

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
24 June 2010 (24.06.2010)

(10) International Publication Number
WO 2010/071880 A1

PCT

(51) International Patent Classification:
A61K 39/395 (2006.01)

(21) International Application Number:
PCT/US2009/068966

(22) International Filing Date:
21 December 2009 (21.12.2009)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/139,098 19 December 2008 (19.12.2008) US

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))

(54) Title: TNFR25 AGONISTS TO ENHANCE IMMUNE RESPONSES TO VACCINES

(57) Abstract: TNFR25 compositions enhance the immune response against antigens. Administration of TNFR25 agonists was found to enhance tumor rejection, responses against viral diseases and other infectious organisms.



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TNFR25 AGONISTS TO ENHANCE IMMUNE RESPONSES TO VACCINES

STATEMENT AS TO FEDERALLY FUNDED RESEARCH

[0001] This invention was made with United States government support under grant number CA109094 awarded by the National Cancer Institute. The United States government has certain rights in the invention.

CROSS-REFERENCE TO RELATED APPLICATIONS

[0002] The present application claims the priority of U.S. provisional patent application No. 61/139,098 filed December 19, 2008, which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[0003] Embodiments of the invention relate to novel compositions and methods utilizing immunomodulating agents that can modulate the immune system or in other cases have an immunosuppressive effect. TNFR25 agonists disclosed herein enhance immune response to vaccines.

BACKGROUND

[0004] An immune response to antigen requires the presence of an antigen-presenting cell (APC), (usually either a macrophage or dendritic cell) in combination with a B cell or T cell. When an APC presents an antigen on its cell surface to a B cell, the B cell is signaled to proliferate and produce antibodies that specifically bind to that antigen. If the antibodies bind to antigens on bacteria or parasites it acts as a signal for granulocytes or polymorphonuclear leukocytes (PMNs) or macrophages to phagocytose and kill them. Another important function of antibodies is to initiate the "complement destruction cascade." When antibodies bind to cells or bacteria, serum proteins called complement bind to the immobilized antibodies and destroy the bacteria by creating holes in them. Antibodies can also signal natural killer cells and macrophages to kill viral or bacterial-infected cells.

[0005] If the APC presents the antigen to T cells, the T cells become activated. Activated T cells proliferate and become secretory in the case of CD4⁺ T cells, or, if they are CD8⁺ T

cells, they become activated to kill target cells that specifically express the antigen presented by the APC. The production of antibodies and the activity of CD8⁺ killer T cells are highly regulated by the CD4⁺ helper T cell subset. The CD4⁺ T cells provide growth factors or signals to these cells that signal them to proliferate and function more efficiently. This multitude of interleukins or cytokines that are produced and secreted by CD4⁺ T cells are often crucial to ensure the activation of natural killer cells, macrophages, CD8⁺ T cells, and PMNs.

[0006] T lymphocytes play a central role in regulating immune responses. Helper T cells express the CD4 surface marker and provide help to B cells for antibody production and help CD8 T cells to develop cytotoxic activity. Other CD4 T cells inhibit antibody production and cytotoxicity. T cells regulate the equilibrium between attack of infected or tumorigenic cells and tolerance to the body's cells. A dysregulated immune attack can lead to autoimmunity, while diminished immune responsiveness results in chronic infection and cancer.

[0007] Tumor Necrosis Factor Receptor 25 (TNFR25) also interchangeably referred to herein as Death receptor 3 (DR3), is a regulator of T cell function. Death receptor 3 (DR3) (Chinnaiyan *et al.*, *Science* 274:990, 1996) is a member of the TNF-receptor family. It is also known as TRAMP (Bodmer *et al.*, *Immunity* 6:79, 1997), wsl-1 (Kitson *et al.*, *Nature* 384:372, 1996), Apo-3 (Marsters *et al.*, *Curr Biol* 6:1669, 1996), and LARD (Screaton *et al.*, *Proc Natl Acad Sci USA* 94:4615, 1997) and contains a typical death domain. Transfection of 293 cells with human DR3 (hDR3) induced apoptosis and activated NF- κ B. Multiple spliced forms of human DR3 mRNA have been observed, indicating regulation at the post transcriptional level (Screaton *et al.*, *Proc Natl Acad Sci USA* 94:4615, 1997).

[0008] Many TNF-receptor family members have the ability to induce cell death by apoptosis or induce costimulatory signals for T cell function. The regulation of these opposing pathways has recently been clarified for TNF-R1, the prototypic death domain-containing receptor that can cause apoptosis or proliferation of receptor positive T cells (Micheau and Tschopp. *Cell* 114:181, 2003). NF- κ B activation by a signaling complex composed of TNF-R1 via TRADD, TRAF2 and RIP induces FLIPL association with a second signaling complex composed of TNFR1, TRADD and FADD, preventing caspase 8 activation as long as the NF- κ B signaling persists. DR3 has been shown to be able to induce apoptosis in transfected cells and to induce NF- κ B and all three MAP-kinase pathways (Chinnaiyan *et al.*, *Science* 274:990, 1996; Bodmer *et al.*, *Immunity* 6:79, 1997; Kitson *et al.*, *Nature* 384:372, 1996; Marsters *et al.*, *Curr Biol* 6:1669, 1996; Screaton *et al.*, *Proc Natl*

Acad Sci USA 94:4615, 1997; Wen *et al.*, *J Biol Chem* 25:25, 2003). Blocking of NF- κ B, but not of MAP-kinase and inhibition of protein synthesis resulted in DR3-mediated cell death, indicating that NF- κ B signals mediate anti-apoptotic effects through the synthesis of anti-apoptotic proteins.

[0009] Expression of human DR3 mRNA is pronounced in lymphoid tissues, mainly in the spleen, lymph nodes, thymus, and small intestine, indicating an important role for DR3 in lymphocytes. Murine DR3 has been deleted by homologous recombination in embryonic stem cells (Wang *et al.*, *Mol Cell Biol* 21:3451, 2001). DR3^{-/-} mice show diminished negative selection by anti-CD3 in the thymus but normal negative selection by superantigens and unimpaired positive selection of thymocytes. Mature peripheral T cells were unaffected by DR3 deficiency. Despite a significant amount of preliminary research, the physiological function of DR3 remains poorly characterized.

SUMMARY

[0010] This Summary is provided to present a summary of the invention to briefly indicate the nature and substance of the invention. It is submitted with the understanding that it will not be used to interpret or limit the scope or meaning of the claims.

[0011] Agonistic TNFR25 compositions improved tumor rejection, enhanced immune responses to HIV by HIV-gp96-Ig vaccination and prevented the generation of antigen specific Treg induced by CD103⁺ dendritic cells. The compositions are of great value in combating cancer, infectious disease and as biological terror-defense.

[0012] Other aspects are described *infra*.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] Figure 1 is a graph showing agonistic TNFR25 antibody 4C12 enhances CD8 CTL expansion mediated by Gp96-Ig vaccines. Lymphocytes: PBL – peripheral blood; SPL – spleen; PEC – peritoneal cavity; PPL – Peyer's Patch lymphocytes; LPL – lamina propria lymphocytes; IEL – intraepithelial lymphocytes.

[0014] Figure 2 is a graph showing TNFR25 agonists enhance expansion of HIV-specific CD8 CTL in response to gp96-Ig vaccination. Abbreviations as in Figure 1.

[0015] Figures 3A, 3B: are graphs showing that TNFR25 agonists synergize with gp96-Ig vaccination to induce CTL expansion. Figure 3A: Tumor naïve C57BL/6 mice were adoptively transferred with OT-I/GFP cells on day -2 (10⁶ cells, intravenous injection). On

day 0, mice were injected with either PBS control, 3T3-ova-gp96 cells (10^6 cells), the TNFR25-agonistic antibody clone 4C12 (20 μ g) or a combination of both the 3T3-ova-gp96 cells and the 4C12 antibody (all injections were given by intraperitoneal injection). Some animals (as indicated) were subsequently treated with rapamycin (75 μ g/kg) daily beginning on day 3. The percentage of OT-I cells out of total CD8⁺ cells was measured by flow cytometry from peripheral blood cells daily. Figure 3B: Following the same protocol described in Figure 3A, some animals were sacrificed on day 5 and peritoneal lavage fluid was collected and analyzed by flow cytometry for the percentage of OT-I cells out of total CD8⁺ cells. Data are represented as mean $\pm$ S.E.M. from a total of 3 experiments with ≥ 2 mice per group per experiment.

[0016] Figures 4A-4C are graphs showing TNFR25 agonists enhance tumor rejection in multiple tumor models. Figure 4A: EG7 tumors were established for 7 days before a course of vaccinations with EG7-gp96-Ig with or without 4C12 was initiated on day 7. Treatments were repeated every 3 days for a total of 5 injections. Survival was determined when tumor size exceeded 225 mm². Figure 4B: Experiments were performed as in Figure 4A except using the LLC-ova tumor model. Figure 4C: Experiments were performed as in Figures 4A and 4B except that TL1A was transfected into the vaccine cell instead of combined administration of 4C12. Tumor size is shown over time.

DETAILED DESCRIPTION

[0017] Several aspects of the invention are described below with reference to example applications for illustration. It should be understood that numerous specific details, relationships, and methods are set forth to provide a full understanding of the invention. One having ordinary skill in the relevant art, however, will readily recognize that the invention can be practiced without one or more of the specific details or with other methods. In other instances, well-known structures or operations are not shown in detail to avoid obscuring the invention. The present invention is not limited by the illustrated ordering of acts or events, as some acts may occur in different orders and/or concurrently with other acts or events. Furthermore, not all illustrated acts or events are required to implement a methodology in accordance with the present invention.

[0018] Unless otherwise defined, all terms (including technical and scientific terms) used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. It will be further understood that terms, such as those defined in

commonly used dictionaries, should be interpreted as having a meaning that is consistent with their meaning in the context of the relevant art and will not be interpreted in an idealized or overly formal sense unless expressly so defined herein.

