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(54) Title: NOVEL SYNTHETIC BIOLOGY-BASED ADCC TECHNOLOGY

(57) Abstract: A novel synthetic biology-based ADCC technology is provided that enhances or enables ADCC response. The novel ADCC technology can be used to prevent or treat cancers, infectious, inflammatory or autoimmune diseases, and other diseases where elimination of diseased cells is desirable.

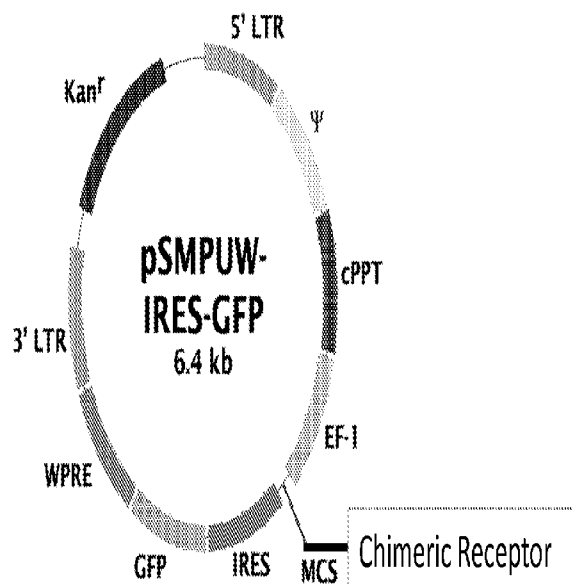


Figure 5



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Novel Synthetic Biology-Based ADCC Technology

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to and the benefit of co-pending U.S. provisional patent application Serial No. 61/925,217, filed on January 8, 2014 and bearing the same title, which application is incorporated herein by reference in its entirety.

TECHNICAL FIELD

[0002] The invention relates to synthetic biology products and processes that can be used to enhance an immune effector cell's ability to mediate antibody-dependent cellular cytotoxicity (ADCC) or to enable cells to mediate ADCC as well as methods of using them in the treatment and prevention of cancer, infectious diseases, inflammatory and autoimmune diseases and other diseases.

BACKGROUND OF INVENTION

[0003] Mammals, especially higher vertebrates including human, have developed highly complex immune systems that use multiple mechanisms and effectors to detect, destroy, or at least contain foreign pathogens as well as diseased or stressed autologous cells. These diseased cells may have been infected by virus or bacteria, or have become cancerous.

[0004] One of the mechanisms for the immune system to recognize and eliminate diseased host cells and invading intracellular microorganisms (e.g., viruses, bacteria or parasites) is through cell-mediated cytotoxicity, which can be carried out by a number of leukocytes and proteins. These potentially cytotoxic effectors include: from the lymphoid lineage--Natural Killer (NK) cells and cytotoxic T lymphocytes (CTLs); and from the myeloid lineage--macrophages, neutrophils and eosinophils.

[0005] An important way for the immune system to unleash cell-mediated cytotoxicity relies on antibodies. Over the past decade, monoclonal antibodies (mAbs) that target tumor-specific cell-surface proteins have become a popular therapeutic approach against cancers. Several mAbs have entered routine clinical practice including rituximab

(Rituxan, Mabthera), trastuzumab (Herceptin) and cetuximab (Erbix). The popularity of mAbs is a result of their bifunctional nature. One end of an antibody (Fab) can be made exquisitely specific to a particular tumor protein without altering the other end (Fc) which recruits a variety of effector cells and proteins that kill the tumor cell.

[0006] Specifically, after recognizing and binding antigens on the surface of a target cell first, antibodies act as an adapter and proceed to activate the cytotoxic capability of immune effector cells through a second binding with certain receptors on those effector cells. This is called the antibody-dependent cellular cytotoxicity (ADCC). For example, in the context of innate immunity against cancer, ADCC is primarily mediated by natural killer (NK) cells (and, to a lesser extent, neutrophils, monocytes and macrophages) that express a relatively low-affinity Fc receptor (FcγRIIIa, also known as CD16a) that is only activated upon binding the Fc (constant) portions of antibodies coating a multivalent antigen on a target diseased cell (e.g., a tumor cell). This binding triggers the release of cytotoxic granules like perforin and granzyme (as well as many cytokines including IFN-γ), leading to the lysis of the target cell. The importance of the ADCC response has been shown both *in vitro* as well as in animal studies. Moreover, several clinical studies have shown that patients carrying a lower affinity variant of CD16 (F158) have worse clinical outcomes.

[0007] However, ADCC efficacy, as primarily mediated by endogenous natural killer (NK) cells, is limited in the body due to a number of physiological as well as pathological reasons as explained below (to the extent that endogenous Cytotoxic T lymphocytes participate in tumor clearance at all, their efficacy has also been found to be very limited and lacking).

[0008] First, most cells involved in the ADCC response such as macrophages and neutrophils do not tend to proliferate when they are activated. NK cells also have limited proliferation potential in response to activation, and they also rapidly die off. Therefore, natural ADCC response in the body risks being overwhelmed by disease progression (e.g., viral infection, cancer) even if the ADCC effectors recognize antibodies coating diseased cells.

[0009] Second, many of the ADCC effector cells also express inhibitory receptors that dampen their immune responsiveness, thereby instituting a system of balances and

checks. These receptors include inhibitory KIRs (killer immunoglobulin-like receptors) for CD56^{low} NK cells, FcγRIIb on monocytes and B cells, and CTLA-4 (CD152) and PD-1 (Programmed-Death-1, CD279) for T cells. Cancer cells and viruses counteract body's ADCC-based defense system by abnormally amplifying such inhibitory pathways.

[00010] Third, the main Fc receptor on ADCC effector cells, FcγRIIIa, has a relatively low affinity ($K_d \approx 10^{-6}$ M) for antibodies--even the V158 variant of the receptor has only a two-fold higher affinity compared to the ineffective F158 form of the receptor. This is one mechanism through which cancer cells become resistant to some therapeutic monoclonal antibodies (mAbs) once the density of the cell surface targets fall below a certain level.

[00011] In view of its natural limitations in proliferation and affinity as well as further depression through inhibitory Fc receptors in the setting of a disease, such as cancer or other diseases, the body's ADCC function has great potential that is never fully realized. Therefore, synthetic biology represents a novel and highly desirable approach to unleash the full potential of ADCC activity in the prevention and treatment of human diseases.

BRIEF SUMMARY OF THE INVENTION

[00012] The present invention ushers in new approaches to improve the immune system's defense against cancer, infections and other diseases. Using tools from synthetic biology and recombinant technology, the present invention aims to design and build an ADCC Enhancer or enhancing system that will greatly improve the effectiveness of ADCC response against many kinds of diseases including cancers. In one aspect, the present invention improves the detection sensitivity and efficiency of the ADCC defense system by enhancing its binding affinity at the "sensor" part of the system without compromising the specificity. In another aspect, the present invention improves the "effector" part of the system by enhancing the proliferation potential of the cytotoxic effector cells. In preferred embodiments, more than one of the above aspects are combined. Other embodiments of the present invention, however, may only reflect one of the afore-mentioned inventive aspects.

[00013] The ADCC Enhancer of the present invention can be used either in combination with an antibody therapy or by itself to target diseased cells recognized or bound by a naturally-occurring antibody or manmade antibody administered for a therapeutic purpose.

[00014] In the first aspect, which focuses on the “sensor” part of the ADCC enhancing system, the present invention sets out to build an ectodomain, or extracellular domain, with higher affinity for an antibody than that of the wildtype human CD16 (e.g., the most common form of human CD16, the F158 variant). This results in a high-affinity transmembrane Fc receptor and its extracellular portion can be based on the ectodomain of an existing Fc receptor. The “chimeric” or “fusion” receptor, in part or in whole, may be borrowed from another macromolecule in the immune system or engineered anew. Having a high-affinity Fc receptor enables an immune effector cell to efficiently bind the same Fc fragment common to different antibodies, which in turn, target a wide variety of cell surface antigens and thereby a wide variety of diseases and indications. This is a great advantage compared to antigen-dependent immunotherapies where each time, a different antibody needs to be built against each specific antigen, a process that is costly in time and other resources.

[00015] In the second aspect, where the focus moves to the “effector” part of the ADCC system, the present invention incorporates module(s) that, once expressed, can promote amplification of the ADCC effector cells.

[00016] Accordingly, in an embodiment, the present invention provides a genetic construct that encodes: a transmembrane chimeric receptor comprising an ectodomain having a higher binding affinity to the Fc of an antibody than that of the F158 variant of human CD16, a transmembrane domain, and an endodomain for mediating ADCC activation and amplification.

[00017] The genetic construct of the invention can include a DNA or RNA molecule. The construct may include substantially the EC3 exon of, e.g., human, CD64 or, in addition, substantially the EC1 and EC2 exons of (human) CD64. Alternatively, the genetic construct of the invention includes an RNA that translates to an ectodomain comprising substantially the ectodomain of CD64. In a feature, the ectodomain is selected based on boundaries of exons or domains in CD64. Preferably, the ectodomain has a

higher affinity for Fc than the V158 variant of human CD16. In another feature, the transmembrane chimeric receptor is expressed as a fusion protein. Also, the endodomain may include amplification module(s), which may be selected from the group consisting of portions or all of intracellular domains of CD3 ζ , of CD28, of CD134, of CD137, of CD27, of CD79a, of CD79b, of CD40 and of the GM-CSF receptor.

[00018] In a further embodiment, the present invention provides a transmembrane chimeric receptor that translates from the above genetic construct. In one feature, the ectodomain of the receptor of the invention comprises substantially the immunoglobulin-like fold in the ectodomain of CD64 that is absent from CD16, or, substantially the ectodomain of CD64. In one feature, the transmembrane domain comprises substantially the transmembrane domain of CD16. The receptor may further include an ADCC amplification module that enhances the survival and proliferation of a host immune effector cell. The amplification module may be selected from the group consisting of portions or all of intracellular domains of CD3 ζ , of CD28, of CD134, of CD137, of CD27, of CD79a, of CD79b, of CD40 and of the GM-CSF receptor.

[00019] In another embodiment, the present invention provides an immune effector cell infected or transfected ex vivo with the genetic construct or the protein of the invention. The immune effector cell can be, e.g., a cytotoxic T lymphocyte, a natural killer cell, an eosinophil, a macrophage, a neutrophil, a basophil, a monocyte, a B cell, and other cells that express or are engineered to express cytotoxic factors.

[00020] In yet another embodiment, the present invention provides a pharmaceutical composition that includes the genetic construct, the protein, or the immune effector cell of the invention, and a pharmaceutically acceptable excipient.

[00021] In a further embodiment, the present invention provides a method of treating a subject in need thereof for a condition therapeutically or prophylactically, where the method includes administering to said subject a therapeutically or prophylactically effective amount of the pharmaceutical composition of the invention. In one feature, the method further includes administering a therapeutic antibody against at least one cell-surface antigen indicative of said condition. The immune effector cell may be autologous to said subject, and the condition selected from the group consisting of a cancer, an inflammatory disease, an autoimmune disease, transplant rejection and an

infection. In a feature, the pharmaceutical composition of the invention is administered as a vaccine against said condition.

[00022] In a yet another embodiment, the present invention provides a method of treating a subject in need thereof for a condition therapeutically or prophylactically by (a) priming dendritic cells isolated from said subject with a source of antigen; (b) infusing said primed dendritic cells back into said subject; (c) administering to said subject a therapeutically or prophylactically effective amount of the pharmaceutical composition of the present invention. The source of the antigen may be autologous or foreign, and the condition may be selected from the group consisting of a cancer, an inflammatory disease, an autoimmune disease, transplant rejection and an infection.

BRIEF DESCRIPTION OF FIGURES

[00023] Figure 1 schematically depicts the embodiment of the chimeric antibody-receptor according to the present invention. The chimeric receptor has an extracellular domain with relatively high affinity for the Fc portion of antibodies as well as an intracellular signaling domain that has relatively high proliferation-stimulating activity.

