 90 Spec No:S228-32); Erb (ErbB1; ErbB3; ErbB4; Zhou, H. *et al.* 2002 *Oncogene* 21(57):8732-40; Rimon, E. *et al.* 2004 Int J Oncol. 24(5):1325-38); GAGE (GAGE-1; GAGE-2; Akcakanat, A. *et al.* 2006 *Int J Cancer.* 118(1):123-8); GD2/GD3/GM2 (Livingston, P.O. *et al.* 2005 Cancer Immunol Immunother. 54(10):1018-25); gp100 (Lotem, M. *et al.* 2006 *J Immunother.* 29(6):616-27); HER-2/neu (Kumar, Pal S *et al.* 2006 Semin Oncol. 33(4):386-91); human papillomavirus-E6/human papillomavirus-E7 (DiMaio, D. *et al.* 2006 *Adv Virus Res.* 66:125-59; KSA (17-1A) (Ragupathi, G. 2005 *Cancer Treat Res.* 123:157-80); MAGE (MAGE-1;

MAGE-3; (Bodey, B. 2002 *Expert Opin Biol Ther.* 2(6):577-84); MART (Kounalakis, N. *et al.* 2005 *Curr Oncol Rep.* 7(5):377-82; MUC-1 (Mathelin, C. 2006 *Gynecol Obstet Fertil.* 34(7-8):638-46); MUM-1 (Castelli, C. *et al.* 2000 *J Cell Physiol.* 182(3):323-31); N-acetylglucosaminyltransferase (Dennis, J.W. 1999 *Biochim Biophys Acta.* 6;1473(1):21-34); p15 (Gil, J. *et al.* 2006 *Nat Rev Mol Cell Biol.* 7(9):667-77); PSA (prostate specific antigen; Cracco, C.M. *et al.* 2005 *Minerva Urol Nefrol.* 57(4):301-11); PSMA (Ragupathi, G. 2005 *Cancer Treat Res.* 123:157-80); sTn (Holmberg, L.A. 2001 *Expert Opin Biol Ther.* 1(5):881-91); TNF-receptor (TNF-α receptor, TNF-ß receptor; or TNF-γ receptor; van Horssen, R. *et al.* 2006 *Oncologist.* 11(4):397-408; Gardnerova, M. *et al.* 2000 *Curr Drug Targets.* 1(4):327-64); or VEGF receptor (O'Dwyer. P.J. 2006 *Oncologist.* 11(9):992-8). Also of interest are antigens specific to particular infectious agents, e.g., viral agents including, but not limited to human immunodeficiency virus (HIV), hepatitis B virus (HBV), influenza, human papilloma virus (HPV), foot and mouth (coxsackieviruses), the rabies virus, herpes simplex virus (HSV), and the causative agents of gastroenteritis, including rotaviruses, adenoviruses, caliciviruses, astroviruses and Norwalk virus; bacterial agents including, but not limited to E. coli, Salmonella thyphimurium, Pseudomonas aeruginosa, Vibrio cholerae, Neisseria gonorrhoeae, Helicobacter pylori, Hemophilus influenzae, Shigella dysenteriae, Staphylococcus aureus, Mycobacterium tuberculosis and Streptococcus pneumoniae, fungal agents and parasites such as Giardia.

[00312] 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; which is incorporated herein by reference in its entirety). 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; which is incorporated herein by reference in its entirety). 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; which is incorporated herein by reference in its entirety). 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.

[00313] In another specific embodiment, the invention encompasses engineering an anti-FcγRIIB antibody including but not limited to any of the antibodies disclosed in U.S. Patent Application Publications 2005/02157667; 2004/0185045; 2005/0260213; or 2006/013810; International Patent Application Publications WO 2005/110474 or WO 2005/115452; U.S. Patent Application 11/305,787 filed December 15, 2005; or Provisional Applications No. 60/809,116; 60/816,126; or 60/816,688 filed on May 26, 2006, June 23, 2006, or June 26, 2006, respectively by modification (*e.g.*, substitution, insertion, deletion) of at least one amino acid residue which modification increases the affinity of the Fc rgion for FcγRIIIA and/or FcγRIIA. Each of the above mentioned references is hereby incorporated by reference in their entirety. Examples of anti-FcγRIIB antibodies, which may or may not be humanized, 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, 1D5 monoclonal antibody having ATCC accession number PTA-5958, 1F2 monoclonal antibody having ATCC accession number PTA-5959, 2D11 monoclonal antibody having ATCC accession number PTA-5960, 2E1 monoclonal antibody having ATCC accession number PTA-5961 and 2H9 monoclonal antibody having ATCC accession number PTA-5962 (all deposited at 10801 University Boulevard, Manassas, VA 02209-2011), which are incorporated herein by reference. 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 some embodiments, a variant Fc 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, Erbitux™ (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® (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, 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); OVAREX™ which is a murine anti-CA 125 antibody (Altarex); PANOREX™ 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 (ImClone System); VITAXIN™ which is a humanized anti-αVβ3 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); RITUXAN™ which is a chimeric anti-CD20 IgG1 antibody (IDEC Pharm/Genentech, Roche/Zettyaku); LYMPHOCIDE™ which is a humanized anti-CD22 IgG antibody (Immunomedics); Smart ID10 which is a humanized anti-HLA antibody (Protein Design Lab); ONCOLYM™ (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); ZEVALIN™ 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-α4β7 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 (FcγR) antibody (Medarex/Centeon);; 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).

[00314] 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-FcγR interactions, including, but not limited to, an ELISA assay, and surface plasmon resonance-based assay for determining the kinetic parameters of Fc-FcγR interaction, *e.g.*, BIAcore assay. The one or more functional assays can be any assay known in the art for characterizing one or more FcγR 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 FcγRs, *e.g.*, FcγRIIIA, FcγRIIA, FcγRIIA; 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 6.2.7, and exemplified herein in Example 7.8.

[00315] 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 FcγR (*e.g.*, FcγRIIIA, FcγRIIB) 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 FcγR.

[00316] Preferably, a variant Fc region of the invention binds FcγRIIIA and/or FcγRIIA 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 FcγRIIIA and/or FcγRIIA with a higher affinity and binds FcγRIIB with a lower affinity than a wild type Fc region as determined in an ELISA assay. In some embodiments, the variant Fc region binds FcγRIIIA and/or FcγRIIA 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 FcγRIIIA and/or FcγRIIA and binds FcγRIIB 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 FcγRIIB as determined in an ELISA assay.

[00317] 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-FcγR interaction, using methods disclosed herein and known to those of skill in the art. Preferably, the Kd of a variant Fc region of the invention for binding to a monomeric FcγRIIIA and/or FcγRIIA as determined by BIAcore analysis is about 100 nM, preferably about 70 nM, most preferably about 40 nM.; and the Kd of the variant Fc region of the invention for binding a dimeric FcγRIIB is about 80 nM, about 100 nM, more preferably about 200 nM.

[00318] In most preferred embodiments, the immunoglobulin comprising the variant Fc regions is further characterized in an animal model for interaction with an FcγR. Preferred animal models for use in the methods of the invention are, for example, transgenic mice expressing human FcγRs, *e.g.*, any mouse model described in U.S. Patent No. 5,877,397, and 6,676,927 which are incorporated herein by reference in their entirety. Transgenic mice for use in the methods of the invention include, but are not limited to, nude knockout FcγRIIIA mice carrying human FcγRIIIA; nude knockout FcγRIIIA mice carrying human FcγRIIA; nude knockout FcγRIIIAmice carrying human FcγRIIB and human FcγRIIIA; nude knockout FcγRIIIA mice carrying human FcγRIIB and human FcγRIIA; nude knockout FcγRIIIA and FcγRIIA mice carrying

human FcγRIIIA and FcγRIIA and nude knockout FcγRIIIA, FcγRIIA and FcγRIIB mice carrying human FcγRIIIA, FcγRIIA and FcγRIIB.

6.2.1 DESIGN STRATEGIES

[00319] The present invention encompasses engineering methods to generate Fc variants including but not limited to computational design strategies, library generation methods, and experimental production and screening methods. These strategies may be applied individually or in various combinations to engineer the Fc variants of the instant invention.

[00320] In most preferred embodiments, the engineering methods of the invention comprise methods in which amino acids at the interface between an Fc region and the Fc ligand are not modified. Fc ligands include but are not limited to FcγRs, C1q, FcRn, C3, mannose receptor, protein A, protein G, mannose receptor, and undiscovered molecules that bind Fc. Amino acids at the interface between an Fc region and an Fc ligand is defined as those amino acids that make a direct and/ or indirect contact between the Fc region and the ligand, play a structural role in determining the conformation of the interface, or are within at least 3 angstroms, preferably at least 2 angstroms of each other as determined by structural analysis, such as x-ray crystallography and molecular modeling. The amino acids at the interface between an Fc region and an Fc ligand include those amino acids that make a direct contact with an FcγR based on crystallographic and structural analysis of Fc-FcγR interactions such as those disclosed by Sondermann et al., (2000, Nature, 406: 267-273; which is incorporated herein by reference in its entirety). 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.

[00321] Preferably, the engineering methods of the invention do not modify any of the amino acids as identified by Shields *et al.*, which are located in the CH2 domain of an Fc region proximal to the hinge region, *e.g.*, Leu234-Pro238; Ala327, Pro329, and affect binding of an Fc region to all human FcγRs.

[00322] In other embodiments, the invention encompasses Fc variants with altered FcγR affinities and/or altered effector functions, such that the Fc variant does not have an amino acid modification at a position at the interface between an Fc region and the Fc ligand. Preferably, such Fc variants in combination with one or more other amino acid modifications which are at the interface between an Fc region and the Fc ligand have a further impact on the particular altered property, e.g. altered FcγR affinity. Modifying amino acids at the interface between Fc and an Fc ligand may be done using methods known in the art, for example based on structural analysis of Fc-ligand complexes. For example but not by way of limitation by exploring energetically favorable substitutions at Fc positions that impact the binding interface, variants can be engineered that sample new interface conformations, some of which may improve binding to the Fc ligand, some of which may reduce Fc ligand binding, and some of which may have other favorable properties. Such new interface conformations could be the result of, for example, direct interaction with Fc ligand residues that form the interface, or indirect effects caused by the amino acid modifications such as perturbation of side chain or backbone conformations

[00323] The invention encompasses engineering Fc variants comprising any of the amino acid modifications disclosed herein in combination with other modifications in which the conformation of the Fc carbohydrate at position 297 is altered. The invention encompasses conformational and compositional changes in the N297 carbohydrate that result in a desired property, for example increased or reduced affinity for an FcγR. Such modifications may further enhance the phenotype of the original amino acid modification of the Fc variants of the invention. Although not intending to be bound by a particular mechanism of actions such a strategy is supported by the observation that the carbohydrate structure and conformation dramatically affect Fc-FcγR and Fc/Cl q binding (Umaha et aL, 1999, Nat Biotechnol 17:176-180; Davies et aL, 2001, Biotechnol Bioeng 74:288-294; Mimura et aL, 2001, J Biol Chem 276:45539 ; Radaev et aL, 2001, J Biol Chem 276:16478-16483; Shields et aL 2002, J Biol Chem 277:26733-26740; Shinkawa et aL, 2003, J Biol Chem 278:3466-3473).

[00324] Another design strategy for generating Fc variants in accordance with the invention is provided in which the Fc region is reengineered to eliminate the structural and functional dependence on glycosylation. This design strategy involves the optimization of Fc structure, stability, solubility, and/or Fc function (for example

affinity of Fc for one or more Fc ligands) in the absence of the N297 carbohydrate. In one approach, positions that are exposed to solvent in the absence of glycosylation are engineered such that they are stable, structurally consistent with Fc structure, and have no tendency to aggregate. Approaches for optimizing aglycosylated Fc may involve but are not limited to designing amino acid modifications that enhance aglycoslated Fc stability and/or solubility by incorporating polar and/or charged residues that face inward towards the Cg2-Cg2 dimer axis, and by designing amino acid modifications that directly enhance the aglycosylated Fc-FcγR interface or the interface of aglycosylated Fc with some other Fc ligand.

[00325] The Fc variants of the present invention may be combined with other Fc modifications, including but not limited to modifications that alter effector function. The invention encompasses combining an Fc variant of the invention with other Fc modifications to provide additive, synergistic, or novel properties in antibodies or Fc fusions. Such modifications may be in the CH1, CH2, or CH3 domains or a combination thereof. Preferably the Fc variants of the invention enhance the property of the modification with which they are combined. For example, if an Fc variant of the invention is combined with a mutant known to bind FcγRIIIA with a higher affinity than a comparable molecule comprising a wild type Fc region; the combination with a mutant of the invention results in a greater fold enhancement in FcγRIIIA affinity.

[00326] In one embodiment, the Fc variants of the present invention may be combined with other known Fc variants such as those disclosed in Duncan et al, 1988, Nature 332:563-564; Lund et al., 1991, J. Immunol 147:2657-2662; Lund et al, 1992, Mol Immunol 29:53-59; Alegre et al, 1994, Transplantation 57:1537-1543; Hutchins et al. , 1995, Proc Natl. Acad Sci U S A 92:11980-11984; Jefferis et al, 1995, Immunol Lett. 44:111-117; Lund et al., 1995, Faseb J 9:115-119; Jefferis et al, 1996, Immunol Lett 54:101-104; Lund et al, 1996, J Immunol 157:49634969; Armour et aL, 1999, Eur J Immunol 29:2613-2624; Idusogie et al, 2000, J Immunol 164:41784184; Reddy et al, 2000, J Immunol 164:1925-1933; Xu et al., 2000, Cell Immunol 200:16-26; Idusogie et al, 2001, J Immunol 166:2571-2575; Shields et al., 2001, J Biol Chem 276:6591-6604; Jefferis et al, 2002, Immunol Lett 82:57-65; Presta et al., 2002, Biochem Soc Trans 30:487-490); US 5,624,821; US 5,885,573; US 6,194,551; PCT WO 00/42072; PCT WO 99/58572; each of which is incorporated herein by reference in its entirety.

6.2.2 FUNCTIONAL ASSAYS OF MOLECULES WITH VARIANT Fc REGIONS

[00327] 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 and FcγR-Fc binding assays disclosed in U.S Patent Application Publications 2005/0037000 and 2005/0064514, and International Patent Application Publication WO 04/063351 (each of which is hereby incorporated by reference in its entirety); 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 FcγR-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, each of which is incorporated by reference herein in its entirety).

[00328] In one embodiment, the molecules of the invention can be assayed for FcγR-mediated phagocytosis in human monocytes. Alternatively, the FcγR-mediated phagocytosis of the molecules of the invention may be assayed in other phagocytes, *e.g.*, neutrophils (polymorphonuclear leuckocytes; 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 FcγRIIIA, comprises of: treating THP-1 cells

with a molecule of the invention or with a control antibody that does not bind to FcγRIIIA, 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.

[00329] 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 FcγR-dependent ADCP; or (ii) 4-4-20 antibody harboring the D265A mutation that knocks out binding to FcγRIII, as a background control for FcγR-dependent ADCP (iii) 4-4-20 antibody carrying variant Fc regions identified by the methods of the invention and produced as exemplified in Example 7.6; and forming the opsonized particle; adding any of the osponized particles described (i-iii) to THP-1 effector cells (a monocytic cell line available from ATCC) in a 60:1 ratio to allow FcγR-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 fluoroscence of the bacteria that are adhered to the outside of the cell surface without being internalized; transfering 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 FcγR on the cell surface. THP-1 cells express both CD32A and CD64. CD64 is a high affinity FcγR 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 μg/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 FcγR-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).

[00330] 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 ^{51}Cr release assay comprising of: labeling target cells with $[^{51}Cr]Na_2CrO_4$ (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); osponizing 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 analzying 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.

[00331] 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; which is incorporated herein by reference in its entirety.

[00332] 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 FcγRIIIA and FcγRIIB, to determine if Fc antibody mutants show increased ADCC activity and phagocytosis relative to wild type IgG1 antibodies.

[00333] 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 (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 FcγRIIIA (Pearse *et al.*, 1993 *PNAS USA* 90: 4314-8). The 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 differentiate into macrophages in the presence of IFNγ and TNF (Koren *et al.*, 1979, *Nature* 279: 328-331).

[00334] 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 efficiency Fc mutants to trigger cell cytotoxicity of target cells in both phagocytosis and ADCC assays. Expression patterns of FcγRI, FcγRIIIA, 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 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.

[00335] 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 embodiments, the invention encompasses the mouse macrophage cell line RAW264.7(ATCC) which can be transfected with human FcγRIIIA 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 FcγRIIIA 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.

[00336] 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.

[00337] 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.

[00338] 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, $[^{51}Cr]Na_2CrO_4$; and the acetoxymethyl ester of the fluorescence enhancing ligand, 2,2':6',2"-terpyridine-6-6"-dicarboxylate (TDA).

[00339] 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; which is incorporated herein by reference in its entirety. 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% CO_2, 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).

[00340] In another specific embodiment, the ADCC assay used to characterize the molecules of the invention comprising variant Fc regions comprises the following steps: Preferably 4-5x10^6 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 (DELFIA BATDA Reagent, Perkin Elmer/Wallac). For optimal labeling efficiency, the number of target cells used in the ADCC assay should preferably not exceed 5x10^6. BATDA reagent is added to the cells and the mixture is incubated at 37°C preferably under 5% CO_2, 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 6.4. 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 6.4. 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 are 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.

[00341] 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% CO_2. Cell supernatants are harvested and added to an 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 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.

[00342] The invention encompasses characterization of the Fc variants in both NK-dependent and macrophage dependent ADCC assays. Fc variants of the invention have altered phenotypes such as an altered effector function as assayed in an NK dependent or macrophage dependent assay.

[00343] The invention encompasses assays known in the art and exemplified herein, to bind C1q and mediate complement dependent cytotoxicity (CDC). To determine C1q binding, a C1q binding ELISA may be performed. An exemplary assay may comprise the following: assay plates may be coated overnight at 4C with polypeptide variant or starting polypeptide (control) in coating buffer. The plates may then be washed and blocked. Following washing, an aliquot of human C1q may be added to each well and incubated for 2 hrs at room temperature. Following a further wash, 100 uL of a sheep anti-complement C1q peroxidase conjugated antibody may be added to each well and incubated for 1 hour at room temperature. The plate may again be washed with wash buffer and 100 ul of substrate buffer containing OPD (O-phenylenediamine dihydrochloride (Sigma)) may be added to each well. The oxidation reaction, observed by the appearance of a yellow color, may be allowed to proceed for 30 minutes and stopped by the addition of 100 ul of 4.5 NH2 SO4. The absorbance may then read at (492-405) nm.

[00344] A preferred variant in accordance with the invention is one that displays a significant reduction in C1q binding, as detected and measured in this assay or a similar assay. Preferably the molecule comprising an Fc variant displays about 50 fold reduction, about 60 fold, about 80 fold, or about 90 fold reduction in C1q binding compared to a control antibody having a nonmutated IgG1 Fc region. In the most preferred embodiment, the molecule comprising an Fc variant does not bind C1q, i.e. the variant displays about 100 fold or more reduction in C1q binding compared to the control antibody.

[00345] Another exemplary variant is one which has a better binding affinity for human C1q than the molecule comprising wild type Fc region. Such a molecule may display, for example, about two-fold or more, and preferably about five-fold or more, improvement in human C1q binding compared to the parent molecule comprising wild type Fc region. For example, human C1q binding may be about two-fold to about 500-

fold, and preferably from about two-fold or from about five-fold to about 1000-fold improved compared to the molecule comprising wild type Fc region.

[00346] To assess complement activation, a complement dependent cytotoxicity (CDC) assay may be performed, *e.g.* as described in Gazzano-Santoro et al., J. Immunol. Methods 202:163 (1996), which is incorporated herein by reference in its entirety. Briefly, various concentrations of the molecule comprising a variant Fc region and human complement may be diluted with buffer. Cells which express the antigen to which the molecule comprising a variant Fc region binds may be diluted to a density of about $1x10^6$ cells/ml. Mixtures of the molecule comprising a variant Fc region, diluted human complement and cells expressing the antigen may be added to a flat bottom tissue culture 96 well plate and allowed to incubate for 2 hrs at 37C and 5% CO2 to facilitate complement mediated cell lysis. 50 μL of alamar blue (Accumed International) may then be added to each well and incubated overnight at 37 C. The absorbance is measured using a 96-well fluorometer with excitation at 530 nm and emission at 590 nm. The results may be expressed in relative fluorescence units (RFU). The sample concentrations may be computed from a standard curve and the percent activity as compared to nonvariant molecule, i.e., a molecule comprising wild type Fc region, is reported for the variant of interest.

[00347] In some embodiments, an Fc variant of the invention does not activate complement. Preferably, the variant does not appear to have any CDC activity in the above CDC assay. The invention also pertains to a variant with enhanced CDC compared to a parent molecule (a molecule comprising wild type Fc region), e.g., displaying about two-fold to about 100-fold improvement in CDC activity in vitro or in vivo (e.g., at the IC50 values for each molecule being compared). Complement assays may be performed with guinea pig, rabbit or human serum. Complement lysis of target cells may be detected by monitoring the release of intracellular enzymes such as lactate dehydrogenase (LDH), as described in Korzeniewski *et al.*, 1983 *Immunol. Methods* 64(3): 313-20; and Decker *et al.*, 1988 *J. Immunol Methods* 115(1): 61-9, each of which is incorporated herein by reference in its entirety; or the release of an intracellular lable such as europium, chromium 51 or indium 111 in which target cells are labeled as described herein.

6.2.3 OTHER ASSAYS

[00348] The molecules of the invention comprising variant Fc regions may also be 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-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; all of which are incorporated herein by reference in their entirety. 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, all of which are incorporated herein by reference in their entirety.

[00349] 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 occurs 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. 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.

[00350] 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, which are incorporated herein by reference in their entireties). 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, which is incorporated herein by reference in its entirety.

[00351] 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 $NaHCO_3$ pH 9.4; 3M NaCl. Any regeneration technique known in the art is contemplated in the method of the invention.

[00352] 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 BIAevaluaion 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* Myszka, 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; all of which are incorporated herein by reference in their entirety.

[00353] In preferred embodiments, the kinetic parameters determined using an SPR analysis, *e.g.*, BIAcore, may be used as a predictive meaure 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_{off} (wt)/K_{off} (mut) for FcγRIIIA; (2) K_{off} (mut)/K_{off} (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.

6.3 METHODS OF RECOMBINANTLY PRODUCING MOLECULES OF THE INVENTION

6.3.1 POLYNUCLEOTIDES ENCODING MOLECULES OF THE INVENTION

[00354] 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.

[00355] 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, which are both incorporated by reference herein in their entireties), to generate, for example, antibodies having a different amino acid sequence, for example by generating amino acid substitutions, deletions, and/or insertions.

[00356] 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).

[00357] 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.

6.3.2 RECOMBINANT EXPRESSION OF MOLECULES OF THE INVENTION

[00358] 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).

[00359] 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.

[00360] 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).

[00361] 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).

[00362] 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 gluta-thione. 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.

[00363] 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).

[00364] 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).

[00365] 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, VERY, BHK, Hela, COS, MDCK, 293, 293T, 3T3, WI38, BT483, Hs578T, HTB2, BT20 and T47D, CRL7030 and Hs578Bst.

[00366] 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 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.

[00367] 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-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 *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. 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, John Wiley & Sons, NY.; Colberre-Garapin *et al.*, 1981, *J. Mol. Biol.* 150:1; and hygro, which confers resistance to hygromycin (Santerre *et al.*, 1984, *Gene* 30:147).

[00368] 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 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).

[00369] 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. 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.

[00370] 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 for the purification of polypeptides or antibodies.

6.4 PROPHYLACTIC AND THERAPEUTIC METHODS

[00371] 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 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. 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. 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.

[00372] The molecules of the invention may also be advantageously utilized in 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 6.4.1.2 and 6.4.2.1 below.

6.4.1 CANCERS

[00373] 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.

[00374] 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 FcγRIIIA and/or FcγRIIA with a greater affinity than a comparable polypeptide comprising a wild type Fc region binds FcγRIIIA and/or FcγRIIA, and/or said 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 FcγRIIIA and/or FcγRIIA with a greater affinity than a comparable polypeptide comprising a wild type Fc region binds FcγRIIIA and/or FcγRIIA, and further binds FcγRIIB with a lower affinity than a comparable polypeptide comprising a wild-type Fc region binds FcγRIIB, 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.

[00375] In some embodiments, the invention encompasses methods and compositions for the treatment or prevention of cancer in a subject with FcγR polymorphisms such as those homozygous for the FγRIIIA-158V or FcγRIIIA-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 allele of FcγRIIIA (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 FcγRIIIA (158V).

[00376] 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 both of which are incorporated hrein by reference in their entireties). 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.

[00377] 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 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.

[00378] 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.

[00379] 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 immunotherpay 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.

[00380] 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 6.2 and **Table 9**) 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 6.2.2 to produce engineered therapeutic carrying the

identified mutation sites with the desired binding properties. Any of the therapeutic antibodies listed in **Table 10** 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. Other therapeutic antibodies include those against pathogenic agents such as those against Streptococcus pneumoniae Serotype 6B, see, *e.g.,* Sun et al., 1999, Infection and Immunity, 67(3): 1172-9.

[00381] The Fc variants of the invention may be incorporated into therapeutic antibodies such as those disclosed herein or other Fc fusion clinical candidates, *i.e.*, a molecule comprising an Fc regions which has been approved for us in clinical trials or any other molecule that may benefit from the Fc variants of the instant invention, humanized, affinity matured, modified or engineered versions thereof.