Definitions

[0019] The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention. As used herein, the singular forms "a", "an" and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise. Furthermore, to the extent that the terms "including", "includes", "having", "has", "with", or variants thereof are used in either the detailed description and/or the claims, such terms are intended to be inclusive in a manner similar to the term "comprising."

[0020] Throughout this application, the term "about" is used to indicate that a value includes the standard deviation of error for the device or method being employed to determine the value.

[0021] The terms "TNFR-SF25", "TNFR25" or "DR3" are all used interchangeably herein for a member of the TNF receptor family whose complete biological function was previously not known. See U.S. Pat. No. 6,713,061, and Borysenko, *et al.*, *Biochem Biophys Res Commun.* 2005 Mar. 18;328(3):794-9, Sheikh, *et al.*, *Curr. Cancer Drug Targets.* 2004 February;4(1):97-104, Podack *et al.*, US publication number 20070128184, which are incorporated by reference in their entirety.

[0022] "TNFR25 agonist" is referred to herein as a substance that binds to the TNFR25 receptor and triggers a response in the cell on which the TNFR25 receptor is expressed similar to a response that would be observed by exposing the cell to a natural TNFR25 ligand, e.g., TL1A. An agonist is the opposite of an antagonist in the sense that while an antagonist may also bind to the receptor, it fails to activate the receptor and actually completely or partially blocks it from activation by endogenous or exogenous agonists. A partial agonist activates a receptor but does not cause as much of a physiological change as does a full agonist. Alternatively, another example of a TNFR25 agonist is an antibody that is capable of binding and activating TNFR25.

[0023] "TNFR25 antagonist" is referred to herein as a substance that inhibits the normal physiological function of a TNFR25 receptor. Such agents work by interfering in the binding

of endogenous receptor agonists/ligands such as TL1A, with TNFR25 receptor. An example of a TNFR25 antagonist is a dominant negative TNFR25 receptor.

[0024] TNFR25 antagonists or agonists may be in the form of aptamers. "Aptamers" are DNA or RNA molecules that have been selected from random pools based on their ability to bind other molecules.

[0025] As used herein, the term "antibody" is inclusive of all species, including human and humanized antibodies and the antigenic target, for example, TNFR25, can be from any species. Thus, an antibody, for example, anti-TNFR25 can be mouse anti-human TNFR25, goat anti-human TNFR25; goat anti-mouse TNFR25; rat anti-human TNFR25; mouse anti-rat TNFR25 and the like. The combinations of antibody generated in a certain species against an antigen target, e.g. TNFR25, from another species, or in some instances the same species (for example, in autoimmune or inflammatory response) are limitless and all species are embodied in this invention. The term antibody is used in the broadest sense and includes fully assembled antibodies, monoclonal antibodies (including human, humanized or chimeric antibodies), polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments that can bind antigen (e.g., Fab', F'(ab)₂, Fv, single chain antibodies, diabodies), comprising complementarity determining regions (CDRs) of the foregoing as long as they exhibit the desired biological activity.

[0026] Depending on the amino acid sequence of the constant domain of their heavy chains, human immunoglobulins can be assigned to different classes. There are five major classes, IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses or isotypes, e.g. IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called alpha, delta, epsilon, gamma and mu respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known. Different isotypes have different effector functions; for example, IgG1 and IgG3 isotypes have ADCC activity. The invention contemplates that antibodies of any class or subclass may be prepared, including IgA, IgD, IgE, IgG and IgM, although IgG is preferred.

[0027] The term "heat shock protein", as used herein, refers to any protein which exhibits increased expression in a cell when the cell is subjected to a stress. In preferred non-limiting embodiments, the heat shock protein is originally derived from a eukaryotic cell; in more preferred embodiments, the heat shock protein is originally derived from a mammalian cell. For example, but not by way of limitation, heat shock proteins which may be used according

to the invention include BiP (also referred to as grp78), hsp/hsc70, gp96(grp94), hsp60, hsp40, and hsp90. Especially preferred heat shock proteins are BiP, gp96, and hsp70. In preferred embodiments, the heat shock protein is gp96. Naturally occurring or recombinantly derived mutants of heat shock proteins may also be used according to the invention.

[0028] An "immunogenic polypeptide" or "antigen" is a polypeptide derived from the cell or organism that elicits in a subject an antibody-mediated immune response (i.e., a "B cell" response or humoral immunity), a cell-mediated immune response (i.e. a "T cell" response), or a combination thereof. A cell-mediated response can involve the mobilization helper T cells, cytotoxic T-lymphocytes (CTLs), or both. Preferably, an immunogenic polypeptide elicits one or more of an antibody-mediated response, a CD4⁺ Th1-mediated response (Th1: type 1 helper T cell), and a CD8⁺ T cell response. It should be understood that the term "polypeptide" as used herein refers to a polymer of amino acids and does not refer to a specific length of a polymer of amino acids. Thus, for example, the terms peptide, oligopeptide, and protein are included within the definition of polypeptide.

[0029] As used herein, "contacting" means placing the biological sample in sufficient proximity to the agent and under the appropriate conditions of, e.g., concentration, temperature, time, ionic strength, to allow the specific interaction between the agent and tumor associated nucleic acid or polypeptide that are present in the biological sample. In general, the conditions for contacting the agent with the biological sample are conditions known by those of ordinary skill in the art to facilitate a specific interaction between a molecule and its cognate (e.g., a protein and its receptor cognate, an antibody and its protein antigen cognate, a nucleic acid and its complementary sequence cognate) in a biological sample. Exemplary conditions for facilitating a specific interaction between a molecule and its cognate are described in U.S. Pat. No. 5,108,921, issued to Low *et al.*

TNFR25 Compositions

[0030] Unlike that of any other member of the TNF-R family, DR3 expression was found to be controlled by alternative mRNA splicing. Resting T cells express little or no DR3 protein, but contained high levels of randomly spliced DR3 mRNA. Upon T cell activation via the T cell receptor, protein kinase C (PKC) is activated. PKC activation in turn mediates correct splicing of full-length DR3 and surface expression of the protein. This unique regulation of DR3 expression allows for rapid DR3 protein expression on T cells and enables environmental regulation of DR3 expression via influencing PKC levels responsible for DR3

splicing and expression. DR3 is also involved in co-stimulating T cell polarization and in stimulating the production of IL-13 and IL-10 in Th2 polarized cells.

[0031] Transgenic expression of TNFR25 in T cells mediates Th2 polarization of cytokine and antibody production upon T cell activation and antigen exposure. In addition transgenic TNFR25 partially inhibits TCR driven proliferation of CD4 and CD8 cells and reduced total T cell numbers in lymphoid organs without inducing apoptosis. CD8 cells were more affected by TNFR25 than CD4 cells. As such, TNFR25 signals are important in effector responses to pathogens by shaping the ensuing polarization towards Th2 or towards a mixed Th1/Th2 response.

[0032] TNFR25 transgenic mice are highly susceptible to antigen induced airway inflammation in an asthma model in mice and produced increased quantities of IL-13 and eosinophils in the lung upon antigen exposure by inhalation. Transgenic mice expressing a dominant negative form of TNFR25 showed increased resistance to airway hyper reactivity when compared to wild type mice.

[0033] In a preferred embodiment a TNFR25 agonist modulates an immune response against an antigen. Preferably, the modulation enhances or up-regulates the immune response against a desired antigen. An example of an anti-TNFR antibody is 4C12 (BUDAPEST RESTRICTED CERTIFICATE OF DEPOSIT; BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE INTERNATIONAL FORM RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3 AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2. Deposited under the Budapest Treaty on Behalf of: University of Miami; Date of Receipt of seeds/strain(s) by the ATCC®: May 5, 2009; ATCC ®Patent Deposit Designation: PTA-10000. Identification Reference by Depositor: Hybridoma cell line; 4C12; The deposit was tested June 4, 2009 and on that date, the seeds/strain(s) were viable. International Depository Authority: American Type Culture Collection (ATCC®), Manassas, VA, USA).

[0034] The amount of a TNFR25 agonist, for example anti-TNFR25 antibody dose is selected as an amount which induces a robust immune response without significant, adverse side effects. Such amount will vary depending upon the age, condition, sex, and the like of a patient, including routes of administration, and, if required, adjuvants used. In general, the dose administered to a patient, in the context of the present invention should be sufficient to effect a beneficial therapeutic response in the patient over time, or to induce the production of

antigen-specific immune cells. Thus, the composition is administered to a patient in an amount sufficient to elicit an immune response to the specific antigens and/or to alleviate, reduce, or cure symptoms and/or complications from the disease or infection. An amount adequate to accomplish this is defined as a "therapeutically effective dose."

[0035] As used herein, the term "TNFR25 composition" comprises agonists and antagonists.

[0036] In another preferred embodiment, the immune response comprises one or more immune components: cytokines, T cells (T lymphocytes), B cells (B lymphocytes), antigen presenting cells, dendritic cells, monocytes, macrophages, myeloid suppressor cells, natural killer (NK) cells, cytotoxic T lymphocytes (CTLs), CTL lines, CTL clones, CTLs from tumor, inflammatory, or other infiltrates and subsets thereof.

[0037] In one preferred embodiment, a TNFR25 agonist comprises an antibody, aptamer, ligand, peptide, oligonucleotide, organic molecule, inorganic molecule, protein or nucleic acid molecule.

[0038] In another preferred embodiment, a TNFR25 agonist comprises an anti-TNFR25 antibody. The TNFR25 antibody can be from any species and the target antigen can be from any species.

[0039] In another preferred embodiment, the anti-TNFR25 can target antigens from more than one species, or be bispecific targeting different epitopes from different species.