[00024] Figure 2 schematically compares the protein domain structures of CD16 and CD64 where the extracellular domains of CD16 and CD64 consist of two and three immunoglobulin folds, respectively. Similar shadings represent homologous folds.

[00025] Figure 3 schematically compares the genomic exon maps of CD16 and CD64: the secretory signal sequence is in white, the extracellular regions are crosshatched and the transmembrane and intracellular regions are in black. Neither the intron nor the exon regions are drawn to scale.

[00026] Figure 4 schematically illustrates the cloning strategy for generating chimeric receptors of the invention according to an embodiment. The flanking letters denotes restriction enzyme sites used during cloning: H-HindIII (“H”), B-BamHI (“B”), and Bg-BglII (“Bg”).

[00027] Figure 5 is a map of a commercially available lentiviral vector used for cloning the receptor according to an embodiment of the invention.

[00028] Figure 6 are maps of alternate vector constructions that can be used for cloning the receptor according to the invention.

[00029] Figure 7 shows the flow cytometry results of Expi293 cells transfected with (a) negative control, (b) an empty vector, (c) vector expressing CD16V, and (d) vector expressing CD64-16 (Exon) constructs, as measured by the GFP signals emitted by expressed vectors.

[00030] Figure 8 shows the flow cytometry results of Expi293 cells transfected with (a) negative control, (b) an empty vector, (c) vector expressing CD16V, and (d) vector expressing CD64-16 (Exon) constructs, as measured by fluorescence emitted by fluorescently tagged anti-CD16 (SMPUW and CD16V) or anti-CD64 (CD64-16 based on exon-fusion).

[00031] Figure 9 shows the flow cytometry results of Expi293 cells stained with Rituximab, a therapeutic antibody, after the cells were transfected with (a) negative control, (b) an empty vector, (c) vector expressing CD16V, and (d) vector expressing CD64-16 (Exon) constructs, as measured by fluorescence emitted by fluorescently tagged anti-human IgG.

[00032] Figure 10 lists a DNA sequence (SEQ ID NO:1) (top) that would translate into chimeric receptors according to embodiments of the invention where an extracellular region of human CD64 is fused to regions of human CD16 using a domain-based strategy (point of fusion marked by a dash). The corresponding protein sequence (SEQ ID NO:2) is listed in the bottom.

[00033] Figure 11 lists a DNA sequence (SEQ ID NO:3) (top) that would translate into chimeric receptors according to embodiments of the invention where an extracellular region of human CD64 is fused to regions of human CD16 using an exon-based strategy (point of fusion marked by a dash). The corresponding protein sequence (SEQ ID NO:4) is listed in the bottom.

[00034] Figure 12 lists a DNA sequence (SEQ ID NO:5) of an intracellular region of a chimeric receptor according to an embodiment of the invention where the region consists of amplification modules from human CD28, human CD134, human CD137 and human CD3ζ. The corresponding protein sequence (SEQ ID NO:6) is listed in the bottom. Spacer sequences are written in small letters.

[00035] Figure 13 lists a DNA sequence (SEQ ID NO:7) (top) that would translate into chimeric receptors according to embodiments of the invention where an extracellular

region of human CD64 is fused to regions of human CD16 using an domain-based strategy is further fused to amplification modules depicted in FIG. 13. The corresponding protein sequence (SEQ ID NO:8) is listed in the bottom. Spacer sequences are written in small letters.

[00036] Figure 14 lists a DNA sequence (SEQ ID NO:9) (top) that would translate into chimeric receptors according to embodiments of the invention where an extracellular region of human CD64 is fused to regions of human CD16 using an exon-based strategy is further fused to amplification modules depicted in FIG. 13. The corresponding protein sequence (SEQ ID NO:10) is listed in the bottom. Spacer and mutation sequences are written in small letters.

DETAILED DESCRIPTION OF INVENTION

I. Definition

[00037] Unless otherwise noted, technical terms are used according to conventional usage. Definitions of common terms in molecular biology may be found, for example, in Benjamin Lewin, *Genes VII*, published by Oxford University Press, 2000 (ISBN 019879276X); Kendrew et al. (eds.); *The Encyclopedia of Molecular Biology*, published by Blackwell Publishers, 1994 (ISBN 0632021829); and Robert A. Meyers (ed.), *Molecular Biology and Biotechnology: a Comprehensive Desk Reference*, published by Wiley, John & Sons, Inc., 1995 (ISBN 0471186341); and other similar technical references.

[00038] As used herein, “a” or “an” may mean one or more. As used herein when used in conjunction with the word “comprising,” the words “a” or “an” may mean one or more than one. As used herein “another” may mean at least a second or more. Furthermore, unless otherwise required by context, singular terms include pluralities and plural terms include the singular.

[00039] As used herein, “about” refers to a numeric value, including, for example, whole numbers, fractions, and percentages, whether or not explicitly indicated. The term “about” generally refers to a range of numerical values (e.g., +/- 5 to 10% of the recited value) that one of ordinary skill in the art would consider equivalent to the recited value

(e.g., having the same function or result). In some instances, the term “about” may include numerical values that are rounded to the nearest significant figure.

[00040] CD16 is expressed as two distinct forms, CD16a and CD16b, which are products of two different yet highly homologous genes. CD16a is a polypeptide-anchored transmembrane protein while CD16b is a glycosylphosphatidylinositol-anchored protein. As used herein, CD16 refers to both forms of the protein, unless inappropriate as would be apparent to one skilled artisan.

[00041] The ADCC Enhancer of the present invention can be incorporated in any immune effector with cytotoxic capability, including but are not limited to: T cells including cytotoxic T lymphocytes (CTLs) and helper T cells, NK cells (large granular lymphocytes), eosinophils, macrophages, neutrophils, basophils, monocytes, and B cells. Effector cells of the present invention may be autologous, syngeneic or allogeneic, with the selection dependent upon the disease to be treated and means available.

II. Composition

[00042] The present invention develops an ADCC enhancing system that has one or more of the following distinguishing features: (a) a genetic material that encodes a chimeric receptor having improved affinity for the Fc fragment of antibodies bound to the surface of a target cell; (b) a genetic material that encodes protein and/or RNA module(s) that enhance proliferation and survival of ADCC effector cells upon engagement with a targeted diseased cell that is tagged by a naturally occurring or therapeutic antibody; (c) a genetic material that encodes protein and/or RNA module(s) that amplify the efficiency of ADCC response;. Towards this end, a genetic construct that encodes such component(s) are introduced, as the ADCC-enhancing system, either *in vivo* or *ex vivo*, into the cells of a host, e.g., a patient subject:

[00043] A chimeric receptor that contains a high-affinity, Fc-binding ectodomain fused to a transmembrane and an intracellular domain that activates ADCC and leads to effector proliferation (FIG. 1);

(1) Chimeric Receptor

[00044] Depending on the effector, the chimeric receptor can include portions of natural constituents of the effector, for instance, parts or all of the ectodomain,

transmembrane domain and/or the intracellular domain of a native receptor. The host contemplated by the present invention is high vertebrate, preferably mammalian, further preferably human. The chimeric receptor of the present invention includes an extracellular domain (ectodomain), a transmembrane domain, and an intracellular domain (cytoplasmic domain or endodomain).

(1)(a) Ectodomain

[00045] Referring to FIG. 1, in one embodiment (left), the chimeric receptor of the present invention incorporates parts or all of the ectodomain of CD64 (FcγRI). While both CD64 and CD16 bind to the Fc region of antibodies, the former has about a 100- to a 1000-fold higher affinity. FcγRI (CD64), the high-affinity Fc receptor ($K_d \approx 10^{-9}$ M for IgG1 and IgG3), is present on all cells of the mononuclear phagocyte lineage (e.g., macrophages and neutrophils), and is responsible for antibody-mediated phagocytosis and mediator release. As shown in FIG. 2, FcγRI includes a glycoprotein α chain whose extracellular domain is comprised of three immunoglobulin domains that are responsible for binding to antibodies, and the presence of all three have been shown to be critical for high affinity interaction with antibodies. In contrast, CD16's ectodomain only has two immunoglobulin-like domains. The presence of the extra immunoglobulin-like fold in CD64 may be a strong determinant of its high Fc affinity. To that extent, the present invention also contemplates inserting only this extra immunoglobulin-like fold in CD64 or a mutant version of it into CD16's ectodomain in order to construct a high-affinity receptor for antibodies.

[00046] According to the present embodiment, the ectodomain of CD64 (FcγRI) is fused to any suitable transmembrane and intracellular domains to generate a high-affinity Fc receptor that mediates ADCC. In a preferred embodiment, the ectodomain of human CD64 (FcγRI) is fused to parts of human CD16 (FcγRIII), e.g., both the transmembrane and intracellular domains of CD16, preferably, of CD16a (FcγRIIIa). Advantageously, with the ectodomain being native to the body, the chimeric receptor in this embodiment is syngeneic and, therefore, non-immunogenic.

[00047] As shown in FIG. 1, there may be additional modules in the intracellular domain of the chimeric receptor of the present invention. This could include one or more modules that can amplify the proliferation and expansion signal (e.g., CD28), one or

more modules that promote the effector cell survival and prevent activation-induced apoptosis (e.g., CD134 and CD137), one or more modules that activate or boost signal transduction for proliferation and cytotoxicity (e.g., CD3 ζ). Details of these amplificatory and stimulatory modules are further described in the following sections.

(1)(b) Transmembrane Domain

[00048] The transmembrane domain of the chimeric receptor of the present invention can be any suitable hydrophobic region that can span the cellular membrane, preferably in a stable fashion. In a preferred embodiment, the transmembrane domain is natural to its neighboring component of the intracellular domain. For example, as shown in FIG. 1, in an embodiment where the component or region of the intracellular domain that is closest to the membrane derives from CD16, the transmembrane domain is preferably the transmembrane domain of CD16.

(1)(c) Endodomain

(1)(c)(i) Signaling for ADCC

[00049] Referring again to FIG. 1, in various embodiments of the present invention, the chimeric receptor includes one or more signaling modules for ADCC. CD16 is the natural antibody receptor that activates the effector cells responsible for most of the ADCC activities in our body, particularly the NK cells. Accordingly, in various embodiments, the endodomain of the chimeric receptor of the present invention includes parts or the entire intracellular domain of CD16, preferably, CD16a. Other modules such as CD3 ζ , which share similar ITAM domains with CD16a, also may be included to contribute to the ADCC signaling. In some embodiments, CD3 ζ may not be needed for ADCC signaling as long as the CD16a intracellular domain is expressed in tandem with other co-stimulatory modules or elements. Conversely, modules like CD3 ζ might be able to replace the function of the intracellular domain of CD16, rendering it unnecessary.

(1)(c)(ii) Amplification Module

[00050] As shown in FIG. 1, the chimeric receptor of the present invention may include elements for amplifying the ADCC response. Depending on the effector cell, one or more amplification modules might be included in the chimeric receptor, serving varying functions. The following modules are described as non-limiting examples and for illustrative purposes.

[00051] In an embodiment, the endodomain of the chimeric receptor of the present invention includes parts or the entire intracellular domain of CD3 ζ . A marker native to the T cell surface, CD3 ζ has been shown to have stimulatory effect on lymphocyte (e.g., T cell) activation and proliferation, bringing about a much amplified cytotoxic T cell response.

[00052] In various embodiments, one or more additional costimulatory modules are added to the chimeric receptor of the invention so that the activation effect is sustained for a significantly longer time, thereby increasing the proliferation and expansion potential. An example is CD28's signaling domain, which has been shown to enhance the survival and proliferation of T lymphocytes when expressed with the TCR ζ domain in an *antigen*-dependent receptor (Krause et al, J. Exp. Med. 188:619-26, 1998). Other optional modules that can be incorporated into the chimeric receptor of the present invention include the intracellular domain of members of the tumor necrosis factor receptor family such as CD134 (OX40), CD137 (4-1BB), CD27, for boosting the survival of the effector cells by preventing activation-induced apoptosis (see, e.g., Finney et al., *J Immunol.*, 2004, 172:104-13). Other intracellular domains that could aid in proliferation in some or multiple cell types are found in CD79a, CD79b, CD40, and the GM-CSF receptor.