[00382] The invention also encompasses engineering any other polypeptide comprising an Fc region which has therapeutic utility, including but not limited to 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 10. 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	Bivatuzumab	head & neck cancer	CD44
Centocor/J&J	Panorex™	Colorectal cancer	17-1A
	ReoPro™	PTCA	gp IIb/IIIa
	ReoPro™	Acute MI	gp IIb/IIIa
	ReoPro™	Ischemic stroke	gp IIb/IIIa
Corixa	Bexocar™	NHL	CD20
CRC Technology	MAb, idiotypic 105AD7	colorectal cancer	gp72

Company	Product	Disease	Target
		vaccine	
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 & high-grade NHL	CD20
	MAb-VEGF	NSCLC, metastatic	VEGF
	MAb-VEGF	Colorectal cancer, metastatic	VEGF
	AMD™ Fab	age-related macular degeneration	CD18
	E-26™ (2nd gen. IgE)	allergic asthma & rhinitis	IgE
IDEC	Zevalin™ (Rituxan™ + yttrium-90)	low grade of follicular, relapsed or refractory, CD20-positive, B-cell NHL and Rituximab-refractory NHL	CD20
ImClone	Cetuximab™ + innotecan	refractory colorectal carcinoma	EGF receptor
	Cetuximab™ + cisplatin & radiation	newly diagnosed or recurrent head & neck cancer	EGF receptor
	Cetuximab™ + gemcitabine	newly diagnosed metastatic pancreatic carcinoma	EGF receptor
	Cetuximab™ + cisplatin + 5FU or Taxol	recurrent or metastatic head & neck cancer	EGF receptor
	Cetuximab™ + carboplatin + paclitaxel	newly diagnosed non-small cell lung carcinoma	EGF receptor
	Cetuximab™ + cisplatin™	head & neck cancer (extensive incurable local-regional disease & distant metasteses)	EGF receptor
	Cetuximab + radiation	locally advanced head & neck	EGF receptor

Company	Product	Disease	Target
		carcinoma	
	BEC2 + Bacillus Calmette Guerin	small cell lung carcinoma	mimics ganglioside GD3
	BEC2 + Bacillus Calmette Guerin	melanoma	mimics ganglioside GD3
	IMC-1C11	colorectal cancer with liver metasteses	VEGF-receptor
ImmonoGen	nuC242-DM1	Colorectal, gastric, and pancreatic cancer	nuC242
ImmunoMedics	LymphoCide™	Non-Hodgkins lymphoma	CD22
	LymphoCide Y-90™	Non-Hodgkins lymphoma	CD22
	CEA-Cide™	metastatic solid tumors	CEA
	CEA-Cide Y-90™	metastatic solid tumors	CEA
	CEA-Scan™ (Tc-99m-labeled arcitumomab)	colorectal cancer (radioimaging)	CEA
	CEA-Scan™ (Tc-99m-labeled arcitumomab)	Breast cancer (radioimaging)	CEA
	CEA-Scan™ (Tc-99m-labeled arcitumomab)	lung cancer (radioimaging)	CEA
	CEA-Scan™ (Tc-99m-labeled arcitumomab)	intraoperative tumors (radio imaging)	CEA
	LeukoScan™ (Tc-99m-labeled sulesomab)	soft tissue infection (radioimaging)	CEA
	LymphoScan™ (Tc-99m-labeled)	lymphomas (radioimaging)	CD22
	AFP-Scan™ (Tc-99m-labeled)	liver 7 gem-cell cancers (radioimaging)	AFP
Intracel	HumaRAD-HN (+ yttrium-90)	head & neck cancer	NA
	HumaSPECT	colorectal imaging	NA
Medarex	MDX-101 (CTLA-4)	Prostate and other cancers	CTLA-4
	MDX-210 (her-2 overexpression)	Prostate cancer	HER-2
	MDX-210/MAK	Cancer	HER-2
MedImmune	Vitaxin™	Cancer	$\alpha v\beta_3$
Merck KGaA	MAb 425	Various cancers	EGF receptor
	IS-IL-2	Various cancers	Ep-CAM

Company	Product	Disease	Target
Millennium	Campath® (alemtuzumab)	chronic lymphocytic leukemia	CD52
NeoRx	CD20-streptavidin (+ biotin-yttrium 90)	Non-Hodgkins lymphoma	CD20
	Avidicin (albumin + NRLU13)	metastatic cancer	NA
Peregrine	Oncolym™ (+ iodine-131)	Non-Hodgkins lymphoma	HLA-DR 10 beta
	Cotara™ (+ iodine-131)	unresectable malignant glioma	DNA-associated proteins
Pharmacia Corporation	C215 (+ staphylococcal enterotoxin)	pancreatic cancer	NA
	MAb, lung/kidney cancer	lung & kidney cancer	NA
	nacolomab tafenatox (C242 + staphylococcal enterotoxin)	colon & pancreatic cancer	NA
Protein Design Labs	Nuvion™	T cell malignancies	CD3
	SMART M195™	AML	CD33
	SMART 1D10™	NHL	HLA-DR antigen
Titan	CEAVac™	colorectal cancer, advanced	CEA
	TriGem™	metastatic melanoma & small cell lung cancer	GD2-ganglioside
	TriAb™	metastatic breast cancer	MUC-1
Trilex	CEAVa™c	colorectal cancer, advanced	CEA
	TriGem™	metastatic melanoma & small cell lung cancer	GD2-ganglioside
	TriAb™	metastatic breast cancer	MUC-1
Viventia Biotech	NovoMAb-G2 radiolabeled	Non-Hodgkins lymphoma	NA
	Monopharm C™	colorectal & pancreatic carcinoma	SK-1 antigen
	GlioMAb-H™ (+ gelonin toxin)	gliorna, melanoma & neuroblastoma	NA
Xoma	Rituxan™	Relapsed/refractory low-grade or follicular NHL	CD20
	Rituxan™	intermediate & high-grade NHL	CD20

Company	Product	Disease	Target
	ING-1	adenomcarcinoma	Ep-CAM

[00383] Accordingly, the invention provides methods of preventing or treating cancer characterized by a cancer antigen, using a therapeutic antibody that binds a cancer antigen and is cytotoxic and has been modified at one or more sites in the Fc region, according to the invention, to bind FcγRIIIA and/or FcγRIIA with a higher affinity than the parent therapeutic antibody, and/or mediates effector function (*e.g.*, ADCC, phagocytosis) more effectively. In another embodiment, the invention provides methods of preventing or treating cancer characterized by a cancer antigen, using a therapeutic antibody that binds a cancer antigen and is cytotoxic, and has been engineered according to the invention to bind FcγRIIIA and/or FcγRIIA with a higher affinity and bind FcγRIIB with a lower affinity than the parent therapeutic antibody , and/or mediates effector function (*e.g.*, ADCC, phagocytosis) more effectively. The therapeutic antibodies that have been engineered according to the invention are useful for prevention or treatment of cancer, since they have an enhanced cytotoxic activity (*e.g.*, enhanced tumor cell killing and/or enhanced for example, ADCC activity or CDC activity).

[00384] Cancers associated with a cancer antigen may be treated or prevented by administration of a therapeutic antibody that binds a cancer antigen and is cytotoxic, and has been engineered according to the methods of the invention to have, for example, an enhanced effector function. In one particular embodiment, the therapeutic antibodies engineered according to the methods of the invention enhance the antibody-mediated cytotoxic effect of the antibody directed at the particular cancer antigen. For example, but not by way of limitation, cancers associated with the following cancer antigens may be treated or prevented by the methods and compositions of the invention: KS 1/4 pan-carcinoma antigen (Perez and Walker, 1990, *J. Immunol.* 142:32-37; Bumal, 1988, *Hybridoma* 7(4):407-415), ovarian carcinoma antigen (CA125) (Yu *et al.*, 1991, *Cancer Res.* 51(2):48-475), prostatic acid phosphate (Tailor *et al.*, 1990, *Nucl. Acids Res.* 18(1):4928), prostate specific antigen (Henttu and Vihko, 1989, *Biochem. Biophys. Res. Comm.* 10(2):903-910; Israeli *et al.*, 1993, *Cancer Res.* 53:227-230), melanoma-associated antigen p97 (Estin *et al.*, 1989, *J. Natl. Cancer Instit.* 81(6):445-44), 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* 59:55-3; 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 erthrocytes and primary endoderm, I(Ma) found in gastric adencarcinomas, M18 and M39 found in breast epithelium, SSEA-1 found in myeloid cells, VEP8, VEP9, Myl, VIM-D5,and $D_1$56-22 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 , E_1 series (blood group B) found in pancreatic cancer, FC10.2 found in embryonal carcinoma cells, gastric adenocarcinoma, CO-514 (blood group Le^a) found in adenocarcinoma, NS-10 found in adenocarcinomas, CO-43 (blood group Le^b), G49, EGF receptor, (blood group ALe^b/Le^y) found in colonic adenocarcinoma, 19.9 found in

colon cancer, gastric cancer mucins, T_5A_7 found in myeloid cells, R_{24} found in melanoma, 4.2, G_{D3}, D1.1, OFA-1, G_{M2}, OFA-2, G_{D2}, M1:22:25:8 found in embryonal carcinoma cells and SSEA-3, SSEA-4 found in 4-8-cell stage embryos. In another embodiment, the antigen is a T cell receptor derived peptide from a cutaneous T cell lymphoma (*see* Edelson, 1998, *The Cancer Journal* 4:62).

[00385] Cancers and related disorders that can be treated or prevented by methods and compositions of the present invention include, but are not limited to, the following: Leukemias including, but not limited to, acute leukemia, acute lymphocytic leukemia, acute myelocytic leukemias such as myeloblastic, promyelocytic, myelomonocytic, monocytic, erythroleukemia leukemias and myelodysplastic syndrome, chronic leukemias such as but not limited to, chronic myelocytic (granulocytic) leukemia, chronic lymphocytic leukemia, hairy cell leukemia; polycythemia vera; lymphomas such as but not limited to Hodgkin's disease, non-Hodgkin's disease; multiple myelomas such as but not limited to smoldering multiple myeloma, nonsecretory myeloma, osteosclerotic myeloma, plasma cell leukemia, solitary plasmacytoma and extramedullary plasmacytoma; Waldenström's macroglobulinemia; monoclonal gammopathy of undetermined significance; benign monoclonal gammopathy; heavy chain disease; bone and connective tissue sarcomas such as but not limited to bone sarcoma, osteosarcoma, chondrosarcoma, Ewing's sarcoma, malignant giant cell tumor, fibrosarcoma of bone, chordoma, periosteal sarcoma, soft-tissue sarcomas, angiosarcoma (hemangiosarcoma), fibrosarcoma, Kaposi's sarcoma, leiomyosarcoma, liposarcoma, lymphangiosarcoma, neurilemmoma, rhabdomyosarcoma, synovial sarcoma; brain tumors including but not limited to, glioma, astrocytoma, brain stem glioma, ependymoma, oligodendroglioma, nonglial tumor, acoustic neurinoma, craniopharyngioma, medulloblastoma, meningioma, pineocytoma, pineoblastoma, primary brain lymphoma; breast cancer including, but not limited to, adenocarcinoma, lobular (small cell) carcinoma, intraductal carcinoma, medullary breast cancer, mucinous breast cancer, tubular breast cancer, papillary breast cancer, Paget's disease, and inflammatory breast cancer; adrenal cancer, including but not limited to, pheochromocytom and adrenocortical carcinoma; thyroid cancer such as but not limited to papillary or follicular thyroid cancer, medullary thyroid cancer and anaplastic thyroid cancer; pancreatic cancer, including but not limited to, insulinoma, gastrinoma, glucagonoma, vipoma, somatostatin-secreting tumor, and carcinoid or islet cell tumor; pituitary cancers including but not limited to, Cushing's disease, prolactin-secreting

pituitary cancers including but not limited to, Cushing's disease, prolactin-secreting tumor, acromegaly, and diabetes insipius; eye cancers including but not limited to, ocular melanoma such as iris melanoma, choroidal melanoma, and cilliary body melanoma, and retinoblastoma; vaginal cancers, including but not limited to, squamous cell carcinoma, adenocarcinoma, and melanoma; vulvar cancer, including but not limited to, squamous cell carcinoma, melanoma, adenocarcinoma, basal cell carcinoma, sarcoma, and Paget's disease; cervical cancers including but not limited to, squamous cell carcinoma, and adenocarcinoma; uterine cancers including but not limited to, endometrial carcinoma and uterine sarcoma; ovarian cancers including but not limited to, ovarian epithelial carcinoma, borderline tumor, germ cell tumor, and stromal tumor; esophageal cancers including but not limited to, squamous cancer, adenocarcinoma, adenoid cyctic carcinoma, mucoepidermoid carcinoma, adenosquamous carcinoma, sarcoma, melanoma, plasmacytoma, verrucous carcinoma, and oat cell (small cell) carcinoma; stomach cancers including but not limited to, adenocarcinoma, fungating (polypoid), ulcerating, superficial spreading, diffusely spreading, malignant lymphoma, liposarcoma, fibrosarcoma, and carcinosarcoma; colon cancers; rectal cancers; liver cancers including but not limited to hepatocellular carcinoma and hepatoblastoma, gallbladder cancers including but not limited to, adenocarcinoma; cholangiocarcinomas including but not limited to, pappillary, nodular, and diffuse; lung cancers including but not limited to, non-small cell lung cancer, squamous cell carcinoma (epidermoid carcinoma), adenocarcinoma, large-cell carcinoma and small-cell lung cancer; testicular cancers including but not limited to, germinal tumor, seminoma, anaplastic, classic (typical), spermatocytic, nonseminoma, embryonal carcinoma, teratoma carcinoma, choriocarcinoma (yolk-sac tumor), prostate cancers including but not limited to, adenocarcinoma, leiomyosarcoma, and rhabdomyosarcoma; penal cancers; oral cancers including but not limited to, squamous cell carcinoma; basal cancers; salivary gland cancers including but not limited to, adenocarcinoma, mucoepidermoid carcinoma, and adenoidcystic carcinoma; pharynx cancers including but not limited to, squamous cell cancer, and verrucous; skin cancers including but not limited to, basal cell carcinoma, squamous cell carcinoma and melanoma, superficial spreading melanoma, nodular melanoma, lentigo malignant melanoma, acral lentiginous melanoma; kidney cancers including but not limited to, renal cell cancer, adenocarcinoma, hypernephroma, fibrosarcoma, transitional cell cancer (renal pelvis and/ or ureter); Wilms' tumor; bladder cancers including but not limited to, transitional cell carcinoma, squamous cell

osteogenic sarcoma, endotheliosarcoma, lymphangioendotheliosarcoma, mesothelioma, synovioma, hemangioblastoma, epithelial carcinoma, cystadenocarcinoma, bronchogenic carcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma and papillary adenocarcinomas (for a review of such disorders, *see* Fishman *et al.*, 1985, *Medicine*, 2d Ed., J.B. Lippincott Co., Philadelphia and Murphy *et al.*, 1997, *Informed Decisions: The Complete Book of Cancer Diagnosis, Treatment, and Recovery*, Viking Penguin, Penguin Books U.S.A., Inc., United States of America).

[00386] Accordingly, the methods and compositions of the invention are also useful in the treatment or prevention of a variety of cancers or other abnormal proliferative diseases, including (but not limited to) the following: carcinoma, including that of the bladder, breast, colon, kidney, liver, lung, ovary, pancreas, stomach, prostate, cervix, thyroid and skin; including squamous cell carcinoma; hematopoietic tumors of lymphoid lineage, including leukemia, acute lymphocytic leukemia, acute lymphoblastic leukemia, B-cell lymphoma, T-cell lymphoma, Burketts lymphoma; hematopoietic tumors of myeloid lineage, including acute and chronic myelogenous leukemias and promyelocytic leukemia; tumors of mesenchymal origin, including fibrosarcoma and rhabdomyoscarcoma; other tumors, including melanoma, seminoma, tetratocarcinoma, neuroblastoma and glioma; tumors of the central and peripheral nervous system, including astrocytoma, neuroblastoma, glioma, and schwannomas; tumors of mesenchymal origin, including fibrosafcoma, rhabdomyoscarama, and osteosarcoma; and other tumors, including melanoma, xenoderma pegmentosum, keratoactanthoma, seminoma, thyroid follicular cancer and teratocarcinoma. It is also contemplated that cancers caused by aberrations in apoptosis would also be treated by the methods and compositions of the invention. Such cancers may include but not be limited to follicular lymphomas, carcinomas with p53 mutations, hormone dependent tumors of the breast, prostate and ovary, and precancerous lesions such as familial adenomatous polyposis, and myelodysplastic syndromes. In specific embodiments, malignancy or dysproliferative changes (such as metaplasias and dysplasias), or hyperproliferative disorders, are treated or prevented by the methods and compositions of the invention in the ovary, bladder, breast, colon, lung, skin, pancreas, or uterus. In other specific embodiments, sarcoma, melanoma, or leukemia is treated or prevented by the methods and compositions of the invention.

[00387] In a specific embodiment, a molecule of the invention (*e.g.*, an antibody comprising a variant Fc region, or a therapeutic monoclonal antibody engineered

according to the methods of the invention) inhibits or reduces the growth of primary tumor or metastasis of cancerous cells by at least 99%, at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 60%, at least 50%, at least 45%, at least 40%, at least 45%, at least 35%, at least 30%, at least 25%, at least 20%, or at least 10% relative to the growth of primary tumor or metastasis in the absence of said molecule of the invention.

6.4.1.1 COMBINATION THERAPY

[00388] The invention further encompasses administering the molecules of the invention in combination with other therapies known to those skilled in the art for the treatment or prevention of cancer, including but not limited to, current standard and experimental chemotherapies, hormonal therapies, biological therapies, immunotherapies, radiation therapies, or surgery. In some embodiments, the molecules of the invention may be administered in combination with a therapeutically or prophylactically effective amount of one or more anti-cancer agents, therapeutic antibodies (*e.g.*, antibodies listed in **Table 10**), or other agents known to those skilled in the art for the treatment and/or prevention of cancer (*See* Section 6.4.1.2).

[00389] In certain embodiments, one or more molecule of the invention is administered to a mammal, preferably a human, concurrently with one or more other therapeutic agents useful for the treatment of cancer. The term "concurrently" is not limited to the administration of prophylactic or therapeutic agents at exactly the same time, but rather it is meant that a molecule of the invention and the other agent are administered to a mammal in a sequence and within a time interval such that the molecule of the invention can act together with the other agent to provide an increased benefit than if they were administered otherwise. For example, each prophylactic or therapeutic agent (*e.g.*, chemotherapy, radiation therapy, hormonal therapy or biological therapy) may be administered at the same time or sequentially in any order at different points in time; however, if not administered at the same time, they should be administered sufficiently close in time so as to provide the desired therapeutic or prophylactic effect. Each therapeutic agent can be administered separately, in any appropriate form and by any suitable route. In various embodiments, the prophylactic or therapeutic agents are administered less than 1 hour apart, at about 1 hour apart, at about 1 hour to about 2 hours apart, at about 2 hours to about 3 hours apart, at about 3 hours to about 4 hours apart, at about 4 hours to about 5 hours apart, at about 5 hours to

about 6 hours apart, at about 6 hours to about 7 hours apart, at about 7 hours to about 8 hours apart, at about 8 hours to about 9 hours apart, at about 9 hours to about 10 hours apart, at about 10 hours to about 11 hours apart, at about 11 hours to about 12 hours apart, no more than 24 hours apart or no more than 48 hours apart. In preferred embodiments, two or more components are administered within the same patient visit.

[00390] In other embodiments, the prophylactic or therapeutic agents are administered at about 2 to 4 days apart, at about 4 to 6 days apart, at about 1 week part, at about 1 to 2 weeks apart, or more than 2 weeks apart. In preferred embodiments, the prophylactic or therapeutic agents are administered in a time frame where both agents are still active. One skilled in the art would be able to determine such a time frame by determining the half life of the administered agents.

[00391] In certain embodiments, the prophylactic or therapeutic agents of the invention are cyclically administered to a subject. Cycling therapy involves the administration of a first agent for a period of time, followed by the administration of a second agent and/or third agent for a period of time and repeating this sequential administration. Cycling therapy can reduce the development of resistance to one or more of the therapies, avoid or reduce the side effects of one of the therapies, and/or improves the efficacy of the treatment.

[00392] In certain embodiments, prophylactic or therapeutic agents are administered in a cycle of less than about 3 weeks, about once every two weeks, about once every 10 days or about once every week. One cycle can comprise the administration of a therapeutic or prophylactic agent by infusion over about 90 minutes every cycle, about 1 hour every cycle, about 45 minutes every cycle. Each cycle can comprise at least 1 week of rest, at least 2 weeks of rest, at least 3 weeks of rest. The number of cycles administered is from about 1 to about 12 cycles, more typically from about 2 to about 10 cycles, and more typically from about 2 to about 8 cycles.

[00393] In yet other embodiments, the therapeutic and prophylactic agents of the invention are administered in metronomic dosing regimens, either by continuous infusion or frequent administration without extended rest periods. Such metronomic administration can involve dosing at constant intervals without rest periods. Typically the therapeutic agents, in particular cytotoxic agents, are used at lower doses. Such dosing regimens encompass the chronic daily administration of relatively low doses for extended periods of time. In preferred embodiments, the use of lower doses can minimize toxic side effects and eliminate rest periods. In certain embodiments, the

therapeutic and prophylactic agents are delivered by chronic low-dose or continuous infusion ranging from about 24 hours to about 2 days, to about 1 week, to about 2 weeks, to about 3 weeks to about 1 month to about 2 months, to about 3 months, to about 4 months, to about 5 months, to about 6 months. The scheduling of such dose regimens can be optimized by the skilled oncologist.

[00394] In other embodiments, courses of treatment are administered concurrently to a mammal, *i.e.*, individual doses of the therapeutics are administered separately yet within a time interval such that molecules of the invention can work together with the other agent or agents. For example, one component may be administered one time per week in combination with the other components that may be administered one time every two weeks or one time every three weeks. In other words, the dosing regimens for the therapeutics are carried out concurrently even if the therapeutics are not administered simultaneously or within the same patient visit.

[00395] When used in combination with other prophylactic and/or therapeutic agents, the molecules of the invention and the prophylactic and/or therapeutic agent can act additively or, more preferably, synergistically. In one embodiment, a molecule of the invention is administered concurrently with one or more therapeutic agents in the same pharmaceutical composition. In another embodiment, a molecule of the invention is administered concurrently with one or more other therapeutic agents in separate pharmaceutical compositions. In still another embodiment, a molecule of the invention is administered prior to or subsequent to administration of another prophylactic or therapeutic agent. The invention contemplates administration of a molecule of the invention in combination with other prophylactic or therapeutic agents by the same or different routes of administration, *e.g.*, oral and parenteral. In certain embodiments, when a molecule of the invention is administered concurrently with another prophylactic or therapeutic agent that potentially produces adverse side effects including, but not limited to, toxicity, the prophylactic or therapeutic agent can advantageously be administered at a dose that falls below the threshold that the adverse side effect is elicited.

[00396] The dosage amounts and frequencies of administration provided herein are encompassed by the terms therapeutically effective and prophylactically effective. The dosage and frequency further will typically vary according to factors specific for each patient depending on the specific therapeutic or prophylactic agents administered, the severity and type of cancer, the route of administration, as well as age, body weight,

response, and the past medical history of the patient. Suitable regimens can be selected by one skilled in the art by considering such factors and by following, for example, dosages reported in the literature and recommended in the *Physician's Desk Reference* (56th ed., 2002).

6.4.1.2 OTHER THERAPEUTIC/PROPHYLACTIC AGENTS

[00397] In a specific embodiment, the methods of the invention encompass the administration of one or more molecules of the invention with one or more therapeutic agents used for the treatment and/or prevention of cancer. In one embodiment, angiogenesis inhibitors may be administered in combination with the molecules of the invention. Angiogenesis inhibitors that can be used in the methods and compositions of the invention include but are not limited to: Angiostatin (plasminogen fragment); antiangiogenic antithrombin III; Angiozyme; ABT-627; Bay 12-9566; Benefin; Bevacizumab; BMS-275291; cartilage-derived inhibitor (CDI); CAI; CD59 complement fragment; CEP-7055; Col 3; Combretastatin A-4; Endostatin (collagen XVIII fragment); Fibronectin fragment; Gro-beta; Halofuginone; Heparinases; Heparin hexasaccharide fragment; HMV833; Human chorionic gonadotropin (hCG); IM-862; Interferon alpha/beta/gamma; Interferon inducible protein (IP-10); Interleukin-12; Kringle 5 (plasminogen fragment); Marimastat; Metalloproteinase inhibitors (TIMPs); 2-Methoxyestradiol; MMI 270 (CGS 27023A); MoAb IMC-1C11; Neovastat; NM-3; Panzem; PI-88; Placental ribonuclease inhibitor; Plasminogen activator inhibitor; Platelet factor-4 (PF4); Prinomastat; Prolactin 16kD fragment; Proliferin-related protein (PRP); PTK 787/ZK 222594; Retinoids; Solimastat; Squalamine; SS 3304; SU 5416; SU6668; SU11248; Tetrahydrocortisol-S; tetrathiomolybdate; thalidomide; Thrombospondin-1 (TSP-1); TNP-470; Transforming growth factor-beta (TGF-b); Vasculostatin; Vasostatin (calreticulin fragment); ZD6126; ZD 6474; farnesyl transferase inhibitors (FTI); and bisphosphonates.

[00398] Anti-cancer agents that can be used in combination with the molecules of the invention in the various embodiments of the invention, including pharmaceutical compositions and dosage forms and kits of the invention, include. but are not limited to: acivicin; aclarubicin; acodazole hydrochloride; acronine; adozelesin; aldesleukin; altretamine; ambomycin; ametantrone acetate; aminoglutethimide; amsacrine; anastrozole; anthramycin; asparaginase; asperlin; azacitidine; azetepa; azotomycin;

batimastat; benzodepa; bicalutamide; bisantrene hydrochloride; bisnafide dimesylate; bizelesin; bleomycin sulfate; brequinar sodium; bropirimine; busulfan; cactinomycin; calusterone; caracemide; carbetimer; carboplatin; carmustine; carubicin hydrochloride; carzelesin; cedefingol; chlorambucil; cirolemycin; cisplatin; cladribine; crisnatol mesylate; cyclophosphamide; cytarabine; dacarbazine; dactinomycin; daunorubicin hydrochloride; decitabine; dexormaplatin; dezaguanine; dezaguanine mesylate; diaziquone; docetaxel; doxorubicin; doxorubicin hydrochloride; droloxifene; droloxifene citrate; dromostanolone propionate; duazomycin; edatrexate; eflornithine hydrochloride; elsamitrucin; enloplatin; enpromate; epipropidine; epirubicin hydrochloride; erbulozole; esorubicin hydrochloride; estramustine; estramustine phosphate sodium; etanidazole; etoposide; etoposide phosphate; etoprine; fadrozole hydrochloride; fazarabine; fenretinide; floxuridine; fludarabine phosphate; fluorouracil; flurocitabine; fosquidone; fostriecin sodium; gemcitabine; gemcitabine hydrochloride; hydroxyurea; idarubicin hydrochloride; ifosfamide; ilmofosine; interleukin II (including recombinant interleukin II, or rIL2), interferon alfa-2a; interferon alfa-2b; interferon alfa-n1 ; interferon alfa-n3; interferon beta-I a; interferon gamma-I b; iproplatin; irinotecan hydrochloride; lanreotide acetate; letrozole; leuprolide acetate; liarozole hydrochloride; lometrexol sodium; lomustine; losoxantrone hydrochloride; masoprocol; maytansine; mechlorethamine hydrochloride; megestrol acetate; melengestrol acetate; melphalan; menogaril; mercaptopurine; methotrexate; methotrexate sodium; metoprine; meturedepa; mitindomide; mitocarcin; mitocromin; mitogillin; mitomalcin; mitomycin; mitosper; mitotane; mitoxantrone hydrochloride; mycophenolic acid; nocodazole; nogalamycin; ormaplatin; oxisuran; paclitaxel; pegaspargase; peliomycin; pentamustine; peplomycin sulfate; perfosfamide; pipobroman; piposulfan; piroxantrone hydrochloride; plicamycin; plomestane; porfimer sodium; porfiromycin; prednimustine; procarbazine hydrochloride; puromycin; puromycin hydrochloride; pyrazofurin; riboprine; rogletimide; safingol; safingol hydrochloride; semustine; simtrazene; sparfosate sodium; sparsomycin; spirogermanium hydrochloride; spiromustine; spiroplatin; streptonigrin; streptozocin; sulofenur; talisomycin; tecogalan sodium; tegafur; teloxantrone hydrochloride; temoporfin; teniposide; teroxirone; testolactone; thiamiprine; thioguanine; thiotepa; tiazofurin; tirapazamine; toremifene citrate; trestolone acetate; triciribine phosphate; trimetrexate; trimetrexate glucuronate; triptorelin; tubulozole hydrochloride; uracil mustard; uredepa; vapreotide; verteporfin; vinblastine sulfate; vincristine sulfate;

trimetrexate; trimetrexate glucuronate; triptorelin; tubulozole hydrochloride; uracil mustard; uredepa; vapreotide; verteporfin; vinblastine sulfate; vincristine sulfate; vindesine; vindesine sulfate; vinepidine sulfate; vinglycinate sulfate; vinleurosine sulfate; vinorelbine tartrate; vinrosidine sulfate; vinzolidine sulfate; vorozole; zeniplatin; zinostatin; zorubicin hydrochloride. Other anti-cancer drugs include, but are not limited to: 20-epi-1,25 dihydroxyvitamin D3; 5-ethynyluracil; abiraterone; aclarubicin; acylfulvene; adecypenol; adozelesin; aldesleukin; ALL-TK antagonists; altretamine; ambamustine; amidox; amifostine; aminolevulinic acid; amrubicin; amsacrine; anagrelide; anastrozole; andrographolide; angiogenesis inhibitors; antagonist D; antagonist G; antarelix; anti-dorsalizing morphogenetic protein-1; antiandrogen, prostatic carcinoma; antiestrogen; antineoplaston; antisense oligonucleotides; aphidicolin glycinate; apoptosis gene modulators; apoptosis regulators; apurinic acid; ara-CDP-DL-PTBA; arginine deaminase; asulacrine; atamestane; atrimustine; axinastatin 1; axinastatin 2; axinastatin 3; azasetron; azatoxin; azatyrosine; baccatin III derivatives; balanol; batimastat; BCR/ABL antagonists; benzochlorins; benzoylstaurosporine; beta lactam derivatives; beta-alethine; betaclamycin B; betulinic acid; bFGF inhibitor; bicalutamide; bisantrene; bisaziridinylspermine; bisnafide; bistratene A; bizelesin; breflate; bropirimine; budotitane; buthionine sulfoximine; calcipotriol; calphostin C; camptothecin derivatives; canarypox IL-2; capecitabine; carboxamide-amino-triazole; carboxyamidotriazole; CaRest M3; CARN 700; cartilage derived inhibitor; carzelesin; casein kinase inhibitors (ICOS); castanospermine; cecropin B; cetrorelix; chlorins; chloroquinoxaline sulfonamide; cicaprost; cis-porphyrin; cladribine; clomifene analogues; clotrimazole; collismycin A; collismycin B; combretastatin A4; combretastatin analogue; conagenin; crambescidin 816; crisnatol; cryptophycin 8; cryptophycin A derivatives; curacin A; cyclopentanthraquinones; cycloplatam; cypemycin; cytarabine ocfosfate; cytolytic factor; cytostatin; dacliximab; decitabine; dehydrodidemnin B; deslorelin; dexamethasone; dexifosfamide; dexrazoxane; dexverapamil; diaziquone; didemnin B; didox; diethylnorspermine; dihydro-5-azacytidine; dihydrotaxol, 9-; dioxamycin; diphenyl spiromustine; docetaxel; docosanol; dolasetron; doxifluridine; droloxifene; dronabinol; duocarmycin SA; ebselen; ecomustine; edelfosine; edrecolomab; eflornithine; elemene; emitefur; epirubicin; epristeride; estramustine analogue; estrogen agonists; estrogen antagonists; etanidazole; etoposide phosphate; exemestane; fadrozole; fazarabine; fenretinide;