[0040] In another preferred embodiment, an anti-TNFR25 antibody is administered with one or more vaccines or therapeutic agents. For example, an agonistic anti TNFR25 antibody i. improves tumor rejection by gp96-vaccine; ii. enhances immune responses to HIV by HIV-gp96-Ig vaccination; iii. prevents the generation of antigen specific Treg induced by CD103⁺ dendritic cells (DC). See, for example, Figures 1 and 2. The findings described herein, provide illustrative examples of the utility of TNFR25 agonists in, for example, cancer vaccine, infectious disease vaccine including HIV, and as potential use in therapeutic efforts for biological terror-defense, and the like.

[0041] In another preferred embodiment, a method of modulating an antigen specific immune response *in vivo*, comprising: administering to a patient, a therapeutically effective amount of an agent and an agonistic anti-TNFR25 antibody. In preferred embodiments, the agonistic anti-TNFR25 antibody up-regulates an agent-induced immune response as compared to the agent-induced immune response in the absence of the agonistic anti-TNFR25 antibody.

[0042] In another preferred embodiment, the anti-TNFR25 is administered to a patient prior to, concurrently with, or after administration of the agent.

[0043] In a preferred embodiment, the agent induces an antigen specific immune response.

[0044] In another preferred embodiment, an anti-TNFR25 antibody decreases or prevents generation of antigen specific Treg cells.

[0045] In another preferred embodiment, the agent is a vaccine and induces an antigen specific response which is enhanced by the anti-TNFR25 composition. For example, the agent is a tumor vaccine and administration of the anti-TNFR25 antibody increases an anti-tumor immune response as compared to the anti-tumor immune response in the absence of the anti-TNFR25 antibody.

[0046] In another preferred embodiment, there is an anti-HIV vaccine and administration of the anti-TNFR25 antibody enhances an anti-HIV immune response as compared to the anti-HIV immune response in the absence of the anti-TNFR25 antibody. In one example, an anti-HIV vaccine comprises HIV-gp96-Ig.

[0047] In another preferred embodiment, the anti-TNFR25 antibody enhances antigen specific immune responses to diseases or disorders comprising tumors, infectious disease organisms, parasites or fungus.

[0048] In another preferred embodiment, a method of enhancing an antigen specific immune response comprising: obtaining a sample from a patient; separating immune cells from the sample; culturing the immune cells with a TNFR25 agonist and/or antigen; expanding the immune cells and re-introducing said cells to a patient. Examples of immune cells comprise antigen presenting cells, T cells, B cells and natural killer cells. The immune cells are preferably cultured with antigen, prior to, concurrently with or after contact with the TNFR25 agonist.

[0049] In another preferred embodiment, the administration of an anti-TNFR25 antibody enhances an immune response to a specific antigen as compared to the antigen specific immune response in the absence of the anti-TNFR25 antibody.

[0050] In another preferred embodiment, the administration of the anti-TNFR25 antibody inhibits generation of antigen specific Treg cells induced by CD103⁺ dendritic cells.

[0051] In another preferred embodiment, the specific antigen comprises at least one of: viral antigen(s), tumor antigen(s), parasitic antigen(s), bacterial antigen(s), protozoan antigen(s) or combinations thereof.

[0052] In yet another preferred embodiment, a method of preventing or treating cancer, comprising: administering to a patient in need thereof, a tumor antigen, an anti-TNFR25 antibody. Preferably, a tumor antigen is derived from a patient tumor comprising at least one of: tumor cell, tumor cell membranes, tumor proteins, tumor nucleic acids or combinations thereof. The patient specific tumor antigen can be obtained via any method, such as for example, biopsy, surgery, fluids etc.

[0053] In another preferred embodiment, a tumor antigen comprises a tumor vaccine.

[0054] In another preferred embodiment, the anti-TNFR25 antibody is administered in conjunction, prior to or after administration of a tumor antigen or vaccine.

[0055] In another preferred embodiment, a method of prevent or treating a disease caused by a biological agent, comprising administering to a patient in need thereof, a vaccine or a biological agent antigen, an anti-TNFR25 antibody. Preferably, the biological agent antigen comprises at least one of: viral antigen(s), tumor antigen(s), parasitic antigen(s), bacterial antigen(s), protozoan antigen(s) or combinations thereof.

[0056] In one embodiment, the biological agent antigen comprises a viral vaccine, such as, for example, an HIV vaccine, influenza vaccine etc.

[0057] In another preferred embodiment, a method of enhancing an antigen specific immune response *in vivo*, comprises administering to a patient in need thereof, an antigen, a TNFR25 agonist. In one embodiment, the TNFR25 agonist is an anti-TNFR25 antibody.

[0058] In another preferred embodiment, the administration of an anti-TNFR25 antibody enhances an immune response to a specific antigen as compared to the antigen specific immune response in the absence of the anti-TNFR25 antibody. The administration of the anti-TNFR25 antibody preferably inhibits generation of antigen specific Treg cells induced by CD103⁺ dendritic cells. The specific antigen comprises at least one of: viral antigen(s), tumor antigen(s), parasitic antigen(s), bacterial antigen(s), protozoan antigen(s) or combinations thereof.

[0059] In one embodiment, the TNFR25 compositions of the present invention are targeted to the cells involved in modulation of the immune system, such as, for example, immune effector cells, cells involved in the regulation of the immune system, *e.g.* T regulatory cells (Treg), MSC, antigen presenting cells and the like. Examples of antigen presenting cells include, dendritic cells, B cells, monocytes/macrophages.

[0060] *Immune System:* Immune systems are classified into two general systems, the "innate" or "primary" immune system and the "acquired/adaptive" or "secondary" immune

system. It is thought that the innate immune system initially keeps the infection under control, allowing time for the adaptive immune system to develop an appropriate response. Studies have suggested that the various components of the innate immune system trigger and augment the components of the adaptive immune system, including antigen-specific B and T lymphocytes (Kos, *Immunol. Res.* 1998, 17:303; Romagnani, *Immunol. Today*. 1992, 13: 379; Banchereau and Steinman, *Nature*. 1988, 392:245).

[0061] A primary immune response refers to an innate immune response that is not affected by prior contact with the antigen. The main protective mechanisms of primary immunity are the skin (protects against attachment of potential environmental invaders), mucous (traps bacteria and other foreign material), gastric acid (destroys swallowed invaders), antimicrobial substances such as interferon (IFN) (inhibits viral replication) and complement proteins (promotes bacterial destruction), fever (intensifies action of interferons, inhibits microbial growth, and enhances tissue repair), natural killer (NK) cells (destroy microbes and certain tumor cells, and attack certain virus infected cells), and the inflammatory response (mobilizes leukocytes such as macrophages and dendritic cells to phagocytose invaders).

[0062] Some cells of the innate immune system, including macrophages and dendritic cells (DC), function as part of the adaptive immune system as well by taking up foreign antigens through pattern recognition receptors, combining peptide fragments of these antigens with major histocompatibility complex (MHC) class I and class II molecules, and stimulating naive CD8⁺ and CD4⁺ T cells respectively (Banchereau and Steinman, *supra*; Holmskov *et al.*, *Immunol. Today*. 1994, 15:67; Ulevitch and Tobias *Annu. Rev. Immunol.* 1995, 13:437). Professional antigen-presenting cells (APCs) communicate with these T cells, leading to the differentiation of naive CD4⁺ T cells into T-helper 1 (Th1) or T-helper 2 (Th2) lymphocytes that mediate cellular and humoral immunity, respectively (Trinchieri *Annu. Rev. Immunol.* 1995, 13:251; Howard and O'Garra, *Immunol. Today*. 1992, 13:198; Abbas *et al.*, *Nature*. 1996, 383:787; Okamura *et al.*, *Adv. Immunol.* 1998, 70:281; Mosmann and Sad, *Immunol. Today*. 1996, 17:138; O'Garra *Immunity*. 1998, 8:275).

[0063] A secondary immune response or adaptive immune response may be active or passive, and may be humoral (antibody based) or cellular that is established during the life of an animal, is specific for an inducing antigen, and is marked by an enhanced immune response on repeated encounters with said antigen. A key feature of the T lymphocytes of the adaptive immune system is their ability to detect minute concentrations of pathogen-derived peptides presented by MHC molecules on the cell surface. Upon activation, naïve CD4 T

cells differentiate into one of at least two cell types, Th1 cells and Th2 cells, each type being characterized by the cytokines it produces. "Th1 cells" are primarily involved in activating macrophages with respect to cellular immunity and the inflammatory response, whereas "Th2 cells" or "helper T cells" are primarily involved in stimulating B cells to produce antibodies (humoral immunity). CD4 is the receptor for the human immunodeficiency virus (HIV). Effector molecules for Th1 cells include, but are not limited to, IFN- γ , GM-CSF, TNF- α , CD40 ligand, Fas ligand, IL-3, TNF- β , and IL-2. Effector molecules for Th2 cells include, but are not limited to, IL-4, IL-5, CD40 ligand, IL-3, GS-CSF, IL-10, TGF- β , and eotaxin. Activation of the Th1 type cytokine response can suppress the Th2 type cytokine response, and reciprocally, activation of the Th2 type cytokine response can suppress the Th1 type response.

[0064] In adaptive immunity, adaptive T and B cell immune responses work together with innate immune responses. The basis of the adaptive immune response is that of clonal recognition and response. An antigen selects the clones of cell which recognize it, and the first element of a specific immune response must be rapid proliferation of the specific lymphocytes. This is followed by further differentiation of the responding cells as the effector phase of the immune response develops. In T-cell mediated non-infective inflammatory diseases and conditions, immunosuppressive drugs inhibit T-cell proliferation and block their differentiation and effector functions.

[0065] In a preferred embodiment, the TNFR25 compositions modulate T cell responses. Preferably, the TNFR25 enhances or up-regulates the T cell response, as compared to a control, such as for example, in the absence of a TNFR25 composition.

[0066] In another preferred embodiment, the T cell response is directed to a specific antigen, e.g. viral tumor, bacterial and the like.

[0067] The phrase "T cell response" means an immunological response involving T cells. The T cells that are "activated" divide to produce memory T cells or cytotoxic T cells. The cytotoxic T cells bind to and destroy cells recognized as containing the antigen. The memory T cells are activated by the antigen and thus provide a response to an antigen already encountered. This overall response to the antigen is the T cell response.