(2) Cellular Expression

[00053] Using standard recombinant technologies, one or more of the expression cassettes for the chimeric receptor are constructed and cloned onto a vector, e.g., that of a plasmid, adenovirus-derived vectors, retrovirus, or lentivirus. The vector is transfected (or, transduced in the case of viral-mediated gene integration) into any type of immune effector cells. Retroviral transduction may be performed using known techniques, such as that of Johnson et al. (Blood 114, 535-46, 2009). Successful transfection and surface display of the chimeric receptor is confirmed using convention means, e.g., by flow cytometry.

[00054] In an embodiment, immune effector cells are extracted from whole blood from a human patient as peripheral blood mononucleated cells (PBMCs), which include lymphocytes (CD4⁺ T cells, CD8⁺ T cells, B cells and NK cells), monocytes, macrophages, and so on. Optionally, effector cells are further prepared by enriching

selected subset(s) of PBMCs for practicing the invention. In a particular embodiment, B cells are removed from the cell mixture.

[00055] The ability of transduced cells to bind antibodies is revealed using flow cytometry. Cells' ability to bind antibody-coated cells, release cytokines, perform ADCC and proliferate is tested and confirmed *ex vivo* and *in vivo* using standard assays and models (e.g., mouse xenograft models) well known to one skilled in the art.

III. Therapeutics and Vaccines

[00056] Clinical implications of the ADCC Enhancer for cancer can be revealed using human cancer cells in conjunction with therapeutic antibodies. For example, Daudi cells are treated with (1) Rituximab (trade name Rituxan®) which targets CD20 implicated in lymphoma, autoimmune diseases and transplant rejection, resulting in effector cell activation, degranulation and proliferation, and (2) immune effector cells transduced with the ADCC Enhancer. Target cell killing is also observed. *In vivo* testing is performed using commercially available NOD.scid. IL2R $\gamma^{-/-}$ mice which have very low T and B cells and no NK cells. Alternatively, NOD.Scid mice which have very low T and B cells and reduced NK cells are used. These mice are engrafted with labeled Daudi cells and tumor growth is observed and measured using any suitable imaging technique. In mice receiving Rituximab and immune effector cells transduced with the ADCC Enhancer, sustained periods of tumor remission, regression, or long-term non-progression are observed.

[00057] In another example, SK-BR-3 or MCF-7 cells are treated with (1) immune effector cells transduced with the ADCC Enhancer and (2) Trastuzumab (trade name Herceptin®) which targets the HER2/neu implicated in breast cancers, resulting in effector cell activation, degranulation and proliferation. Target cell killing is also observed. *In vivo* anti-tumor potency of the ADCC Enhancer is observed in mice models similar to the example described immediately above.

[00058] The clinical use of the ADCC Enhancer in autoimmunity can be revealed using one of the well-established mouse models for this kind of diseases. For instance, antibody-mediated B cell deletion has been shown to prevent and even reverse type-1 diabetes in NOD mice. However, this effect is limited by the low affinity of the Fc

receptors (Hu et al. *J Clin Invest.* 2007, 117(12):3857-67; Xiu et al. *J Immunol.* 2008, 180(5):2863-75). Control mice are compared with mice receiving either anti-CD19 or anti-CD20 antibodies alone or in combination with murine immune effector cells transduced with or otherwise expressing the ADCC Enhancer of the present invention. The mice receiving the ADCC Enhancer show delayed onset of disease or sustained reversal of symptoms.

[00059] Similar experiments can be performed in other mouse models where antibody-mediated depletion has been shown to impact diseases such as multiple sclerosis, or experimental autoimmune encephalomyelitis (Barr et al. *J Exp Med.* 2012, 209(5): 1001-10)), arthritis (Yanaba et al. *J Immunol.* 2007, 179(2): 1369-80), and so on.

[00060] The clinical use of the ADCC Enhancer in viral infections such as HIV infection can be revealed using well-established humanized mouse model where treatment with a combination of antibodies has been shown to control HIV replication (*Nature*, 2012, 492(7427): 118-22). Humanized mice are first generated by reconstituting NOD.RAG1^{-/-}. IL2R γ ^{-/-} mice with human fetal liver-derived CD34⁺ hematopoietic stem cells. These mice have a completely human immune system, can be infected by HIV and do not negatively react to human antibodies. Infected control mice are compared with mice receiving either neutralizing antibody cocktail alone or in combination with human immune effector cells transduced with the ADCC Enhancer of the present invention. The mice receiving the ADCC enhancer show sustained reduction in viremia and recovery of T cell numbers.

[00061] An alternate model system to test the clinical efficacy of the ADCC Enhancer is the simian-human immunodeficiency virus (SHIV)-infected infant rhesus macaque model where neutralizing antibodies have been shown to prevent rapid onset of the disease (Jaworski et al. *J Virol.* 2013, 87(19): 10447-59). Infected control macaques are compared with those receiving either neutralizing antibody cocktail alone or in combination with immune effector cells transduced with the ADCC Enhancer of the present invention. The macaques receiving the ADCC enhancer similarly show sustained reduction in viremia and recovery of T cell numbers.

[00062] DNA and RNA constructs that encode the ADCC Enhancing system of the present invention may be formulated for administration to a subject using techniques

known to the skilled artisan. Formulations comprising DNA and RNA constructs that encode the ADCC Enhancing system may include pharmaceutically acceptable excipient(s). Excipients included in the formulations will have different purposes depending, for example, on the kind of gene construct or effector cells used, and the mode of administration. Examples of generally used excipients include, without limitation: saline, buffered saline, dextrose, water-for-infection, glycerol, ethanol, and combinations thereof, stabilizing agents, solubilizing agents and surfactants, buffers and preservatives, tonicity agents, bulking agents, and lubricating agents. The populations of immune effector cells expressing the ADCC Enhancer are typically prepared and cultured *ex vivo*.

[00063] In one method embodiment of the invention, immune effector cells extracted from whole blood from a subject is first infected *ex vivo* with the recombinant lentivirus (or any other gene therapy vector e.g. herpes virus, adenovirus, AAV, etc.) encoding the ADCC Enhancer of the invention. The infected PBMC cells are then infused back into the same patient subject after confirmation of success in the transfection. The immune effector cells transfused into the patient subject then proceed to find and destroy diseased cells including tumor cells that are coated with antibodies. To practice this method embodiment, the present invention provides a kit to be used by a physician that include the formulated gene constructs of the invention, and, optionally, agents for preparing the PBMC cells as well as instructions. A modified embodiment of this method entails infecting or transfecting a subset of cells from the PBMCs. This can be achieved by culturing the PBMCs under conditions that preferentially support growth of a particular cell type, or by selecting cells through positive or negative selection techniques like fluorescent activated cell sorting or magnetic activated cell sorting, or a combination of both. An alternative method embodiment of the invention is transfection of the DNA construct or RNA for the receptor into PBMCs or purified subsets of cells using standard transfection techniques.

[00064] In another embodiment of the invention, the formulations comprise gene constructs encoding the ADCC Enhancer delivered by liposome or nanoparticle-based technology, and may be administered to a subject using modes and techniques known to the skilled artisan. Exemplary modes include, but are not limited to, intravenous

injection. Other modes include, without limitation, intratumoral, intradermal, subcutaneous (s.c., s.q., sub-Q, Hypo), intramuscular (i.m.), intraperitoneal (i.p.), intra-arterial, intramedullary, intracardiac, intra-articular (joint), intrasynovial (joint fluid area), intracranial, intraspinal, and intrathecal (spinal fluids). Any known device useful for parenteral injection or infusion of the formulations can be used to effect such administration.

[00065] The formulations comprising gene constructs encoding the ADCC Enhancer that are administered to a subject comprise a number of gene constructs or effector cells that are effective for the treatment and/or prophylaxis of the specific indication or disease. Thus, therapeutically effective gene constructs encoding the ADCC Enhancer are administered to subjects when the methods of the present invention are practiced. In general, cell-based formulations are administered that comprise between about 1×10^4 and about 1×10^{10} effector cells. In most cases, the formulation will comprise between about 1×10^5 and about 1×10^9 effector cells. However, the number of effector cells administered to a subject will vary between wide limits, depending upon the location, source, identity, extent and severity of the cancer or disease, the age and condition of the individual to be treated, etc. A physician will ultimately determine appropriate dosages to be used.

[00066] As used herein, the terms “treat”, “treating”, and “treatment” have their ordinary and customary meanings, and include one or more of: blocking, ameliorating, or decreasing in severity and/or frequency a symptom of a disease (e.g., cancer) in a subject, and/or inhibiting the growth, division, spread, or proliferation of cancer cells, or progression of cancer (e.g., emergence of new tumors) in a subject. Treatment means blocking, ameliorating, decreasing, or inhibiting by about 1% to about 100% versus a subject in which the methods of the present invention have not been practiced. Preferably, the blocking, ameliorating, decreasing, or inhibiting is about 100%, 99%, 98%, 97%, 96%, 95%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%, 5% or 1% versus a subject in which the methods of the present invention have not been practiced.

[00067] The clinical potency, both as a therapeutic and a prophylactic, of immune effector cells expressing the ADCC Enhancer of the present invention may be optionally enhanced through the use of dendritic cells (DCs). In lymphoid organs, DCs present

antigen to T-helper cells, which in turn, regulate immune effectors including CTLs, B cells, macrophages, eosinophils and NK cells. It has been reported that autologous DC engineered to express an HIV antigen or pulsed with exogenous HIV protein was able to prime CTLs *in vitro* against HIV (Wilson et al., J Immunol., 1999, 162:3070-78).

Therefore, in an embodiment of the present invention, DCs are first isolated from the subject patient, and then primed *ex vivo* through incubation with a source of target antigen(s), e.g., certain tumors-associated antigens or other surface markers of a disease which can be from the subject patient or a foreign source. These DCs are eventually infused back into the patient prior to treatment by autologous CTL and/or other effector cells transfected with the ADCC Enhancing system of the present invention or by formulations comprising DNA and RNA constructs that encode the ADCC Enhancing system. This provides a model of enhanced treatment as well as vaccine using the ADCC Enhancer with the help of DCs.

[00068] The invention also provides a kit comprising one or more containers filled with quantities of gene constructs encoding the ADCC Enhancer of the present invention with pharmaceutically acceptable excipients. The kit may also include instructions for use. Associated with the kit may further 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.

IV. EXAMPLES

[00069] (1) *Chimeric Receptor*

[00070] According to embodiments of the present invention, ectodomains of the chimeric ADCC-enhancing receptors were generated in two ways:

[00071] (a) Domainal fusion: Based on the predicted amino acid boundaries between the ectodomain and transmembrane region of FcγRI (CD64) and FcγRIII (CD16), a predicted ectodomain of human FcγRI was fused to the transmembrane and intracellular regions of human FcγRIII. DNA and amino acid sequences for the resulting regions in the fusion receptor, SEQ ID NOs:1 and 2, respectively, are shown in FIG. 10. Obviously, DNA sequences besides SEQ ID NO:1 can also translate into the protein of

SEQ ID NO:2, and those DNA sequences are also contemplated by the present invention in this and similar instances throughout the application.

[00072] (b) Exon-based fusion: Based on the exon boundaries in FcγRI and FcγRIII, a predicted ectodomain of human FcγRI was fused to the transmembrane and intracellular regions of human FcγRIII. As shown in FIG. 3, both proteins have two exons coding a secretory peptide (S1 and S2), followed by three (CD64) or two (CD16) exons coding for the extracellular regions. Specifically, exons from S1 to EC3 from CD64 were fused with the TM/C exon in CD16. The DNA and amino acid sequences for the resulting regions in the fusion receptor, SEQ ID NOs:3 and 4, respectively, are shown in FIG. 11.

[00073] To make the chimeric receptor, cDNA clones of human CD64 and CD16 were obtained from Origene (also available from a variety of other commercial vendors). Flanking primers were designed and synthesized to amplify selected portions of the cDNA and recombinantly combine into one vector using PCR. Primers included restriction sites that facilitated cloning to generate the fusion construct and subcloning into various commercial vectors. Standard restriction digestion and ligation procedures were used throughout.