gadolinium texaphyrin; gallium nitrate; galocitabine; ganirelix; gelatinase inhibitors; gemcitabine; glutathione inhibitors; hepsulfam; heregulin; hexamethylene bisacetamide; hypericin; ibandronic acid; idarubicin; idoxifene; idramantone; ilmofosine; ilomastat; imidazoacridones; imiquimod; immunostimulant peptides; insulin-like growth factor-1 receptor inhibitor; interferon agonists; interferons; interleukins; iobenguane; iododoxorubicin; ipomeanol, 4-; iroplact; irsogladine; isobengazole; isohomohalicondrin B; itasetron; jasplakinolide; kahalalide F; lamellarin-N triacetate; lanreotide; leinamycin; lenograstim; lentinan sulfate; leptolstatin; letrozole; leukemia inhibiting factor; leukocyte alpha interferon; leuprolide+estrogen+progesterone; leuprorelin; levamisole; liarozole; linear polyamine analogue; lipophilic disaccharide peptide; lipophilic platinum compounds; lissoclinamide 7; lobaplatin; lombricine; lometrexol; lonidamine; losoxantrone; lovastatin; loxoribine; lurtotecan; lutetium texaphyrin; lysofylline; lytic peptides; maitansine; mannostatin A; marimastat; masoprocol; maspin; matrilysin inhibitors; matrix metalloproteinase inhibitors; menogaril; merbarone; meterelin; methioninase; metoclopramide; MIF inhibitor; mifepristone; miltefosine; mirimostim; mismatched double stranded RNA; mitoguazone; mitolactol; mitomycin analogues; mitonafide; mitotoxin fibroblast growth factor-saporin; mitoxantrone; mofarotene; molgramostim; monoclonal antibody, human chorionic gonadotrophin; monophosphoryl lipid A+myobacterium cell wall sk; mopidamol; multiple drug resistance gene inhibitor; multiple tumor suppressor 1-based therapy; mustard anticancer agent; mycaperoxide B; mycobacterial cell wall extract; myriaporone; N-acetyldinaline; N-substituted benzamides; nafarelin; nagrestip; naloxone+pentazocine; napavin; naphterpin; nartograstim; nedaplatin; nemorubicin; neridronic acid; neutral endopeptidase; nilutamide; nisamycin; nitric oxide modulators; nitroxide antioxidant; nitrullyn; O6-benzylguanine; octreotide; okicenone; oligonucleotides; onapristone; ondansetron; ondansetron; oracin; oral cytokine inducer; ormaplatin; osaterone; oxaliplatin; oxaunomycin; paclitaxel; paclitaxel analogues; paclitaxel derivatives; palauamine; palmitoylrhizoxin; pamidronic acid; panaxytriol; panomifene; parabactin; pazelliptine; pegaspargase; peldesine; pentosan polysulfate sodium; pentostatin; pentrozole; perflubron; perfosfamide; perillyl alcohol; phenazinomycin; phenylacetate; phosphatase inhibitors; picibanil; pilocarpine hydrochloride; pirarubicin; piritrexim; placetin A; placetin B; plasminogen activator inhibitor; platinum complex; platinum compounds; platinum-triamine complex; porfimer sodium; porfiromycin; prednisone; propyl

bis-acridone; prostaglandin J2; proteasome inhibitors; protein A-based immune modulator; protein kinase C inhibitor; protein kinase C inhibitors, microalgal; protein tyrosine phosphatase inhibitors; purine nucleoside phosphorylase inhibitors; purpurins; pyrazoloacridine; pyridoxylated hemoglobin polyoxyethylene conjugate; raf antagonists; raltitrexed; ramosetron; ras farnesyl protein transferase inhibitors; ras inhibitors; ras-GAP inhibitor; retelliptine demethylated; rhenium Re 186 etidronate; rhizoxin; ribozymes; RII retinamide; rogletimide; rohitukine; romurtide; roquinimex; rubiginone B1; ruboxyl; safingol; saintopin; SarCNU; sarcophytol A; sargramostim; Sdi 1 mimetics; semustine; senescence derived inhibitor 1; sense oligonucleotides; signal transduction inhibitors; signal transduction modulators; single chain antigen binding protein; sizofiran; sobuzoxane; sodium borocaptate; sodium phenylacetate; solverol; somatomedin binding protein; sonermin; sparfosic acid; spicamycin D; spiromustine; splenopentin; spongistatin 1; squalamine; stem cell inhibitor; stem-cell division inhibitors; stipiamide; stromelysin inhibitors; sulfinosine; superactive vasoactive intestinal peptide antagonist; suradista; suramin; swainsonine; synthetic glycosaminoglycans; tallimustine; tamoxifen methiodide; tauromustine; tazarotene; tecogalan sodium; tegafur; tellurapyrylium; telomerase inhibitors; temoporfin; temozolomide; teniposide; tetrachlorodecaoxide; tetrazomine; thaliblastine; thiocoraline; thrombopoietin; thrombopoietin mimetic; thymalfasin; thymopoietin receptor agonist; thymotrinan; thyroid stimulating hormone; tin ethyl etiopurpurin; tirapazamine; titanocene bichloride; topsentin; toremifene; totipotent stem cell factor; translation inhibitors; tretinoin; triacetyluridine; triciribine; trimetrexate; triptorelin; tropisetron; turosteride; tyrosine kinase inhibitors; tyrphostins; UBC inhibitors; ubenimex; urogenital sinus-derived growth inhibitory factor; urokinase receptor antagonists; vapreotide; variolin B; vector system, erythrocyte gene therapy; velaresol; veramine; verdins; verteporfin; vinorelbine; vinxaltine; vitaxin; vorozole; zanoterone; zeniplatin; zilascorb; and zinostatin stimalamer. Preferred additional anti-cancer drugs are 5-fluorouracil and leucovorin.

[00399] Examples of therapeutic antibodies that can be used in methods of the invention include but are not limited to ZENAPAX® (daclizumab) (Roche Pharmaceuticals, Switzerland) which is an immunosuppressive, humanized anti-CD25 monoclonal antibody for the prevention of acute renal allograft rejection; PANOREX™ which is a murine anti-17-IA cell surface antigen IgG2a antibody (Glaxo Wellcome/Centocor); BEC2 which is a murine anti-idiotype (GD3 epitope) IgG

antibody (ImClone System); IMC-C225 which is a chimeric anti-EGFR IgG antibody (ImClone System); VITAXIN™ which is a humanized anti-αVβ3 integrin antibody (Applied Molecular Evolution/MedImmune); Smart M195 which is a humanized anti-CD33 IgG antibody (Protein Design Lab/Kanebo); LYMPHOCIDE™ which is a humanized anti-CD22 IgG antibody (Immunomedics); ICM3 is a humanized anti-ICAM3 antibody (ICOS Pharm); IDEC-114 is a primatied anti-CD80 antibody (IDEC Pharm/Mitsubishi); 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); D2E7 is a humanized anti-TNF-α antibody (CAT/BASF); CDP870 is a humanized anti-TNF-α Fab fragment (Celltech); 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-α4β7 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); and CAT-152 is a human anti-TGF-β_2 antibody (Cambridge Ab Tech). Other examples of therapeutic antibodies that can be used in accordance with the invention are presented in **Table 10**.

6.4.2 AUTOIMMUNE DISEASE AND INFLAMMATORY DISEASES

[00400] In some embodiments, molecules of the invention comprise a variant Fc region, having one or more amino acid modifications in one or more regions, which modification increases the affinity of the variant Fc region for FcγRIIB but decreases the affinity of the variant Fc region for FcγRIIIA and/or FcγRIIA. Molecules of the invention with such binding characteristics are useful in regulating the immune response, *e.g.*, in inhibiting the immune response in connection with autoimmune diseases or inflammatory diseases. Although not intending to be bound by any mechanism of action, molecules of the invention with an enhanced affinity for FcγRIIB and a decreased affinity for FcγRIIIA and/or FcγRIIA may lead to dampening of the activating response to FcγR and inhibition of cellular responsiveness.

[00401] In some embodiments, a molecule of the invention comprising a variant Fc region is not an immunoglobulin, and comprises at least one amino acid modification which modification increases the affinity of the variant Fc region for FcγRIIB relative to a molecule comprising a wild-type Fc region. In other embodiments, said molecule further comprises one or more amino acid modifications, which modifications decreases the affinity of the molecule for an activating FcγR. In some embodiments, the molecule is a soluble (*i.e.*, not membrane bound) Fc region. The invention contemplates other amino acid modifications within the soluble Fc region which modulate its affinity for various Fc receptors, including those known to one skilled in the art as described herein. In other embodiments, the molecule (*e.g.*, the Fc region comprising at least one or more amino acid modification) is modified using techniques known to one skilled in the art and as described herein to increase the in vivo half life of the Fc region. Such molecules have therapeutic utility in treating and/or preventing an autoimmune disorder. Although not intending to be bound by any mechanism of actions, such molecules with enhanced affinity for FcγRIIB will lead to a dampening of the activating receptors and thus a dampening of the immune response and have therapeutic efficacy for treating and/or preventing an autoimmune disorder.

[00402] In certain embodiments, the one or more amino acid modifications, which increase the affinity of the variant Fc region for FcγRIIB but decrease the affinity of the variant Fc region for FcγRIIIA comprise 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 217 with serine, at position 378 with valine, and at position 408 with arginine; or a substitution at position 375 with cysteine and at position 396 with leucine; or a substitution at position 246 with isolcucine and at position 334 with asparagine. In one embodiment, the one or more amino acid modifications, which increase the affinity of the variant Fc region for FcγRIIB but decrease the affinity of the variant Fc region for FcγRIIIA comprise a substitution at position 247 with leucine. In another embodiment, the one or more amino acid modification, which increases the affinity of the variant Fc region for FcγRIIB but decreases the affinity of the variant Fc region for FcγRIIIA comprise a substitution at position 372 with tyrosine. In yet another embodiment, the one or more amino acid modification, which increases the affinity of the variant Fc region for FcγRIIB but decreases the affinity of the variant Fc region for FcγRIIIA comprise a substitution at position 326 with glutamic acid. In one embodiment, the one or more

amino acid modification, which increases the affinity of the variant Fc region for FcγRIIB but decreases the affinity of the variant Fc region for FcγRIIIA comprise a substitution at position 224 with leucine.

[00403] The variant Fc regions that have an enhanced affinity for FcγRIIB and a decreased affinity for FcγRIIIA and/or FcγRIIA relative to a comparable molecule comprising a wild-type Fc region, may be used to treat or prevent autoimmune diseases or inflammatory diseases. The present invention provides methods of preventing, treating, or managing one or more symptoms associated with an autoimmune or inflammatory disorder in a subject, comprising administering to said subject a therapeutically or prophylactically effective amount of one or more molecules of the invention with variant Fc regions that have an enhanced affinity for FcγRIIB and a decreased affinity for FcγRIIIA and or FcγRIIA relative to a comparable molecule comprising a wild type Fc region.

[00404] The invention also provides methods for preventing, treating, or managing one or more symptoms associated with an inflammatory disorder in a subject further comprising, administering to said subject a therapeutically or prophylactically effective amount of one or more anti-inflammatory agents. The invention also provides methods for preventing, treating, or managing one or more symptoms associated with an autoimmune disease further comprising, administering to said subject a therapeutically or prophylactically effective amount of one or more immunomodulatory agents. Section 6.4.2.1 provides non-limiting examples of anti-inflammatory agents and immunomodulatory agents.

[00405] Examples of autoimmune disorders that may be treated by administering the molecules of the present invention include, but are not limited to, alopecia areata, ankylosing spondylitis, antiphospholipid syndrome, autoimmune Addison's disease, autoimmune diseases of the adrenal gland, autoimmune hemolytic anemia, autoimmune hepatitis, autoimmune oophoritis and orchitis, autoimmune thrombocytopenia, Behcet's disease, bullous pemphigoid, cardiomyopathy, celiac sprue-dermatitis, chronic fatigue immune dysfunction syndrome (CFIDS), chronic inflammatory demyelinating polyneuropathy, Churg-Strauss syndrome, cicatrical pemphigoid, CREST syndrome, cold agglutinin disease, Crohn's disease, discoid lupus, essential mixed cryoglobulinemia, fibromyalgia-fibromyositis, glomerulonephritis, Graves' disease, Guillain-Barre, Hashimoto's thyroiditis, idiopathic pulmonary fibrosis, idiopathic thrombocytopenia purpura (ITP), IgA neuropathy, juvenile arthritis, lichen planus,

lupus erthematosus, Ménière's disease, mixed connective tissue disease, multiple sclerosis, type 1 or immune-mediated diabetes mellitus, myasthenia gravis, pemphigus vulgaris, pernicious anemia, polyarteritis nodosa, polychrondritis, polyglandular syndromes, polymyalgia rheumatica, polymyositis and dermatomyositis, primary agammaglobulinemia, primary biliary cirrhosis, psoriasis, psoriatic arthritis, Raynauld's phenomenon, Reiter's syndrome, Rheumatoid arthritis, sarcoidosis, scleroderma, Sjögren's syndrome, stiff-man syndrome, systemic lupus erythematosus, lupus erythematosus, takayasu arteritis, temporal arteristis/ giant cell arteritis, ulcerative colitis, uveitis, vasculitides such as dermatitis herpetiformis vasculitis, vitiligo, and Wegener's granulomatosis. Examples of inflammatory disorders include, but are not limited to, asthma, encephilitis, inflammatory bowel disease, chronic obstructive pulmonary disease (COPD), allergic disorders, septic shock, pulmonary fibrosis, undifferentitated spondyloarthropathy, undifferentiated arthropathy, arthritis, inflammatory osteolysis, and chronic inflammation resulting from chronic viral or bacteria infections. As described herein in Section 3.2.2, some autoimmune disorders are associated with an inflammatory condition. Thus, there is overlap between what is considered an autoimmune disorder and an inflammatory disorder. Therefore, some autoimmune disorders may also be characterized as inflammatory disorders. Examples of inflammatory disorders which can be prevented, treated or managed in accordance with the methods of the invention include, but are not limited to, asthma, encephilitis, inflammatory bowel disease, chronic obstructive pulmonary disease (COPD), allergic disorders, septic shock, pulmonary fibrosis, undifferentitated spondyloarthropathy, undifferentiated arthropathy, arthritis, inflammatory osteolysis, and chronic inflammation resulting from chronic viral or bacteria infections.

[00406] Molecules of the invention with variant Fc regions that have an enhanced affinity for FcγRIIB and a decreased affinity for FcγRIIIA relative to a comparable molecule comprising a wild-type Fc region can also be used to reduce the inflammation experienced by animals, particularly mammals, with inflammatory disorders. In a specific embodiment, a molecule of the invention reduces the inflammation in an animal by at least 99%, at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 60%, at least 50%, at least 45%, at least 40%, at least 45%, at least 35%, at least 30%, at least 25%, at least 20%, or at least 10% relative to the inflammation in an animal, which is not administered the said molecule.

[00407] Molecules of the invention with variant Fc regions that have an enhanced affinity for FcγRIIB and a decreased affinity for FcγRIIIA relative to a comparable molecule comprising a wild-type Fc region can also be used to prevent the rejection of transplants.

[00408] The invention further contemplates engineering any of the antibodies known in the art for the treatment and/or prevention of autoimmune disease or inflammatory disease, so that the antibodies comprise a variant Fc region comprising one or more amino acid modifications, which have been identified by the methods of the invention to have an enhanced affinity for FcγRIIB and a decreased affinity for FcγRIIIA relative to a comparable molecule comprising a wild type Fc region. A non-limiting example of the antibodies that are used for the treatment or prevention of inflammatory disorders which can be engineered according to the invention is presented in **Table 11**, and a non-limiting example of the antibodies that are used for the treatment or prevention of autoimmune disorder is presented in **Table 12**.

TABLE 11: ANTIBODIES FOR INFLAMMATORY DISEASES AND AUTOIMMUNE DISEASES THAT CAN ENGINEERED IN ACCORDANCE WITH THE INVENTION.

Antibody Name	Target Antigen	Product Type	Isotype	Sponsors	Indication
5G1.1	Complement (C5)	Humanized	IgG	Alexion Pharm Inc	Rheumatoid Arthritis
5G1.1	Complement (C5)	Humanized	IgG	Alexion Pharm Inc	SLE
5G1.1	Complement (C5)	Humanized	IgG	Alexion Pharm Inc	Nephritis
5G1.1-SC	Complement (C5)	Humanized	ScFv	Alexion Pharm Inc	Cardiopulmonary Bypass
5G1.1-SC	Complement (C5)	Humanized	ScFv	Alexion Pharm Inc	Myocardial Infarction
5G1.1-SC	Complement (C5)	Humanized	ScFv	Alexion Pharm Inc	Angioplasty
ABX-CBL	CBL	Human		Abgenix Inc	GvHD
ABX-CBL	CD147	Murine	IgG	Abgenix Inc	Allograft rejection
ABX-IL8	IL-8	Human	IgG2	Abgenix Inc	Psoriasis
Antegren	VLA-4	Humanized	IgG	Athena/Elan	Multiple Sclerosis
Anti-CD11a	CD11a	Humanized	IgG1	Genentech Inc/Xoma	Psoriasis
Anti-CD18	CD18	Humanized	Fab'2	Genentech Inc	Myocardial infarction

Antibody Name	Target Antigen	Product Type	Isotype	Sponsors	Indication
Anti-LFA1	CD18	Murine	Fab'2	Pasteur-Merieux/ Immunotech	Allograft rejection
Antova	CD40L	Humanized	IgG	Biogen	Allograft rejection
Antova	CD40L	Humanized	IgG	Biogen	SLE
BTI-322	CD2	Rat	IgG	Medimmune Inc	GvHD, Psoriasis
CDP571	TNF-alpha	Humanized	IgG4	Celltech	Crohn's
CDP571	TNF-alpha	Humanized	IgG4	Celltech	Rheumatoid Arthritis
CDP850	E-selectin	Humanized		Celltech	Psoriasis
Corsevin M	Fact VII	Chimeric		Centocor	Anticoagulant
D2E7	TNF-alpha	Human		CAT/BASF	Rheumatoid Arthritis
Hu23F2G	CD11/18	Humanized		ICOS Pharm Inc	Multiple Sclerosis
Hu23F2G	CD11/18	Humanized	IgG	ICOS Pharm Inc	Stroke
IC14	CD14			ICOS Pharm Inc	Toxic shock
ICM3	ICAM-3	Humanized		ICOS Pharm Inc	Psoriasis
IDEC-114	CD80	Primatised		IDEC Pharm/Mitsubish i	Psoriasis
IDEC-131	CD40L	Humanized		IDEC Pharm/Eisai	SLE
IDEC-131	CD40L	Humanized		IDEC Pharm/Eisai	Multiple Sclerosis
IDEC-151	CD4	Primatised	IgG1	IDEC Pharm/GlaxoSmi thKline	Rheumatoid Arthritis
IDEC-152	CD23	Primatised		IDEC Pharm	Asthma/Allergy
Infliximab	TNF-alpha	Chimeric	IgG1	Centocor	Rheumatoid Arthritis
Infliximab	TNF-alpha	Chimeric	IgG1	Centocor	Crohn's
LDP-01	beta2-integrin	Humanized	IgG	Millennium Inc (LeukoSite Inc.)	Stroke
LDP-01	beta2-integrin	Humanized	IgG	Millennium Inc (LeukoSite Inc.)	Allograft rejection
LDP-02	alpha4beta7	Humanized		Millennium Inc (LeukoSite Inc.)	Ulcerative Colitis
MAK-195F	TNF alpha	Murine	Fab'2	Knoll Pharm, BASF	Toxic shock
MDX-33	CD64 (FcR)	Human		Medarex/Centeo n	Autoimmune haematogical disorders
MDX-CD4	CD4	Human	IgG	Medarex/Eisai/ Genmab	Rheumatoid Arthritis
MEDI-507	CD2	Humanized		Medimmune Inc	Psoriasis
MEDI-507	CD2	Humanized		Medimmune Inc	GvHD

Antibody Name	Target Antigen	Product Type	Isotype	Sponsors	Indication
OKT4A	CD4	Humanized	IgG	Ortho Biotech	Allograft rejection
OrthoClone OKT4A	CD4	Humanized	IgG	Ortho Biotech	Autoimmune disease
Orthoclone/ anti-CD3 OKT3	CD3	Murine	mIgG2a	Ortho Biotech	Allograft rejection
RepPro/ Abciximab	gpIIbIIIa	Chimeric	Fab	Centocor/Lilly	Complications of coronary angioplasty
rhuMab-E25	IgE	Humanized	IgG1	Genentech/Novartis/Tanox Biosystems	Asthma/Allergy
SB-240563	IL5	Humanized		GlaxoSmithKline	Asthma/Allergy
SB-240683	IL-4	Humanized		GlaxoSmithKline	Asthma/Allergy
SCH55700	IL-5	Humanized		Celltech/Scherin g	Asthma/Allergy
Simulect	CD25	Chimeric	IgG1	Novartis Pharm	Allograft rejection
SMART a-CD3	CD3	Humanized		Protein Design Lab	Autoimmune disease
SMART a-CD3	CD3	Humanized		Protein Design Lab	Allograft rejection
SMART a-CD3	CD3	Humanized	IgG	Protein Design Lab	Psoriasis
Zenapax	CD25	Humanized	IgG1	Protein Design Lab/Hoffman-La Roche	Allograft rejection

TABLE 12: ANTIBODIES FOR AUTOIMMUNE DISORDERS THAT CAN BE ENGINEERED IN ACCORDANCE WITH THE INVENTION

Antibody	Indication	Target Antigen
ABX-RB2		antibody to CBL antigen on T cells, B cells and NK cells fully human antibody from the Xenomouse
5c8 (Anti CD-40 ligand antibody)	Phase II trials were halted in Oct. 99 examine "adverse events"	CD-40
IDEC 131	systemic lupus erythyematous (SLE)	anti CD40 humanized
IDEC 151	rheumatoid arthritis	primatized ; anti-CD4
IDEC 152	Asthma	primatized; anti-CD23
IDEC 114	Psoriasis	primatized anti-CD80
MEDI-507	rheumatoid arthritis; multiple sclerosis Crohn's disease Psoriasis	anti-CD2
LDP-02 (anti-b7 mAb)	inflammatory bowel disease Chron's disease ulcerative colitis	a4b7 integrin receptor on white blood cells (leukocytes)
SMART Anti-Gamma Interferon antibody	autoimmune disorders	Anti-Gamma Interferon
Verteportin	rheumatoid arthritis	
MDX-33	blood disorders caused by autoimmune reactions Idiopathic Thrombocytopenia Purpurea (ITP) autoimmune hemolytic anemia	monoclonal antibody against FcRI receptors
MDX-CD4	treat rheumatoid arthritis and other autoimmunity	monoclonal antibody against CD4 receptor molecule
VX-497	autoimmune disorders multiple sclerosis rheumatoid arthritis inflammatory bowel disease lupus psoriasis	inhibitor of inosine monophosphate dehydrogenase (enzyme needed to make new RNA and DNA used in production of nucleotides needed for lymphocyte proliferation)
VX-740	rheumatoid arthritis	inhibitor of ICE interleukin-1 beta (converting enzyme controls pathways leading to aggressive immune response)

Antibody	Indication	Target Antigen
VX-745	specific to inflammation involved in chemical signalling of immune response onset and progression of inflammation	inhibitor of P38MAP kinase mitogen activated protein kinase
Enbrel (etanercept)		targets TNF (tumor necrosis factor)
IL-8		fully human monoclonal antibody against IL-8 (interleukin 8)
Apogen MP4		recombinant antigen selectively destroys disease associated T-cells induces apoptosis T-cells eliminated by programmed cell death no longer attack body's own cells specific apogens target specific T-cells

6.4.2.1 IMMUNOMODULATORY AGENTS AND ANTI-INFLAMMATORY AGENTS

[00409] The present invention provides methods of treatment for autoimmune diseases and inflammatory diseases comprising administration of the molecules with variant Fc regions having an enhanced affinity for FcγRIIB and a decreased affinity for FcγRIIIA and/or FcγRIIA in conjunction with other treatment agents. Examples of immunomodulatory agents include, but are not limited to, methotrexate, ENBREL, REMICADE™, leflunomide, cyclophosphamide, cyclosporine A, and macrolide antibiotics (*e.g.*, FK506 (tacrolimus)), methylprednisolone (MP), corticosteroids, steriods, mycophenolate mofetil, rapamycin (sirolimus), mizoribine, deoxyspergualin, brequinar, malononitrilamides (*e.g.*, leflunamide), T cell receptor modulators, and cytokine receptor modulators.

[00410] Anti-inflammatory agents have exhibited success in treatment of inflammatory and autoimmune disorders and are now a common and a standard treatment for such disorders. Any anti-inflammatory agent well-known to one of skill in the art can be used in the methods of the invention. Non-limiting examples of anti-inflammatory agents include non-steroidal anti-inflammatory drugs (NSAIDs), steroidal anti-inflammatory drugs, beta-agonists, anticholingeric agents, and methyl xanthines. Examples of NSAIDs include, but are not limited to, aspirin, ibuprofen, celecoxib (CELEBREX™), diclofenac (VOLTAREN™), etodolac (LODINE™),

fenoprofen (NALFON™), indomethacin (INDOCIN™), ketoralac (TORADOL™), oxaprozin (DAYPRO™), nabumentone (RELAFEN™), sulindac (CLINORIL™), tolmentin (TOLECTIN™), rofecoxib (VIOXX™), naproxen (ALEVE™, NAPROSYN™), ketoprofen (ACTRON™) and nabumetone (RELAFEN™). Such NSAIDs function by inhibiting a cyclooxgenase enzyme (*e.g.*, COX-1 and/or COX-2). Examples of steroidal anti-inflammatory drugs include, but are not limited to, glucocorticoids, dexamethasone (DECADRON™), cortisone, hydrocortisone, prednisone (DELTASONE™), prednisolone, triamcinolone, azulfidine, and eicosanoids such as prostaglandins, thromboxanes, and leukotrienes.

6.4.3 INFECTIOUS DISEASE

[00411] The invention also encompasses methods for treating or preventing an infectious disease in a subject comprising administering a therapeutically or prophylatically effective amount of one or more molecules of the invention. 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, protozae, and viruses.

[00412] Viral diseases that can be treated or prevented using the molecules of the invention in conjunction with the methods of the present invention include, but are not limited to, those caused by hepatitis type A, hepatitis type B, hepatitis type C, influenza, varicella, adenovirus, herpes simplex type I (HSV-I), herpes simplex type II (HSV-II), rinderpest, rhinovirus, echovirus, rotavirus, respiratory syncytial virus, papilloma virus, papova virus, cytomegalovirus, echinovirus, arbovirus, huntavirus, coxsackie virus, mumps virus, measles virus, rubella virus, polio virus, small pox, Epstein Barr virus, human immunodeficiency virus type I (HIV-I), human immunodeficiency virus type II (HIV-II), and agents of viral diseases such as viral miningitis, encephalitis, dengue or small pox.

[00413] Bacterial diseases that can be treated or prevented using the molecules of the invention in conjunction with the methods of the present invention, that are caused by bacteria include, but are not limited to, mycobacteria rickettsia, mycoplasma, neisseria , S. pneumonia, Borrelia burgdorferi (Lyme disease), Bacillus anthracis (anthrax), tetanus, streptococcus, staphylococcus, mycobacterium, tetanus, pertussis, cholera, plague, diptheria, chlamydia, S. aureus and legionella.

[00414] Protozoal diseases that can be treated or prevented using the molecules of the invention in conjunction with the methods of the present invention, that are caused by protozoa include, but are not limited to, leishmania, kokzidioa, trypanosoma or malaria.