[0068] In another preferred embodiment, the TNFR25 compositions modulate immune cells. Preferably, the TNFR25 compositions increase or enhance the response of the immune cells to a specific antigen, for example, viral antigen, tumor antigen and the like.

[0069] "Cells of the immune system" or "immune cells", is meant to include any cells of the immune system that may be assayed, including, but not limited to, B lymphocytes, also called B cells, T lymphocytes, also called T cells, natural killer (NK) cells, natural killer T (NK) cells, lymphokine-activated killer (LAK) cells, monocytes, macrophages, neutrophils, granulocytes, mast cells, platelets, Langerhan's cells, stem cells, dendritic cells, peripheral blood mononuclear cells, tumor-infiltrating (TIL) cells, gene modified immune cells including hybridomas, drug modified immune cells, antigen presenting cells and derivatives, precursors or progenitors of the above cell types.

[0070] In another preferred embodiment, the TNFR25 compositions modulate the response of immune effector cells. Preferably, the immune effector cells are up-regulated or enhanced and directed to a specific antigen.

[0071] "Immune effector cells" refers to cells, and subsets thereof, e.g. Treg, Th1, Th2, capable of binding an antigen and which mediate an immune response selective for the antigen. These cells include, but are not limited to, T cells (T lymphocytes), B cells (B lymphocytes), antigen presenting cells, such as for example dendritic cells, monocytes, macrophages; myeloid suppressor cells, natural killer (NK) cells and cytotoxic T lymphocytes (CTLs), for example CTL lines, CTL clones, and CTLs from tumor, inflammatory, or other infiltrates.

[0072] In another preferred embodiment, the TNFR25 compositions modulate T regulatory cells. Preferably, regulation of Treg cells induces an increase or enhancement of immune cell response to a specific antigen.

[0073] A "T regulatory cell" or "Treg cell" or "Tr cell" refers to a cell that can inhibit a T cell response. Treg cells express the transcription factor *Foxp3*, which is not upregulated upon T cell activation and discriminates Tregs from activated effector cells. Tregs are identified by the cell surface markers CD25, CD45RB, CTLA4, and GITR. Treg development is induced by MSC activity. Several Treg subsets have been identified that have the ability to inhibit autoimmune and chronic inflammatory responses and to maintain immune tolerance in tumor-bearing hosts. These subsets include interleukin 10- (IL-10-) secreting T regulatory type 1 (Tr1) cells, transforming growth factor- β - (TGF- β -) secreting T helper type 3 (Th3) cells, and "natural" CD4⁺/CD25⁺ Tregs (Trn) (Fehervari and Sakaguchi. *J. Clin. Invest.* 2004, 114:1209-1217; Chen *et al. Science*. 1994, 265: 1237-1240; Groux *et al. Nature*. 1997, 389: 737-742).

[0074] The term "myeloid suppressor cell (MSC)" refers to a cell that is of hematopoietic lineage and expresses Gr-1 and CD11b; MSCs are also referred to as immature myeloid cells and were recently renamed to myeloid-derived suppressor cells (MDSCs). MSCs may also express CD115 and/or F4/80 (see Li *et al.*, *Cancer Res.* 2004, 64:1130-1139). MSCs may also express CD31, c-kit, vascular endothelial growth factor (VEGF)-receptor, or CD40 (Bronte *et al.*, *Blood.* 2000, 96:3838-3846). MSCs may further differentiate into several cell types, including macrophages, neutrophils, dendritic cells, Langerhan's cells, monocytes or granulocytes. MSCs may be found naturally in normal adult bone marrow of human and animals or in sites of normal hematopoiesis, such as the spleen in newborn mice. Upon distress due to graft-versus-host disease (GVHD), cyclophosphamide injection, or γ -irradiation, for example, MSCs may be found in the adult spleen. MSCs can suppress the immunological response of T cells, induce T regulatory cells, and produce T cell tolerance. Morphologically, MSCs usually have large nuclei and a high nucleus-to-cytoplasm ratio. MSCs can secrete TGF- β and IL-10 and produce nitric oxide (NO) in the presence of IFN- γ or activated T cells. MSCs may form dendriform cells; however, MSCs are distinct from dendritic cells (DCs) in that DCs are smaller and express CD11c; MSCs do not express CD11c. T cell inactivation by MSCs *in vitro* can be mediated through several mechanisms: IFN- γ -dependent nitric oxide production (Kusmartsev *et al.* *J Immunol.* 2000, 165: 779-785); Th2-mediated-IL-4/IL-13-dependent arginase 1 synthesis (Bronte *et al.* *J Immunol.* 2003, 170: 270-278); loss of CD3 ξ signaling in T cells (Rodriguez *et al.* *J Immunol.* 2003, 171: 1232-1239); and suppression of the T cell response through reactive oxygen species (Bronte *et al.* *J Immunol.* 2003, 170: 270-278; Bronte *et al.* *Trends Immunol.* 2003, 24: 302-306; Kusmartsev *et al.* *J Immunol.* 2004, 172: 989-999; Schmielau and Finn, *Cancer Res.* 2001, 61: 4756-4760).

[0075] In another preferred embodiment, modulation of immune cells and subsequent responses comprises a method of treating a patient with a disease such as for example, cancer, viral disease, or disease caused by any infectious organism wherein an anti-TNFR25 composition, is administered to a patient, and modulates the functions of the immune cells, for example, proliferation of a lymphocyte wherein that lymphocyte had been previously suppressed or attenuated, or in cases where the immune response is normal but the enhancement of the immune response results in more effective and faster treatment of a patient. Negative regulatory pathway, and not lack of inherent tumor immunogenicity (*i.e.*, the ability of the unmanipulated tumors to stimulate protective

immunity), play an important role in preventing the immune-mediated control of tumor progression. The therapeutic implication is that countering immune-attenuating/suppressive regulatory circuits contributes to successful immune control of cancer and is as, if not more, important than developing potent vaccination protocols.

[0076] *Tumor vaccines:* As such, TNFR25 agonists are effective biological response modifiers, in for example, for tumor vaccines because they boost T cell activation and the cellular immune response to a tumor specific antigen, whereas TNFR25 antagonists block or inhibit T cell activation. Therefore, another aspect of the invention relates to methods and therapeutic agents that increase the effectiveness of a tumor vaccine.

[0077] Tumor vaccines attempt to the use of elements of the body's natural immune system to fight cancer. Tumor vaccines contain one or more tumor specific antigens and may contain an adjuvant and biological response modifiers. A tumor specific antigen is a polypeptide that is substantially limited to expression in or on tumor cells and which can be used to stimulate an immune response intended to target those tumor cells. Different types of vaccines are used to treat different types of cancer. For an antigenic composition to be useful as a vaccine, an antigenic composition must induce an immune response to the antigen in a cell or tissue. As used herein, an "antigenic composition" may comprise an antigen (e.g., a peptide or polypeptide), a nucleic acid encoding an antigen (e.g., an antigen expression vector), or a cell expressing or presenting an antigen.

[0078] The enhancement of the immune response to a vaccine or other antigenic stimulant can be measured by any conventional method, such as for example proliferation assays, cytokine secretion, types of cytokines secreted, cytotoxic T lymphocyte assays, ELISAS, RIA and the like. The enhanced immune response can also be detected by monitoring the treatment. For example, in the case of treating cancer, an enhanced immune response could also be monitored by observing one or more of the following effects: (1) inhibition, to some extent, of tumor growth, including, (i) slowing down (ii) inhibiting angiogenesis and (ii) complete growth arrest; (2) reduction in the number of tumor cells; (3) maintaining tumor size; (4) reduction in tumor size; (5) inhibition, including (i) reduction, (ii) slowing down or (iii) complete prevention, of tumor cell infiltration into peripheral organs; (6) inhibition, including (i) reduction, (ii) slowing down or (iii) complete prevention, of metastasis; (7) enhancement of anti-tumor immune response, which may result in (i) maintaining tumor size, (ii) reducing tumor size, (iii) slowing the growth of a tumor, (iv)

reducing, slowing or preventing invasion and/or (8) relief, to some extent, of the severity or number of one or more symptoms associated with the disorder.

[0079] In another preferred embodiment, the anti-TNFR25 can be administered as a vector construct expressing anti-TNFR25 antibodies. In addition, the vector construct can contain nucleotide sequences encoding cytokines, such as granulocyte macrophage colony stimulating factor (GM-CSF), interleukin-12 (IL-12) and co-stimulatory molecules such B7-1, B7-2, CD40. The cytokines can be used in various combinations to fine-tune the response of the subject's immune system, including both antibody and cytotoxic T lymphocyte responses, to bring out the specific level of response needed to control or eliminate the infection or disease state. The polynucleotide can also encode a fusion product containing an antigenic polypeptide, for example, an anti-tumor antigen, anti-viral antigen and the like, and a co-stimulatory molecule, such as CTLA-4. Examples of suitable vectors comprise viral vectors which include polio virus, pox viruses such as vaccinia, canary pox, and fowl pox, herpes viruses, including catfish herpes virus, adenovirus-associated vector, and retroviruses. Exemplary bacterial vectors include attenuated forms of *Salmonella*, *Shigella*, *Edwardsiella ictaluri*, *Yersinia ruckerii*, and *Listeria monocytogenes*.

[0080] *Antigens:* An antigen may be from a pathogen or may be a self antigen in the case of a cancer vaccine or other self antigen associated with a non-infectious, non-cancer chronic disorder such as allergy. The vaccine may be a nucleic acid alone or it may also comprise an adjuvant or other stimulant to improve and/or direct the immune response, and may also further comprise pharmaceutically acceptable excipient(s).