[00074] Referring now to FIG. 4, once the ectodomain of human CD64 was fused to the transmembrane and intracellular domains of human CD16, they were further fused to various amplification modules found in various human proteins, specifically, CD28 (a.a. 162-202), CD134 (a.a. 213-249), CD137 (a.a. 187-232) and CD3ζ (a.a. 31-142) (all amino acid positions are based on mature protein sequences). Mutations were introduced into the sequence as marked by asterisks as follows: Commercially available CD16 cDNA has a polymorphism that was reverted. In addition, a dileucine motif in CD28 that is known to down-modulate surface expression was mutated. Each module was separated by a -GGATCT- sequence that introduced two spacer amino acids. The DNA and amino acid sequences for the resulting regions in the fusion receptor, SEQ ID NOs:5 and 6, respectively, are shown in FIG. 12. The DNA and amino acid sequences for the entire fusion/chimeric receptor with regions from CD64, CD16 and the four amplification modules described above, SEQ ID NOs:7 to 10, are shown in FIGS. 13 (domainal fusion)

and 14 (exon-based fusion), respectively. The “higher-affinity” mutant of human CD16 (V158) was used as control.

[00075] The chimeric receptors were then cloned into a commercially available lentiviral vector either with a surrogate marker (e.g., pSMPUW-IRES-GFP shown in FIG. 5) or without (e.g., pSMPUW-puro shown in FIG. 6).

[00076] In pSMPUW-IRES-GFP (FIG. 5), GFP expression was linked to the chimeric receptor through an internal ribosome entry site. The puromycin cassette in the pSMPUW-puro vector could be used to select stable and successful clones. For therapeutic uses contemplated by the present invention, however, both the GFP and Puro cassettes would be removed to minimize the size of the vector, as shown being crossed out in FIG. 6. Receptor expression correlated with GFP expression only when the chimeric receptor was generated using exon-based fusion, and therefore, that version of the chimeric receptor became the preferred and default embodiment herein.

[00077] *(2) Cellular Expression*

[00078] Lentiviral vectors (pSMPUW) were transfected in 293T or Expi293 cells to test expression of the chimeric receptor described above by flow cytometry. Transfection was performed with standard procedures similar to procedures noted above. In the case of pSMPUW-IRES-GFP, transfection was first confirmed through GFP expression 2-3 days after transfection (FIG. 7). Next, expression of the chimeric receptor of the present invention was confirmed by staining the cells with commercially available fluorescently tagged anti-CD16 or anti-CD64 antibodies and measuring resulting fluorescence (FIG. 8).

[00079] Since expression of the receptor is linked to GFP expression with the IRES element, receptor expression was correlated to GFP expression. Staining with antibodies was typically achieved by harvesting and washing 10^6 cells with phosphate buffered saline (PBS) with or without 5% fetal bovine serum (FBS). Cells were incubated for 30 minutes at 4°C in PBS-FBS in the presence of an appropriate amount of antibodies. Cells were again washed in PBS and analyzed by flow cytometry.

[00080] The CD64-CD16 chimeric receptors made using domainal fusion strategy showed much weaker expression (data not shown) than those made using the exon-based fusion.

[00081] The pSMPUW vectors were co-transfected with commercially available helper plasmids to make lentiviral particles. Successful transfection was confirmed by analyzing GFP and/or receptor expression.

[00082] *(3) Therapeutic Affinity*

[00083] To test the ability of the chimeric receptors in terms of binding human antibodies, cells were first “stained” with commercially available rituximab (Rituxan) antibody as above. Briefly, 10^6 cells were harvested, washed with PBS and incubated for 30 minutes at 4°C with 0.1 ug/ml of rituximab. Cells were again washed in PBS and stained with a fluorescently tagged goat anti-human IgG – an antibody that would bind to rituximab or any other human IgG.

[00084] Under our test conditions, the CD64-CD16 chimeric receptor made with exon-based fusion strategy showed significantly better binding to rituximab than the benchmark V158 version of CD16 (FIG. 9). Similar experiments are carried out with other therapeutic antibodies as well.

[00085] An additional level of functionality is the ability to bind antibody-coated target cells. To test, target cells, e.g., Daudi (Burkitt's lymphoma line) cells, are first fluorescently labeled with a cell-tracing reagent, e.g., CellTrace Far Red DDAO-SE. This is typically done by harvesting 5×10^6 cells, washing them with PBS and then incubating for 5 min with appropriate amount of reagent. The reaction is stopped by adding excess amount of PBS-FBS and then the cells are washed twice with PBS. The labeled cells are then coated with rituximab as described above. Rituximab binds to the cell surface CD20 molecules expressed on Daudi cells. The rituximab-coated cells are then incubated with cells expressing different chimeric receptors for 60 min at 37°C to test their ability to form heterologous aggregates (GFP+, CellTrace+) as measured by flow cytometry. When using a lentiviral vector without a surrogate marker like GFP, cells expressing the chimeric receptor also need to be stained with a compatible dye e.g. CFSE.

[00086] Similar experiments can be done with other cell lines with appropriate targeting antibodies, e.g., Raji or Ramos cells with rituximab, SK-BR-3 (breast carcinoma) cells with trastuzumab, etc.

[00087] *(4) Immune Effector Cells*

[00088] After lentiviral particles are made in 293T cells, they can be used to test the functionality of the receptors in T cells. For example, Jurkat (acute T cell leukemia) cells are commonly used surrogate for primary T cells. Functionality of the receptors is revealed in much the same way as described above in 293T cells. Jurkat cells are infected using standard spinoculation method. Typically, 2×10^5 cells in 100 μ l are plated in a 96-well plate. An appropriate amount of viral supernatant is added along with polybrene (4 μ g/ml) and centrifuged for 2 hours at 30°C at 350xg. Cells are analyzed 2 or more days after infection. In the case of puromycin^R-containing lentiviral vectors, stable lines can be generated by selecting the cells with puromycin.

[00089] Similar methods are used to infect naïve primary T cells after activation with anti-CD3/CD28. Apart from the analysis similar to Jurkat or 293 cells, the ability of the receptors of the invention can be revealed for activation, proliferation and for triggering cytotoxicity. Typically, to measure proliferation, 10^6 transduced cells are grown in the presence of 50 IU/ml of IL-2. Rituximab-coated Daudi cells are added to this culture on different days at a ratio of 1:1. As above, other antibody-coated cells can also be used in these experiments. Ideally the target cells (e.g. Daudi) are pretreated with mitomycin C to stall proliferation. T cell proliferation is measured by flow cytometry or other traditional methods e.g. thymidine incorporation, CFSE dilution, etc.

[00090] Lysosomal-associated membrane protein-1 (LAMP-1 or CD107a) has been described as a marker of CD8⁺ T-cell and NK cell degranulation of lytic granules following stimulation. In the co-culture experiments described above, the level of CD107a⁺ T cells can be analyzed as a measure for degranulation by flow cytometry.

[00091] To assess cytotoxicity, T cells are co-cultured for several hours with CellTrace-tagged target cells in the presence of an appropriate therapeutic antibody. Receptor transduction dependent, antibody-dependent depletion of viable target cells are observed. This can be measured by flow cytometry using propidium iodide or 7-AAD.

[00092] *(5) In Vivo Anti-tumor Activity*

[00093] Anti-tumor activity is revealed by standard xenograft tumor models using NSG mice from the Jackson lab (see, Shultz et al. *Nat Rev Immunol.* 2007 Feb;7(2):118-30). NSG mice lack functional T, B or NK cells and are severely immunocompromised and

ideal for engraftment with primary human cells. Other similar mouse strains e.g. NOG mice can also be used.

[00094] For instance, luciferase-expressing target tumor cells are injected intraperitoneally (i.p. 0.3×10^6 cells/mouse). Mice are treated i.p. with rituximab (150 ug) and receptor-transduced T cells (10^7 cells) along with 1000-2000IU of IL-2 after a few days. As controls, either rituximab, the receptor or T cells are excluded in a subset of mice. Tumor engraftment and growth are measured using a Xenogen IVIS system.

[00095] Alternate tumor models include injecting 10^7 Daudi cells s.c. and giving the treatments i.v. Tumor growth is measured directly with calipers or judged by survival.

[00096] Similarly experiments are conducted with other cell line antibody combinations including i.p., s.c. or i.v. injection of Raji cells. In the latter, tumor growth is measured by survival.

[00097] For the purpose of therapy, the puromycin or the GFP marker would be removed to minimize expression of irrelevant genes.

[00098] Another alternate example is to electroporate or transfect mRNA directly. This would be safer than lentiviruses.

[00099] It will be apparent to those skilled in the art that various modifications and variations can be made in the present invention without departing from the scope or spirit of the invention. Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

[000100] Throughout this application, various publications, patents, and/or patent applications are referenced in order to more fully describe the state of the art to which this invention pertains. The disclosures of these publications, patents, and/or patent applications are herein incorporated by reference in their entireties to the same extent as if each independent publication, patent, and/or patent application was specifically and individually indicated to be incorporated by reference.

CLAIMS

1. A genetic construct that encodes a transmembrane chimeric receptor comprising an ectodomain having a higher binding affinity to the Fc of an antibody than that of human CD16, a transmembrane domain, and an endodomain for mediating ADCC activation and amplification.
2. The genetic construct of claim 1, comprising a DNA molecule.
3. The genetic construct of claim 1, comprising an RNA molecule.
4. The genetic construct of claim 1, comprising substantially the EC3 exon of CD64.
5. The genetic construct of claim 4, further comprising substantially the EC1 and EC2 exons of CD64.
6. The genetic construct of claim 1, wherein said ectodomain is selected based on boundaries of exons or domains in CD64.
7. The genetic construct of claim 1, comprising an RNA that translates to an ectodomain comprising substantially the ectodomain of CD64.
8. The genetic construct of claim 1, where said ectodomain has a higher affinity for Fc than that of the V158 variant of human CD16.
9. The genetic construct of claim 1, where said ectodomain has a higher affinity for Fc than that of the F158 variant of human CD16.
10. The genetic construct of claim 1, wherein the endodomain is selected from the group consisting of portions or all of intracellular domains of CD3 ζ , of CD28, of CD134, of CD137, of CD27, of CD79a, of CD79b, of CD40 and of the GM-CSF receptor.
11. The genetic construct of claim 1, comprising a DNA sequence selected from the group consisting of SEQ ID NOs:1, 3, 5, 7, and 9.
12. A transmembrane chimeric receptor comprising an ectodomain having a higher binding affinity to the Fc of an antibody than that of human CD16, a transmembrane domain, and an endodomain for mediating ADCC activation and amplification.
13. The transmembrane chimeric receptor of claim 12, comprising an amino acid sequence selected from the group consisting of SEQ ID NOs:2, 4, 6, 8, and 10.

14. The transmembrane chimeric receptor of claim 12, wherein the ectodomain has a higher binding affinity to the Fc of an antibody than that of the V158 variant of human CD16.
15. The transmembrane chimeric receptor of claim 12, wherein the ectodomain comprises substantially the immunoglobulin-like fold in the ectodomain of CD64 that is absent from CD16.
16. The transmembrane chimeric receptor of claim 12, wherein the ectodomain comprises substantially the ectodomain of CD64.
17. The transmembrane chimeric receptor of claim 12, wherein the transmembrane domain comprises substantially the transmembrane domain of CD16.
18. The transmembrane chimeric receptor of claim 12, further comprising an ADCC amplification module that enhances the survival and proliferation of a host immune effector cell.
19. The transmembrane chimeric receptor of claim 19, wherein said amplification module is selected from the group consisting of portions or all of intracellular domains of CD3 ζ , of CD28, of CD134, of CD137, of CD27, of CD79a, of CD79b, of CD40 and of the GM-CSF receptor.
20. An immune effector cell infected or transfected ex vivo with the genetic construct of claims 1-11 or the protein of claims 12-20.
21. The immune effector cell of claim 21 comprising a cytotoxic T lymphocyte.
22. The immune effector cell of claim 21 comprising a natural killer cell.
23. The immune effector cell of claim 21, selected from the group consisting of an eosinophil, a macrophage, a neutrophil, a basophil, a monocyte, a B cell, and other cells that express or are engineered to express cytotoxic factors.
24. A pharmaceutical composition comprising the genetic construct of claims 1-11 or the receptor of claims 12-20, and a pharmaceutically acceptable excipient.
25. A pharmaceutical composition comprising the immune effector cell of claim 22-24, and a pharmaceutically acceptable excipient.