[00415] Parasitic diseases that can be treated or prevented using the molecules of the invention in conjunction with the methods of the present invention, that are caused by parasites include, but are not limited to, chlamydia and rickettsia.

[00416] 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. Examples of infectious agents include but are not limited to bacteria (*e.g.*, *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Enterococcus faecials*, *Candida albicans*, *Proteus vulgaris*, *Staphylococcus viridans*, and *Pseudomonas aeruginosa)*, a pathogen (*e.g.*, B-lymphotropic papovavirus (LPV); Bordatella pertussis; Borna Disease virus (BDV); Bovine coronavirus; Choriomeningitis virus; Dengue virus; a virus, E. coli; Ebola; Echovirus 1; Echovirus-11 (EV); Endotoxin (LPS); Enteric bacteria; Enteric Orphan virus; Enteroviruses ; Feline leukemia virus; Foot and mouth disease virus; Gibbon ape leukemia virus (GALV); Gram-negative bacteria ; Heliobacter pylori; Hepatitis B virus (HBV); Herpes Simplex Virus; HIV-1; Human cytomegalovirus; Human coronovirus; Influenza A, B & C ; Legionella; Leishmania mexicana; Listeria monocytogenes; Measles virus; Meningococcus; Morbilliviruses; Mouse hepatitis virus; Murine leukemia virus; Murine gamma herpes virus; Murine retrovirus; Murine coronavirus mouse hepatitis virus; Mycobacterium avium-M; Neisseria gonorrhoeae; Newcastle disease virus; Parvovirus B19; Plasmodium falciparum; Pox Virus; Pseudomonas; Rotavirus; Samonella typhiurium; Shigella; Streptococci; T-cell lymphotropic virus 1; Vaccinia virus).

[00417] 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.

[00418] 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. Antibiotics that can be used in combination with the molecules of the invention include, but are not limited to, macrolide (*e.g.*, tobramycin (Tobi®)), a cephalosporin (*e.g.*, cephalexin (Keflex®), cephradine (Velosef®), cefuroxime (Ceftin®), cefprozil (Cefzil®), cefaclor (Ceclor®), cefixime (Suprax®) or cefadroxil (Duricef®)), a clarithromycin (*e.g.*, clarithromycin (Biaxin®)), an erythromycin (*e.g.*, erythromycin (EMycin®)), a penicillin (*e.g.*, penicillin V (V-Cillin K® or Pen Vee K®)) or a quinolone (*e.g.*, ofloxacin (Floxin®), ciprofloxacin (Cipro®) or norfloxacin (Noroxin®)),aminoglycoside antibiotics (*e.g.*, apramycin, arbekacin, bambermycins, butirosin, dibekacin, neomycin, neomycin, undecylenate, netilmicin, paromomycin, ribostamycin, sisomicin, and spectinomycin), amphenicol antibiotics (*e.g.*, azidamfenicol, chloramphenicol, florfenicol, and thiamphenicol), ansamycin antibiotics (*e.g.*, rifamide and rifampin), carbacephems (*e.g.*, loracarbef), carbapenems (*e.g.*, biapenem and imipenem), cephalosporins (*e.g.*, cefaclor, cefadroxil, cefamandole, cefatrizine, cefazedone, cefozopran, cefpimizole, cefpiramide, and cefpirome), cephamycins (*e.g.*, cefbuperazone, cefmetazole, and cefminox), monobactams (*e.g.*, aztreonam, carumonam, and tigemonam), oxacephems (*e.g.*, flomoxef, and moxalactam), penicillins (*e.g.*, amdinocillin, amdinocillin pivoxil, amoxicillin, bacampicillin, benzylpenicillinic acid, benzylpenicillin sodium, epicillin, fenbenicillin, floxacillin, penamecillin, penethamate hydriodide, penicillin o-benethamine, penicillin 0, penicillin V, penicillin V benzathine, penicillin V hydrabamine, penimepicycline, and phenethicillin potassium), lincosamides (*e.g.*, clindamycin, and lincomycin), amphomycin, bacitracin, capreomycin, colistin, enduracidin, enviomycin, tetracyclines (*e.g.*, apicycline, chlortetracycline, clomocycline, and demeclocycline), 2,4-diaminopyrimidines (*e.g.*, brodimoprim), nitrofurans (*e.g.*, furaltadone, and furazolium chloride), quinolones and analogs thereof (*e.g.*, cinoxacin, clinafloxacin, flumequine, and grepafloxacin), sulfonamides (*e.g.*, acetyl sulfamethoxypyrazine, benzylsulfamide, noprylsulfamide, phthalyl-sulfacetamide, sulfachrysoidine, and sulfacytine), sulfones (*e.g.*, diathymosulfone, glucosulfone sodium, and solasulfone), cycloserine, mupirocin and tuberin.

[00419] In certain embodiments, the molecules of the invention can be administered in combination with a therapeutically or prophylactically effective amount of one or more antifungal agents. Antifungal agents that can be used in combination with the

molecules of the invention include but are not limited to amphotericin B, itraconazole, ketoconazole, fluconazole, intrathecal, flucytosine, miconazole, butoconazole, clotrimazole, nystatin, terconazole, tioconazole, ciclopirox, econazole, haloprogrin, naftifine, terbinafine, undecylenate, and griseofuldin.

[00420] In some embodiments, the molecules of the invention can be administered in combination with a therapeutically or prophylactically effective amount of one or more anti-viral agent. Useful anti-viral agents that can be used in combination with the molecules of the invention include, but are not limited to, protease inhibitors, nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors and nucleoside analogs. Examples of antiviral agents include but are not limited to zidovudine, acyclovir, gangcyclovir, vidarabine, idoxuridine, trifluridine, and ribavirin, as well as foscarnet, amantadine, rimantadine, saquinavir, indinavir, amprenavir, lopinavir, ritonavir, the alpha-interferons; adefovir, clevudine, entecavir, pleconaril.

6.5 VACCINE THERAPY

[00421] The invention further encompasses using a composition of the invention to induce an immune response against an antigenic or immunogenic agent, including but not limited to cancer antigens and infectious disease antigens (examples of which are disclosed *infra*). The vaccine compositions of the invention comprise one or more antigenic or immunogenic agents to which an immune response is desired, wherein the one or more antigenic or immunogenic agents is coated with a variant antibody of the invention that has an enhanced affinity to FcγRIIIA. Although not intending to be bound by a particular mechanism of action, coating an antigenic or immunogenic agent with a variant antibody of the invention that has an enhanced affinity to FcγRIIIA, enhances the immune response to the desired antigenic or immunogenic agent by inducing humoral and cell-mediated responses. The vaccine compositions of the invention are particularly effective in eliciting an immune response, preferably a protective immune response against the antigenic or immunogenic agent.

[00422] In some embodiments, the antigenic or immunogenic agent in the vaccine compositions of the invention comprises a virus against which an immune response is desired. The viruses may be recombinant or chimeric, and are preferably attenuated. Production of recombinant, chimeric, and attenuated viruses may be performed using standard methods known to one skilled in the art. The invention encompasses a live

recombinant viral vaccine or an inactivated recombinant viral vaccine to be formulated in accordance with the invention. A live vaccine may be preferred because multiplication in the host leads to a prolonged stimulus of similar kind and magnitude to that occurring in natural infections, and therefore, confers substantial, long-lasting immunity. Production of such live recombinant virus vaccine formulations may be accomplished using conventional methods involving propagation of the virus in cell culture or in the allantois of the chick embryo followed by purification.

[00423] In a specific embodiment, the recombinant virus is non-pathogenic to the subject to which it is administered. In this regard, the use of genetically engineered viruses for vaccine purposes may require the presence of attenuation characteristics in these strains. The introduction of appropriate mutations (*e.g.*, deletions) into the templates used for transfection may provide the novel viruses with attenuation characteristics. For example, specific missense mutations which are associated with temperature sensitivity or cold adaption can be made into deletion mutations. These mutations should be more stable than the point mutations associated with cold or temperature sensitive mutants and reversion frequencies should be extremely low. Recombinant DNA technologies for engineering recombinant viruses are known in the art and encompassed in the invention. For example, techniques for modifying negative strand RNA viruses are known in the art, see, *e.g.*, U.S. Patent No. 5,166,057, which is incorporated herein by reference in its entirety.

[00424] Alternatively, chimeric viruses with "suicide" characteristics may be constructed for use in the intradermal vaccine formulations of the invention. Such viruses would go through only one or a few rounds of replication within the host. When used as a vaccine, the recombinant virus would go through limited replication cycle(s) and induce a sufficient level of immune response but it would not go further in the human host and cause disease. Alternatively, inactivated (killed) virus may be formulated in accordance with the invention. Inactivated vaccine formulations may be prepared using conventional techniques to "kill" the chimeric viruses. Inactivated vaccines are "dead" in the sense that their infectivity has been destroyed. Ideally, the infectivity of the virus is destroyed without affecting its immunogenicity. In order to prepare inactivated vaccines, the chimeric virus may be grown in cell culture or in the allantois of the chick embryo, purified by zonal ultracentrifugation, inactivated by formaldehyde or β-propiolactone, and pooled.

[00425] In certain embodiments, completely foreign epitopes, including antigens derived from other viral or non-viral pathogens can be engineered into the virus for use in the intradermal vaccine formulations of the invention. For example, antigens of non-related viruses such as HIV (gp160, gp120, gp41) parasite antigens (*e.g.*, malaria), bacterial or fungal antigens or tumor antigens can be engineered into the attenuated strain.

[00426] Virtually any heterologous gene sequence may be constructed into the chimeric viruses of the invention for use in the intradermal vaccine formulations. Preferably, heterologous gene sequences are moieties and peptides that act as biological response modifiers. Preferably, epitopes that induce a protective immune response to any of a variety of pathogens, or antigens that bind neutralizing antibodies may be expressed by or as part of the chimeric viruses. For example, heterologous gene sequences that can be constructed into the chimeric viruses of the invention include, but are not limited to, influenza and parainfluenza hemagglutinin neuraminidase and fusion glycoproteins such as the HN and F genes of human PIV3. In yet another embodiment, heterologous gene sequences that can be engineered into the chimeric viruses include those that encode proteins with immuno-modulating activities. Examples of immuno-modulating proteins include, but are not limited to, cytokines, interferon type 1, gamma interferon, colony stimulating factors, interleukin -1, -2, -4, -5, -6, -12, and antagonists of these agents.

[00427] In yet other embodiments, the invention encompasses pathogenic cells or viruses, preferably attenuated viruses, which express the variant antibody on their surface.

[00428] In alternative embodiments, the vaccine compositions of the invention comprise a fusion polypeptide wherein an antigenic or immunogenic agent is operatively linked to a variant antibody of the invention that has an enhanced affinity for FcγRIIIA. Engineering fusion polypeptides for use in the vaccine compositions of the invention is performed using routine recombinant DNA technology methods and is within the level of ordinary skill.

[00429] The invention further encompasses methods to induce tolerance in a subject by administering a composition of the invention. Preferably a composition suitable for inducing tolerance in a subject, comprises an antigenic or immunogenic agent coated with a variant antibody of the invention, wherein the variant antibody has a higher

affinity to FcγRIIB. Although not intending to be bound by a particular mechanism of action, such compositions are effective in inducing tolerance by activating the FcγRIIB mediatated inhibitory pathway.

6.6 COMPOSITIONS AND METHODS OF ADMINISTERING

[00430] The invention provides methods and pharmaceutical compositions comprising molecules of the invention (*i.e.*, antibodies, polypeptides) comprising variant Fc regions. The invention also provides methods of treatment, prophylaxis, and amelioration of one or more symptoms associated with a disease, disorder or infection by administering to a subject an effective amount of a fusion protein or a conjugated molecule of the invention, or a pharmaceutical composition comprising a fusion protein or a conjugated molecule of the invention. In a preferred aspect, an antibody, a fusion protein, or a conjugated molecule, is substantially purified (*i.e.*, substantially free from substances that limit its effect or produce undesired side-effects). In a specific embodiment, the subject is an animal, preferably a mammal such as non-primate (*e.g.*, cows, pigs, horses, cats, dogs, rats *etc.*) and a primate (*e.g.*, monkey such as, a cynomolgous monkey and a human). In a preferred embodiment, the subject is a human. In yet another preferred embodiment, the antibody of the invention is from the same species as the subject.

[00431] Various delivery systems are known and can be used to administer a composition comprising molecules of the invention (*i.e.*, antibodies, polypeptides), comprising variant Fc regions, *e.g.*, encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the antibody or fusion protein, receptor-mediated endocytosis (*See*, *e.g.*, Wu and Wu, 1987, *J. Biol. Chem.* 262:4429-4432), construction of a nucleic acid as part of a retroviral or other vector, *etc.* Methods of administering a molecule of the invention include, but are not limited to, parenteral administration (*e.g.*, intradermal, intramuscular, intraperitoneal, intravenous and subcutaneous), epidural, and mucosal (*e.g.*, intranasal and oral routes). In a specific embodiment, the molecules of the invention are administered intramuscularly, intravenously, or subcutaneously. The compositions may be administered by any convenient route, for example, by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, *etc.*) and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, pulmonary administration can

also be employed, *e.g.*, by use of an inhaler or nebulizer, and formulation with an aerosolizing agent. *See, e.g.*, U.S. Patent Nos. 6,019,968; 5,985, 320; 5,985,309; 5,934,272; 5,874,064; 5,855,913; 5,290,540; and 4,880,078; and PCT Publication Nos. WO 92/19244; WO 97/32572; WO 97/44013; WO 98/31346; and WO 99/66903, each of which is incorporated herein by reference in its entirety.

[00432] The invention also provides that the molecules of the invention (*i.e.*, antibodies, polypeptides) comprising variant Fc regions, are packaged in a hermetically sealed container such as an ampoule or sachette indicating the quantity of antibody. In one embodiment, the molecules of the invention are supplied as a dry sterilized lyophilized powder or water free concentrate in a hermetically sealed container and can be reconstituted, *e.g.*, with water or saline to the appropriate concentration for administration to a subject. Preferably, the molecules of the invention are supplied as a dry sterile lyophilized powder in a hermetically sealed container at a unit dosage of at least 5 mg, more preferably at least 10 mg, at least 15 mg, at least 25 mg, at least 35 mg, at least 45 mg, at least 50 mg, or at least 75 mg. The lyophilized molecules of the invention should be stored at between 2 and 8°C in their original container and the molecules should be administered within 12 hours, preferably within 6 hours, within 5 hours, within 3 hours, or within 1 hour after being reconstituted. In an alternative embodiment, molecules of the invention are supplied in liquid form in a hermetically sealed container indicating the quantity and concentration of the molecule, fusion protein, or conjugated molecule. Preferably, the liquid form of the molecules of the invention are supplied in a hermetically sealed container at least 1 mg/ml, more preferably at least 2.5 mg/ml, at least 5 mg/ml, at least 8 mg/ml, at least 10 mg/ml, at least 15 mg/kg, at least 25 mg/ml, at least 50 mg/ml, at least 100 mg/ml, at least 150 mg/ml, at least 200 mg/ml of the molecules.

[00433] The amount of the composition of the invention which will be effective in the treatment, prevention or amelioration of one or more symptoms associated with a disorder can be determined by standard clinical techniques. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the condition, and should be decided according to the judgment of the practitioner and each patient's circumstances. Effective doses may be extrapolated from dose-response curves derived from in vitro or animal model test systems.

[00434] For antibodies encompassed by the invention, the dosage administered to a patient is typically 0.0001 mg/kg to 100 mg/kg of the patient's body weight.

Preferably, the dosage administered to a patient is between 0.0001 mg/kg and 20 mg/kg, 0.0001 mg/kg and 10 mg/kg, 0.0001 mg/kg and 5 mg/kg, 0.0001 and 2 mg/kg, 0.0001 and 1 mg/kg, 0.0001 mg/kg and 0.75 mg/kg, 0.0001 mg/kg and 0.5 mg/kg, 0.0001 mg/kg to 0.25 mg/kg, 0.0001 to 0.15 mg/kg, 0.0001 to 0.10 mg/kg, 0.001 to 0.5 mg/kg, 0.01 to 0.25 mg/kg or 0.01 to 0.10 mg/kg of the patient's body weight. Generally, human antibodies have a longer half-life within the human body than antibodies from other species due to the immune response to the foreign polypeptides. Thus, lower dosages of human antibodies and less frequent administration is often possible. Further, the dosage and frequency of administration of antibodies of the invention or fragments thereof may be reduced by enhancing uptake and tissue penetration of the antibodies by modifications such as, for example, lipidation.

[00435] In one embodiment, the dosage of the molecules of the invention administered to a patient are 0.01mg to 1000mg/day, when used as single agent therapy. In another embodiment the molecules of the invention are used in combination with other therapeutic compositions and the dosage administered to a patient are lower than when said molecules are used as a single agent therapy.

[00436] In a specific embodiment, it may be desirable to administer the pharmaceutical compositions of the invention locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion, by injection, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. Preferably, when administering a molecule of the invention, care must be taken to use materials to which the molecule does not absorb.

[00437] In another embodiment, the compositions can be delivered in a vesicle, in particular a liposome (*See* Langer, *Science* 249:1527-1533 (1990); Treat *et al.*, in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353- 365 (1989); Lopez-Berestein, ibid., pp. 3 17-327; *see* generally ibid.).

[00438] In yet another embodiment, the compositions can be delivered in a controlled release or sustained release system. Any technique known to one of skill in the art can be used to produce sustained release formulations comprising one or more molecules of the invention. *See, e.g.*, U.S. Patent No. 4,526.938; PCT publication WO 91/05548; PCT publication WO 96/20698; Ning *et al.*, 1996, "Intratumoral Radioimmunotheraphy of a Human Colon Cancer Xenograft Using a Sustained-Release Gel," *Radiotherapy &*

Oncology 39:179-189, Song *et al.*, 1995, "Antibody Mediated Lung Targeting of Long-Circulating Emulsions," *PDA Journal of Pharmaceutical Science & Technology* 50:372-397; Cleek *et al.*, 1997, "Biodegradable Polymeric Carriers for a bFGF Antibody for Cardiovascular Application," *Pro. Int'l. Symp. Control. Rel. Bioact. Mater.* 24:853-854; and Lam *et al.*, 1997, "Microencapsulation of Recombinant Humanized Monoclonal Antibody for Local Delivery," *Proc. Int'l. Symp. Control Rel. Bioact. Mater.* 24:759-760, each of which is incorporated herein by reference in its entirety. In one embodiment, a pump may be used in a controlled release system (*See* Langer, *supra*; Sefton, 1987, *CRC Crit. Ref. Biomed. Eng.* 14:20; Buchwald *et al.*, 1980, Surgery 88:507; and Saudek *et al.*, 1989, *N. Engl. J. Med.* 321:574). In another embodiment, polymeric materials can be used to achieve controlled release of antibodies (*see e.g.*, Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, 1983, J., *Macromol. Sci. Rev. Macromol. Chem.* 23:61; *See* also Levy *et al.*, 1985, *Science* 228:190; During *et al.*, 1989, Ann. Neurol. 25:351; Howard *et al.*, 1989, *J. Neurosurg.* 7 1:105); U.S. Patent No. 5,679,377; U.S. Patent No. 5,916,597; U.S. Patent No. 5,912,015; U.S. Patent No. 5,989,463; U.S. Patent No. 5,128,326; PCT Publication No. WO 99/15154; and PCT Publication No. WO 99/20253). Examples of polymers used in sustained release formulations include, but are not limited to, poly(2-hydroxy ethyl methacrylate), poly(methyl methacrylate), poly(acrylic acid), poly(ethylene-co-vinyl acetate), poly(methacrylic acid), polyglycolides (PLG), polyanhydrides, poly(N-vinyl pyrrolidone), poly(vinyl alcohol), polyacrylamide, poly(ethylene glycol), polylactides (PLA), poly(lactide-co-glycolides) (PLGA), and polyorthoesters. In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target (*e.g.*, the lungs), thus requiring only a fraction of the systemic dose (*see, e.g.*, Goodson, in Medical Applications of Controlled Release, *supra*, vol. 2, pp. 115-138 (1984)). In another embodiment, polymeric compositions useful as controlled release implants are used according to Dunn *et al.* (*See* U.S. 5,945,155). This particular method is based upon the therapeutic effect of the in situ controlled release of the bioactive material from the polymer system. The implantation can generally occur anywhere within the body of the patient in need of therapeutic treatment. In another embodiment, a non-polymeric sustained delivery system is used, whereby a non-polymeric implant in the body of the subject is

used as a drug delivery system. Upon implantation in the body, the organic solvent of the implant will dissipate, disperse, or leach from the composition into surrounding tissue fluid, and the non-polymeric material will gradually coagulate or precipitate to form a solid, microporous matrix (*See* U.S. 5,888,533).

[00439] Controlled release systems are discussed in the review by Langer (1990, *Science* 249:1527-1533). Any technique known to one of skill in the art can be used to produce sustained release formulations comprising one or more therapeutic agents of the invention. *See, e.g.*, U.S. Patent No. 4,526,938; International Publication Nos. WO 91/05548 and WO 96/20698; Ning *et al.*, 1996, *Radiotherapy & Oncology* 39:179-189; Song *et al.*, 1995, *PDA Journal of Pharmaceutical Science & Technology* 50:372-397; Cleek *et al.*, 1997, *Pro. Int'l. Symp. Control. Rel. Bioact. Mater.* 24:853-854; and Lam *et al.*, 1997, *Proc. Int'l. Symp. Control Rel. Bioact. Mater.* 24:759-760, each of which is incorporated herein by reference in its entirety.

[00440] In a specific embodiment where the composition of the invention is a nucleic acid encoding an antibody, the nucleic acid can be administered *in vivo* to promote expression of its encoded antibody, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, *e.g.*, by use of a retroviral vector (*See* U.S. Patent No. 4,980,286), or by direct injection, or by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, or by administering it in linkage to a homeobox-like peptide which is known to enter the nucleus (*See e.g.*, Joliot *et al.*, 1991, *Proc. Natl. Acad. Sci. USA* 88:1864-1868), *etc.* Alternatively, a nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression by homologous recombination.

[00441] For antibodies, the therapeutically or prophylactically effective dosage administered to a subject is typically 0.1 mg/kg to 200 mg/kg of the subject's body weight. Preferably, the dosage administered to a subject is between 0.1 mg/kg and 20 mg/kg of the subject's body weight and more preferably the dosage administered to a subject is between 1 mg/kg to 10 mg/kg of the subject's body weight. The dosage and frequency of administration of antibodies of the invention may be reduced also by enhancing uptake and tissue penetration (*e.g.*, into the lung) of the antibodies or fusion proteins by modifications such as, for example, lipidation.

[00442] Treatment of a subject with a therapeutically or prophylactically effective amount of molecules of the invention can include a single treatment or, preferably, can

include a series of treatments. In a preferred example, a subject is treated with molecules of the invention in the range of between about 0.1 to 30 mg/kg body weight, one time per week for between about 1 to 10 weeks, preferably between 2 to 8 weeks, more preferably between about 3 to 7 weeks, and even more preferably for about 4, 5, or 6 weeks. In other embodiments, the pharmaceutical compositions of the invention are administered once a day, twice a day, or three times a day. In other embodiments, the pharmaceutical compositions are administered once a week, twice a week, once every two weeks, once a month, once every six weeks, once every two months, twice a year or once per year. It will also be appreciated that the effective dosage of the molecules used for treatment may increase or decrease over the course of a particular treatment.

6.6.1 PHARMACEUTICAL COMPOSITIONS

[00443] The compositions of the invention include bulk drug compositions useful in the manufacture of pharmaceutical compositions (*e.g.*, impure or non-sterile compositions) and pharmaceutical compositions (*i.e.*, compositions that are suitable for administration to a subject or patient) which can be used in the preparation of unit dosage forms. Such compositions comprise a prophylactically or therapeutically effective amount of a prophylactic and/or therapeutic agent disclosed herein or a combination of those agents and a pharmaceutically acceptable carrier. Preferably, compositions of the invention comprise a prophylactically or therapeutically effective amount of one or more molecules of the invention and a pharmaceutically acceptable carrier.

[00444] In one particular embodiment, the pharmaceutical composition comprises a therapeutically effective amount of one or more molecules of the invention comprising a variant Fc region, wherein said variant Fc region binds FcγRIIIA and/or FcγRIIA with a greater affinity than a comparable molecule comprising a wild-type Fc region binds FcγRIIIA and/or FcγRIIA and/or said variant Fc region mediates an effector function at least 2-fold more effectively than a comparable molecule comprising a wild-type Fc region, and a pharmaceutically acceptable carrier. In another embodiment, the pharmaceutical composition comprises a therapeutically effective amount of one or more molecules of the invention comprising a variant Fc region, wherein said variant Fc region binds FcγRIIIA with a greater affinity than a comparable molecule comprising a wild-type Fc region binds FcγRIIIA, and said variant Fc region binds

FcγRIIB with a lower affinity than a comparable molecule comprising a wild-type Fc region binds FcγRIIB, and/or said variant Fc region mediates an effector function at least 2-fold more effectively than a comparable molecule comprising a wild-type Fc region, and a pharmaceutically acceptable carrier. In another embodiment, said pharmaceutical compositions further comprise one or more anti-cancer agents.

[00445] The invention also encompasses pharmaceutical compositions comprising a therapeutic antibody (*e.g.*, tumor specific monoclonal antibody) that is specific for a particular cancer antigen, comprising one or more amino acid modifications in the Fc region as determined in accordance with the instant invention, and a pharmaceutically acceptable carrier.

[00446] In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant (*e.g.*, Freund's adjuvant (complete and incomplete), excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like.

[00447] Generally, the ingredients of compositions of the invention are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by

injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

[00448] The compositions of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include, but are not limited to those formed with anions such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, *etc.*, and those formed with cations such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, *etc.*

6.6.2 KITS

[00449] The invention provides a pharmaceutical pack or kit comprising one or more containers filled with the molecules of the invention (*i.e.*, antibodies, polypeptides comprising variant Fc regions). Additionally, one or more other prophylactic or therapeutic agents useful for the treatment of a disease can also be included in the pharmaceutical pack or kit. The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

[00450] The present invention provides kits that can be used in the above methods. In one embodiment, a kit comprises one or more molecules of the invention. In another embodiment, a kit further comprises one or more other prophylactic or therapeutic agents useful for the treatment of cancer, in one or more containers. In another embodiment, a kit further comprises one or more cytotoxic antibodies that bind one or more cancer antigens associated with cancer. In certain embodiments, the other prophylactic or therapeutic agent is a chemotherapeutic. In other embodiments, the prophylactic or therapeutic agent is a biological or hormonal therapeutic.

6.7 CHARACTERIZATION AND DEMONSTRATION OF THERAPEUTIC UTILITY

[00451] Several aspects of the pharmaceutical compositions, prophylactic, or therapeutic agents of the invention are preferably tested *in vitro*, in a cell culture system, and in an animal model organism, such as a rodent animal model system, for the desired therapeutic activity prior to use in humans. For example, assays which can

be used to determine whether administration of a specific pharmaceutical composition is desired, include cell culture assays in which a patient tissue sample is grown in culture, and exposed to or otherwise contacted with a pharmaceutical composition of the invention, and the effect of such composition upon the tissue sample is observed. The tissue sample can be obtained by biopsy from the patient. This test allows the identification of the therapeutically most effective prophylactic or therapeutic molecule(s) for each individual patient. In various specific embodiments, *in vitro* assays can be carried out with representative cells of cell types involved in an autoimmune or inflammatory disorder (*e.g.*, T cells), to determine if a pharmaceutical composition of the invention has a desired effect upon such cell types.

[00452] Combinations of prophylactic and/or therapeutic agents can be tested in suitable animal model systems prior to use in humans. Such animal model systems include, but are not limited to, rats, mice, chicken, cows, monkeys, pigs, dogs, rabbits, *etc.* Any animal system well-known in the art may be used. In a specific embodiment of the invention, combinations of prophylactic and/or therapeutic agents are tested in a mouse model system. Such model systems are widely used and well-known to the skilled artisan. Prophylactic and/or therapeutic agents can be administered repeatedly. Several aspects of the procedure may vary. Said aspects include the temporal regime of administering the prophylactic and/or therapeutic agents, and whether such agents are administered separately or as an admixture.

[00453] Preferred animal models for use in the methods of the invention are, for example, transgenic mice expressing human FcγRs on mouse effector cells, *e.g.*, any mouse model described in U.S. 5,877,396 (which is incorporated herein by reference in its entirety) can be used in the present invention. Transgenic mice for use in the methods of the invention include, but are not limited to, mice carrying human FcγRIIIA; mice carrying human FcγRIIA; mice carrying human FcγRIIB and human FcγRIIIA; mice carrying human FcγRIIB and human FcγRIIA.