[0081] Diseases against which a subject may be treated include viral diseases, allergic manifestations, diseases caused by bacterial or other pathogens, such as parasitic organisms, AIDS, autoimmune diseases such as Systemic Lupus Erythematosus, Alzheimer's disease and cancers. Suitable antigens comprise bacterial, viral, fungal and protozoan antigens derived from pathogenic organisms, as well as allergens, and antigens derived from tumors and self-antigens. Typically, the antigen will be a protein, polypeptide or peptide antigen.

[0082] The methods and compositions described herein provide a means for treating a variety of malignant cancers. For example, the system of the present invention can be used to mount both humoral and cell-mediated immune responses to particular proteins specific to the cancer in question, such as an activated oncogene, a fetal antigen, or an activation marker. Such tumor antigens include any of the various MAGEs (melanoma associated antigen E), including MAGE 1, 2, 3, 4, etc.; any of the various tyrosinases; MART 1 (melanoma antigen

recognized by T cells), mutant ras; mutant p53; p97 melanoma antigen; HER2/*neu*; CEA (carcinoembryonic antigen), among others.

[0083] Other examples of antigens include a wide variety of proteins from the herpesvirus family, including proteins derived from herpes simplex virus (HSV) types 1 and 2, such as HSV-1 and HSV-2 glycoproteins gB, gD and gH; antigens derived from varicella zoster virus (VZV), Epstein-Barr virus (EBV) and cytomegalovirus (CMV) including CMV gB and gH; and antigens derived from other human herpesviruses such as HHV6 and HHV7; human papilloma viruses (HPV), etc.

[0084] Antigens derived from other viruses, include without limitation, proteins from members of the families Picornaviridae (e.g., polioviruses, etc.); Caliciviridae; Togaviridae (e.g., rubella virus, dengue virus, etc.); Flaviviridae; Coronaviridae; Reoviridae; Birnaviridae; Rhabdoviridae (e.g., rabies virus, etc.); Filoviridae; Paramyxoviridae (e.g., mumps virus, measles virus, respiratory syncytial virus, etc.); Orthomyxoviridae (e.g., influenza virus types A, B and C, etc.); Bunyaviridae; Arenaviridae; Retroviridae (e.g., HTLV-I; HTLV-II; HIV-1 (also known as HTLV-III, LAV, ARV, hTLR, etc.)), including but not limited to antigens from the isolates HIV (III) b' HIV (SF2), HIV (LAV), HIV (LAI), HIV (MN)); HIV-1 (CM235), HIV-1 (US4); HIV-2; simian immunodeficiency virus (SIV) among others.

[0085] Numerous bacterial antigens, such as those derived from organisms that cause diphtheria, cholera, tuberculosis, tetanus, pertussis, meningitis, and other pathogenic states, including, without limitation, *Bordetella pertussis*, *Neisseria meningitides* (A, B, C, Y), *Hemophilus influenza* type B (HIB), and *Helicobacter pylori*. Examples of parasitic antigens include those derived from organisms causing malaria and Lyme disease.

[0086] In some embodiments, the antigen can be whole cells, organisms, fragments of membranes, nucleic acids, peptides, polypeptides, organic or inorganic molecules, etc.

[0087] In some embodiments, the tumor cells are autologous tumor cells. An "autologous tumor cell" is one obtained from a tumor found in the patient, or is a primary descendent of such a cell. In other embodiments, the tumor cells are allogeneic. An "allogeneic tumor cell" is a tumor cell of the same type as that being harbored by the subject but is derived from an established tumor cell line derived from an unrelated subject, or alternatively, is a tumor derived cell originating from an unrelated tumor type but sharing common tumor-associated antigens.

[0088] In one embodiment, the subject is a mammal. In preferred embodiments, the subject is human harboring a neoplasm. In particular embodiments of the invention, the

subject is a human subject and the biological sample and the tumor cells are of human origin. In one embodiment, the human subject has prostate cancer and the tumor cells are from one or more prostate tumor cell lines. In another embodiment, the human subject has colon cancer and the tumor cells are from one or more colon cancer tumor cell lines. In yet another embodiment, the human subject has breast cancer and the tumor cells are from one or more breast cancer cell lines. In still another embodiment, the human subject has ovarian cancer and the tumor cells are from one or more ovarian cancer tumor cell lines.

[0089] In another aspect, the invention provides a method of screening for the presence of a tumor-associated antigen in a biological specimen. In this method, a tumor-associated antigen identified as described above is isolated. An antibody directed to the tumor-associated antigen is prepared, the tumor-associated antigen being reactive with the serum of a subject treated with a vaccine comprising an immune system potentiator and/or enhancer and proliferation-incompetent tumor cells which express the tumor-associated antigen, and not being reactive with the serum of the untreated subject. The biological specimen is then contacted with the antibody. A detection is made of whether there is an antigen-antibody reaction, the presence of such a reaction being indicative of the presence of the tumor-associated antigen in the tissue specimen, and the absence of such a reaction being indicative of no tumor-associated antigen being present. In some embodiments, the tumor-associated antigen in the biological specimen is on a tumor cell. In one embodiment, the antibody is a monoclonal antibody. In another embodiment, the antibody comprises polyclonal antibodies. In some embodiments, the biological specimen is blood, serum, a tissue biopsy, spinal fluid, saliva, lacrimal secretions, semen, vaginal secretions, feces, urine, ascites fluid, or a tumor cell line.

[0090] In one embodiment, the specimen is taken from a subject with carcinoma and the antibody is directed to a tumor-associated antigen having a molecular weight of about 150 kD as determined by SDS-PAGE. In some embodiments, the specimen is taken from a subject with prostate carcinoma, breast carcinoma, lung carcinoma, colon carcinoma, ovarian cancer, or leukemia.

[0091] The invention also provides isolated, tumor-associated antigens. In some embodiments, the novel, isolated tumor-associated antigens have molecular weights of about 250 kD, 160 kD, 150 kD, 130 kD, 105 kD, 60 kD, 32 kD, 31 kD, 27 kD, 26 kD, 14 kD, and 12 kD, as determined by SDS-PAGE, and do not cross-react immunologically with prostate specific antigen (PSA). It is understood that the fact that a tumor-associated antigen does not

cross-react with PSA becomes important where the serum is from a prostate cancer patient that may be expressing PSA. In one embodiment of the invention, the antigen is carcinoma-associated and has a molecular weight of about 150 kD as determined by SDS-PAGE.

Combination Therapies

[0092] In a preferred embodiment, the enhancement or up-regulation of an immune response can be combined with one or more therapies. The anti-TNFR25 antibody, for example, can be administered prior to, concurrently with, or after a course of treatment with one or more agents or methods of treatment.

[0093] In another embodiment, the TNFR25 compositions can be administered to autologous cells, allow the cells to expand and then re-infuse the cells into the patient. In another preferred embodiment a desired antigen, e.g. patient specific tumor antigen, is also administered such that the immune response is specific for the administered antigens. In this way, treatment of a tumor is individualized for the patient allowing for the more efficient destruction of the tumor.

[0094] In another preferred embodiment, an antigen is administered to a patient concurrently with a TNFR25 composition. Preferably, the administration of the antigen activates an antigen specific immune response and the TNFR25 composition enhances the antigen specific immune response. The antigen can be from any source, e.g. tumor, bacterial, viral etc and can include one or more antigens. Administration of the TNFR25 composition enhances the antigen specific immune response by at least about 10 fold as compared to the absence of the TNFR25 composition, preferably, at least about 20-fold as compared to an immune response to a specific antigen in the absence of administration of a TNFR25 composition; preferably, at least about 50-fold enhancement of an antigen specific immune response as compared to an immune response to a specific antigen in the absence in the administration of a TNFR25 composition; preferably, a at least about 100-fold, 500-fold, 1000-fold, 10,000 fold enhancement of an antigen specific immune response as compared to an immune response to a specific antigen in the absence of administration of a TNFR25 composition. The "enhancement" of an immune response not only refers to the specificity of an immune response, but also the efficiency, strength and temporally. That is the immune response is induced faster, stronger and with greater specificity to an antigen. The enhanced immune response can be measured in many ways. The antigen can be immunogenic, however, the antigen used can also be non-immunogenic. "Immunogenicity" is used herein

to refer to the innate ability of an antigen or organism to elicit an immune response in an animal when the antigen or organism is administered to the animal. Thus, "enhancing the immune response" refers to increasing the ability of an antigen or organism to elicit an immune response in an animal when the antigen or organism is administered to an animal. The increased ability or enhancement of an immune response can be measured by, among other things, a greater number of antibodies to an antigen or organism, a greater diversity of antibodies to an antigen or organism, a greater number of T-cells specific for an antigen or organism, a greater cytotoxic or helper T-cell response to an antigen or organism, and the like. The assays can be conducted over time and the type, strength, faster or more efficient immune response can be measured over different time periods in the presence or absence of the TNFR25 compositions.

[0095] The anti-TNFR25 agents can be administered in a pharmaceutical composition, as a polynucleotide in a vector, liposomes, nucleic acids peptides and the like.

[0096] *Chemotherapy:* The TNFR25 compositions can be administered with chemotherapy. Administration of for example, anti-TNFR25 would likely result in the decreased need of chemotherapy, or if chemotherapy is still required or recommended, the doses would be lower, thereby alleviating some of the adverse side effects of these chemotherapeutic agents. Cancer therapies also include a variety of combination therapies with both chemical and radiation based treatments. Combination chemotherapies include, for example, cisplatin (CDDP), carboplatin, procarbazine, mechlorethamine, cyclophosphamide, camptothecin, ifosfamide, melphalan, chlorambucil, busulfan, nitrosurea, dactinomycin, daunorubicin, doxorubicin, bleomycin, plicomycin, mitomycin, etoposide (VP16), tamoxifen, raloxifene, estrogen receptor binding agents, taxol, gemcitabine, navelbine, farnesyl-protein transferase inhibitors, transplatin, 5-fluorouracil, vincristin, vinblastin and methotrexate, or any analog or derivative variant of the foregoing.