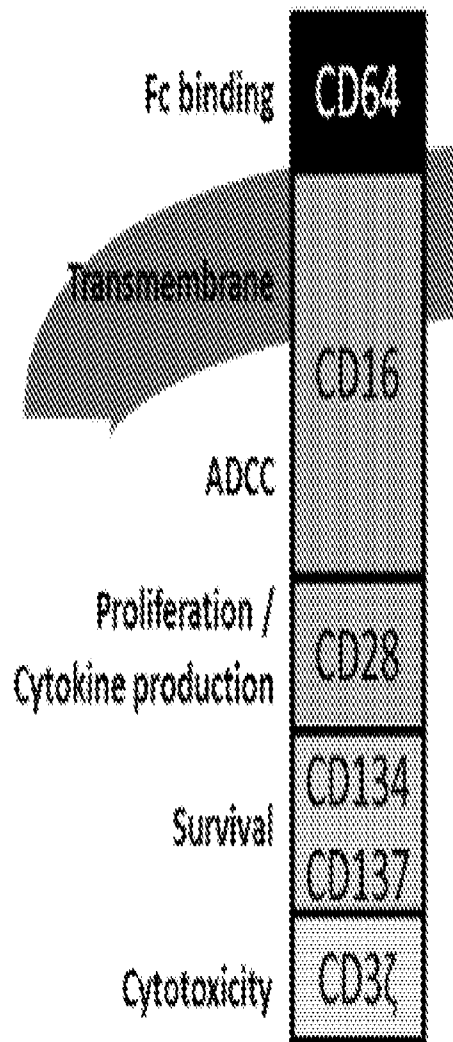


Figure 1

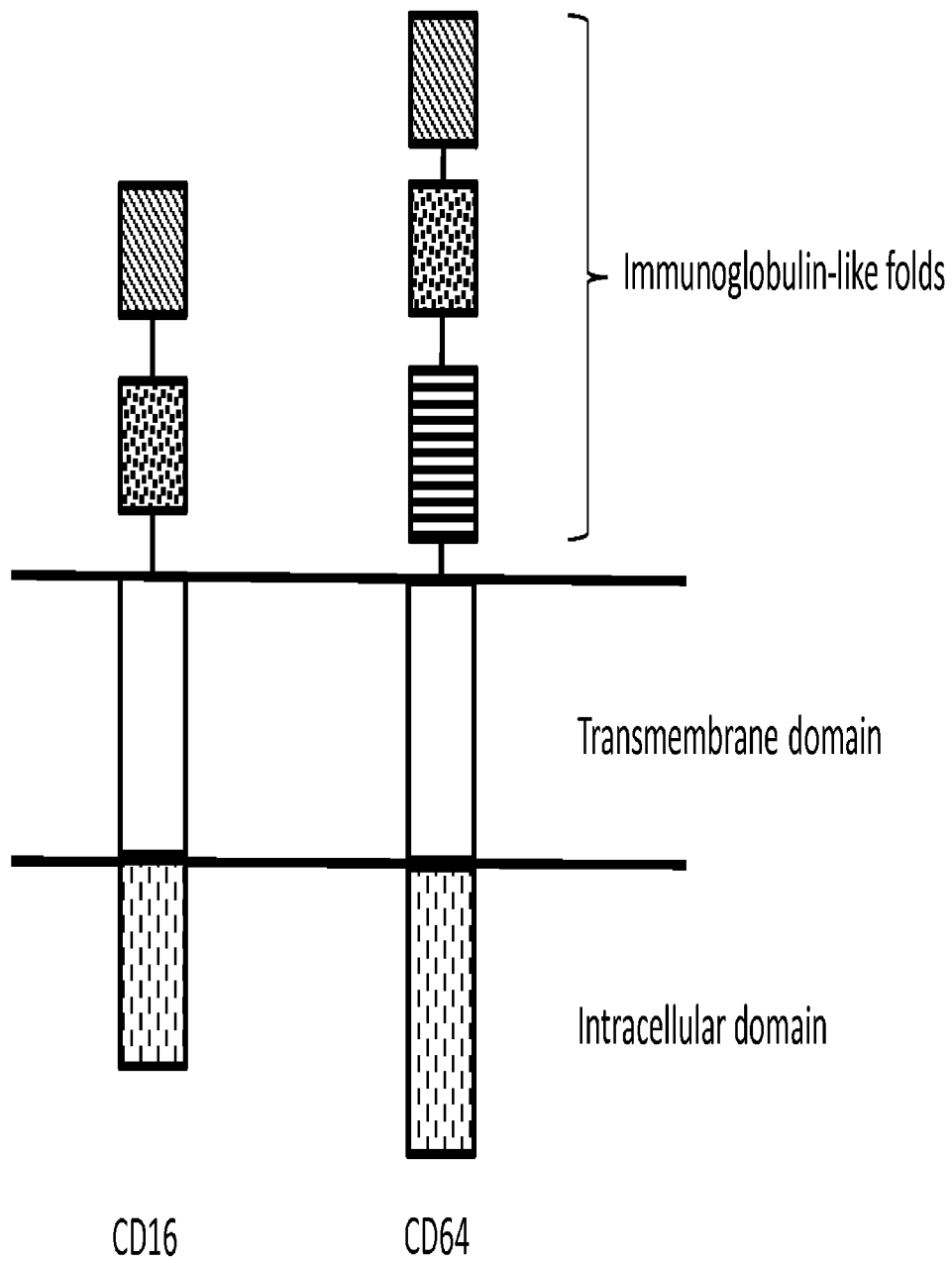


Figure 2

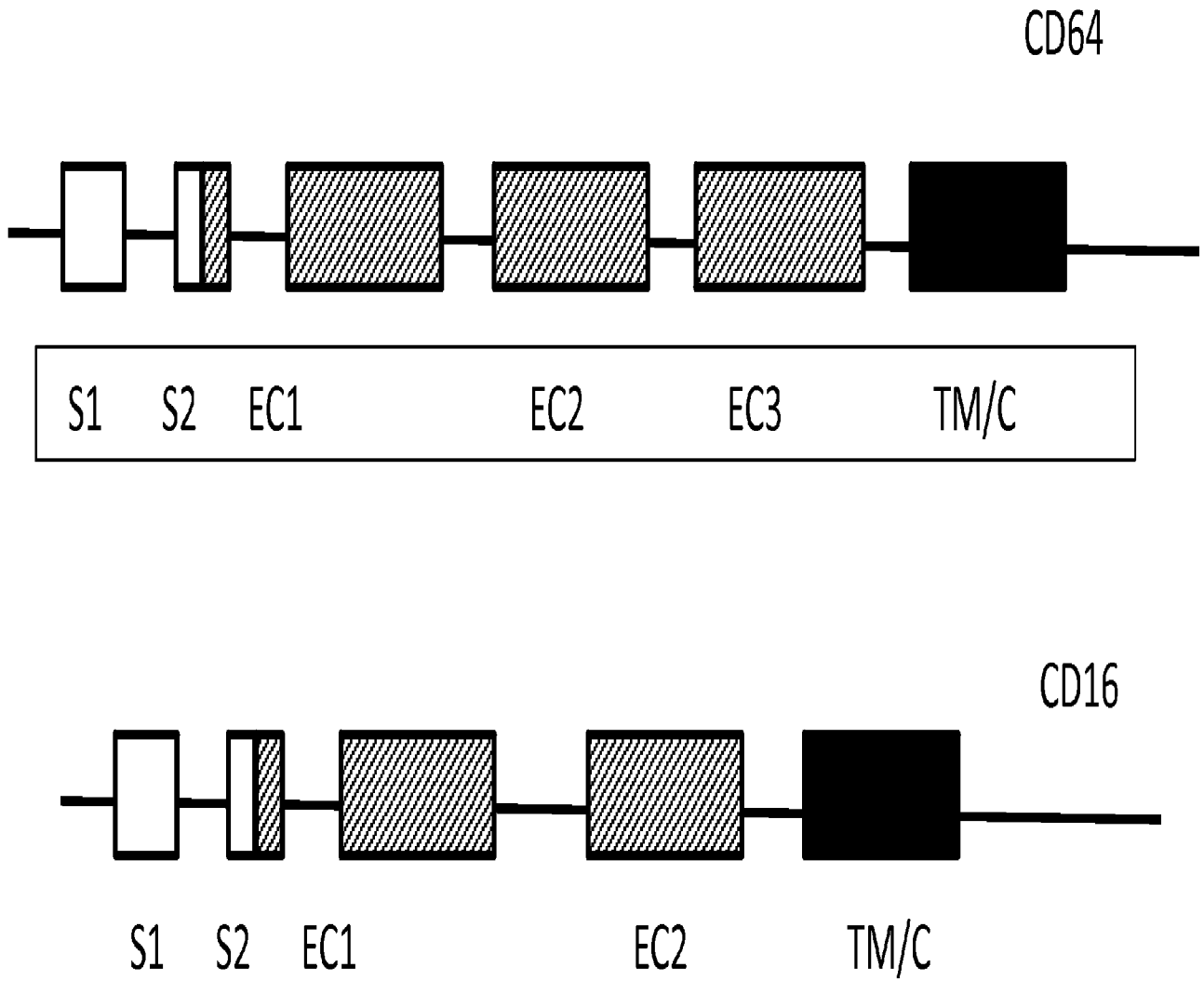


Figure 3

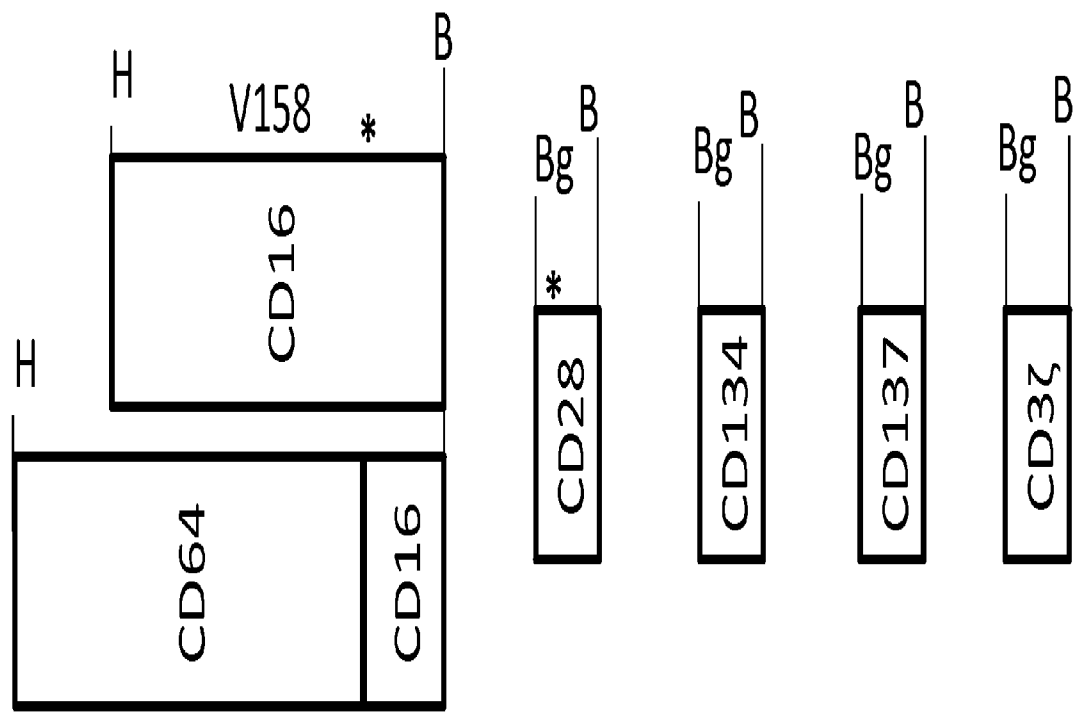


Figure 4

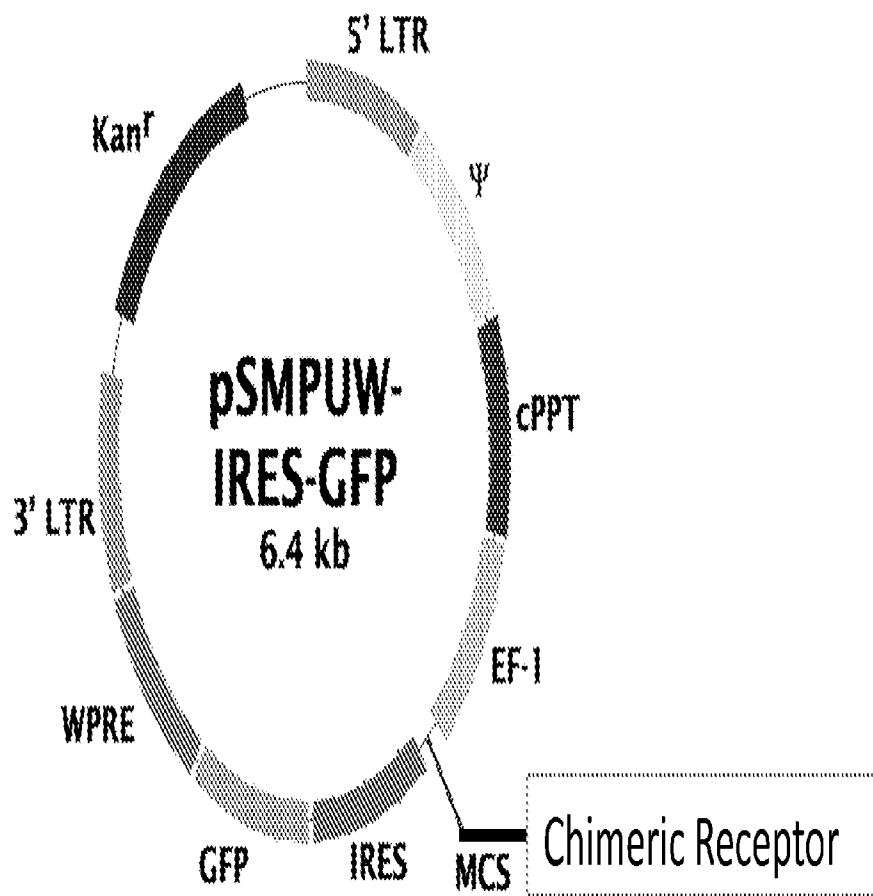


Figure 5

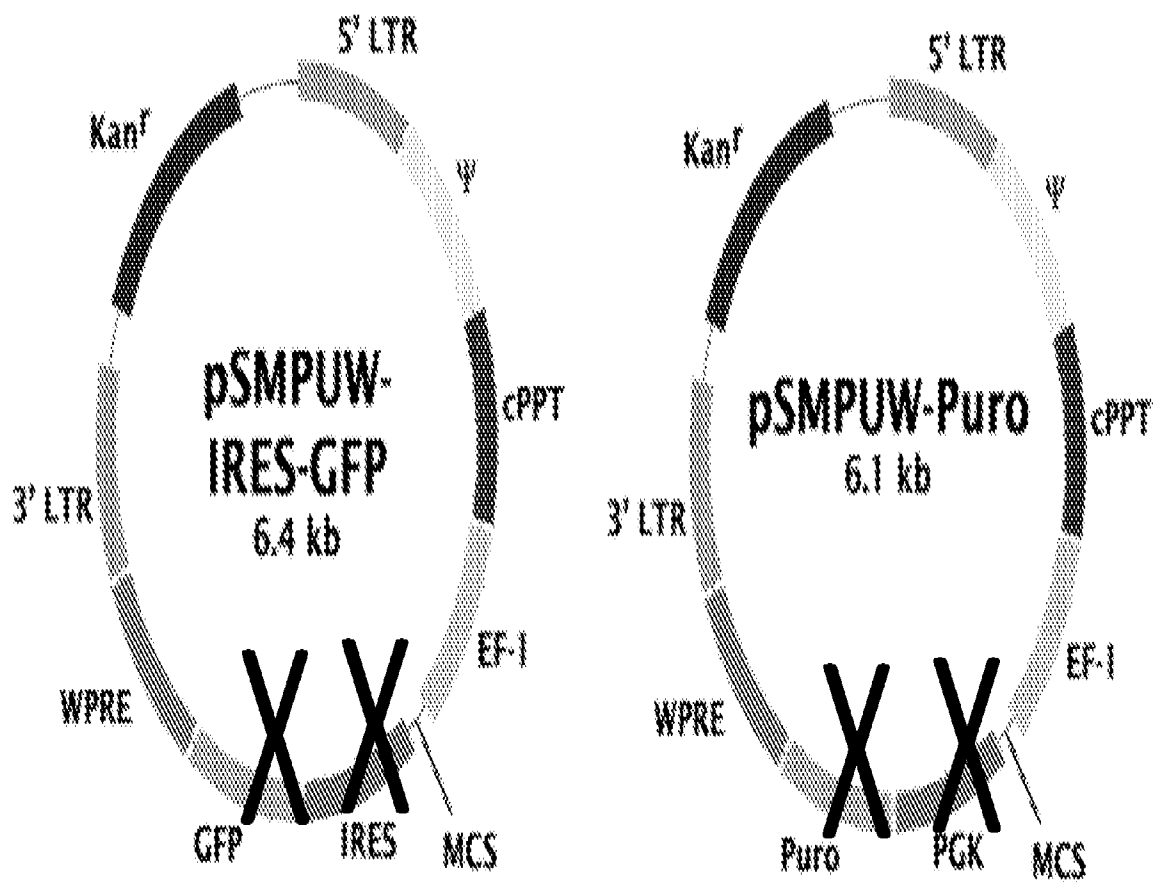


Figure 6

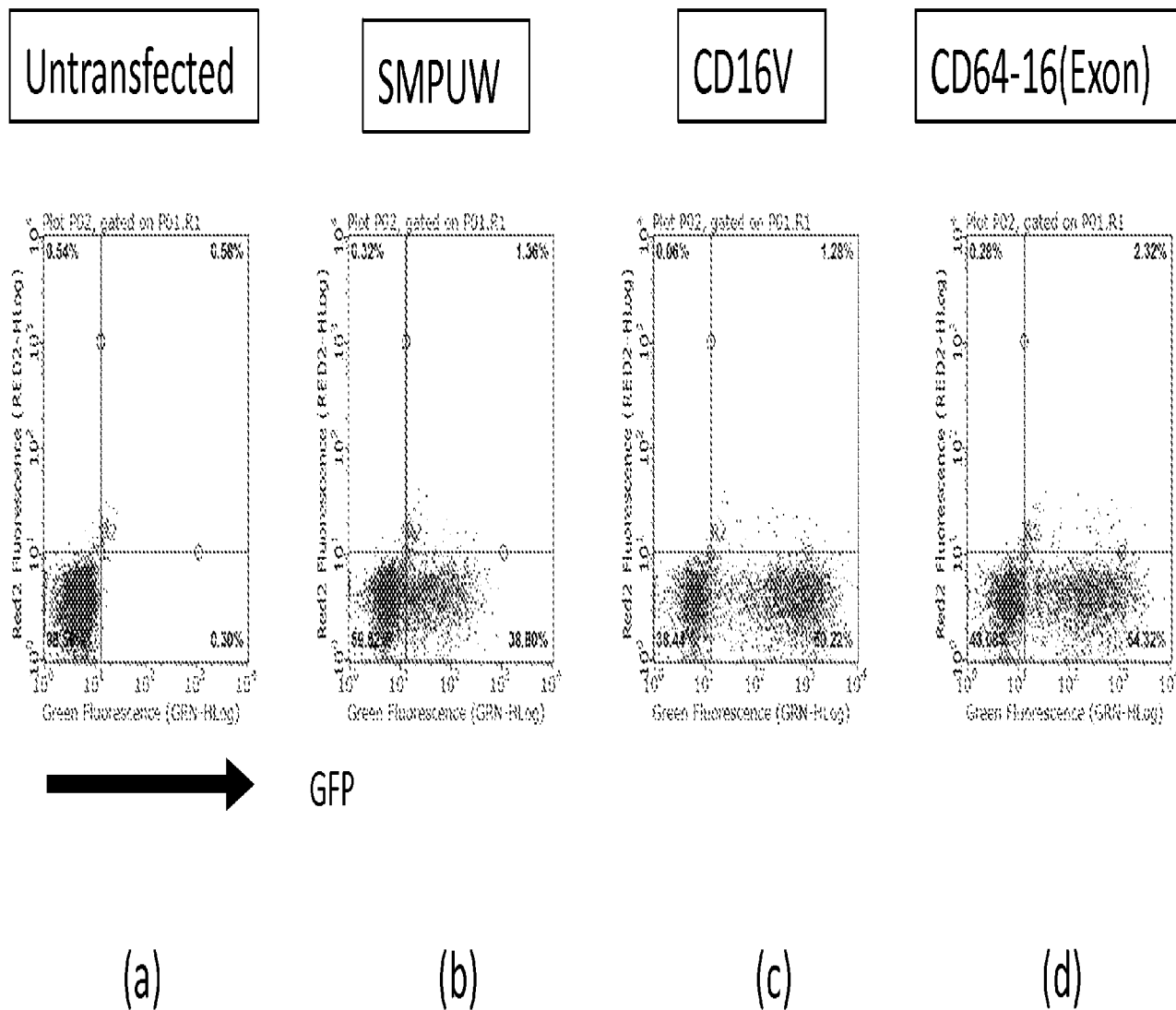


Figure 7

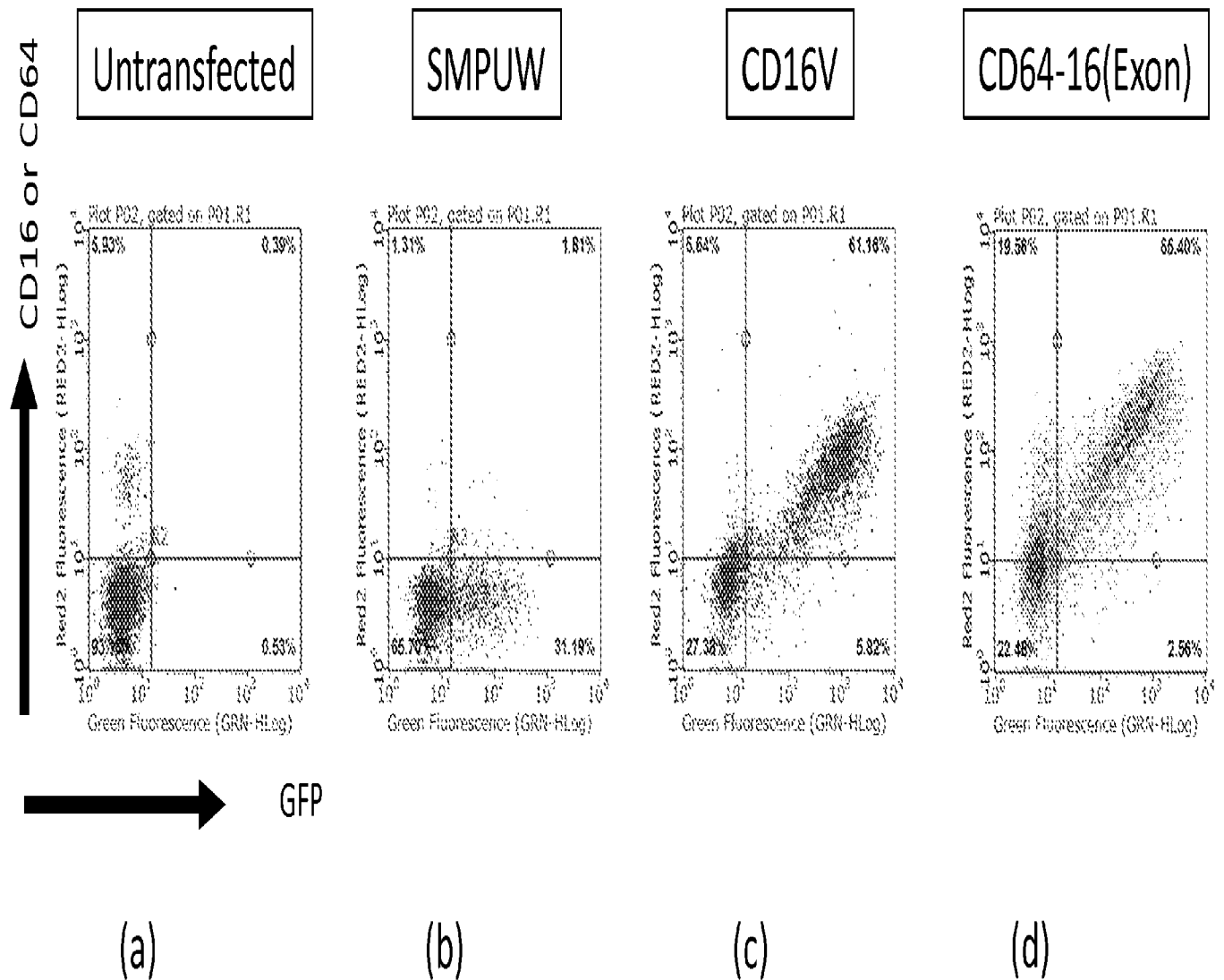


Figure 8

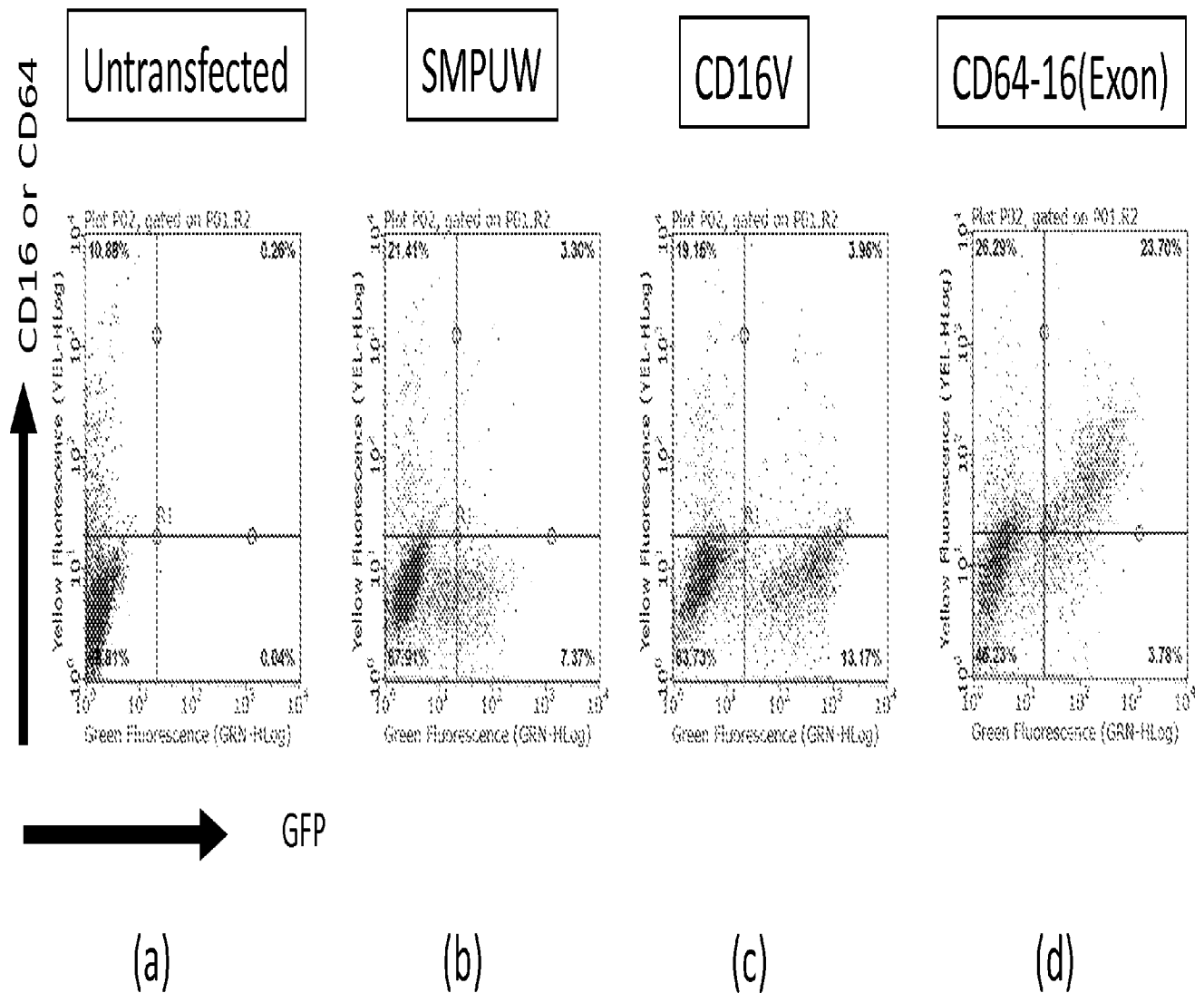


Figure 9

CD64-CD16 Chimeric Receptor DNA (Domain-based construction)

CTTGGAGACAACATGTGGTCTTGACAACTCTGCTCCTTTGGGTTCCAGTTGATGGGCAAGTGGACACCACAAAGGCAGTGATCACTTTGCAGCCTCCATG
GGTCAGCGTGTTCCAAGAGGAAACCGTAACCTTGCAATTGTGAGGTGCTCCATCTGCCTGGGAGCAGCTCTACACAGTGGTTTCTCAATGGCACAGCCACT
CAGACCTCGACCCCAGCTACAGAATCACCTCTGCCAGTGTCAATGACAGTGGTGAATACAGGTGCCAGAGAGGTCTCTCAGGGCGAAGTGACCCCATAC
AGCTGGAAATCCACAGAGGCTGGCTACTACTGCAGGTCTCCAGCAGAGTCTTCACGGAAGGAGAACCTCTGGCCTTGAGGTGTCATGCGTGGAAGGATA
AGCTGGTGTAATGTGCTTTACTATCGAAATGGCAAAGCCTTTAAGTTTTTCCACTGGAATCTAACCTCACCATTCTGAAAACCAACATAAGTCACAATGG