[00454] Preferably, mutations showing the highest levels of activity in the functional assays described above will be tested for use in animal model studies prior to use in humans. Antibodies harboring the Fc mutants identified using the methods of the invention and tested in ADCC assays, including ch4D5 and ch520C9, two anti-Erb-B2 antibodies, and chCC49, an anti-TAG72 antibody, are preferred for use in animal models since they have been used previously in xenograft mouse model (Hudsiak *et al.*, 1989, *Mol. Cell Biol.* 9: 1165-72; Lewis *et al.*, 1993, *Cancer Immunol. Immunother.* 37:

255-63; Bergman *et al.*, 2001 *Clin. Cancer Res.* 7: 2050-6; Johnson *et al.*, 1995, *Anticancer Res.* 1387-93). Sufficient quantities of antibodies may be prepared for use in animal models using methods described supra, for example using mammalian expression systems and IgG purification methods disclosed and exemplified herein. A typical experiment requires at least about 5.4 mg of mutant antibody. This calculation is based on average quantities of wild type antibody required to protect 8-10 30 g mice following a loading dose of 4 μg/g and a weekly maintenance dose, 2 μg/g, for ten weeks. invention encompasse tumor cell lines as a source for xenograft tumors, such as SK-BR-3, BT474 and HT29 cells which are derived from patients with breast adenocarcinoma. These cells have both Erb-B2 and the prolactin receptors on their surface. The SK-BR-3 cells have been used successfully in both ADCC and xenograft tumor models. In other assays OVCAR3 cells derived from a human ovarian adenocarcinoma may be used. These cells express the antigen TAG72 on the cell surface and can be used in conjunction with the chCC49 antibody. The use of different antibodies and multiple tumor models will circumvent loss of any specific mutations due to an antibody specific Fc mutant incompatibility.

[00455] Mouse xenograft models may be used for examining efficacy of mouse antibodies generated against a tumor specific target based on the affinity and specificity of the CDR regions of the antibody molecule and the ability of the Fc region of the antibody to elicit an immune response (Wu et al., 2001, Trends Cell Biol. 11: S2-9). Transgenic mice expressing human FcγRs on mouse effector cells are unique and are tailor-made animal models to test the efficacy of human Fc-FcγR interactions. Pairs of FcγRIIIA, FcγRIIIB and FcγRIIA transgenic mouse lines generated in the lab of Dr. Jeffrey Ravetch (Through a licensing agreement with Rockefeller U. and Sloan Kettering Cancer center) can be used such as those listed in the **Table 13** below.

Table 13: Mice Strains

Strain Background	Human FcR
Nude / CD16A KO	none
Nude / CD16A KO	FcγRIIIA
Nude / CD16A KO	FcγR IIA
Nude / CD16A KO	FcγR IIA and IIIA
Nude / CD32B KO	none
Nude / CD32B KO	FcγR IIB

[00456] Preferably Fc mutants showing both enhanced binding to FcγRIIIA and reduced binding to FcγRIIB, increased activity in ADCC and phagocytosis assays are

tested in animal model experiments. The animal model experiments examine the increase in efficacy of Fc mutant bearing antibodies in FcγRIIIA transgenic, nude mCD16A knockout mice compared to a control which has been administered native antibody. Preferably, groups of 8-10 mice are examined using a standard protocol. An exemplary animal model experiment may comprise the following steps: in a breast cancer model, ~2 x 10^6 SK-BR-3 cells are injected subcutaneously on day 1 with 0.1 mL PBS mixed with Matrigel (Becton Dickinson). Initially a wild type chimeric antibody and isotype control are administered to establish a curve for the predetermined therapeutic dose, intravenous injection of 4D5 on day 1 with an initial dose of 4 μg/g followed by weekly injections of 2 μg/g. Tumor volume is monitored for 6-8 weeks to measure progress of the disease. Tumor volume should increase linearly with time in animals injected with the isotype control. In contrast very little tumor growth should occur in the group injected with 4D5. Results from the standard dose study are used to set an upper limit for experiments testing the Fc mutants. These studies are done using subtherapeutic doses of the Fc mutant containing antibodies. A one tenth dose was used on xenograft models in experiments done in FcγRIIB knockout mice, *see*, Clynes *et al.*, 2000, *Nat. Med.* 6: 443-6, with a resultant block in tumor cell growth. Since the mutants of the invention preferrably show an increase in FcγRIIIA activation and reduction in FcγRIIB binding the mutants are examined at one tenth therapeutic dose. Examination of tumor size at different intervals indicates the efficacy of the antibodies at the lower dose. Statistical analysis of the data using *t* test provides a way of determining if the data is significant. Fc mutants that show increased efficacy are tested at incrementally lower doses to determine the smallest possible dose as a measure of their efficacy.

[00457] The anti-inflammatory activity of the combination therapies of invention can be determined by using various experimental animal models of inflammatory arthritis known in the art and described in Crofford L.J. and Wilder R.L., "Arthritis and Autoimmunity in Animals", in Arthritis and Allied Conditions: A Textbook of Rheumatology, McCarty *et al.*(eds.). Chapter 30 (Lee and Febiger, 1993). Experimental and spontaneous animal models of inflammatory arthritis and autoimmune rheumatic diseases can also be used to assess the anti-inflammatory activity of the combination therapies of invention. The following are some assays provided as examples, and not by limitation.

[00458] The principle animal models for arthritis or inflammatory disease known in the art and widely used include: adjuvant-induced arthritis rat models, collagen-induced arthritis rat and mouse models and antigen-induced arthritis rat, rabbit and hamster models, all described in Crofford L.J. and Wilder R.L., "Arthritis and Autoimmunity in Animals", in Arthritis and Allied Conditions: A Textbook of Rheumatology, McCarty *et al.*(eds.), Chapter 30 (Lee and Febiger, 1993), incorporated herein by reference in its entirety.

[00459] The anti-inflammatory activity of the combination therapies of invention can be assessed using a carrageenan-induced arthritis rat model. Carrageenan-induced arthritis has also been used in rabbit, dog and pig in studies of chronic arthritis or inflammation. Quantitative histomorphometric assessment is used to determine therapeutic efficacy. The methods for using such a carrageenan-induced arthritis model is described in Hansra P. *et al.*, "Carrageenan-Induced Arthritis in the Rat," *Inflammation*, 24(2): 141-155, (2000). Also commonly used are zymosan-induced inflammation animal models as known and described in the art.

[00460] The anti-inflammatory activity of the combination therapies of invention can also be assessed by measuring the inhibition of carrageenan-induced paw edema in the rat, using a modification of the method described in Winter C. A. *et al.*, "Carrageenan-Induced Edema in Hind Paw of the Rat as an Assay for Anti-inflammatory Drugs" *Proc. Soc. Exp. Biol Med.* 111, 544-547, (1962). This assay has been used as a primary *in vivo* screen for the anti-inflammatory activity of most NSAIDs, and is considered predictive of human efficacy. The anti-inflammatory activity of the test prophylactic or therapeutic agents is expressed as the percent inhibition of the increase in hind paw weight of the test group relative to the vehicle dosed control group.

[00461] Additionally, animal models for inflammatory bowel disease can also be used to assess the efficacy of the combination therapies of invention (Kim *et al.*, 1992, Scand. *J. Gastroentrol.* 27:529-537; Strober, 1985, *Dig. Dis. Sci.* 30(12 Suppl):3S-10S). Ulcerative cholitis and Crohn's disease are human inflammatory bowel diseases that can be induced in animals. Sulfated polysaccharides including, but not limited to amylopectin, carrageen, amylopectin sulfate, and dextran sulfate or chemical irritants including but not limited to trinitrobenzenesulphonic acid (TNBS) and acetic acid can be administered to animals orally to induce inflammatory bowel diseases.

[00462] Animal models for autoimmune disorders can also be used to assess the efficacy of the combination therapies of invention. Animal models for autoimmune disorders such as type 1 diabetes, thyroid autoimmunity, sytemic lupus eruthematosus, and glomerulonephritis have been developed (Flanders *et al.*, 1999, *Autoimmunity* 29:235-246; Krogh *et al.*, 1999, *Biochimie* 81:511-515; Foster, 1999, *Semin. Nephrol.* 19:12-24).

[00463] Further, any assays known to those skilled in the art can be used to evaluate the prophylactic and/or therapeutic utility of the combinatorial therapies disclosed herein for autoimmune and/or inflammatory diseases.

[00464] Toxicity and efficacy of the prophylactic and/or therapeutic protocols of the instant invention can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Prophylactic and/or therapeutic agents that exhibit large therapeutic indices are preferred. While prophylactic and/or therapeutic agents that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such agents to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

[00465] The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage of the prophylactic and/or therapeutic agents for use in humans. The dosage of such agents lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any agent used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} (*i.e.*, the concentration of the test compound that achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

[00466] The anti-cancer activity of the therapies used in accordance with the present invention also can be determined by using various experimental animal models for the study of cancer such as the SCID mouse model or transgenic mice or nude mice with

human xenografts, animal models, such as hamsters, rabbits, *etc.* known in the art and described in *Relevance of Tumor Models for Anticancer Drug Development* (1999, eds. Fiebig and Burger); *Contributions to Oncology* (1999, Karger); *The Nude Mouse in Oncology Research* (1991, eds. Boven and Winograd); and *Anticancer Drug Development Guide* (1997 ed. Teicher), herein incorporated by reference in their entireties.

[00467] Preferred animal models for determining the therapeutic efficacy of the molecules of the invention are mouse xenograft models. Tumor cell lines that can be used as a source for xenograft tumors include but are not limited to, SKBR3 and MCF7 cells, which can be derived from patients with breast adenocarcinoma. These cells have both erbB2 and prolactin receptors. SKBR3 cells have been used routinely in the art as ADCC and xenograft tumor models. Alternatively, OVCAR3 cells derived from a human ovarian adenocarcinoma can be used as a source for xenograft tumors.

[00468] The protocols and compositions of the invention are preferably tested *in vitro*, and then *in vivo*, for the desired therapeutic or prophylactic activity, prior to use in humans. Therapeutic agents and methods may be screened using cells of a tumor or malignant cell line. Many assays standard in the art can be used to assess such survival and/or growth; for example, cell proliferation can be assayed by measuring ^{3}H-thymidine incorporation, by direct cell count, by detecting changes in transcriptional activity of known genes such as proto-oncogenes (*e.g.*, fos, myc) or cell cycle markers; cell viability can be assessed by trypan blue staining, differentiation can be assessed visually based on changes in morphology, decreased growth and/or colony formation in soft agar or tubular network formation in three-dimensional basement membrane or extracellular matrix preparation, etc.

[00469] Compounds for use in therapy can be tested in suitable animal model systems prior to testing in humans, including but not limited to in rats, mice, chicken, cows, monkeys, rabbits, hamsters, *etc.*, for example, the animal models described above. The compounds can then be used in the appropriate clinical trials.

[00470] Further, any assays known to those skilled in the art can be used to evaluate the prophylactic and/or therapeutic utility of the combinatorial therapies disclosed herein for treatment or prevention of cancer, inflammatory disorder, or autoimmune disease.

[00471] Having now generally described the invention, the same will be more readily understood through reference to the following examples, which are provided by way of illustration and are not intended to be limiting of the present invention unless specified.

7. EXAMPLES

7.1 ANALYSIS OF KINETIC PARAMETERS OF Fc MUTANTS

[00472] Fc mutants exhibiting altered affinity to FcγRIIIA and FcγRIIB were determined from yeast display technology and FcγR-Fc interaction assays as disclosed in U.S Patent Application Publications 2005/0037000 and 2005/0064514, and International Patent Application Publication WO 04/063351, each of which is hereby incorporated by reference in its entirety. Effects of the mutant in *in vitro* assays was assed by determining kinetic parameters of the binding of ch4-4-20 antibodies harboring the Fc mutants using a BIAcore assay (BIAcore instrument 1000, BIAcore Inc., Piscataway, N.J.). The FcγRIIIA used in this assay was a soluble monomeric protein, the extracellular region of FcγRIIIA joined to the linker-AVITAG sequence, while the FcγRIIB used in this assay was a soluble dimeric protein, both were prepared as described in U.S Patent Application Publications 2005/0037000 and 2005/0064514, and International Patent Application Publication WO 04/063351. Briefly, the FcγRIIB used was the extracellular domain of FcγRIIB fused to the hinge-CH2-CH3 domain of human IgG2.

[00473] BSA-FITC (36 μg/mL in 10mM Acetate Buffer at pH 5.0) was immobilized on one of the four flow cells (flow cell 2) of a sensor chip surface through amine coupling chemistry (by modification of carboxymethyl groups with mixture of NHS/EDC) such that about 5000 response units (RU) of BSA-FITC was immobilized on the surface. Following this, the unreacted active esters were "capped off" with an injection of 1M Et-NH2. Once a suitable surface was prepared, ch 4-4-20 antibodies carrying the Fc mutations were passed over the surface by one minute injections of a 20 μg/mL solution at a 5 μL/mL flow rate. The level of ch-4-4-20 antibodies bound to the surface ranged between 400 and 700 RU. Next, dilution series of the receptor (FcγRIIIA and FcγRIIB-Fc fusion protein) in HBS-P buffer (10mM HEPES, 150 mM NaCl, .005% Surfactant P20, 3mM EDTA, pH 7.4) were injected onto the surface at 100 μL/min. Antibody regeneration between different receptor dilutions was carried out by single 5 second injections of 100mM $NaHCO_3$ pH 9.4; 3M NaCl.

[00474] The same dilutions of the receptor were also injected over a BSA-FITC surface without any ch-4-4-20 antibody at the beginning and at the end of the assay as reference injections.

[00475] Once an entire data set was collected, the resulting binding curves were globally fitted using computer algorithms supplied by the manufacturer, 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 BIAevaluaion Software Handbook (BIAcore, Inc., Piscataway, NJ).

[00476] Binding curves for two different concentrations (200 nM and 800 nM for FcγRIIIA and 200 nM and 400nM for FcγRIIB fusion protein) were aligned and responses adjusted to the same level of captured antibodies, and the reference curves were subtracted from the experimental curves. Association and dissociation phases were fitted separately. Dissociation rate constant was obtained for interval 32-34 sec of the dissociation phase; association phase fit was obtained by a 1:1 Langmuir model and base fit was selected on the basis R_{max} and chi^2 criteria.

RESULTS

[00477] FIG. 4 shows the capture of ch 4-4-20 antibodies with mutant Fc regions on the BSA-FTIC-immobilized sensor chip. 6 μL of antibodies at a concentration of about 20 μg/mL were injected at 5 μL/min over the BSA-FITC surface. FIG. 5 is a sensogram of real time binding of FcγRIIIA to ch-4-4-20 antibodies carrying variant Fc regions. Binding of FcγRIIIA was analyzed at 200 nM concentration and resonance signal responses were normalized at the level of the response obtained for the wild type ch-4-4-20 antibody. Kinetic parameters for the binding of FcγRIIIA to ch-4-4-20 antibodies were obtained by fitting the data obtained at two different FcγRIIIA concentrations, 200 and 800 nM (FIG. 6). The solid line represents the association fit which was obtained based on the K_{off} values calculated for the dissociation curves in interval 32-34 seconds. K_D and K_{off} represent the average calculated from the two different FcγRIIIA concentrations used. FIG. 7 is a sensogram of real time binding of FcγRIIB-Fc fusion protein to ch-4-4-20 antibodies carrying variant Fc regions. Binding of FcγRIIB-Fc fusion protein was analyzed at 200 nM concentration and resonance

signal responses were normalized at the level of the response obtained for the wild type ch-4-4-20 antibody. Kinetic parameters for the binding of FcγRIIB-Fc fusion protein to ch-4-4-20 antibodies were obtained by fitting the data obtained at two different FcγRIIB-Fc fusion protein concentrations, 200 and 800 nM (FIG. 8). The solid line represents the association fit which was obtained based on the K_{off} calculated for the dissociation curves in interval 32-34 seconds. K_D and K_{off} represent the average from the two different FcγRIIB-Fc fusion protein concentrations used.

[00478] The kinetic parameters (K_{on} and K_{off}) that were determined from the BIAcore analysis correlated with the binding characteristic of the mutants as determined by an ELISA assay and the functional activity of the mutants as determined in an ADCC assay. Specifically, as seen in **Table 14**, mutants that had an enhanced ADCC activity relative to the wild-type protein, and had an enhanced binding to FcγRIIIA as determined by an ELISA assay had an improved K_{off} for FcγRIIIA (*i.e.*, a lower K_{off}). Therefore, a lower K_{off} value for FcγRIIIA for a mutant Fc protein relative to a wild type protein may be likely to have an enhanced ADCC function. On the other hand, as seen in **Table 15**, mutants that had an enhanced ADCC activity relative to the wild-type protein, and had a reduced binding for FcγRIIB-Fc fusion protein as determined by an ELISA assay had a higher K_{off} for FcγRIIB -Fc fusion protein.

[00479] Thus, the K_{off} values for FcγRIIIA and FcγRIIB can be used as predictive measures of how a mutant will behave in a functional assay such as an ADCC assay. In fact, ratios of K_{off} values for FcγRIIIA and FcγRIIB-Fc fusion protein of the mutants to the wild type protein were plotted against ADCC data (FIG. 9). Specifically, in the case of K_{off} values for FcγRIIIA, the ratio of K_{off} (wt)/ K_{off} (mutant) was plotted against the ADCC data; and in the case of K_{off} values for FcγRIIB, the ratio of K_{off} (mut)/ K_{off} (wt) was plotted against the ADCC data. Numbers higher than one (1) show a decreased dissociation rate for FcγRIIIA and an increased dissociation rate for FcγRIIB -Fc relative to wild type. Mutants that fall within the indicated box have a lower off rate for FcγRIIIA binding and a higher off-rate for FcγRIIB -Fc binding, and possess an enhanced ADCC function.

Table 14. Kinetic parameters of FcRIIIA binding to ch4-4-20Ab obtained by "separate fit" of 200nM and 800nM binding curves

Ch4-4-20Ab	BIAcore Kd,nM	K_{on} 1/Ms	K_{off}, 1/s	ELISA,OD	ADCC, %
Wt(0225)	319	6.0×10^5	0.170	0.5	17.5
Mut11(0225)	90	8.22×10^5	0.075	0.37	32
Mut5(0225)	214	8.2×10^5	0.172	0.75	26
Mut6(0225)	264	6.67×10^5	0.175	0.6	23
Mut8(0225)	234	8.3×10^5	0.196	0.5	22
Mut10(0225)	128	9.04×10^5	0.115	1.0	41
Mut12(0225)	111	1.04×10^6	0.115	1.0	37
Mut15(0225)	67.9	1.97×10^6	0.133	1.0	15
Mut16(0225)	84.8	1.60×10^6	0.133	1.0	15
Mut18(0225)	92	1.23×10^6	0.112	1.0	28
Mut25(0225)	48.6	2.05×10^6	0.1	1.0	41
Mut14(0225)	75.4	1.37×10^6	0.1	1.1	28
Mut17(0225)	70.5	1.42×10^6	0.1	1.25	30
Mut19(0225)	100	1.20×10^6	0.120	0.75	11
Mut20(0225)	71.5	1.75×10^6	0.126	0.5	10
Mut23(0225)	70.2	1.43×10^6	0.105	1.25	25

Highlighted mutants do not fit to the group by ELISA or ADCC data.

Table 15. Kinetic parameters of FcRIIB-Fc binding to wild type and mutant ch4-4-20Ab obtained by "separate fit" of 200 nM and 800 nM binding curves.

Ch4-4-20Ab	BIAcore Kd,nM	K_{on} 1/Ms	K_{off}, 1/s	ELISA,OD	ADCC, %
Wt(0225)	61.4		0.085	0.4	17.5
Mut11(0225)	82.3		0.1	0.08	32
Mut5(0225)	50		0.057	0.6	26
Mut6(0225)	66.5		0.060	0.35	23
Mut8(0225)	44.2		0.068	0.25	22
Mut10(0225)	41.3		0.05	1.2	41
Mut12(0225)	40.1		0.051	0.4	37
Mut15(0225)	37.8		0.040	1.55	15
Mut16(0225)	40		0.043	1.55	15
Mut18(0225)	51.7		0.043	1.25	28
Mut25(0225)			0.112	0.08	41
Mut14(0225)	95.6		0.089	0.13	28
Mut17(0225)	55.3		0.056	0.38	30
Mut19(0225)	45.3		0.046	1.0	11
Mut20(0225)	24.1		0.028	0.8	10
Mut23(0225)	108		0.107	0.1	25

7.2 SCREENING FOR Fc MUTANTS USING MULTIPLE ROUNDS OF ENRICHMENT USING A SOLID PHASE ASSAY

[00480] The following mutant screens were aimed at identifying additional sets of mutants that show improved binding to FcγRIIIA and reduced binding to FcγRIIB. Secondary screening of selected Fc variants was performed by ELISA followed by testing for ADCC in the 4-4-20 system. Mutants were than selected primarily based on their ability to mediate ADCC via 4-4-20 using Fluorescein coated SK-BR3 cells as targets and isolated PBMC from human donors as the effector cell population. Fc mutants that showed a relative increase in ADCC, *e.g.*, an enhancedment by a factor of 2 were than cloned into anti-HER2/neu or anti-CD20 chAbs and tested in an ADCC assay using the appropriate tumor cells as targets. The mutants were also analyzed by BIAcore and their relative K_{off} were determined.

[00481] Screen 1: Sequential solid phase depletion and selection using Magnetic beads coated with FcγRIIB followed by selection with magnetic beads coated with FcγRIIIA. The aim of this screen was identification of Fc mutants that either no longer bind FcγRIIB or show reduced binding to FcγRIIB. A 10-fold excess of the naïve library ($\sim 10^7$ cells) was incubated with magnetic beads ("My One", Dynal) coated with FcγRIIB. Yeast bound to beads were separated from the non-bound fraction by placing the tube containing the mixture in a magnetic field. Those yeast cells that were not bound to the beads were removed and placed in fresh media. They were next bound to beads that were coated with FcγRIIIA. Yeast bound to beads were separated from the nonbound fraction by placing the tube containing the mixture in a magnetic field. Nonbound yeast were removed and the bound cells were removed by vigorous vortexing. The recovered cells were regrown in glucose containing media and reinduced in selective media containing galactose. The selection process was repeated. The final culture was than used to harvest DNA. Inserts containing the Fc domain were amplified by PCR and cloned into 4-4-20. Approximately 90 Fc mutants were screened by 4-4-20 ELISA and ADCC assays and the resultant positive mutants are shown in **Table 16**.

Table 16: Mutants selected by sequential solid phase depletion and selection using Magnetic beads coated with FcγRIIB followed by selection with magnetic beads coated with FcγRIIIA.

Mutant	Amino Acid changes
MgFc37	K248M
MgFc38	K392T, P396L
MgFc39	E293V, Q295E, A327T
MgFc41	H268N, P396LN
MgFc43	Y319F, P352L, P396L
MgFc42	D221E, D270E, V308A, Q311H, P396L, G402D

[00482] *Screens 2&3: Mutants Selected by FACS, Equilibrium and Kinetic Screening:* The first library screen identified a mutation at position 396, changing the amino acid from Proline to Leucine (P396L). This Fc variant showed increased binding to both FcγRIIIA and FcγRIIB. A second library was constructed using P396L as a base line. PCR mutagenesis was used to generate ~10^7 mutants each of which contained the P396L mutation and contained additional nucleotide changes. The P396L library was screened using two sets of conditions.

[00483] An equilibrium screen was performed using biotinylated FcγRIIIA -linker-avitag as a monomer, using methods already described. Approximately 10-fold excess of library (10^8 cells) was incubated in a 0.5 mL of approximately 7 nM FcγRIIIA for 1 hr. The mixture was sorted by FACS, selecting top 1.2% of binders. Selected yeast cells were grown in selective media containing glucose and reinduced in selective media containing galactose. The equilibrium screen was repeated a second time and the sort gate was set to collect the top 0.2% of binders. The selected yeast cells were then grown under selective conditions in glucose. This culture was than used to harvest DNA. Inserts containing the Fc domain were amplified by PCR and cloned into the nucleotide sequence encoding 4-4-20 variable domain using methods already described. Approximately 90 Fc mutants were screened by 4-4-20 ELISA and ADCC and the resultant positive mutants are shown in **Table 17**.

Table 17
Mutants selected by FACS using an Equilibrium screen with concentrations of FcRIIIA of approximately 7 nM.

Mutant	Amino Acid changes
MgFc43b	K288R, T307A, K344E, P396L
MgFc44	K334N, P396L
MgFc46	P217S, P396L
MgFc47	K210M, P396L
MgFc48	V379M, P396L
MgFc49	K261N, K210M, P396L
MgFc60	P217S, P396L

[00484] A kinetic screen was also implemented to identify mutants with improved K_{off} in binding FcγRIIIA. Conditions were established for screening the P396L library using a strain with the P396L Fc variant displayed on the yeast surface. Briefly cells grown under inducing conditions were incubated with 0.1 μM biotinylated FcγRIIIA - linker-avitag monomer for 1 hr. The cells were washed to remove the labeled ligand. Labeled cells were then incubated for different times with 0.1 μM unlabeled FcγRIIIA-linker-avitag monomer, washed and then stained with SA:PE for FACS analysis (FIG. 10). Cells were also stained with goat anti-human Fc to show that the Fc display was maintained during the experiment.

[00485] Based on the competition study it was determined that a 1 minute incubation resulted in approximately 50% loss of cell staining. This time point was chosen for the kinetic screen using the P396L library. Approximately 10-fold excess of library (10^8 cells) was incubated with 0.1 μM biotinylated FcγRIIIA-linker-avitag monomer in a 0.5 mL volume. Cells were washed and then incubated for 1 minute with unlabeled ligand. Subsequently the cells were washed and labeled with SA:PE. The mixture was sorted by FACS, selecting the top 0.3% of binders. Selected yeast cells were grown in selective media containing glucose and reinduced in selective media containing galactose. The kinetic screen was repeated a second time and the sort gate was set to collect the top 0.2% of binders. The nonselcted P396L library was compared to the yeast cells selected for improved binding by FACS (FIG. 11). The histograms show the percentage of cells that are costained with both FcγRIIIA /PE and goat anti-human Fc/FITC (upper right).

[00486] The selected yeast cells from the second sort were then grown under selective conditions in glucose. This culture was than used to harvest DNA. Inserts containing the Fc domain were amplified by PCR and cloned into the nucleotide sequence

encoding 4-4-20 variable domain using methods described above. Approximately 90 Fc mutants were screened by 4-4-20 ELISA and ADCC and the resultant positive mutants are shown in **Table 18**.

Table 18
Mutants selected by FACS using a Kinetic screen using equimolar amounts of unlabeled CD16A for 1 minute.

Mutants	Amino Acid changes
MgFc50	P247S, P396L
MgFc51	Q419H, P396L
MgFc52	V240A, P396L
MgFc53	L410H, P396L
MgFc54	F243L, V305I, A378D, F404S, P396L
MgFc55	R255l, P396L
MgFc57	L242F, P396L
MgFc59	K370E, P396L

Screens 4 and 5: Combining the Solid Phase FcγRIIB Depletion Step with FcγRIIIA Selection by FACs Sort, using the FcγRIIIA 158V allele

[00487] Analysis of Fc variants from Screen 1 showed that the mutations that were selected from the secondary screen had improved binding to both FcγRIIIA and FcγRIIB. Therefore, the data suggested that sequential depletion and selection using magnetic beads (solid phase) under the established conditions did not efficiently select for differential binding of FcγRIIIA and FcγRIIB. Therefore, in order to screen more effectively for mutants that bind FcγRIIIA, while having reduced or no binding to FcγRIIB, the solid phase FcγRIIB depletion step was combined with FcγRIIIA selection by FACs sort. This combination identified Fc variants that bind FcγRIIIA with greater or equal affinity than wild-type Fc.

[00488] A 10-fold excess of the naïve library ($\sim 10^7$) was incubated with magnetic beads coated with FcγRIIB. Yeast bound to beads were separated from the non-bound fraction by placing the tube containing the mixture in a magnetic field. Those yeast cells that were not bound to the beads were removed and placed in fresh media and subsequently reinduced in media containing galactose. The FcγRIIB 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 were stained with FcγRIIIA. These cells were then selected twice by a FACS sort using 0.1 μM biotinylated FcγRIIIA linker-avitag (data not shown). The

FcγRIIIA was the 158V allotype. Yeast cells were analyzed for both FcγRIIIA and FcγRIIB binding after each sort and compared to binding by wild-type Fc domain (FIGS. 12 A-B).