[0097] *Radiotherapy:* The compositions can be combined with radiotherapy. Other factors that cause DNA damage and have been used extensively include what are commonly known as gamma-rays, X-rays, and/or the directed delivery of radioisotopes to tumor cells. Other forms of DNA damaging factors are also contemplated such as microwaves, proton beam irradiation (U.S. Pat. No. 5,760,395 and U.S. Pat. No. 4,870,287) and UV-irradiation. It is most likely that all of these factors effect a broad range of damage on DNA, on the precursors of DNA, on the replication and repair of DNA, and on the assembly and maintenance of chromosomes. Dosage ranges for X-rays range from daily doses of 50 to 200

roentgens for prolonged periods of time (3 to 4 wk), to single doses of 2000 to 6000 roentgens. Dosage ranges for radioisotopes vary widely, and depend on the half-life of the isotope, the strength and type of radiation emitted, and the uptake by the neoplastic cells.

[0098] *Immunotherapy:* The anti-TNFR25 agents can be combined with other forms of immunotherapy. For example, in the context of cancer treatment, immunotherapeutics, generally, rely on the use of immune effector cells and molecules (e.g., monoclonal antibodies) to target and destroy cancer cells. Trastuzumab (HerceptinTM) is such an example. The immune effector may be, for example, an antibody specific for some marker on the surface of a tumor cell. The antibody alone may serve as an effector of therapy or it may recruit other cells to actually effect cell killing. The antibody also may be conjugated to a drug or toxin (chemotherapeutic, radionuclide, ricin A chain, cholera toxin, pertussis toxin, etc.) and serve merely as a targeting agent. Alternatively, the effector may be a lymphocyte carrying a surface molecule that interacts, either directly or indirectly, with a tumor cell target. Various effector cells include cytotoxic T cells and NK cells. The combination of therapeutic modalities, i.e., direct cytotoxic activity and enhancement of the immune effector response by, for example, anti-TNFR25 antibody, would provide therapeutic benefit in the treatment of cancer.

[0099] The antigen specific immune response would target one or more tumor antigens and the administration of the TNFR25 compositions would enhance the immune response directed to these tumor antigens. Many tumor markers exist and any of these may be suitable for targeting in the context of the present invention. Common tumor markers include carcinoembryonic antigen, prostate specific antigen, urinary tumor associated antigen, fetal antigen, tyrosinase (p97), gp68, TAG-72, HMFG, Sialyl Lewis Antigen, MucA, MucB, PLAP, estrogen receptor, laminin receptor, erb B and p155. An alternative aspect of immunotherapy is to combine anticancer effects with immune stimulatory effects. Immune stimulating molecules also exist including: cytokines such as IL-2, IL-4, IL-12, GM-CSF, gamma-IFN, chemokines such as MIP-1, MCP-1, IL-8 and growth factors such as FLT3 ligand. Combining immune stimulating molecules, either as proteins or using gene delivery in combination with a tumor suppressor such as MDA-7 enhance anti-tumor effects.

[00100] A number of different approaches for passive immunotherapy of cancer exist. They may be broadly categorized into the following: injection of antibodies alone; injection of antibodies coupled to toxins or chemotherapeutic agents; injection of antibodies coupled to radioactive isotopes; injection of anti-idiotypic antibodies; and finally, purging of tumor cells

in bone marrow. Preferably, human monoclonal antibodies are employed in passive immunotherapy, as they produce few or no side effects in the patient.

[00101] In active immunotherapy, an antigenic peptide, polypeptide or protein, or an autologous or allogeneic tumor cell composition or "vaccine" is administered, generally with a distinct bacterial adjuvant. In melanoma immunotherapy, those patients who elicit high IgM response often survive better than those who elicit no or low IgM antibodies. IgM antibodies are often transient antibodies and the exception to the rule appears to be anti-ganglioside or anticarbohydrate antibodies.

[00102] In adoptive immunotherapy, the patient's circulating lymphocytes, or tumor infiltrated lymphocytes, are isolated *in vitro*, activated by, lymphokines such as IL-2 or transduced with genes for tumor necrosis. The TNFR25 compositions, for example anti-TNFR25 antibody, are administered or cultured with the cells which are then re-infused. To achieve this, one would administer to an animal, or human patient, an immunologically effective amount of activated lymphocytes in combination with anti-TNFR25 and, optionally, with an adjuvant-incorporated antigenic peptide composition. The activated lymphocytes will most preferably be the patient's own cells that were earlier isolated from a blood or tumor sample and activated (or "expanded") *in vitro*. This form of immunotherapy has produced several cases of regression of melanoma and renal carcinoma, but the percentage of responders were few compared to those who did not respond. The anti-TNFR25 can be administered to a patient, after re-infusion to the cells under a regimen that can be determined by the treating physician or nurse practitioner.

[00103] *Surgery*: Approximately 60% of persons with cancer will undergo surgery of some type, which includes preventative, diagnostic or staging, curative and palliative surgery. Curative surgery is a cancer treatment that may be used in conjunction with other therapies, such as the treatment of the present invention, chemotherapy, radiotherapy, hormonal therapy, gene therapy, immunotherapy and/or alternative therapies.

[00104] Curative surgery includes resection in which all or part of cancerous tissue is physically removed, excised, and/or destroyed. Tumor resection refers to physical removal of at least part of a tumor. In addition to tumor resection, treatment by surgery includes laser surgery, cryosurgery, electrosurgery, and microscopically controlled surgery (Mohs' surgery). It is further contemplated that the present invention may be used in conjunction with removal of superficial cancers, precancers, or incidental amounts of normal tissue.

[00105] Upon excision of part or all of cancerous cells, tissue, or tumor, a cavity may be formed in the body. Treatment may be accomplished by perfusion, direct injection or local application of the area with an additional anti-cancer therapy. Such treatment may be repeated, for example, every 1, 2, 3, 4, 5, 6, or 7 days, or every 1, 2, 3, 4, and 5 weeks or every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 months. These treatments may be of varying dosages as well

[00106] *Other Agents:* It is contemplated that other agents may be used in combination with the present invention to improve the therapeutic efficacy of treatment. These additional agents include immunomodulatory agents that affect the upregulation of cell surface receptors and GAP junctions, cytostatic and differentiation agents, the inhibition of cell adhesion, and the increase in sensitivity of the hyperproliferative cells to apoptotic inducers or other agents. Immunomodulatory agents include tumor necrosis factor; interferon alpha, beta, and gamma; IL-2 and other cytokines; F42K and other cytokine analogs; or MIP-1, MIP-1beta, MCP-1, RANTES, and other chemokines. It is further contemplated that the upregulation of cell surface receptors or their ligands such as Fas/Fas ligand, DR4 or DR5/TRAIL (Apo-2 ligand) would potentiate the enhancing abilities of the present invention. Increases in intercellular signaling by elevating the number of GAP junctions would increase the proliferative effects on desired cell populations.

[00107] Apo2 ligand (Apo2L, also called TRAIL) is a member of the tumor necrosis factor (TNF) cytokine family. TRAIL activates rapid apoptosis in many types of cancer cells, yet is not toxic to normal cells. TRAIL mRNA occurs in a wide variety of tissues. Most normal cells appear to be resistant to TRAIL's cytotoxic action, suggesting the existence of mechanisms that can protect against apoptosis induction by TRAIL. The first receptor described for TRAIL, called death receptor 4 (DR4), contains a cytoplasmic "death domain"; DR4 transmits the apoptosis signal carried by TRAIL. Additional receptors have been identified that bind to TRAIL. One receptor, called DR5, contains a cytoplasmic death domain and signals apoptosis much like DR4. The DR4 and DR5 mRNAs are expressed in many normal tissues and tumor cell lines. Decoy receptors such as DcR1 and DcR2 have been identified that prevent TRAIL from inducing apoptosis through DR4 and DR5. These decoy receptors thus represent a novel mechanism for regulating sensitivity to a pro-apoptotic cytokine directly at the cell's surface. The preferential expression of these inhibitory receptors in normal tissues suggests that TRAIL may be useful as an anticancer agent that induces apoptosis in cancer cells while sparing normal cells.

[00108] There have been many advances in the therapy of cancer following the introduction of cytotoxic chemotherapeutic drugs. However, one of the consequences of chemotherapy is the development/acquisition of drug-resistant phenotypes and the development of multiple drug resistance. The development of drug resistance remains a major obstacle in the treatment of such tumors and therefore, an enhancement of the immune response provides an alternative approach.

[00109] Another form of therapy includes hyperthermia, which is a procedure in which a patient's tissue is exposed to high temperatures (up to 106° F). External or internal heating devices may be involved in the application of local, regional, or whole-body hyperthermia. Local hyperthermia involves the application of heat to a small area, such as a tumor. Heat may be generated externally with high-frequency waves targeting a tumor from a device outside the body. Internal heat may involve a sterile probe, including thin, heated wires or hollow tubes filled with warm water, implanted microwave antennae, or radio frequency electrodes.

[00110] A patient's organ or a limb is heated for regional therapy, which is accomplished using devices that produce high energy, such as magnets. Alternatively, some of the patient's blood may be removed and heated before being perfused into an area that will be internally heated. Whole-body heating may also be implemented in cases where cancer has spread throughout the body. Warm-water blankets, hot wax, inductive coils, and thermal chambers may be used for this purpose.

[00111] Hormonal therapy may also be used in conjunction with the present invention or in combination with any other cancer therapy previously described. The use of hormones may be employed in the treatment of certain cancers such as breast, prostate, ovarian, or cervical cancer to lower the level or block the effects of certain hormones such as testosterone or estrogen. This treatment is often used in combination with at least one other cancer therapy as a treatment option or to reduce the risk of metastases.

Methods of Stimulating or Enhancing an Immune Response

[00112] In a typical immunization regime employing the TNFR25 compositions of the present invention, the formulations may be administered in several doses (e.g. 1-4). The dose will be determined by the immunological activity the composition produced and the condition of the patient, as well as the body weight or surface areas of the patient to be treated. The size of the dose also will be determined by the existence, nature, and extent of any adverse

side effects that may accompany the administration of a particular composition in a particular patient.