CACCTACCATTGCTCAGGCATGGGAAAGCATCGCTACACATCAGCAGGAATATCTGTCACTGTGAAAGAGCTATTTCCAGCTCCAGTGCTGAATGCATCTGT
GACATCCCACTCCTGGAGGGGAATCTGGTCACCTGAGCTGTGAAACAAAGTTGCTCTTGAGAGGCCTGGTTTGCAGCTTTACTTCTCCTTCTACATGG
GCAGCAAGACCCTGCGAGGCAGGAACACATCCTCTGAATACCAATACTAAGTCTAGAGAGAGAGTCTGGGTTATACTGGTGCGAGGCTGCCACAGA
GGATGGAAATGTCCTTAAGCGCAGCCCTGAGTTGGAGCTTCAAGTGCTTGGCCTCCAGTTACCAACTCCTGTCTGGTTTCAT-
TACCAAGTCTCTTTCTGCTTGGTGATGGTACTCCTTTTTGCAGTGGACACAGGACTATATTTCTGTGAAGACAAACATTGGAAGCTCAACAAGAGACTGG
AAGGACCATAAATTTAATGGAGAAAGGACCCTCAAGACAAA

Protein sequence (340 a.a.)

MWFLTLLLWVPVDGQVDTTKAVITLQPPWVSVFQEETVLHCEVLHLPSSSTQWFLNGTATQTSTPSYRITSASVNDSGEYRCQRGLSGRSDPIQLEIHRGW
LLLQVSSRVFTEGEPLALRCHAWKDKLVYNVLYRNGKAFKFFHWNSNLTILKTNISHNGTYHCSGMGKHRYTSAGISVTVKELFPAPVLNASVTSPLLEGNLVT
SCETKLLQRPGLQLYFSFYMGSKTLRGRNTSSEYQILTARREDSGLYWCEATEDGNVLKRSPLELQVLGLQLPTPVWFHYQVSFCLVMVLLFAVDGLYFSVKT
NIRSSTRDWKDHKFKWRKDPQDK

Figure 10

CD64-CD16 Chimeric Receptor DNA (Exon-based construction)