[00489] The selected yeast cells from the second sort were then grown under selective conditions in glucose. This culture was then used to harvest DNA. Inserts containing the Fc domain were amplified by PCR and cloned into the nucleotide sequence encoding 4-4-20 variable domain. Approximately 90 Fc mutants were screened by 4-4-20 ELISA and ADCC and the resultant positive mutants are shown in **Table 19** (mutants 61-66).

Table 19: Mutants selected by magnetic bead depletion using beads coated with CD32B and final selection by FACS using FcγRIIIA 158Valine or 158Phenylalanine

Mutants	Amino Acid Changes
MgFc61	A330V
MgFc62	R292G
MgFc63	S298N, K360R, N361D
MgFc64	E233G
MgFc65	N276Y
MgFc66	A330V, V427M
MgFc67	V284M, S298N, K334E, R355W, R416T

[00490] *Screening of Fc mutants using the 158F allele of FcγRIIIA:* Two different alleles of FcγRIIIA receptor exist that have different binding affinities for the IgG1 Fc domain (Koene *et al.*, 1997, *Blood* 90: 1109-1114; Wu *et al.*, 1997, *J. Clin. Invest.* 100: 1059-70). The 158F allele binds to the Fc domain with a binding constant 5-10 fold lower than the 158V allele. Previously all of the Fc screens using yeast display were done using the high binding 158V allele as a ligand. In this experiment, Fc mutants were selected from the FcγRIIB depleted yeast population using biotinylated FcγRIIIA158F-linker-avitag monomer as a ligand. The sort gate was set to select the top 0.25 percent FcγRIIIA 158F binders. The resulting enriched population was analyzed by FACS (FIG. 12B). Individual clones were then isolated and their binding to different FcγRs were analyzed by FACS (FIG. 12B). Analysis of individual clones from the population resulted in the identification of a single mutant harboring 5 mutations MgFc67 (V284M, S298N, K334E, R355W, R416S), which had an enhanced binding to FcγRIIIA and a reduced binding to FcγRIIB.

[00491] *Secondary Screen of Mutants by an ADCC assay For Screens 1, 2, and 3:*Mutants that were selected in the above screens were then analyzed using a standard

ADCC assay to determine the relative rates of lysis mediated by ch4-4-20 harboring the Fc mutants. ch4-4-20 antibodies carrying the Fc variants were constructed using methods already described above. SK-BR3 cells were used as targets and effector cells were PBMC that were isolated from donors using a Ficoll gradient, as described supra (Section 7.7). The ADCC activity results for the mutants are summarized in **Table 20**.

[00492] As seen in **Table 20**, mutants isolated using the above primary and secondary screens based on FcγRIIB depletion and FcγRIIIA selection showed enhanced ADCC activity relative to wild-type.

Table 20
Analysis of ADCC mediated by 4-4-20 anti-Fluorescein antibody on SKBR3 cells coated with fluorescein.

Mutant	Amino Acid Change	Relative rate of lysis
MgFc37	K248M	3.83
MgFc38	K392T, P396L	3.07
MgFc39	E293V, Q295E, A327T	4.29
MgFc41	H268N, P396LN	2.24
MgFc43	Y319F, P352L, P396L	1.09
MgFc42	D221E, D270E, V308A, Q311H, P396L, G402D	3.17
MgFc43b	K288R, T307A, K344E, P396L	3.3
MgFc44	K334N, P396L	2.43
MgFc46	P217S, P396L	2.04
MgFc47	K210M, P396L	2.02
MgFc48	V379M, P396L	2.01
MgFc49	K261N, K210M, P396L	2.06
MgFc50	P247S, P396L	2.1
MgFc51	Q419H, P396L	2.24
MgFc52	V240A, P396L	2.35
MgFc53	L410H, P396L	2
MgFc54	F243L, V305I, A378D, F404S, P396L	3.59
MgFc55	R255I, P396L	2.79
MgFc57	L242F, P396L	2.4
MgFc59	K370E, P396L	2.47
MgFc60	P217S, P396L	1.44

[00493] Mutants 37, 38, 39, 41, 43 were analyzed using 0.5 μg/mL ch4-4-20. All other antibodies were tested at 1 μg/mL. All rates were normalized to wild type ch4-4-20 (IgG1).

[00494] Mutants were additionally cloned into the heavy chain of antitumor monoclonal antibody 4D5 (anti-HER2/neu) and anti-CD20 monoclonal antibody 2H7 by replacing the Fc domain of these monoclonal antibodies. These chimeric monoclonal antibodies were expressed and purified and tested in an ADCC assay using standard methods by transient transfection into 293H cells and purification over protein G column. The chimeric 4D5 antibodies were tested in an ADCC assay using SK-BR3 cells as targets (FIG. 13), whereas the chimeric 2H7 antibodies were tested in an ADCC assay using Daudi cells as targets (FIG. 14).

[00495] *Secondary Screen of Mutants via BIAcore:* Mutants that were selected in the above screens were then analyzed by BIAcore to determine the kinetic parameters for binding FcγRIIIA(158V) and FcγRIIB. The method used was similar to that disclosed in Section 7.1, supra.

[00496] The data displayed are K_{off} values relative to wild type off rates as determined from experiments using the Fc mutants in the ch4-4-20 monoclonal antibody. Relative numbers greater than one indicate a decrease in K_{off} rate. Numbers less than one indicate an increase in off rate.

[00497] Mutants that showed a decrease in off rates for FcγRIIIA were MgFc38 (K392, P396L), MgFc43(Y319F, P352L, P396L), MgFc42(D221E, D270E, V308A, Q311H, P396L, G402D), MgFc43b (K288R, T307A, K344E, P396L), MgFc44 (K334N, P396L), MgFc46 (P217S, P396L), MgFc49 (K261N, K210M, P396L). Mutants that showed a decrease in off rate for FcγRIIB were, MgFc38(K392, P396L), MgFc39 (E293V, Q295E, A327T), MgFc43 (K288R, T307A, K344E, P396L), MgFc44 (K334N, P396L). The Biacore data is summarized in **Table 21**.

Table 21: BIAcore data

Fc mutant	AA residues	FcγRIIIA158V (K_{off} WT/ Mut)	FcγRIIB (K_{off} WT/ Mut)
MgFc37	K248M	0.977	1.03
MgFc38	K392T, P396L	1.64	2.3
MgFc39	E293V, Q295E. A327T	0.86	1.3
MgFc41	H268N, P396LN	0.92	1.04
MgFc43	Y319F, P352L. P396L	1.23	2.29
MgFc42	D221E, D270E. V308A, Q311H. P396L, G402D	1.38	
MgFc43b	K288R, T307A.	1.27	0.89

Fc mutant	AA residues	FcγRIIIA158V (K_{off} WT/ Mut)	FcγRIIB (K_{off} WT/ Mut)
	K344E, P396L		
MgFc44	K334N, P396L	1.27	1.33
MgFc46	P217S, P396L	1.17	0.95
MgFc47	K210M, P396L		
MgFc48	V379M, P396L		
MgFc49	K261N, K210M, P396L	1.29	0.85
MgFc50	P247S, P396L		
MgFc51	Q419H, P396L		
MgFc52	V240A, P396L		
MgFc53	L410H, P396L		
MgFc54	F243L, V305I, A378D, F404S, P396L		
MgFc55	R255l, P396L		
MgFc57	L242F, P396L		
MgFc59	K370E, P396L		
MgFc60	P217S, P396L		
MgFc61	A330V	1	0.61
MgFc62	R292G	1	0.67
MgFc63	S298N, K360R, N361D	1	0.67
MgFc64	E233G	1	0.54
MgFc65	N276Y	1	0.64
MgFc66	A330V, G427M,	1	0.62
MgFc67	V284M, S298N, K334E, R355W, R416T		

7.3 PBMC MEDIATED ADCC ASSAYS

[00498] MATERIALS AND METHODS

[00499] Fc variants that show improved binding to FcγRIIIA were tested by PBMC based ADCC using 60:1 effector:target ratio. Two different tumor model systems were used as targets, SK-BR3 (anti-HER2/neu) and Daudi (anti-CD20). Percent specific Lysis was quantitated for each mutant. Linear regression analysis was used to plot the data setting the maximal percent lysis at 100%.

[00500] ADCC is activated on immune system effector cells via a signal transduction pathway that is triggered by an interaction between low affinity FcγR and an immune complex. Effector cell populations were derived from either primary blood or activated monocyte derived macrophages (MDM). Target cells were loaded with europium and incubated with chimeric MAb and subsequently incubated with effector cell

populations. Europium works the same way as ^{51}Cr, but it is non-radioactive and the released europium is detected in a fluorescent plate reader. Lymphocytes harvested from peripheral blood of donors (PBM) using a Ficoll-Paque gradient (Pharmacia) contain primarily natural killer cells (NK). The majority of the ADCC activity will occur via the NK containing FcγRIIIA but not FcγRIIB on their surface.

[00501] Experiments were performed using two different target cell populations, SK-BR- 3 and Daudi, expressing HER2/neu and CD20, respectively. ADCC assays were set up using Ch4-4-20/ FITC coated SK-BR-3, Ch4D5/SKBR3, and Rituxan/Daudi (data not shown). Chimeric MAbs were modified using Fc mutations identified. Fc mutants were cloned into ch4D5. Purified Ab was used to opsonize SK-BR-3 cells or Daudi cells. Fc mutants were cloned into ch4D5.

[00502] **RESULTS.** Fc mutants showed improved PBMC mediated ADCC activity in SK BR3 cells (FIG. 13). The plot shows linear regression analysis of a standard ADCC assay. Antibody was titrated over 3 logs using an effector to target ratio of 75:1. % lysis = (Experimental release – SR)/(MR-SR) * 100.

[00503] Fc mutants showed improved PBMC mediated ADCC activity in Daudi cells (FIG. 14).

7.4 MONOCYTE DERIVED MACROPHAGE (MDM) BASED ADCC ASSAYS

[00504] FcγR dependent tumor cell killing is mediated by macrophage and NK cells in mouse tumor models (Clynes et al., 1998, PNAS USA, 95: 652-6). Elutriated monocytes from donors were used as effector cells to analyze the efficiency Fc mutants to trigger cell cytotoxicity of target cells in ADCC assays. Expression patterns of FcγRI, FcγR3A, and FcγR2B are affected by different growth conditions. FcγR expression from frozen monocytes cultured in media containing different combinations of cytokines and human serum were examined by FACS using FcR specific MAbs. (FIG. 15). Cultured cells were stained with FcγR specific antibodies and analyzed by FACS to determine MDM FcγR profiles. Conditions that best mimic macrophage *in vivo* FcγR expression, *i.e.*, showed the greatest fraction of cells expressing CD16 and CD32B were used in a monocyte derived macrophage (MDM) based ADCC assay. For the experiment in FIG. 15, frozen elutriated monocytes were grown for 8 days in DMEM and 20% FBS containing either M-CSF (condition 1) or GM-CSF (condition 2). For the experiment in FIG. 16, frozen elutriated monocytes were cultured for 2 days

in DMEM and 20% FBS containing GM-CSF, IL-2 and IFNγ prior to ADCC assay. Serum free conditions have also been developed which allow for high levels of CD16 and CD32B expression (data not shown). Briefly, purified monocytes were grown for 6-8 days in Macrophage-SFM (Invitrogen) containing GM-CSF, M-CSF, IL-6, IL-10, and IL-1β. While the incidence of CD32B+/CD16+ cells in these cultures is highest using a mixture of cytokines, combinations of two of more cytokines will also enhance FcγR expression (M-CSF/IL-6, M-CSF/IL-10; or M-CSF/IL-1β). For ADCC assays, IFNγ is added for the final 24-48 hours.

[00505] MDM based ADCC required incubation times of >16 hrs to observe target cell killing. Target cells were loaded with Indium-111 which is retained for long incubations within the target cells. Indium release was quantitated using a gamma counter. All other reagents, Abs and target cells, were similar to the PBMC based ADCC assay. ADCC activity due to FcγRI can be efficiently blocked using the anti-FcRI blocking antibody (M21, Ancell). The assay conditions differ slightly from the PBMC based assay. 20:1 target to effector; 18-14 hr incubation at 37C.

[00506] Fc mutants that show improved PBMC ADCC, increased binding to FcγRIIIA, or decreased binding to FcγRIIB were tested (FIG. 16).

7.5 EFFECT OF Fc MUTANTS ON COMPLEMENT ACTIVITY

[00507] Fc mutants were originally identified based on their increased binding to FcγRIIIA. These mutants were subsequently validated for their improved affinity for all low affinity receptors and in many cases improved activity in ADCC mediated by PBMC. *In vivo* antibody mediated cytotoxicity can occur through multiple mechanisms. In addition to ADCC other possible mechanisms include complement dependent cytotoxicity (CDC) and apoptosis. The binding of C1q to the Fc region of an immunoglobulin initiates as cascade resulting in cell lysis by CDC. The interaction between C1q and the Fc has been studied in a series of Fc mutants. The results of these experiments indicate that C1q and the low affinity FcR bind to overlapping regions of the Fc, however the exact contact residues within the Fc vary.

[00508] Mutants that showed improved ADCC in the PBMC based assay were examined for their effect in CDC. Antibodies were analyzed in the anti CD20 Ch-mAb, 2H7. We detected improved CDC for each mutant ch-mAb tested. Interestingly even

though these mutants were selected for their improved ADCC they also show enhanced CDC

[00509] MATERIALS AND METHODS. CDC assay was used to test the Fc mutants using anti-CD20 and Daudi cells as targets. Guinea Pig Serum was used as the source for complement (US Biological). The CDC assay was similar to PBMC based ADCC. Target cells were loaded with europium and opsonized with ChMAb. However complement, guinea pig serum, was added instead of effector cells. FIG. 17 shows a flow chart of the assay. Anti-CD20 ChMab over 3 orders of magnitude was titrated. % lysis was calculated. Daudi cells, (3×10^6) were labeled with BADTA reagent. 1×10^4 cells were aliquoted into wells in a 96 well plate. Antibodies were titrated into the wells using 3 fold dilutions. The opsonization reaction was allowed to proceed for 30-40 minutes at 37° C in 5% CO_2. Guinea pig serum was added to a final conc. of 20%. The reaction was allowed to proceed for 3.5 hrs at 37° C in 5% CO_2. Subsequently, 100 uls of cell media was added to the reaction and cells were spun down. For detection 20 uls of the supernatant was added to 200 uls of the Europium solution and the plates were read in the Victor2(Wallac).

[00510] RESULTS: All mutants that show improved binding for either activating FcR or C1q were placed in the CDC assay (FIG. 18). Fc mutants that showed enhanced binding to FcγRIIIA also showed improved complement activity Each of the mutants show enhanced complement activity compared to wild type. The mutants tested are double mutants. In each case one of the mutations present is P396L.

[00511] To determine whether the increase in CDC correlated with increased binding of C1q to IgG1 Fc binding between the two proteins was measured in realtime using surface plasmon resonance. In order to examine the binding between C1q and an IgG1 Fc the Fc variants were cloned into an anti-CD32B ch-mAb, 2B6. This allowed us to capture the wt and mutant antibodies to the glass slide via soluble CD32B protein (FIG. 19A). Three of the four mutants tested in CDC were also examined in the Biacore. All 3 showed greatly enhanced K_{off} compare to wild type Fc (FIG. 19B). BIAcore format for C1q binding to 2B6 mutants demonstrate enhanced binding of mutants with P396L mutation (FIG. 20). Mutation D270E can reduce C1q binding at different extent. A summary of the kinetic analysis of FcγR and C1q binding is depicted in the **Table 22** below.

TABLE 22 KINETIC ANALYSIS OF FcgR and C1q binding to mutant 2B6

2B6Mutants	3aV158	3aF158	2bfcagl	2aR131Fcagl	2aH131Fcagl	C1q
WT	0.192	0.434	0.056	0.070	0.053	0.124
MgFc38 (K392T,P396L)	0.114	0.243	0.024	0.028	0.024	0.096
MgFc51 (Q419H,P396L)	0.142	0.310	0.030	0.036	0.028	0.074
MgFc55 (R255I,P396L)	0.146	0.330	0.030	0.034	0.028	0.080
MgFc59 (K370E,P396L)	0.149	0.338	0.028	0.033	0.028	0.078
MgFc31/60	0.084	0.238	0.094	0.127	0.034	0.210
MgFc51/60	0.112	0.293	0.077	0.089	0.028	0.079
MgFc55/60	0.113	0.288	0.078	0.099	0.025	0.108
MgFc59/60	0.105	0.296	0.078	0.095	0.024	0.107

7.6 DESIGNING Fc VARIANTS WITH DECREASED BINDING TO FcγRIIB

[00512] Based on a selection for Fc mutants that reduce binding to FcγRIIB and increase binding to FcγRIIA 131H a number of mutations including D270E were identified. Each mutation was tested individually for binding to the low affinity Fc receptors and their allelic variants.

[00513] D270E had the binding characteristics that suggested it would specifically reduce FcγRIIB binding. D270E was tested in combination with mutations that were previously identified based on their improved binding to all FcR.

[00514] RESULTS. As shown in **Tables 23 and 24** and FIGS. 21 and 22 addition of D270E mutation enhances FcγRIIIA and FcγRIIA H131 binding and reduces binding to FcγRIIB. FIG. 23 shows the plot of MDM ADCC data against the K_{off} as determined for CD32A H131H binding for select mutants.

TABLE 23
OFF RATE (1/s) of FcγR BINDING TO WILD TYPE AND MUTANT CHIMERIC 4D5 Ab OBTAINED BY BIACORE ANALYSIS

4D5 Mutants	3aV158	3aF158	2bfcagl	2aR131Fcagl	2aH131Fcagl
Wt pure	0.175	0.408	0.078	0.067	0.046
MgFc55	0.148	0.381	0.036	0.033	0.029
MgFc55/60	0.120	0.320	0.092	0.087	0.013
MgFc55/60+R292G	0.116	0.405	0.124	0.112	0.037
MgFc55/60+Y300L	0.106	0.304	0.092	0.087	0.015
MgFc52	0.140	0.359	0.038	0.040	0.026
MgFc52/60	0.122	0.315	0.094	0.087	0.013
MgFc59	0.145	0.378	0.039	0.047	0.033
MgFc59/60	0.117	0.273	0.088	0.082	0.012
MgFc31	0.125	0.305	0.040	0.043	0.030
MgFc31/60	0.085	0.215	0.139	0.132	0.020
MgFc51	0.135	0.442	0.060	0.047	0.062
MgFc51/60	0.098	0.264	0.118	0.106	0.023
MgFc38	0.108	0.292	0.034	0.025	0.032
MgFc38/60	0.089	0.232	0.101	0.093	0.021

TABLE 24 KINETIC CHARACTERISTICS OF 4D5 MUTANTS

4D5Mutants	3aV158	3aF158	2bfcagl	2aR131Fcagl	2aH131Fcagl
MgFc70	0.101	0.250	0.030	0.025	0.025
MgFc71	0.074	0.212	0.102	0.094	0.020
MgFc73	0.132	0.306	0.190	-----	0.024
MgFc74	0.063	0.370	n.b.	0.311	0.166
WT023stable	0.150	0.419	0.071	0.068	0.043

7.7 ANALYSIS OF KINETIC PARAMETERS OF Fc MUTANTS

[00515] Kinetic parameters of binding of chimeric 4D5 antibodies harboring Fc mutants to the two allotypes of FcγRIIIA, FcγRIIA 131H and FcγRIIB were analyzed by BIAcore using a method similar to that disclosed in Section 7.8 *supra*. The two allotypes of FcγRIIIA, FcγRIIIA 158V and FcγRIIIA 158F, are described in further detail in Section 7.9 *supra*.

[00516] Materials and Methods

[00517] Both allotypes of FcγRIIIA used in this assay were soluble monomeric proteins, the extracellular region of FcγRIIIA joined to the linker-AVITAG sequence as described in Section 7.1. The FcγRIIB and FcγRIIA used in this assay were soluble dimeric proteins, *i.e.* the extracellular domain of FcγRIIB or FcγRIIA fused to the hinge-CH2-CH3 domain of human IgG2 as described in Section 7.1 *supra.*

[00518] Details of BIAcore methodology and analysis are found in Section 7.1. In this assay, variant Fc regions were cloned into a chimeric 4D5 antibody, which is specific for human epidermal growth factor receptor 2 (HER2/neu). The antigen, HER2/neu, was immobilized on one of the flow cells of the sensor chip. The chimeric 4D5 antibodies carrying the Fc mutations were then passed over the surface by 3 minute injections of a 300 nM solution at 5 µl/min flow rate. Next, dilution series of the receptor in HBS-P buffer (10 mM HEPES, 150 mM NaCL, .005% Surfactant P20, 3 mMEDTA, pH7.4) were injected onto the surface at 100 µl/min.

[00519] Binding curves for two different concentrations of receptor(400 nM and 800 nM for both FcγRIIIA V158 and FcγRIIIA 158F; 100 nM and 200 nM for both FcγRIIA and FcγRIIB) were aligned and responses adjusted to the same levels of captured antibodies, and reference curves subtracted from experimental curves. Association and dissociation phases were separately fitted.

[00520] Results

[00521] Binding of FcγRIIIA, allotype 158 V and 158F, FcγRIIB and FcγRIIA 131H were analyzed and resonance responses were normalized at the level of response obtained for a wild type chimeric 4D5 antibody. Kinetic parameters for the binding of the FcγRs to the chimeric 4D5 antibody were obtained by fitting the data at two different FcγR concentrations: 400 nM and 800 nM for both FcγRIIIA V158 and FcγRIIIA 158F; 100 nM and 200 nM for both FcγRIIA and FcγRIIB.

[00522] **Table** 25 presents the off rate for each of the four receptors analyzed in association with the indicated variant Fc regions.

Table 25
Off rate (1/s) of FcγR binding to wild type and mutant chimeric 4D5 Ab obtained by BIAcore analysis

Chimeric 4D5 Fc Region	Amino Acid at Position					FcγR Receptor			
	243	292	300	305	396	3A 158V	3A 158F	2B	2A 131H
Wild Type	**F**	**R**	**Y**	**V**	**P**	**0.186**	**0.294**	**0.096**	**0.073**
MgFc0088	L	P	L	I	L	0.016	0.064	0.058	0.035
MGFc0143	I	P	L	I	L	0.017	0.094	0.091	0.049
Quadruple									
MGFc0088A	L	P	L		L	0.016	0.094	0.075	0.044
MGFc0084	L	P		I	L	0.048	0.133	0.278	0.083
MGFc0142	L		L	I	L				
Triple									
MGFc0155	L	P	L			0.029	0.135	0.155	0.057
MGFc0074	L	P		I		0.063	0.37	NB	0.166
MGFc0093		P		I	L	0.080	0.197	0.125	0.190
Double									
MGFc0162	L	P				0.041	0.515	0.900	0.18
MGFc0091	L				L	0.108	0.330	0.036	0.026
MGFc0070		P		I		0.101	0.250	0.030	0.025
Single									
SV12/F243L	L					0.048	0.255	0.112	0.100
MGFc0161		P				0.067	0.485	0.421	0.117
		G				0.124	NT	0.384	NT
MGFc0092			L			0.211	NT	0.058	0.02
MGFc0089					L	0.127	0.306	0.031	0.039

[00523] **Table 26** presents the results of an duplicate study wherein the K_{off} values of the chimeric 4D5 antibodies were computed relative to wild type off rates. Relative numbers greater than one indicate a decrease in K_{off} rate. Numbers less than one indicate an increase in K_{off} rate.

Table 26. Relative Off-Rate of ch4D5 antibodies obtained by BIAcore analysis

Chimeric 4D5 Fc Region	FcγR Receptor Relative Off Rate (K_{off} WT/K_{off} MUT)			
	3A 158V	3A 158 F	2B	2A 131H
F243L, R292P, Y300L, V305I, P396L	**10.06**	**8.25**	**1.38**	**1.11**
F243L, R292P, Y300L	**6.69**	**2.3**	**0.32**	**0.65**
F243L, R292P, P396L	**5.37**	**3.52**	**0.32**	**0.65**
F243L, R292P, Y300L, P396L	**10.06**	**5.62**	**1.07**	**0.89**
F243L, R292P, V305I	**2.56**	**1.43**	**nb***	**0.23**
F243L	**4.79**	**3.44**	**0.84**	**0.57**

***nb, no binding**

7.8 ADCC ASSAY OF Fc MUTANTS

[00524] Fc mutations identified in Example 7.7 as comprising increased affinity for FcγIIIA and/or FcγIIA were analyzed for their relative ADCC activity.

[00525] Materials and Methods

[00526] Details regarding ADCC assays are found in Section 7.1 and in U.S Patent Application Publications 2005/0037000 and 2005/0064514, and International Patent Application Publication WO 04/063351, each of which is hereby incorporated by reference in its entirety. In this assay, HT29 colon carcinoma cells (ATCC Accession No. HTB-38) loaded with Indium-111 were used as targets and effector cells were PBMC that were isolated from donors using a Ficoll gradient. Target cells were opsonized with chimeric 4D5 antibodies comprising the variant Fc regions at final concentrations of 2-5000 ng/ml. Opsonized target cells were then added to effector cells to produce an effector:target ratio of 50:1 and incubated for 18 hours at 37°C, 5% CO_2. After incubation, cells were centrifuged at ~220xg for five minutes at 10°C. The level of Indium-111 in the supernatant was recorded by a gamma counter.

[00527] Results

[00528] Chimeric 4D5 antibodies comprising variant Fc regions MGFc88 (F243L,R292P,Y300L,V305I,P396L), MGFc88A (F243L,R292P,Y300L, P396L) and MGFc155 (F243L,R292P,Y300L) were selected based on enhanced affinity for FcγRIIIA and/or FcγIIA and tested for their ADCC activity. FIGS. 24 A & B show that the Fc variants tested exhibit enhanced ADCC activity relative to wild type antibody at opsonization concentrations above 20 ng/ml in a concentration dependent manner. The data indicate that Fc mutants identified as comprising increased affinity for FcγRIIIA are also likely to exhibit enhanced ADCC activity.

7.9 Fc MUTANT MEDIATED TUMOR GROWTH CONTROL IN AN IN VIVO TUMOR MODEL

[00529] Fc mutations identified as comprising enhanced affinity for FcγIIIA and/or FcγIIA were further analyzed for relative efficacy of tumor control using an *in vivo* tumor model system.

[00530] Materials and Methods

[00531] Antibodies harboring Fc mutants were tested for anti-tumor activity in a murine xenograft system. Balbc/nude mice are subcutaneously injected with $5x10^6$ Daudi cells and subsequently monitored for general signs of illness, *e.g.*. weight

gain/loss and grooming activity. Without treatment, this model system results in 100 % mortality with an average survival time of approximately 2 weeks post tumor cell inoculation. Treatment consists of doses of wild-type antibody or antibody comprising a variant Fc region administered at weekly intervals. Animals administered buffer alone at the same intervals serve as a control. Tumor weight is calculated based on the estimated volume of the subcutaneous tumor according to the formula ($width^2$ X length)/2.

[00532] Results

[00533] At weekly intervals, mice inoculated with Daudi cells received wild-type humanized 2B6 ("h2B6"), humanized 2B6 comprising mutant FcMG0088 (F243L, R292P, Y300L, V305I P396L) ("h2B6 0088") or buffer alone. Wild-type and Fc mutant h2B6 antibody showed similar levels of tumor suppression at the highest dose schedule tested, weekly doses of 25 μg (FIGS. 25 A and B). However, significant differences in antibody efficacy were observed when dosages were reduced. 100 and 10 fold reduction in wild-type h2B6 dosages provided no greater tumor control than administration of buffer alone (FIG. 42 A). In contrast, h2B6 0088 provided significant protection at weekly doses of 2.5 μg and at least limited protection at weekly doses of .25 μg (FIG. 25 B).

[00534] The protection conferred by even the lowest dose of Fc mutant antibody was confirmed in survival comparisons. At 11 weeks, 4 out of 7 mice remained alive in the group treated with 0.25 μg doses of h2B6 0088 compared to only 1 out of 7 in the group treated with the same dose of wild-type h2B6 (FIGS. 26 A & B)

7.10 Fc MUTANT MEDIATED TUMOR GROWTH CONTROL IN A HUMAN Fc RECEPTOR EXPRESSING TRANSGENIC MOUSE TUMOR MODEL

[00535] Fc mutations identified as comprising enhanced affinity for FcγIIIA and/or FcγIIA were further analyzed for relative efficacy of tumor control using an *in vivo* xenograft human Fc receptor transgenic mouse tumor model system.