[00113] The compositions of the present invention may be administered via a non-mucosal or mucosal route. These administrations may include *in vivo* administration via parenteral injection (e.g. intravenous, subcutaneous, and intramuscular) or other traditional direct routes, such as buccal/sublingual, rectal, oral, nasal, topical (such as transdermal and ophthalmic), vaginal, pulmonary, intraarterial, intraperitoneal, intraocular, or intranasal routes or directly into a specific tissue. Alternatively, the compositions of the invention may be administered by any of a variety of routes such as oral, topical, subcutaneous, mucosal, intravenous, intramuscular, intranasal, sublingual, transcutaneous, subdermal, intradermal and via suppository. Administration may be accomplished simply by direct administration using a patch, needle, catheter or related device, at a single time point or at multiple time points.

[00114] Immunization via the mucosal surfaces offers numerous potential advantages over other routes of immunization. The most obvious benefits are 1) mucosal immunization does not require needles or highly-trained personnel for administration, and 2) immune responses are raised at the site(s) of pathogen entry, as well as systemically (Isaka *et al.* 1999; Kozlowski *et al.* 1997; Mestecky *et al.* 1997; Wu *et al.* 1997).

[00115] The present invention also contemplates the provision of means for dispensing intranasal formulations of the compositions hereinbefore defined, and at least one adjuvant or at least one delivery agent as hereinbefore defined. A dispensing device may, for example, take the form of an aerosol delivery system, and may be arranged to dispense only a single dose, or a multiplicity of doses. Such a device would deliver a metered dose of the vaccine or antigenic formulation to the nasal passage. Other examples of appropriate devices include, but are not limited to, droppers, swabs, aerosolizers, insufflators (e.g. Valois Monopowder Nasal Administration Device, Bepak UniDose DP), nebulizers, and inhalers. The devices may deliver the antigenic or vaccine formulation by passive means requiring the subject to inhale the formulation into the nasal cavity. Alternatively, the device may actively deliver the formulation by pumping or spraying a dose into the nasal cavity. The antigenic formulation or vaccine may be delivered into one or both nostrils by one or more such devices. Administration could include two devices per subject (one device per nostril). Actual dose of active ingredient (may be about 5-1000 µg. In a preferred embodiment, the composition is administered to the nasal mucosa by rapid deposition within the nasal passage from a device containing the formulation held close to or inserted into the nasal passageway.

[00116] The invention involves the use of various materials disclosed herein to "immunize" subjects or as "vaccines". As used herein, "immunization" or "vaccination" means increasing or activating an immune response against an antigen. It does not require elimination or eradication of a condition but rather contemplates the clinically favorable enhancement of an immune response toward an antigen. Generally accepted animal models can be used for testing of immunization against cancer using a tumor associated antigen nucleic acid. For example, human cancer cells can be introduced into a mouse to create a tumor, and one or more tumor associated nucleic acids can be delivered by the methods described herein. The effect on the cancer cells (e.g., reduction of tumor size) can be assessed as a measure of the effectiveness of the tumor associated nucleic acid immunization. Of course, testing of the foregoing animal model using more conventional methods for immunization include the administration of one or more tumor associated polypeptides or peptides derived therefrom, optionally combined with one or more adjuvants and/or cytokines to boost the immune response. Methods for immunization, including formulation of a vaccine composition and selection of doses, route of administration and the schedule of administration (e.g. primary and one or more booster doses), are well known in the art. The tests also can be performed in humans, where the end point is to test for the presence of enhanced levels of circulating CTLs against cells bearing the antigen, to test for levels of circulating antibodies against the antigen, to test for the presence of cells expressing the antigen and so forth.

[00117] As part of the immunization compositions, one or more tumor associated polypeptides or stimulatory fragments thereof can be administered with one or more doses of TNFR25 compositions and, if desired adjuvants, to induce an immune response or to increase an immune response. An adjuvant is a substance incorporated into or administered with antigen which potentiates the immune response. Adjuvants may enhance the immunological response by providing a reservoir of antigen (extracellularly or within macrophages), activating macrophages and stimulating specific sets of lymphocytes. Adjuvants of many kinds are well known in the art. Specific examples of adjuvants include monophosphoryl lipid A (MPL, SmithKline Beecham), a congener obtained after purification and acid hydrolysis of *Salmonella minnesota* Re 595 lipopolysaccharide; saponins including QS21 (SmithKline Beecham), a pure QA-21 saponin purified from *Quillja saponaria* extract; DQS21, described in PCT application WO96/33739 (SmithKline Beecham); QS-7, QS-17, QS-18, and QS-L1 (So *et al.*, *Mol. Cells.* 7:178-186, 1997); incomplete Freund's adjuvant;

complete Freund's adjuvant; montanide; and various water-in-oil emulsions prepared from biodegradable oils such as squalene and/or tocopherol. Other adjuvants are known in the art and can be used in the invention (see, e.g. Goding, *Monoclonal Antibodies: Principles and Practice*, 2nd Ed., 1986). Methods for the preparation of mixtures or emulsions of peptide and adjuvant are well known to those of skill in the art of vaccination.

[00118] Other agents which stimulate the immune response of the subject can also be administered to the subject. For example, other cytokines are also useful in vaccination protocols as a result of their lymphocyte regulatory properties. Many other cytokines useful for such purposes will be known to one of ordinary skill in the art, including interleukin-12 (IL-12) which has been shown to enhance the protective effects of vaccines (see, e.g., *Science* 268: 1432-1434, 1995), GM-CSF and IL-18. Thus cytokines can be administered in conjunction with antigens and adjuvants to increase the immune response to the antigens.

[00119] There are a number of additional immune response potentiating compounds that can be used in vaccination protocols. These include costimulatory molecules provided in either protein or nucleic acid form. Such costimulatory molecules include the B7-1 and B7-2 (CD80 and CD86 respectively) molecules which are expressed on dendritic cells (DC) and interact with the CD28 molecule expressed on the T cell. This interaction provides costimulation (signal 2) to an antigen/MHC/TCR stimulated (signal 1) T cell, increasing T cell proliferation and effector function. B7 also interacts with CTLA4 (CD152) on T cells and studies involving CTLA4 and B7 ligands indicate that the B7-CTLA4 interaction can enhance antitumor immunity and CTL proliferation (Zheng *et al.*, *Proc. Nat'l Acad. Sci. USA* 95:6284-6289, 1998).

[00120] B7 typically is not expressed on tumor cells so they are not efficient antigen presenting cells (APCs) for T cells. Induction of B7 expression would enable the tumor cells to stimulate more efficiently CTL proliferation and effector function. A combination of B7/IL-6/IL-12 costimulation has been shown to induce IFN-gamma and a Th1 cytokine profile in the T cell population leading to further enhanced T cell activity (Gajewski *et al.*, *J. Immunol.* 154:5637-5648, 1995). Tumor cell transfection with B7 has been discussed in relation to *in vitro* CTL expansion for adoptive transfer immunotherapy by Wang *et al.* (*J. Immunother.* 19:1-8, 1996). Other delivery mechanisms for the B7 molecule would include nucleic acid (naked DNA) immunization (Kim *et al.*, *Nature Biotechnol.* 15:7:641-646, 1997) and recombinant viruses such as adeno and pox (Wendtner *et al.*, *Gene Ther.* 4:726-735, 1997). These systems are all amenable to the construction and use of expression cassettes for

the coexpression of B7 with other molecules of choice such as the antigens or fragment(s) of antigens discussed herein (including polytopes) or cytokines. These delivery systems can be used for induction of the appropriate molecules *in vitro* and for *in vivo* vaccination situations. The use of anti-CD28 antibodies to directly stimulate T cells *in vitro* and *in vivo* could also be considered.

[00121] Thus, if desired, the anti-TNFR25 antibody can be administered with one or more other molecules resulting in the enhancement of an immune response.

[00122] Lymphocyte function associated antigen-3 (LFA-3) is expressed on APCs and some tumor cells and interacts with CD2 expressed on T cells. This interaction induces T cell IL-2 and IFN-gamma production and can thus complement but not substitute, the B7/CD28 costimulatory interaction (Parra *et al.*, *J. Immunol.*, 158:637-642, 1997; Fenton *et al.*, *J. Immunother.*, 21:95-108, 1998).

[00123] Lymphocyte function associated antigen-1 (LFA-1) is expressed on leukocytes and interacts with ICAM-1 expressed on APCs and some tumor cells. This interaction induces T cell IL-2 and IFN-gamma production and can thus complement but not substitute, the B7/CD28 costimulatory interaction. LFA-1 is thus a further example of a costimulatory molecule that could be provided in a vaccination protocol in the various ways discussed above.

[00124] Complete CTL activation and effector function requires Th cell help through the interaction between the Th cell CD40L (CD40 ligand) molecule and the CD40 molecule expressed by DCs (Ridge *et al.*, *Nature* 393:474, 1998; Bennett *et al.*, *Nature* 393:478, 1998; Schoenberger *et al.*, *Nature* 393:480, 1998). This mechanism of this costimulatory signal is likely to involve upregulation of B7 and associated IL-6/IL-12 production by the DC (APC). The CD40-CD40L interaction thus complements the signal 1 (antigen/MHC-TCR) and signal 2 (B7-CD28) interactions.

[00125] The use of anti-CD40 antibodies to stimulate DC cells directly, would be expected to enhance a response to tumor associated antigens which are normally encountered outside of an inflammatory context or are presented by non-professional APCs (tumor cells). In these situations Th help and B7 costimulation signals are not provided. This mechanism might be used in the context of antigen pulsed DC based therapies or in situations where Th epitopes have not been defined within known tumor associated antigen precursors.