CTTGGAGACAACATGTGGTTCTTGACAACTCTGCTCCTTTGGGTTCCAGTTGATGGGCAAGTGGACACCACAAAGGCAGTGATCACTTTGCAGCCTCCATG
GGTCAGCGTGTTCCAAGAGGAAACCGTAACCTTGCAATTGTGAGGTGCTCCATCTGCCTGGGAGCAGCTCTACACAGTGGTTTCTCAATGGCACAGCCACT
CAGACCTCGACCCCAGCTACAGAATCACCTCTGCCAGTGTCATGACAGTGGTGAATACAGGTGCCAGAGAGGTCTCTCAGGGCGAAGTGACCCCATAC
AGCTGGAAATCCACAGAGGCTGGCTACTACTGCAGGTCTCCAGCAGAGTCTTCACGGAAGGAGAACCTCTGGCCTTGAGGTGTCATGCGTGGAAGGATA
AGCTGGTGTAATGTGCTTTACTATCGAAATGGCAAAGCCTTTAAGTTTTCCACTGGAATTCTAACCTCACCATTCTGAAAACCAACATAAGTCACAATGG
CACCTACCATTGCTCAGGCATGGGAAAGCATCGCTACACATCAGCAGGAATATCTGTCACTGTGAAAGAGCTATTTCCAGCTCCAGTGCTGAATGCATCTGT
GACATCCCCTCTGGAGGGGAATCTGGTCACCTGAGCTGTGAAACAAAGTTGCTCTGCAGAGGCCTGGTTTGCAGCTTTACTTCTCCTTCTACATGG
GCAGCAAGACCCTGCGAGGCAGGAACACATCCTCTGAATACCAATACTAACTGCTAGAAGAGAAGACTCTGGGTTTACTGGTGCGAGGCTGCCACAGA
GGATGGAAATGTCCTTAAGCGCAGCCCTGAGTTGGAGCTTCAAGTGCTTG-
GTTTGGCAGTGCAACCATCTCATCTCTTTCCACCTGGGTACCAAGTCTCTTCTGCTTGGTGATGGTACTCCTTTTGCAGTGGACACAGGACTATATTC
TCTGTGAAGACAAACATTGAAGCTCAACAAGAGACTGGAAGGACCATAAATTTAAATGGAGAAAGGACCCTCAAGACAAA

Protein seq (343 a.a.)