[00536] Materials and Methods

[00537] Humanized antibodies against human CD32B (h2B6) or HER2/neu (h4D5) harboring Fc mutations were tested for anti-tumor activity in a murine xenograft system, in which mouse FcγIIIA (CD16) was replaced with its human orthologue, CD16A (huCD16A). Immunodeficient mice were injected with $5x10^6$ tumor cells and subsequently monitored for general signs of illness, *e.g.*. weight gain/loss and grooming

activity. Treatment consists of doses of wild-type antibody or antibody comprising a variant Fc region administered at daily or weekly intervals (as stated). Animals administered buffer alone or antibody comprising mutation N297Q (which abrogates binding to any FcγR)at the same intervals serve as a control. Tumor weight was calculated based on the estimated volume of the subcutaneous tumor according to the formula (width^2 X length)/2.

[00538] Results

[00539] h2B6: Humanized anti-CD32B and Fc variants

[00540] At weekly intervals beginning two weeks subsequent to tumor injection, RAG1-/- C57BI/6 mice subcutaneously injected with Raji cells (CD32B-expressing tumor cells) received wild-type humanized 2B6 ("h2B6"; Rituxan), humanized 2B6 comprising mutant FcMG0088 (F243L, R292P, Y300L, V305I P396L) ("h2B6 0088"; "FcMg88", or MGA321) or buffer alone. Wild-type and Fc mutant h2B6 antibody showed similar levels of tumor growth suppression at weekly doses of 250 μg and 25μg (FIG. 27). However, significant differences in antibody efficacy were observed when the dosage was reduced to 2.5 μg: At this dosage, wild-type h2B6 provided limited tumor growth control compared with administration of buffer alone; in contrast, the 2.5 μg dosage of h2B6 0088 delayed tumor progression by as much as one week (FIG. 27). In another experiment, the efficacy of the lower dosages of Fc-optimized MGA321 tested were equivalent to controls (PBS or Rituxin at equivalent dosages); however, administration of the highest dosage (250 μg) of Fc-optimized h2B6 antibody provided significant tumor growth control compared with wild-type h2B6- and buffer alone-treated mice (FIGS. 28A-2B).

[00541] The protection conferred by the Fc mutant antibody was confirmed in survival comparisons. Nude (FoxN1) mice were intraperitoneally injected with EL4-CD32B cells and then treated on days 0, 1, 2, 3, and 6 with IP administered humanized 2B6 1.3 or humanized 2B6 1.3 comprising mutant 31/60 (P247L, D270E, N421K) ("h2B6 1.3 3160"). At 14 weeks, at least 90% of mice treated with h2B6 1.3 3160 survived compared with 55% or less in the group treated with the same dose of wild-type h2B6 1.3 (FIG. 29 and FIG. 30).

[00542] In a further survival experiments in the same system, mice were treated with IP administered humanized 2B6 3.5, humanized 2B6 3.5 comprising mutant FcMG0088 (F243L, R292P, Y300L, V305I, P396L) ("h2B6 3.5 0088"), humanized 2B6 3.5 N297Q (negative control) or buffer alone. At 14 weeks, all mice treated with

h2B6 3.5 0088 survived compared with 30% in the group treated with the same dose of wild-type h2B6 3.5 and <20% in the groups treated with the N297Q mutant or PBS (FIG. 31A; treatment on day 0, 1, 2, 3); the same result was achieved for a dose as low as 4 μg/g body weight (FIG. 31B; treatment on day 0, 1, 2, 3, 4)

[00543] The protective effects of antibodies comprising variant Fc regions was further tested in transgenic mice carrying human CD32A in addition to the mCD16 -/- huCD16A+ genotype using the EL4-CD32B model described *supra*. Treatment with wild-type h2B6 or negative control, h2B6 3.5 N297Q, resulted in only 20% at 100 days post tumor inoculation; treatment with Fc optimized antibody, h2B6 3.5 0088, increased survival by 10%, with 30% survival at 100 days post inoculation (FIG. 32).

[00544] The effect of the expression of hCD16A and/or hCD32A in the transgenic murine EL4-CD32B tumor model on treatment with h2B6 antibodies was further investigated using nude (FoxN1) mice positive for hCD16A, positive for both hCD16A and hCD32A, or positive for hCD32A, each on a mCD16 -/- background. Transgenic were intraperitoneally injected with EL4-CD32B cells and then treated at day 0, 1, 2, and 3 with wild-type humanized 2B6 3.5 ("h2B6 3.5"), humanized 2B6 3.5 comprising mutant FcMG0088 (F243L, R292P, Y300L, V305I, P396L) ("h2B6 3.5 88"), h2B6 N297Q ("h2B6 3.5 N297Q;" negative control) or buffer alone. At 100 days post tumor inoculation, all CD16A positive mice treated with h2B6 3.5 88 survived compared with 50% or less survival in the groups treated with the same dose of wild-type h2B6 3.5 or controls (FIG. 33A). In mice harboring human CD32A in addition to the mCD16 -/- huCD16A+ genotype, survival was not more than 25% at 100 days post inoculation regardless of treatment (FIG. 33B). In mice expressing hCD32A but not hCD16, only mice in the Fc-optimized treatment group, treated with h2B6 3.5 88, survived longer than 1 month, with 25% survival at 100 days post inoculation (FIG. 33C).

[00545] The effect of different time courses of treatment with Fc-optimized antibodies (h2B6 0088; "MGA321") on mouse survival was also investigated. Intraperitoneal injection of mice with MGA321 immediately after tumor injection conferred survival on 75%-100% of mice, either at a single or multiple doses over consecutive days or weeks (FIG. 34). When MGA321 was first administered a day or later subsequent to tumor introduction, it conferred a maximum of 40% survival, even when administered in multiple doses over days or weeks (FIG. 34).

[00546] ch4D5: Chimeric anti-HER2/neu and Fc variants.

[00547] A HER2/neu positive tumor model was established by IP injection of mSKOV3 cells (HER2/neu-expressing ovarian tumor cells) into mCD16 knockout nude (FoxN1) mice that also carried, and expressed, human CD16A or both human CD16A and human CD32A. At 8 weekly intervals, starting at time 0, inoculated mice were subcutaneously injected with "wild type" chimeric antibody against human HER2/neu (ch4D5), ch4D5 comprising FcMG0088 (F243L, R292P, Y300L, V305I P396L) ("ch4D5 0088"), ch4D5 comprising N297Q (aglycosylated negative control; "ch4D5 N297Q"), or buffer alone. Treatment with ch4D5 0088 suppressed tumor growth for the entire course of the experiment (10 weeks) in transgenic mice positive for human CD16A on the mCD16-/- background or in transgenic mice harboring human CD32A in addition to the mCD16 -/- huCD16A+ genotype (FIG. 35A or 35B, respectively).

[00548] The protection conferred by the Fc mutant ch4D5 antibody was confirmed in survival comparisons. Knockout mCD16 (mCD16 -/-) nude (N/N) mice transgenic for human CD16A were intraperitoneally injected with mSKOV3 cells and then treated with wild-type chimeric 4D5 ("ch4D5"), ch4D5 comprising FcMG0088 (F243L, R292P, Y300L, V305I P396L), ch4D5 N297Q (negative control), or buffer alone. Tumor inoculated mice received six doses beginning at day 0 (day of tumor inoculation) of 100 μg or 1 μg of the antibody delivered IP (FIG. 36A and 36B, respectively). At 14 weeks, ~60% of mice treated with 100 μg ch4D5 0088 survived compared with ~40% in the group treated with the same dose of wild-type ch4D5 and <10% of mice treated with ch4D5 N297Q or buffer alone (FIG. 36A). At doses of 1 μg, the overall duration of survival of mice treated with wild-type or Fc-optimized antibodies was reduced relative to those treated with 100 μg. However, at six weeks, more than 80% of ch4D5 0088-treated mice were still alive, compared with ~10% of mice that received the other treatments (FIG. 36B), confirming that a moderate dose of Fc-optimized ch4D5 confers a significant improvement over the wild-type antibody in enhancing viability.

[00549] Other Fc variants of ch4D5 were tested in similar survival experiments.

[00550] A chimeric 4D5 comprising mutant MGFc0155 (F243L, R292P, Y300L) ("ch4D5 0155") or mutant MCFc3160 (P247L, D270E, N421K) ("ch4D5 3160") were tested alongside wild-type ch4D5, ch4D5 N297Q, and ch4D5 0088 as described *supra*. Mice received eight weekly intraperitoneal treatments, starting at day 0 (tumor inoculation), with 100 μg antibody. In mice receiving doses of 100 μg, 100% of the group treated with ch4D5 0155 were alive at 130 days post inoculation, compared with

~85% of those treated with ch4D5 0088 and 50% of those treated with ch4D5 3160. In contrast, only approximately 30% of mice given wild-type ch4D5 were still alive at day 130. All mice treated with buffer alone or ch4D5 N297Q died within 14 weeks and 10 weeks, respectively, after tumor injection (FIG. 37A). At a 10-fold lower dosage, Fc-optimized antibodies were less efficacious in enhancing survival compared with wild-type ch4D5. While ch4D5 0155 was most effective at enhancing survival at earlier time points, at 18 weeks, ~60% of mice in the group treated with ch4D5 0155 or ch4D5 0088 survived, compared with 50% of wild-type-treated and 25% of ch 4D5 3160-treated (FIG. 37B).

[00551] In another set of survival experiments, Knockout mCD16 (mCD16 -/-) nude (N/N) mice transgenic for human CD16A or transgenic for both human CD16A ("hCD16") and human CD32A ("hCD32A") were intraperitoneally injected with mSKOV3 cells and then, starting at day 0, given eight weekly treatments with wild-type ch4D5, ch4D5 0088, ch4D5 N297Q or buffer alone. At seven weeks post-tumor injection, all ch4D5 0088-treated and hCD16 positive mice were alive, whereas only ~30% of Ch4D5 N297Q and ~10% of buffer-treated hCD16 positive mice survived (FIG. 38A). In nude mice harboring human CD32A in addition to the mCD16 -/- huCD16A+ genotype, 50% or the mice treated with ch4D5 0088 were alive after eight weeks, compared with those that received ch4D5 N297Q (15% alive at eight weeks) or buffer alone (all dead before six weeks) (FIG. 38B).

7.11 Fc VARIANTS EXHIBITING ALTERED RATIOS OF AFFINITIES

[00552] Immunoglobulins whose Fc regions had been mutated in the manner described above are screened for Fc variants having altered Ratios of Affinities to FcγRIII and FcγRII by assessing the K_{off} of the variants and their wild-type immunoglobulin progenitors. Testing is done using both the V158 and F158 isotypes of FcγRIII, and against FcγRIIB and FcγRIIAH131. The results are summarized in **Table 27**.

TABLE 27
COMPARISON OF K_{OFF} OF FC MUTANTS TO WILD TYPE ANTIBODY (K_{OFF} WT / K_{OFF} MUTANT)

Fc sequence	CD16A V158	CD16A F158	CD32B	CD32A H131	Ratio of Affinities CD16A/CD32B	
					V158	F158
WT	1.00	1.00	1.00	1.00	1.00	1.00
F243L	4.79	3.44	0.84	0.57	5.70	4.10
D270E	1.25	1.48	0.39	2.24	3.21	3.79
R292P	2.90	0.64	0.25	0.53	11.60	2.56
R292G	1.54	--	0.25	0.14	6.2	--
Y300L	1.01	1.17	1.18	1.86	0.86	0.99
P396L	1.27	1.73	2.58	1.63	0.49	0.67
P396L D270E	1.38	1.65	0.89	2.44	1.55	1.85
P396L F243L	1.49	1.60	2.22	1.50	0.67	0.72
P247L N421K	1.29	1.73	2.00	1.30	0.65	0.87
P247L N421K D270E	1.89	2.46	0.58	1.95	3.26	4.24
P247L N421K F243L	1.89	1.71	0.17	0.39	11.12	10.06
P247L N421K D270E F243L	2.30	3.45	0.32	0.98	7.19	10.78
P247L N421K D270E Y300L	2.44	1.16	0.8	1.84	3.05	1.45
R255L P396L	1.09	1.39	2.22	1.34	0.49	0.63
R255L P396L D270E	1.34	1.65	0.87	3.00	1.54	1.90
R255L P396L D270E F243L	1.75	1.64	0.38	1.44	4.61	4.32
R255L P396L D270E R292G	1.39	1.30	0.65	1.05	2.14	2.00
R255L P396L D270E Y300L	1.52	1.74	0.87	2.60	1.75	2.00
K392T P396L	1.49	1.81	2.35	1.22	0.63	0.77
K392T P396L D270E	1.81	2.28	0.79	1.86	2.29	2.89
K392T P396L D270E F243L	3.16	2.44	0.44	1.70	7.18	5.55
Q419H P396L	1.19	1.19	1.33	0.63	0.89	0.89
Q419H P396L D270E	1.64	2.00	0.68	1.70	2.41	2.94
Q419H P396L D270E F243L	1.46	1.15	0.26	1.11	5.62	4.42
R292P F243L	4.73	0.6	0.12	0.34	39.4	5.00
R292P F243L	4	1.67	0.16	0.52	25	10.44
R292P V284M K370N	1.14	1.37	0.37	1.79	3.1	3.7
R292P V305I	1.59	2.11	2.67	1.56	0.60	0.79
R292P V305I	1.32	1.28	0.37	0.75	3.6	3.46
R292P V305I F243L	2.56	1.43	ND	0.23	>25	>25
R292P V305I F243L P396L	5.37	2.53	0.40	0.78	13.43	6.33
R292P V305I F243L P396L Y300L	10.06	8.25	1.38	1.11	7.29	5.98
R292P V305I F243I P396L Y300L	10.9	3.12	1.05	1.49	10.4	2.97
R292P F243L P396L Y300L	10.06	5.62	1.07	0.89	9.40	5.25
R292P F243L P300L	6.69	2.3	0.32	0.65	20.9	7.19
R292P V305I P396L	1.85	1.90	0.92	1.50	2.01	2.07
R292P V305I P396L F243L	5.37	2.53	0.40	0.78	13.43	6.33
G316D R416G D270E	2.18	2.49	0.78	1.95	2.79	3.19
G316D R416G D270E F243L	1.50	1.34	0.20	1.22	7.50	6.70
G316D R416G D270E P396L	1.22	0.94	1.07	0.95	1.14	0.88

Fc mutants analyzed in this study are shown in the left hand column. The dissociation rate constants for binding of the Fc to the different FcR were determined by Biacore analysis. ND= no detectable binding

[00553] The results show that the methods of the present invention are capable of producing both molecules that possess Fc regions having a Ratio of Affinities greater

than wild-type (i.e., >1) as well as molecules that possess Fc regions having a Ratio of Affinities less than wild-type (i.e., <1). An analysis of the Fc variants shows that the variant Fc-containing molecules fall into various classes, as shown in **Table 28**.

Table 28

Fc sequence	CD16A V158	CD16A F158	CD32B	Ratio of Affinities CD16A/CD32B V158	Ratio of Affinities CD16A/CD32B F158
Ratio of Affinities > 1					
Class I: Increased Binding to CD16; Decreased Binding to CD32B					
F243L	4.79	3.44	0.84	5.70	4.10
D270E	1.25	1.48	0.39	3.21	3.79
R292P	2.90		0.25	11.60	
R292G	1.54		0.25	6.2	
P396L D270E	1.38	1.65	0.89	1.55	1.85
P247L N421K D270E	1.89	2.46	0.58	3.26	4.24
P247L N421K F243L	1.89	1.71	0.17	11.12	10.06
P247L N421K D270E F243L	2.30	3.45	0.32	7.19	10.78
R255L P396L D270E	1.34	1.65	0.87	1.54	1.90
R255L P396L D270E F243L	1.75	1.64	0.38	4.61	4.32
R255L P396L D270E R292G	1.39	1.30	0.65	2.14	2.00
R255L P396L D270E Y300L	1.52	1.74	0.87	1.75	2.00
K392T P396L D270E	1.81	2.28	0.79	2.29	2.89
K392T P396L D270E F243L	3.16	2.44	0.44	7.18	5.55
Q419H P396L D270E	1.64	2.00	0.68	2.41	2.94
Q419H P396L D270E F243L	1.46	1.15	0.26	5.62	4.42
R292P F243L	4.73		0.12	39.4	
R292P F243L	4	1.67	0.16	25	10.44
R292P V284M K370N	1.14	1.37	0.37	3.1	3.7
R292P V305I	1.32	1.28	0.37	3.6	3.46
R292P V305I F243L	2.56	1.43	ND	>25	>25
R292P V305I F243L P396L	5.37	2.53	0.40	13.43	6.33
R292P F243L P300L	6.69	2.3	0.32	20.9	7.19
R292P V305I P396L F243L	5.37	2.53	0.40	13.43	6.33
G316D R416G D270E	2.18	2.49	0.78	2.79	3.19
G316D R416G D270E F243L	1.50	1.34	0.20	7.50	6.70
G316D R416G D270E P396L	1.22		1.07	1.14	
Class II: Decreased Binding to CD16; Greatly Decreased Binding to CD32B					
R292P		0.64	0.25		2.56
R292P F243L		0.6	0.12		5.00
Class III: Increased Binding to CD16; Unchanged Binding to CD32B					
R292P V305I F243I P396L Y300L	10.9	3.12	1.05	10.4	2.97
R292P F243L P396L Y300L	10.06	5.62	1.07	9.40	5.25
R292P V305I P396L	1.85	1.90	0.92	2.01	2.07
Class IV: Greatly Increased Binding to CD16; Increased Binding to CD32B					
R292P V305I F243L P396L Y300L	10.06	8.25	1.38	7.29	5.98
G316D R416G D270E P396L	1.22		1.07	1.14	
Ratio of Affinities < 1					
Class V: Unchanged Binding to CD16; Increased Binding to CD32B					
Y300L	1.01		1.18		0.99
R255L P396L	1.09		2.22	0.49	
Class VI: Increased Binding to CD16; Greatly Increased Binding to CD32B					

Table 28					
Fc sequence	CD16A V158	CD16A F158	CD32B	Ratio of Affinities CD16A/CD32B V158	F158
P396L	1.27	1.73	2.58	0.49	0.67
R255L P396L		1.39	2.22		0.63
P396L F243L	1.49	1.60	2.22	0.67	0.72
P247L N421K	1.29	1.73	2.00	0.65	0.87
R255L P396L		1.39	2.22	0.49	0.63
K392T P396L	1.49	1.81	2.35	0.63	0.77
Q419H P396L	1.19	1.19	1.33	0.89	0.89
R292P V305I	1.59	2.11	2.67	0.60	0.79
Class VII: Decreased Binding to CD16; Increased / Unchanged Binding to CD32B					
G316D R416G D270E P396L		0.94	1.07		0.88

7.12 PREDICTIVE EFFICACY OF RATIOS OF AFFINITIES

[00554] Fc domains of Fc variants that exhibited improved Ratios of Affinities in the context of the spectrum of murine FcγRs are evaluated to determine their in vivo efficacy. For such purpose, the Fc domains were incorporated into a prototype therapeutic antibody and tested in xenograft mouse models of B-cell lymphoma and in tumor models in FcγRIII-knock-out mice that express the low-binding allele of human CD16A. The impact of Fc engineering on tumor clearance was investigated by using WT or human FcγR-transgenic mice. Hu2B6 was used as the model mAb, since this antibody does not induce complement lysis or apoptosis, but inhibits tumor growth in mice by mechanisms that are exquisitely Fcγ dependent (Rankin, C.T. *et al.* 2006 *Blood* 108:2384-2391). Because hu2B6 does not cross-react with murine (m) FcγRII or other endogenous murine proteins, there is no antibody target other than the implanted CD32B-positive tumor cells in this model (Rankin, C.T. *et al.* 2006 *Blood* 108:2384-2391). Furthermore, hu2B6 completely blocks human CD32B, thus eliminating binding of the hu2B6 Fc region to the target cells as a confounding factor. Fc variants 088, 3160, 5660, 3860 and 0071 were selected for this purpose.

[00555] Fc variants 088 and 3160 are tested for treatment of B cell tumors in 16A tg mice. Fc variants 088, 3160, 5660, 3860 and 0071 were tested for treatment of B cell tumors in Balb/c mice.

Mouse Tumor Models

[00556] **Xenograft models:** Female athymic Balb/c nude (nu/nu) mice, 8-10 weeks old, are purchased from Taconic. Daudi cells ($5x10^6$ per mouse) are suspended in PBS + Matrigel and injected subcutaneously into the right flank of Balb/c nude mice. Tumor development is monitored twice per week, using calipers, and tumor weight is

estimated by the following formula: tumor weight = (length x width^2)/2. Intraperitoneal (IP) injections of antibodies at various concentrations (1 μg/g, or 0.1 μg/g) are performed weekly for 6 weeks, starting at day 0.

[00557] ***EL4/CD32B Model:*** Male and female athymic mFcγRIII $^{-/-}$, hCD16A$^+$ nude mice, are bred in MacroGenics, Inc. animal facility. EL4/CD32B cells (1x10^4 per mouse) are suspended in PBS and injected IP at day 0. IP injections of antibodies (10 μg/g or 4 μg/g) are performed on Days 0, 1, 2, and 3. Mouse body weight is measured twice a week. Mice showing excessive body weight gain as well as signs of ascites tumors are sacrificed by CO_2 asphyxiation. Survival was recorded accordingly. Data is analyzed using PRISM (Graphpad Software, San Diego California) for calculation of standard deviation (ADCC, tumor model) and statistical significance using T-tests and log rank analysis (tumor models).

[00558] For a complete mechanistic interpretation of the data, the binding profiles of the engineered Fcγ to the mouse (m) FcγRs were fully characterized. Of the mFcγR, mFcγRII is structurally and functionally homologous to human CD32B, while mFcγRIII and mFcγRIV are receptors functionally related to human activating FcγRs expressed on NK cells and monocyte/macrophages, respectively (Nimmerjahn, F. *et al.* 2005 *Science* 310:1510-1512; Nimmerjahn, F. 2005 *Immunity* 23:41-51). Since the ratio of Fcγ-binding to activating and inhibitory FcγRs is shown to be important in determining antibody-dependent outcomes *in vivo* (Nimmerjahn, F. *et al.* 2005 *Science* 310:1510-1512; Nimmerjahn, F. 2005 *Immunity* 23:41-51), mutants are selected based on their binding profiles (**Table 29**) using human FcRs. Data in **Table 29** is expressed as fold changes relative to wild-type affinities.

Table 29

MGFc No.	Fc Mutant	CD16A^H	CD16A^L	CD32A^H	CD32B	CD16/CD32B
0193	F243L	+3.8	+2.4	-0.8	-0.2	5.7
0089	P396L	+0.3	+0.7	+0.6	+1.6	0.5
3160	P247L D270E N421K	+0.9	+1.5	+1.0	-0.7	3.3
5560	R255L D270E: P396L	+0.3	+0.7	+2.0	-0.1	1.5
3860	D270E K392T P396L	+0.8	+1.3	+0.9	-0.3	2.3
0074	F243L R292P v3051	+1.6	+0.4	-3.3	-13.3	36.6
0071	D270E G316D R416G	+0.4	+0.1	+0.4	-1.7	3.8
0155	F243L R292P Y300L	+6.4	+3.6	0.0	-0.7	12.3
0031	P247L N421K	+0.3	+0.7	+0.3	+1.0	0.6
0161	R292P	+1.4	+0.6	-0.5	-2.7	8.7
0162	F243L R292P	+3.0	+0.7	-0.9	-5.3	24.7
0170	F243L R292P P396L	+5.3	+2.4	+0.4	-1.6	16.0

0092 Y300L	0.0	+0.2	+1.9	+0.2	0.9
0088 F243L R292F Y300L V305I P396L	+9.1	+7.3	+2:2	+0.4	7.3
0084 F243L R292P V305I P396L	-3.0	+1.3	-0.3	-1.6	10.6
Controls					
Wild-Type	0.0	0.0	0.0	0.0	1.0
AAA* E333A K334A S298A	+0.6	+0.1	-3.3	-2.5	5.7
XmAb** S239D I332E 330L	+13.9	+8.1	0.0	+0.74	8.5

Shading indicates Fc versions tested in mouse tumor models.
* see, Presta, L., United States Patent No. 6,737,056; Shields *et al.* 2002, J Biol Chem 277:26733-26740
** see, Lazar, G.A. *et al.* Proc. Natl. Acad. Sci. (USA) 103:4005-4010 (2006)

[00559] Mutants MGFc3160 (P247E/D270E/N421K) and MGFc0088 showed increased binding to mFcγRIII and mFcγRIV, respectively. MGFc3160, however, showed concomitantly increased mFcγRII binding, while MFc0088 exhibited a more favorable activating-to-inhibitory profile, with no increase in binding to the inhibitory receptor. Mutant MGFc0071 (D270E/G316D/R416G) demonstrated WT Fcγ-level binding to all mFcγRs (**Table 29**) and was included as a control.

[00560] Sub-cutaneous inoculation of Daudi cells in nude mice produces localized, progressively expanding tumors whose growth is significantly reduced by weekly intra-peritoneally (i.p.) injections of 1 μg/g WT hu2B6 (**FIG. 39A**). Weekly 0.1 μg/g doses, however, are ineffective. At the higher dose, treatment with hu2B6-MGFc3160 is indistinguishable from that with WT hu2B6, but a modest improvement over WT hu2B6 is detectable at the lower dose. hu2B6-MGFc0088, however, resulted in a significant reduction in tumor growth at all doses tested, consistent with its enhanced mFcγRIV binding and highly favorable activating-to-inhibitory ratio due to no corresponding increase in mFcγRII binding. Hu2B6-MGFc0071, which showed WT level binding to all mFcγRs, behaved similarly to WT hu2B6 (**FIG. 39B**).

Enhanced tumor clearance in human CD16A transgenic mice

[00561] The activity of Fc-engineered mAbs is further analyzed in mFcγRIII-knockout mice expressing the transgene for the low-affinity allele of human CD16A (Li, M. *et al.* 1996 *J. Exp. Med.* 183:1259-1263). In these mice, human CD16A-158^{phe} is expressed by NK cells and mononuclear phagocytes, similarly to its cell-type specific expression in humans (Perussia, B. *et al. Eur. J. Immunol.*21:425-429). The murine cell line, EL4 (Gorer, P.A. (1950) *Brit. J. Canc.* 4(4):372-379), was transduced with human CD32B and used in place of Daudi cells, whose tumor take was poor in these transgenic mice. Knock-out transgenic mice, mFcγRIII$^{-/-}$/CD16A-158^{phe+}, injected i.p. with CD32B-EL4 cells, died eight weeks after inoculation. Treatment with hu2B6-WT rescued 40% of the animals, whereas 90% survived after receiving hu2B6-MGFc3160

(**FIG 39C**). In a separate experiment, a regimen of hu2B6-MGFc0088 that did not prevent tumor growth as a WT Fcγ showed 100% mouse survival for the duration of the experiment (**FIG 39D**). Therefore, the potency of hu2B6-MGFc3160 and hu2B6-MGFc0088 *in vivo* ranks consistently with their relative improvement in binding to FcγRs expressed in the mice (**Table 29**).

[00562] Thus, the Ratio of Affinities of an Fc variant is found to be predictive of the *in vivo* efficacy of molecules comprising the Fc variant region. Both hu2B6-MGFc3160 and hu2B6-MGFc0088 showed enhanced inhibition of tumor cell growth compared to WT mAb. Since MGFc3160 showed an isolated enhancement in mFcγRIII binding in the absence of improved mFcγRIV interaction, the increased activity could be attributable to both NK cells and mononuclear phagocytes. On the contrary, the properties of MGFc0088, an Fcγ domain with substantially increased affinity to mFcγRIV but mFcγRIII binding properties similar to those of WT, were consistent with the notion that mononuclear phagocytes are the critical cells for improved tumor elimination. In the huFcγR transgenic mouse, the substitution of mFcγRIII with huCD16A-158^{phe} on both NK cells and monocytes resulted in an increase in the activating-to-inhibitory binding ratio for MGFc0088. Again, the greater increase in mouse survival with hu2B6-MGFc0088 correlated with its increased affinity for the second activating receptor, mFcγRIV, expressed by monocytes/macrophages (Nimmerjahn, F. *et al.* 2005 *Immunity* 23:41-51). The ability of Fc variants to bind mixed human/murine FcγRIII and FcγRII receptors was determined. The results (**Table 30**) indicate that the variants bind to chimeric receptors with substantial equivalence.