MWFLTLLLWVPVDGQVDTTKAVITLQPPWVSVFQEETVTLHCEVLHLPSSSTQWFLNGTATQTSTPSYRITSASVNDSGEYRCQRLSGRSDPIQLEIHRG
WLLLQVSSRVFTEGEPLALRCHAWKDKLVYNVLYYRNGKAFKFFHWNSNLTILKTNISHNGTYHCSGMGKHRYTSAGISVTVKELFPAPVLNASVTSPLLEGNL
VTLSCEKLLLRPGLQLYFSFYMGSKTLRGRNTSSEYQILTARREDSGLYWCEATEDGNVLKRSPLELQVLGLAVSTISSFFPPGYQVSFCLVMVLLFAVD TGL
YFSVKTNIRSSTRDWKDHKFKWRKDPQDK

Figure 11

Mod4 DNA (CD28-CD134-CD137-CD3z)

AGGAGTAAGAGGAGCAGGCTCCTGCACAGTGACTACATGAACATGACTCCCCGCCGCCCGGGCCACCCGCAAGCATTACCAGCCCTATGCCCCA
CCACGCGACTTCGCAGCCTATCGCTCCg gatctCGGAGGGACCAGAGGCTGCCCCCGATGCCCAAGCCCCCTGGGGGAGGCAGTTCCGGACC
CCCATCCAAGAGGAGCAGGCCGACGCCCACTCCACCCTGGCCAAGATCg gatctCGTTTCTCTGTAAACGGGGCAGAAAGAACTCCTGTATATATT
CAAACAACCATTTATGAGACCAGTACAACTACTCAAGAGGAAGATGGCTGTAGCTGCCGATTTCCAGAAGAAGAAGGAGGATGTGAAGTg
gatctAGAGTGAAGTTCAGCAGGAGCGCAGACGCCCCGCGTACCAGCAGGGCCAGAACCAGCTCTATAACGAGCTCAATCTAGGACGAAGAGAG
GAGTACGATGTTTTGGACAAGAGACGTGGCCGGGACCCTGAGATGGGGGAAAGCCGCAGAGAAGGAAGAACCCTCAGGAAGGCCTGTACAAT
GAACTGCAGAAAGATAAGATGGCGGAGGCCTACAGTGAGATTGGGATGAAAGGCGAGCGCCGGAGGGGCAAGGGGCACGATGGCCTTTACCAG
GGTCTCAGTACAGCCACCAAGGACACCTACGACGCCCTTCACATGCAGGCCCTGCCCCCTCGCTAA

Mod4 Protein seq (243 a.a.)

RSKRSRLHSDYMNMTPRRPGPTRKHYPYAPPRDFAAYRSGSRRDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKIGSRFSVKRGRKKLLYIFKQP
FMRPVQTTQEEDGCSCRFPEEEEGGCELGSRVKFSRSADAPAYQQGNQLYNELNLGRREEYDVLDRRRGRDPEMGGKPQRRKNPQEGLYNELQKDK
MAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

Figure 12

64-16D-M4G DNA

ATGTGGTTCTTGACAACTCTGCTCCTTTGGGTTCCAGTTGATGGGCAAGTGGACACCACAAAGGCAGTGATCACTTTGCAGCCTCCATGGGTCA
 GCGTGTTCCAAGAGGAAACCGTAACCTTGCAATTGTGAGGTGCTCCATCTGCCTGGGAGCAGCTCTACACAGTGGTTTCTCAATGGCACAGCCAC
 TCAGACCTCGACCCCAGCTACAGAATCACCTCTGCCAGTGTCAATGACAGTGGTGAATACAGGTGCCAGAGAGGTCTCTCAGGGCGAAGTGA
 CCCCATACAGCTGGAAATCCACAGAGGCTGGCTACTACTGCAGGTCTCCAGCAGAGTCTTCACGGAAGGAGAACCTCTGGCCTTGAGGTGTCA
 TGCGTGGAAGGATAAGCTGGTGTACAATGTGCTTTACTATCGAAATGGCAAAGCCTTTAAGTTTTTCCACTGGAATTCTAACCTCACCATTCTGAA
 AACCAACATAAGTCACAATGGCACCTACCATTGCTCAGGCATGGGAAAGCATCGCTACACATCAGCAGGAATATCTGTCACTGTGAAAGAGCTAT
 TTCCAGCTCCAGTGTGAATGCATCTGTGACATCCCCACTCTGGAGGGGAATCTGGTCACCCTGAGCTGTGAAACAAAGTTGCTCTTGACAG
 GCCTGGTTTGACGCTTTACTTCTCTTCTACATGGGCAGCAAGACCCTGCGAGGCAGGAACACATCCTCTGAATACCAAATACTAACTGCTAGAA
 GAGAAGACTCTGGGTATACTGGTGCAGGCTGCCACAGAGGTGAAATGTCCTTAAGCGCAGCCCTGAGTTGGAGCTTCAAGTGCTTGGCC
 TCCAGTTACCAACTCCTGTCTGGTTTCATTACCAAGTCTCTTTCTGCTTGGTGTGTTACTCCTTTTGCAGTGGACACAGGACTATATTTCTCTGT
 GAAGACAAACATTGGAAGCTCAACAAGAGACTGGAAGGACCATAAATTTAAATGGAGAAAGGACCCTCAAGACAAAggatctAGGAGTAAGAG
 GAGCAGGggCggGCACAGTACTACATGAACATGACTCCCCGCCGCCCGGGCCACCCGCAAGCATTACCAGCCCTATGCCCCACCACGCGAC
 TTCGACGCTATCGCTCCggatctCGGAGGGACCAGAGGTGCCCCCGATGCCACAAGCCCCCTGGGGGAGGCAGTTCCGGACCCCATCC
 AAGAGGAGCAGGCCGACGCCCCTCCACCCTGGCCAAGATCggatctCGTTTCTCTGTTAAACGGGGCAGAAAGAACTCCTGTATATATTCAA
 CAACCATTATGAGACCAGTACAACTACTCAAGAGGAAGATGGCTGTAGCTGCCGATTTCCAGAAGAAGAAGGAGGATGTGAACTGggat
 ctAGAGTGAAGTTCAGCAGGAGCGCAGACGCCCCCGGTACCAGCAGGGCCAGAACCAGCTCTATAACGAGCTCAATCTAGGACGAAGAGAG
 GAGTACGATGTTTTGGACAAGAGAGCTGGCCGGGACCCTGAGATGGGGGAAAGCCGAGAGAAGGAAGAACCCTCAGGAAGGCCTGTAC
 AATGAACTGCAGAAAGATAAGATGGCGGAGGCTACAGTGAGATTGGGATGAAAGGCGAGCGCGGAGGGGCAAGGGGCACGATGGCCTTT
 ACCAGGTCTCAGTACAGCCACCAAGGACACCTACGACGCCCTTCACATGCAGGCCCTGCCCCCTCGCTAA

64-16D-M4G Protein (585 a.a.)

MWFLTLLLWVPVDGQVDTTKAVITLQPPWVSVFQEETVTLHCEVLHLPSSSTQWFLNGTATQTSTPSYRITSASVNDSGEYRCQRLSGRSDPIQ
 LEIHRGWLLLQVSSRVFTEGEPLALRCHAWKDKLVNVLYYRNGKAFKFFHWSNLTILKTNISHNGTYHCSGMGKHRYTSAGISVTVKELFPAPVL
 NASVTSPLLEGNLVTLSCTKLLQRPGLQLYFSFYMGSKTLRGRNTSSEYQILTARREDSGLYWCEATEDGNVLKRSPELELQVLGLQLPTPVWFHY
 QVSFCLVMVLLFAVDGLYFSVKTNIRSSTRDWKDHKFKWRKDPQDKGSRKRSRGGHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRSGSRRD
 QRLPPDAHKKPPGGGSFRTPIQEEQADAHSTLAKIGSRFSVKRGRKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELGSRVKFSRSADAPAYQ
 QGQNQLYNELNLGRREEYDVLKRRGRDPEMGGKPQRRKNPQEGLYNELQDKMAEAYSEIGMKGERRRGKHDGLYQGLSTATKDTYDALHM
 QALPPR

Figure 13

64-16E-M4G DNA

ATGTGGTTCTTGACAACTCTGCTCCTTTGGGTTCCAGTTGATGGGCAAGTGGACACCACAAAGGCAGTGATCACTTTGCAGCCTCCATGGGTCAGC
GTGTTCCAAGAGGAAACCGTAACCTTGCAATTGTGAGGTGCTCCATCTGCCTGGGAGCAGCTCTACACAGTGGTTTTCTCAATGGCACAGCCACTCA
GACCTCGACCCCCAGCTACAGAATCACCTCTGCCAGTGTCAATGACAGTGGTGAATACAGGTGCCAGAGAGGTCTCTCAGGGCGAAGTGACCCCA
TACAGCTGGAAATCCACAGAGGCTGGCTACTACTGCAGGTCTCCAGCAGAGTCTTCACGGAAGGAGAACCTCTGGCCTTGAGGTGTCATGCGTGG
AAGGATAAGCTGGTGACAATGTGCTTTACTATCGAAATGGCAAAGCCTTTAAGTTTTTCCACTGGAATCTAACCTCACCATTCTGAAAACCAACAT
AAGTCACAATGGCACCTACCATTGCTCAGGCATGGGAAAGCATCGCTACACATCAGCAGGAATATCTGTCACTGTGAAAGAGCTATTTCCAGCTCC
AGTGCTGAATGCATCTGTGACATCCCCACTCCTGGAGGGGAATCTGGTCACCCTGAGCTGTGAAACAAAGTTGCTCTTGACAGAGGCTGGTTTGC
AGCTTTACTTCTCCTTCTACATGGGCAGCAAGACCTGCGAGGCAGGAACACATCCTCTGAATACCAATACTAAGTCTGCTAGAAGAGAAGACTCTG
GGTTATACTGGTGCGAGGCTGCCACAGAGGATGGAAATGTCTTAAGCGCAGCCCTGAGTTGGAGCTTCAAGTCTTGGTTTGGCAGTGTCAACC
ATCTCATCATTCTTCCACCTGGGTACCAAGTCTCTTTCTGCTTGGTGATGGTACTCCTTTTTGCAGTGGACACAGGACTATATTTCTCTGTGAAGAC
AAACATTCGAAGCTCAACAAGAGACTGGAAGGACCATAAATTTAATGGAGAAAGGACCCTCAAGACAAAggatctAGGAGTAAGAGGAGCAGGg
gCggGCACAGTGACTACATGAACATGACTCCCCGCCGCCCGGGGCCACCCGCAAGCATTACCAGCCCTATGCCCCACCACGCGACTTCGCAGCCT
ATCGTCCggatctCGGAGGGACCAGAGGCTGCCCCCGATGCCACAAGCCCCCTGGGGGAGGCAGTTTCCGGACCCCCATCAAGAGGAGCAG
GCCGACGCCCACTCCACCCTGGCCAAGATCggatctCGTTTCTCTGTAAACGGGGCAGAAAGAACTCCTGTATATATTCAAACAACCATTTATGAG
ACCAAGTACAACTACTCAAGAGGAAGATGGCTGTAGCTGCCGATTTCCAGAAGAAGAAGAAGGAGGATGTGAACTGggatctAGAGTGAAGTTCA
GCAGGAGCGCAGACGCCCCCGCTACCAGCAGGGCCAGAACAGCTCTATAACGAGCTCAATCTAGGACGAAGAGAGGAGTACGATGTTTTGGA
CAAGAGACGTGGCCGGGACCCTGAGATGGGGGAAAGCCGAGAGAAGGAAGAACCCTCAGGAAGGCCTGTACAATGAACTGCAGAAAGATA
AGATGGCGGAGGCCTACAGTGAGATTGGGATGAAAGGCGAGCGCCGGAGGGGCAAGGGGCACGATGGCCTTTACCAGGGTCTCAGTACAGCC
ACCAAGGACACCTACGACGCCCTTACATGCAGGCCCTGCCCCCTCGCTAA

64-16E-M4G Protein (588 a.a.)

MWFLTLLLLWVPVDGQVDTTKAVITLQPPWVSFQEETVLHCEVLHLPSSSTQWFLNGTATQTSTPSYRITSASVNDSGEYRCQRGLSGRSDPIQ
LEIHRGWLLLQVSSRVFTEGEPLALRCHAWKDKLVYNVLYRNGKAFKFFHWNLSNLTKTNISHNGTYHCSGMGKHRYTSAGISVTVKELFPAPVLN
ASVTSPLLEGNLVTLSCETKLLLQRPGLQLYFSFYMGSKTLRGRNTSSEYQILTRREDSGLYWCEAATEDGNVLKRSPELELQVLGLAVSTISSFFPPGY
QVSFCLVMVLLFAVDGTGLYFSVKTNIRSSTRDWKDHKFKWRKDPQDKGSRSKSRGGHSDYMNMTPRRPGPTRKHYPYAPPRDFAAYRSGSRRD
QRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKIGSRFSVKRGRKLLYIFKQPFMRPVQTTQEEDGCSRFPEEEEGGCELGSRVKFSRSADAPAYQ
QQQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPQRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGGHDGLYQGLSTATKDTYDALHM
QALPPR

Figure 14