Table 30

No.	Fc Mutant	hCD16 mCD32	hCD16 mCD32	hCD16 mCD32
0071	D270E G316D R416G		1.0	1.1
3160	P247L D270E N421K	1.7	0.6	2.2
5560	R255L D270E P396L		0.7	1.5
3860	D270E K392T P396L		0.7	1.5
0088	F243L R292F Y300L V305I P396L	10.7	17.3	1.4

The binding of selected Fc variants to Fc receptors, and their Ratios of Affinities is shown in **Table 31**.

Table 31

	3aV	3aF	2bFcagl	2aRFcagl	2aHFcagl	32A-131H:32B	16A-158V:32B	16A-158F:32B
wt	1	1	1	1	1	1	1	1
L234F, F243L, R292P, Y300L	1.7	1.6	0.2	0.2	0.3	1.5	8.5	8
L235I, F243L, R292P, Y300L	2.6	3.3	0.2	0.1	0.5	2.5	13	16.5
L235Q, F243L, R292P, Y300L	1.8	1.3	n.d.	n.d.	0.2	n.d.	n.d.	n.d.
L235V, F243L, R292P, Y300L, P396L	6.1	4.8	0.4	0.4	1.3	3.3	15	12
L235P, F243L, R292P, Y300L, P396L	5.4	2.5	0.2	0.2	0.7	3.5	27	12.5

7.13 FC VARIANTS OF HER2-REACTIVE ANTIBODIES EXHIBITING DIMINISHED FUCOSYLATION

[00563] As discussed above, one aspect of the present invention relates to the recognition that variations in the Fc region of an antibody can interfere with the cellular glycosylation mechanism and thereby yield antibodies that exhibit a decreased extent of glycosylation (and in particular, fucosylation). In order to demonstrate the effect of such variations in the Fc region on the capacity of cells to fucosylate the anti-Her2 antibodies of the present invention, a series of variants of the anti-Her2/neu antibody ch45D4-FcMT2 were constructed (**Table 32**).

[00564] Antibody ch4D5-FcMT2 has a light chain having an amino acid sequence of **SEQ ID NO: 45**, and a heavy chain having an amino acid sequence of **SEQ ID NO: 46**. ch4D5-FcMT2 has an N65S substitution on the light chain, which results in a de-glycosylated light chain, and L235V, F243L, R292P, Y300L, and P396L substitutions on the heavy chain (all numbered according to Kabat). This antibody binds to CD16A

(FcγRIII-A), and binding to CD16-158^{Phe} is enhanced in a proportionally greater fashion than binding to CD16-158^{Val}, but exhibits reduced binding to CD32B (FcγRII-B). Polynucleotides encoding the ch4D5-FcMT2 light and heavy chains are provided in **SEQ ID NO:47** and **48**, respectively.

[00565] **Table 32** shows the ratio of wild-type K_{off} / mutant K_{off} for antibodies having the indicated Fc variations. Results for antibody ch45D4-FcMT2 are shown in boldface in the shaded row of **Table 32** (n.b., no binding; n.d., no detectable binding).

Table 32

Amino Acid Changes						Wild-Type K_{off} / Mutant K_{off}				
						CD16A (158V)	CD16A (158F)	CD32B	CD32A (131H)	CD32A (131R)
L235V		F243L	R292P	Y300L		2.3	2	n.d.	0.5	n.d.
L235R		F243L	R292P	Y300L		1.2	0.3	n.d.	n.d.	n.d.
L235W		F243L	R292P	Y300L		1.4	0.9	0.5	1.4	0.6
L235G		F243L	R292P	Y300L		0.8	0.2	n.d.	n.d.	n.d.
L235P		F243L	R292P	Y300L		2.6	1.1	n.d.	0.2	n.d.
L235N		F243L	R292P	Y300L		1.6	0.7	n.d.	0.2	n.d.
L235T		F243L	R292P	Y300L		2.2	1.5	0.1	0.4	0.1
L235S		F243L	R292P	Y300L		1.7	1	0.1	0.3	0.1
L235H		F243L	R292P	Y300L		2	1.1	0.8	1.4	0.8
L235E		F243L	R292P	Y300L		1.8	1	0.2	0.4	0.2
L235F		F243L	R292P	Y300L		1.8	1	0.4	1.3	0.4
L235Y		F243L	R292P	Y300L		1.9	1.1	0.4	1.4	0.4
L235K		F243L	R292P	Y300L		0.7	0.5	n.b	n.b	n.b.
L235V		**F243L**	**R292P**	**Y300L**	**P396L**	**6.1**	**4.8**	**0.4**	**1.3**	**0.4**
L235P		F243L	R292P	Y300L	P396L	5.4	2.5	0.2	0.7	0.2
L235C		F243L	R292P	Y300L		n.b.	n.b.	n.b.	n.b.	n.d
L235A		F243L	R292P	Y300L		2.4	1.3	n.d.	0.4	0.2
L235D		F243L	R292P	Y300L		1.9	1.1	0.3	0.4	0.3
L234F		F243L	R292P	Y300L		1.7	1.6	0.2	0.3	0.2
L235M		F243L	R292P	Y300L		2.3	1.3	0.2	0.3	0.3
L235I		F243L	R292P	Y300L		2.6	3.3	0.2	0.5	0.1
L235Q		F243L	R292P	Y300L		1.8	1.3	n.d.	0.2	n.d.
L235T		F243L	R292P	Y300L	P396L	4.2	2.8	0.3	0.9	0.3
L235A		F243L	R292P	Y300L	P396L	4.4	2.6	0.4	1.1	0.4
L234F		F243L	R292P	Y300L	P396L	3.5	2.9	0.3	1.2	0.4
L235I		F243L	R292P	Y300L	P396L	4.5	4.3	0.5	1.6	0.6
L235Q		F243L	R292P	Y300L	P396L	3.9	2	0.1	0.7	0.2
L234F	L235V	F243L	R292P	Y300L	P396L	1.9	2	n.d.	0.5	0.2
L234F	L235I	F243L	R292P	Y300L		1.2	1	n.d.	0.3	n.d.
L234F	L235I	F243L	R292P	Y300L	P396L	1.9	2.3	n.d.	0.9	0.2

[00566] For further comparison, **Table 3** shows the ratio of wild-type K_{off} / mutant K_{off} for Ch4D5 anti-CD20 (rituximab) antibodies having the indicated Fc variations (see, Stavenhagen, J.B. et al. (2007) "Fc Optimization of Therapeutic Antibodies Enhances Their Ability to Kill Tumor Cells In vitro and Controls Tumor Expansion In vivo via Low-Affinity Activating Fcγ Receptors," Cancer Res. 67(18):8882-8890).

[00567] As is apparent from **Tables 3** and **32**, antibodies having an Fc variant containing a substitution at position L234 or L235, particularly in combination with a substitution at any one or more of positions F243, R292, Y300, V305, and P396, exhibit altered Fc binding to FcγRIII (*e.g.*, improved binding to the activating receptors (*e.g.*, CD16A, CD32A) and reduced binding to CD32B.

[00568] The glycosylation of antibody ch45D4-FcMT2 was investigated using MAb Neutral Monosaccharide Analysis (3 Hour Hydrolysis)" (to investigate GLY-2-2-4 glycosylation) and by "N-Linked Oligosaccharide Profiling of Neutral Oligosaccharides using an Aqueous Chromatographic Separation" (to investigate GLY-12-3-2 glycosylation). The results of this investigation are shown in **Figure 40, Panels A-D**. **Figure 40, Panel A** shows the assignment of N-linked oligosaccharides as determined using an antibody reference panel. As is apparent from **Figure 40**, the glycan peaks observed for antibody ch45D4-FcMT2 (**Panels B** and **C**) do not correspond to any of the glycans shown in **Panel A**. The identity of the glycans is readily determined by conducting a chromatographic analysis of the antibody ch45D4-FcMT2 glycans in the presence of an added known glycan, and identifying the known glycan that causes an increase in the peak size of any of the peaks shown in **Figure 40**, **Panels B** and **C**.

[00569] The monosaccharide computational analysis of the antibodies is shown in **Table 33** shows the pmol/injection and mol/mol of monosaccharides/antibody for the monosacchrides fucose (Fuc), N-acetylated galactose (GALNAc), N-acetylated glucose (GlcNAc), galactose (Gal) and mannose (Man). In **Table** 9, BLQ denotes that the concentration of monosaccharide was below the limit of quantification. The very high levels of mannose identified in the analysis indicates that elevated extents of manosylation enhance the therapeutic efficacy (as measured, for example by the ratio of mutant K_{off} / wild-type K_{off}) of antibodies having the variant Fc regions of the present invention.

Table 33: Monosaccharide Compositional Analysis						
ch45D4-FcMT2		**Fuc**	**GalNAc**	**GlcNAc**	**Gal**	**Man**
Sample 1	pmol/injection	BLQ*	BLQ	165.8	BLQ	1033.0
		BLQ	BLQ	176.8	BLQ	1014.7
		BLQ	BLQ	205.9	BLQ	1135.8
	mol/mol	**≤ 0.2**	**≤ 0.3**	**1.8**	**≤ 0.5**	**10.5**
Sample 2	pmol/injection	BLQ	BLQ	77.0	BLQ	698.7
		BLQ	BLQ	104.4	BLQ	836.4
		BLQ	BLQ	127.7	BLQ	924.4
	mol/mol	**≤ 0.2**	**≤ 0.3**	**1.0**	**≤ 0.5**	**8.1**

7.14 SITES OF FC VARIATION ASSOCIATED WITH DIMINISHED FUCOSYLATION

[00570] In order to assess the effect of variations at positions 234, 235, 243, 292, 300 and 396, alone or in combination weith one another, on the fucosylation of IgG Fc regions, a series of Fc variants was prepared using the IgG ch4D5 (chimeric anti-Her2/neu), h2B6 3.5 (anti-FcγRIIB; see US 2008/0044417), ch2.4G2 (rat anti-mouse FcγRII-III; Kurlander *et al.* (1984) "*The Blockade Of Fc Receptor-Mediated Clearance Of Immune Complexes In Vivo By A Monoclonal Antibody (2.4G2) Directed Against Fc Receptors On Murine Leukocytes*," J. Immunol. 133(2):855-862; Unkeless, J.C. (1979) "*Characterization of a Mooclonal Antibody Directed Against Mouse Macrophage and Lymphocyte Fc Receptors*," J. Exper. Med. 150:580-596; the sequences for the VH and VL chains of this antibody are available from Genbank (ACP40510 and ACP40511, respectively)) were constructed, and their respective patterns of glycosylation determined. **Table 34** shows the percentages at which the glycosylation species G0F, G1F, G2F, man5, man6, man7, man 8, and man9 were obtained in the Fc variants of such antibodies (man (mannose), cpx (complex oligosaccharide)).

Table 34												
Fc Variant	**Fc Variation**	**Percentage of Total Glycosylation**								**total man**	**total cpx**	**Ratio of man/cpx**
		G0F	**G1F**	**G2F**	**man5**	**man6**	**man7**	**man8**	**man9**			
Antibody ch4D5												
Fc WT	WT	43	26	7	11		2	1	1	16	76	0.2
1	P396L	31	30	6	21	7	6			33	67	0.5
2	Y300L	34	28	5	13	5	3	2		24	67	0.3
3	F243L R292P Y300L P396L	7	6	2	31	12	10	12	9	75	14	5.2
4	F243L R292P Y300L	5	9	11	31	8	10	11	7	66	25	2.6
5	R292P	51	13	1	15	4	3	2	1	24	66	0.4
6	F243L	8	10	5	30	12	8	7	5	62	23	2.7

Table 34

Fc Variant	Fc Variation	Percentage of Total Glycosylation								total man	total cpx	Ratio of man/cpx
		G0F	G1F	G2F	man5	man6	man7	man8	man9			
Antibody ch4D5												
	R292P P396L											
7	F243L R292P P396L	6		28		5	12	16	10	43	33	1.3
8	F243L R292P P396L	4	11	10	26	16	10	9	5	66	25	2.6
9	F243L	7	13	14	20	16	13	6	3	58	33	1.7
10	F243V	6	12	33	19		14	6	2	41	51	0.8
11	F243R	6	4	19	12		24	21	5	62	29	2.1
12	F243C	9	16	29	21		11	5	1	38	54	0.7
13	F243F R292P Y300L	52	12	2	13	4	4	4	1	27	65	0.4
14	F243C R292P Y300L	7	12	8	31	13	9	7	2	63	26	2.4
15	F243R R292P Y300L	3	3	27	5		29	26	1	61	32	1.9
16	F243R R292P Y300L	3	4	23	4		29	29	2	64	29	2.2
17	F243V R292P Y300L	5	15	12	22	10	9	10	6	58	32	1.8
18	F243V R292P Y300L	5	11	7	30	14	8	9	5	66	24	2.7
19	L235V F243L R292P Y300L P396L	4	9	6	22	12	11	15	12	73	19	3.7
20	L235V F243L R292P Y300L P396L	4	7	3	27	14	11	15	11	78	14	5.4
21	F243C R292P	7	23	23	19		9	6	3	36	53	0.7
22	F243R R292P	5	5	19	6		25	27	3	61	28	2.2
23	F243V R292P	5	21	29	16		11	6	3	37	55	0.7
24	F243L R292G Y300L	8	19	15	19	8	9	7	4	47	41	1.2
25	F243L R292L Y300L	7	17	34	7		19	10	3	39	58	0.7
26	F243L	7	21	29	12		12	7	3	34	57	0.6

Table 34

Fc Variant	Fc Variation	Percentage of Total Glycosylation								total man	total cpx	Ratio of man/cpx
		G0F	G1F	G2F	man5	man6	man7	man8	man9			
Antibody ch4D5												
	R292S Y300L											
27	F243L R292M Y300L	6	22	35	8		15	7	2	33	63	0.5
28	F243L R292D Y300L	5	8	27	6		23	19	2	50	40	1.3
29	F243L R292Y Y300L	7	22	32	10		13	6	2	32	61	0.5
30	F243F R292P P396L	43	22	4	9	4	5	5		23	69	0.3
31	F243C R292P P396L	8	21	23	18		9	7	3	37	52	0.7
32	F243R R292P P396L	3	5	22	3		29	28	2	63	31	2.1
33	F243V R292P P396L	5	19	21	18	5	10	8	4	45	45	1.0
34	F243F R292P Y300L P396L	36	13	3	13	6	5	9	4	38	51	0.7
35	F243F R292P Y300L P396L	36	12		14	7	5	9	4	39	48	0.8
36	F243C R292P Y300L P396L	8	14	8	23	10	10	10	6	59	29	2.0
37	F243R R292P Y300L P396L	4	7	26	4		29	25		58	36	1.6
38	F243V R292P Y300L P396L	5	12	7	22	11	10	12	9	65	25	2.6
39	F243L R292P	4	19	28	17		12	8	4	41	51	0.8
40	F243L R292G Y300L P396L	6	18	14	20	9	9	9	5	52	38	1.4
41	F243L R292L Y300L P396L	5	19	38	6		18	9	2	36	61	0.6
42	F243L	6	22	26	13	5	12	7	3	39	54	0.7

Table 34

Fc Variant	Fc Variation	Percentage of Total Glycosylation								total man	total cpx	Ratio of man/cpx
		G0F	G1F	G2F	man5	man6	man7	man8	man9			
Antibody ch4D5												
	R292S Y300L P396L											
43	F243L R292M Y300L P396L	7	23	34	9		14	7	3	32	63	0.5
44	F243L R292M Y300L P396L	7	21	28	9	5	14	7	3	38	55	0.7
45	F243L R292D Y300L P396L	3	10	30	4		24	19	2	49	43	1.1
46	F243L R292Y Y300L P396L	7	23	25	10	5	11	6	3	35	55	0.6
47	S239D A330L I332E	26	47	21			6			6	94	0.1
Antibody 2B6 3.5												
WT Fc	WT	46	28	4	6	4	4	3	2	19	79	0.1
48	F243L R292P V305I	6	16	9	33	14	3	4	9	63	30	0.1
49	F243L R292P Y300L V305I P396L	6	6	2	15	13	14	19	18	79	14	0.1
Antibody ch2.4G2												
WT Fc	WT	57	15		8	7	4	3	2	24	72	0.1
50	F243L R292P V305I	15	14		15	20	10	10	6	61	30	0.1
51	F243L R292P Y300L V305I P396L	8	5		10	14	13	18	25	80	14	0.1

[00571] **Table 35** shows the relative binding ($K_{dissociation}$ wild type / $K_{dissociation}$ variant) of the CH4D5 IgG Fc of **Table 34** to CD16A and Cd32B. Values greater than 1.0 thus indicate that the IgG variant bound to the Fc receptor with greater affinity than wild-type Fc, whereas values less than 1.0 indicate that the IgG variant bound to the Fc receptor with decreased affinity than wild-type Fc.

Table 35

Fc Variant	Fc Variation	Ratio of man/cpx	CD16A(V158) (wt/mut)	CD16A(F158) (wt/mut)	CD32B-G2Ag (wt/mut)	CD32A-G2Ag (wt/mut)
Antibody ch4D5						
WT Fc	WT	0.2	1.0	1.0	1.0	1
1	P396L	0.5	2.2	1.5	2.1	1.8
2	Y300L	0.3	1.6	1.2	0.8	1.5
3	F243L R292P Y300L P396L	5.2	6.9	4.6	1.2	1.8
4	F243L R292P Y300L	2.6	3.7	2.2	0.5	1.1
5	R292P	0.4	1.0	1.0	0.3	0.6
6	F243L R292P P396L	2.7	2.3	1.6	0.4	0.8
7	F243L R292P P396L	1.3	2.3	1.6	0.4	0.8
8	F243L R292P P396L	2.6	2.3	1.6	0.4	0.8
9	F243L	1.7	1.1	1.1	0.8	0.7
10	F243V	0.8	1.0	1.0	0.8	0.7
11	F243R	2.1	0.3	0.3	0.2	0.2
12	F243C	0.7	0.7	0.6	0.3	0.3
13	F243F R292P Y300L	0.4	2.1	2.0	0.7	1.3
14	F243C R292P Y300L	2.4	2.6	2.1	0.4	0.9
15	F243R R292P Y300L	1.9	1.1	0.8	0.4	0.9
16	F243R R292P Y300L	2.2	1.1	0.8	0.4	0.9
17	F243V R292P Y300L	1.8	3.5	2.0	0.5	1.1
18	F243V R292P Y300L	2.7	3.5	2.0	0.5	1.1
19	L235V F243L R292P Y300L P396L	3.7	5.3	4.5	0.5	1.3
20	L235V F243L R292P Y300L P396L	5.4	5.3	4.5	0.5	1.3

Table 35

Fc Variant	Fc Variation	Ratio of man/cpx	CD16A(V158) (wt/mut)	CD16A(F158) (wt/mut)	CD32B-G2Ag (wt/mut)	CD32A-G2Ag (wt/mut)
21	F243C R292P	0.7	2.2	1.1	0.3	0.3
22	F243R R292P	2.2	n.d	n.d	n.d	n.d
23	F243V R292P	0.7	3.0	1.1	0.2	0.4
24	F243L R292G Y300L	1.2	4.4	2.5	0.5	1.3
25	F243L R292L Y300L	0.7	2.6	1.7	0.7	1.6
26	F243L R292S Y300L	0.6	2.9	2.1	0.5	1.4
27	F243L R292M Y300L	0.5	2.7	1.9	0.7	1.7
28	F243L R292D Y300L	1.3	1.0	0.8	0.4	0.0
29	F243L R292Y Y300L	0.5	1.5	1.4	0.5	1.1
30	F243F R292P P396L	0.3	1.6	1.8	0.8	1.4
31	F243C R292P P396L	0.7	2.3	1.5	0.4	0.7
32	F243R R292P P396L	2.1	1.2	0.9	0.3	0.6
33	F243V R292P P396L	1.0	2.4	1.8	0.4	0.9
34	F243F R292P Y300L P396L	0.7	4.5	3.4	1.4	2.2
35	F243F R292P Y300L P396L	0.8	4.5	3.4	1.4	2.2
36	F243C R292P Y300L P396L	2.0	5.4	3.5	1.1	1.8
37	F243R R292P Y300L P396L	1.6	1.6	1.5	1.1	1.9

Table 35

Fc Variant	Fc Variation	Ratio of man/cpx	CD16A(V158) (wt/mut)	CD16A(F158) (wt/mut)	CD32B-G2Ag (wt/mut)	CD32A-G2Ag (wt/mut)
38	F243V R292P Y300L P396L	2.6	6.4	4.1	1.3	2.0
39	F243L R292P	0.8	2.2	1.1	0.3	0.4
40	F243L R292G Y300L P396L	1.4	5.0	3.6	1.1	1.8
41	F243L R292L Y300L P396L	0.6	3.4	2.8	1.2	1.9
42	F243L R292S Y300L P396L	0.7	2.8	2.2	1.4	2.3
43	F243L R292M Y300L P396L	0.5	3.3	2.3	1.3	2.0
44	F243L R292M Y300L P396L	0.7	3.3	2.3	1.3	2.0
45	F243L R292D Y300L P396L	1.1	1.4	1.1	0.8	1.5
46	F243L R292Y Y300L P396L	0.6	2.1	2.0	1.0	1.7
47	S239D A330L I332E	0.1	15.9	11.2	1.5	1.2
Antibody 2B6 3.5						
WT Fc	WT	0.1	1	1	1	1
48	F243L R292P V305I	0.1	2.6	1.4	0.2	0.1
49	F243L R292P Y300L V305I P396L	0.1	10.1	8.3	3.2	1.4
Antibody ch2.4G2						
WT Fc	WT	0.1	1	1	1	1
50	F243L R292P V305I	0.1	2.6	1.4	0.2	0.1

Table 35						
Fc Variant	Fc Variation	Ratio of man/cpx	CD16A(V158) (wt/mut)	CD16A(F158) (wt/mut)	CD32B-G2Ag (wt/mut)	CD32A-G2Ag (wt/mut)
51	F243L R292P Y300L V305I P396L	0.1	10.1	8.3	3.2	1.4

[00572] The results thus indicate that variations involving positions 243 and 292 had pronounced effects on the fucosylation pattern obtained and on Fc binding affinity to CD16A, CD32A and CD32B (**Figures 43-48**). In **Figures 43-48**, in order to maintain a single y-axis, the percentage of observed man5 or man6 glycosylation patterns (%man56) or of observed man7, man8 or man9 glycosylation patterns (%man789) is shown divided by 10. Thus, for example, a reported value of 4.0 in these figures for a %man56 glycosylation pattern indicates that 40% of the glycosylation adducts of the variant were either man5 or man6. Similarly, the values of total percent mannose (%total man) and total complex oligosaccharides (% total cpx) are also shown divided by 10.

[00573] **Figure 43** shows the effect of altering the identity of the residue substituted at position F243 of a tetra variant (F243X, R292P, Y300L, P396L) on the observed glycosylation profile of the variant and on the relative binding ($K_{dissociation}$ wild type / $K_{dissociation}$ variant) of the Fc variant to the Fc receptors. The Figure shows that modifications at position 243 alone are sufficient to cause altered glycosylation and increased affinity to CD16A relative to the wild-type. F243L was particularly capable of increasing affinity to CD16A relative to wild-type Fc.

[00574] **Figure 44** shows the effect of altering the identity of the residue substituted at position R292 of a tetra variant (F243L, R292X, Y300L, P396L) on the observed glycosylation profile of the variant and on the relative binding ($K_{dissociation}$ wild type / $K_{dissociation}$ variant) of the Fc variant to the Fc receptors. The Figure shows that modifications at position 292 alone are sufficient to cause altered glycosylation and increased affinity to CD16A relative to the wild-type. R292P was particularly capable of increasing affinity to CD16A relative to wild-type Fc.

[00575] **Figure 45** shows the effect of progressive alterations in the identity of the residue substituted at position R292, Y300 and P396 of an F243C Fc variant on the observed glycosylation profile of the variant and on the relative binding ($K_{dissociation}$ wild type / $K_{dissociation}$ variant) of the Fc variant to the Fc receptors. The Figure shows

that progressive modifications at these sites synergistically enhanced affinity to CD16A relative to wild-type Fc.

[00576] **Figure 46** shows the effect of progressive alterations in the identity of the residue substituted at position R292, Y300 and P396 of an F243L Fc variant on the observed glycosylation profile of the variant and on the relative binding ($K_{dissociation}$ wild type / $K_{dissociation}$ variant) of the Fc variant to the Fc receptors. The Figure shows that progressive modifications at these sites synergistically enhanced affinity to CD16A relative to wild-type Fc, with the tetra variant F243L, R292P, Y300L, P396L exhibiting the largest increase in relative CD16A binding affinity.

[00577] **Figure 47** shows the effect of progressive alterations in the identity of the residue substituted at position R292, Y300 and P396 of an F243R tetra variant on the observed glycosylation profile of the variant and on the relative binding ($K_{dissociation}$ wild type / $K_{dissociation}$ variant) of the Fc variant to the Fc receptors. The Figure shows that modifications at position Y300 or Y396 synergistically enhanced affinity to CD16A of the F243R variant relative to wild-type Fc.

[00578] **Figure 48** shows the effect of progressive alterations in the identity of the residue substituted at position R292, Y300 and P396 of a tetra variant (F243V, R292X, Y300X, P396X) on the observed glycosylation profile of the variant and on the relative binding ($K_{dissociation}$ wild type / $K_{dissociation}$ variant) of the Fc variant to the Fc receptors. The Figure shows that progressive modifications at these sites synergistically enhanced affinity to CD16A relative to wild-type Fc, with the tetra variant F243V, R292P, Y300L, P396L exhibiting the largest increase in relative CD16A binding affinity.

[00579] All publications and patents mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference in its entirety. While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth.

[00580] While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications

and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth.

WHAT IS CLAIMED IS:

Claim 1. A method of attenuating the post-translational fucosylation of a molecule comprising a modified Fc region, comprising:

(A) modifying an IgG Fc region to comprise an amino acid substitution relative to a wild-type Fc region to yield a modified Fc region;

(B) expressing a candidate molecule comprising said modified Fc region in a host cell capable of mediating normal glycosylation;

(C) assessing a ratio of high mannose oligosaccharide glycosylation to complex oligosaccharide glycosylation exhibited by said modified Fc region;

(D) identifying said candidate molecule comprising said modified Fc region as a suitable molecule if said modified Fc region exhibits said ratio that is greater than 0.2; and

(E) expressing said suitable molecule possessing said modified Fc region in said host cell.

Claim 2. The method of claim 1, wherein said amino acid substitution is at position L234, L235, F243, R292, Y300, V305 or P396.

Claim 3. The method of claim 2, wherein said amino acid substitutions comprise substitutions at positions L234 and L235.

Claim 4. The method of claim 3, wherein said amino acid substitutions comprise L234F and L235V.

Claim 5. The method of any one of claims 1-4, wherein said modified Fc region exhibits altered affinity to a CD16A receptor, a CD16B receptor, a CD32B receptor, or a CD32A receptor.

Claim 6. The method of claim 5, wherein said modified Fc region exhibits decreased affinity to a CD32B receptor.

Claim 7. The method of claim 5, wherein said modified Fc region exhibits increased affinity to a CD16A receptor.

Claim 8. The method of any one of claims 1-7, wherein said modified Fc region is the Fc region of a monoclonal antibody, a chimeric antibody, a humanized antibody, a human antibody or a single chain antibody.

Claim 9. The method of claim 8, wherein said antibody specifically binds CD16A, CD32B, HER2/neu, A33, CD5, CD11c, CD19, CD20, CD22, CD23, CD27, CD40, CD45, CD79a, CD79b, CD103, CTLA4, ErbB1, ErbB3, ErbB4, VEGF receptor, TNF-α receptor, TNF-ß receptor, or TNF-γ receptor.

Claim 10. The method of claim 8, wherein said antibody specifically binds a cancer antigen associated with a cancer.

Claim 11. The method of claim 10, wherein said cancer is a breast, ovarian, prostate, cervical, lung or pancreatic cancer.

Claim 12. The method of claim 10, wherein said cancer is breast cancer and said antigen is HER2/neu.

Claim 13. The method of claim 1, wherein said ratio is: Σ (% Man5 + % Man6 + % Man7 + % Man8 + % Man9) : Σ (% G0F + % G1F + % G2F).

Claim 14. The method of claim 1, wherein said ratio is greater than 0.5 and less than 5.5.