Delivery of TNFR25 Compositions

[00126] The invention contemplates delivery of nucleic acids, polypeptides or peptides of the TNFR25 compositions, for example, anti-TNFR25 antibody. Throughout the description, the term "anti-TNFR25 antibody" will be used as an illustrative example and is not to be construed as limiting the invention to just an antibody. Delivery of polypeptides and peptides can be accomplished according to standard vaccination protocols which are well known in the art.

[00127] In a preferred embodiment, an anti-TNFR25 antibody is administered to a patient via immunization routes. For example, intra-venously, intra-muscularly, intra-peritoneally, and the like.

[00128] In another embodiment, the delivery of nucleic acid, e.g. encoding an anti-TNFR25 antibody, is accomplished by *ex vivo* methods, i.e. by removing a cell from a subject, genetically engineering the cell to include a tumor associated nucleic acid, and reintroducing the engineered cell into the subject. One example of such a procedure is outlined in U.S. Pat. No. 5,399,346. In general, it involves introduction *in vitro* of a functional copy of a gene into a cell(s) of a subject, and returning the genetically engineered cell(s) to the subject. The functional copy of the gene is under operable control of regulatory elements which permit expression of the gene in the genetically engineered cell(s). Numerous transfection and transduction techniques as well as appropriate expression vectors are well known to those of ordinary skill in the art, some of which are described in PCT application WO95/00654. *In vivo* nucleic acid delivery using vectors such as viruses and targeted liposomes also is contemplated according to the invention.

Preparation of Antibodies

[00129] In one embodiment, the TNFR25 composition comprises an anti-TNFR25 antibody. As described above, the antibody can be generated in any species and can be against TNFR25 from any species. Both monoclonal and polyclonal antibodies are contemplated and comprise embodiments of the invention. The antibody further be bispecific and be targeted to one or more TNFR25 from different species.

[00130] *Polyclonal Antibodies:* Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. Alternatively, antigen may be injected directly into the animal's lymph node (see Kilpatrick *et al.*, *Hybridoma*, 16:381-389, 1997). An improved antibody response may be obtained by conjugating the relevant antigen to a protein that is immunogenic in the species

to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride or other agents known in the art.

[00131] *Monoclonal Antibodies*: Monoclonal antibodies may be made using the hybridoma method first described by Kohler *et al.*, Nature, 256:495 (1975), or may be made by recombinant DNA methods.

[00132] In the hybridoma method, a mouse or other appropriate host animal, such as rats, hamster or macaque monkey, is immunized as herein described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, Monoclonal Antibodies: Principles and Practice, pp. 59-103 (Academic Press, 1986)).

[00133] The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

[00134] Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133: 3001 (1984); Brodeur *et al.*, Monoclonal Antibody Production Techniques and Applications, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)). Exemplary murine myeloma lines include those derived from MOP-21 and M.C.-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA.

[00135] Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or

by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA). The binding affinity of the monoclonal antibody can, for example, be determined by BIAcore or Scatchard analysis (Munson *et al.*, *Anal. Biochem.*, 107:220 (1980)).

[00136] After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal. The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

[00137] *Recombinant Production of Antibodies:* The amino acid sequence of an immunoglobulin of interest may be determined by direct protein sequencing, and suitable encoding nucleotide sequences can be designed according to a universal codon table.

[00138] Alternatively, DNA encoding the monoclonal antibodies may be isolated and sequenced from the hybridoma cells using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). Sequence determination will generally require isolation of at least a portion of the gene or cDNA of interest. Usually this requires cloning the DNA or, preferably, mRNA (i.e., cDNA) encoding the monoclonal antibodies. Cloning is carried out using standard techniques (see, e.g., Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Guide*, Vols 1-3, Cold Spring Harbor Press, which is incorporated herein by reference). For example, a cDNA library may be constructed by reverse transcription of polyA⁺ mRNA, preferably membrane-associated mRNA, and the library screened using probes specific for human immunoglobulin polypeptide gene sequences. In a preferred embodiment, however, the polymerase chain reaction (PCR) is used to amplify cDNAs (or portions of full-length cDNAs) encoding an immunoglobulin gene segment of interest (e.g., a light chain variable segment). The amplified sequences can be readily cloned into any suitable vector, e.g., expression vectors, minigene vectors, or phage display vectors. It will be appreciated that the particular method of cloning used is not critical, so long as it is

possible to determine the sequence of some portion of the immunoglobulin polypeptide of interest.

[00139] As used herein, an "isolated" nucleic acid molecule or "isolated" nucleic acid sequence is a nucleic acid molecule that is either (1) identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the nucleic acid or (2) cloned, amplified, tagged, or otherwise distinguished from background nucleic acids such that the sequence of the nucleic acid of interest can be determined. An isolated nucleic acid molecule is other than in the form or setting in which it is found in nature. However, an isolated nucleic acid molecule includes a nucleic acid molecule contained in cells that ordinarily express the antibody where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

[00140] One source for RNA used for cloning and sequencing is a hybridoma produced by obtaining a B cell from the transgenic mouse and fusing the B cell to an immortal cell. An advantage of using hybridomas is that they can be easily screened, and a hybridoma that produces a human monoclonal antibody of interest selected. Alternatively, RNA can be isolated from B cells (or whole spleen) of the immunized animal. When sources other than hybridomas are used, it may be desirable to screen for sequences encoding immunoglobulins or immunoglobulin polypeptides with specific binding characteristics. One method for such screening is the use of phage display technology. Phage display is described in e.g., Dower *et al.*, WO 91/17271, McCafferty *et al.*, WO 92/01047, and Caton and Koprowski, *Proc. Natl. Acad. Sci. USA*, 87:6450-6454 (1990), each of which is incorporated herein by reference. In one embodiment using phage display technology, cDNA from an immunized transgenic mouse (e.g., total spleen cDNA) is isolated, the polymerase chain reaction is used to amplify a cDNA sequences that encode a portion of an immunoglobulin polypeptide, e.g., CDR regions, and the amplified sequences are inserted into a phage vector. cDNAs encoding peptides of interest, e.g., variable region peptides with desired binding characteristics, are identified by standard techniques such as panning.

[00141] The sequence of the amplified or cloned nucleic acid is then determined. Typically the sequence encoding an entire variable region of the immunoglobulin polypeptide is determined, however, it will sometimes be adequate to sequence only a portion of a variable region, for example, the CDR-encoding portion. Typically the portion sequenced will be at least 30 bases in length, more often bases coding for at least about one-third or at least about one-half of the length of the variable region will be sequenced.

[00142] Sequencing can be carried out on clones isolated from a cDNA library, or, when PCR is used, after subcloning the amplified sequence or by direct PCR sequencing of the amplified segment. Sequencing is carried out using standard techniques (see, e.g., Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Guide*, Vols 1-3, Cold Spring Harbor Press, and Sanger, F. *et al.* (1977) *Proc. Natl. Acad. Sci. USA* 74: 5463-5467, which is incorporated herein by reference). By comparing the sequence of the cloned nucleic acid with published sequences of human immunoglobulin genes and cDNAs, one of skill will readily be able to determine, depending on the region sequenced, (i) the germline segment usage of the hybridoma immunoglobulin polypeptide (including the isotype of the heavy chain) and (ii) the sequence of the heavy and light chain variable regions, including sequences resulting from N-region addition and the process of somatic mutation. One source of immunoglobulin gene sequence information is the National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, Md.

[00143] Once isolated, the DNA may be operably linked to expression control sequences or placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to direct the synthesis of monoclonal antibodies in the recombinant host cells. Recombinant production of antibodies is well known in the art.

[00144] Expression control sequences refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

[00145] Nucleic acid is operably linked when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, operably linked means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites

do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

[00146] Cell, cell line, and cell culture are often used interchangeably and all such designations herein include progeny. Transformants and transformed cells include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included.

[00147] The invention also provides isolated nucleic acids encoding specific binding agents or antibodies of the invention, optionally operably linked to control sequences recognized by a host cell, vectors and host cells comprising the nucleic acids, and recombinant techniques for the production of the specific binding agents or antibodies, which may comprise culturing the host cell so that the nucleic acid is expressed and, optionally, recovering the specific binding agent or antibody from the host cell culture or culture medium.

[00148] Many vectors are known in the art. Vector components may include one or more of the following: a signal sequence (that may, for example, direct secretion of the specific binding agent or antibody), an origin of replication, one or more selective marker genes (that may, for example, confer antibiotic or other drug resistance, complement auxotrophic deficiencies, or supply critical nutrients not available in the media), an enhancer element, a promoter, and a transcription termination sequence, all of which are well known in the art.

[00149] Suitable host cells include prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*, e.g., *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, e.g., *Salmonella typhimurium*, *Serratia*, e.g., *Serratia marcescans*, and *Shigella*, as well as Bacilli such as *B. subtilis* and *B. licheniformis*, *Pseudomonas*, and *Streptomyces*. In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for specific binding agent-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Pichia*, e.g. *P. pastoris*, *Schizosaccharomyces pombe*; *Kluyveromyces*, *Yarrowia*; *Candida*; *Trichoderma reesia*; *Neurospora crassa*; *Schwanniomyces* such as *Schwanniomyces*

occidentalis; and filamentous fungi such as, e.g., *Neurospora*, *Penicillium*, *Tolypocladium*, and *Aspergillus* hosts such as *A. nidulans* and *A. niger*.

[00150] Suitable host cells for the expression of glycosylated specific binding agent or antibody are derived from multicellular organisms. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. A variety of viral strains for transfection of such cells are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV.

[00151] However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become routine procedure. Examples of useful mammalian host cell lines are Chinese hamster ovary cells, including CHOK1 cells (ATCC CCL61), DXB-11, DG-44, and Chinese hamster ovary cells/-DHFR (CHO, Urlaub *et al.*, *Proc. Natl. Acad. Sci. USA* 77: 4216 (1980)); monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, [Graham *et al.*, *J. Gen Virol.* 36: 59 (1977)]; baby hamster kidney cells (BHK, ATCC CCL 10); mouse sertoli cells (TM4, Mather, *Biol. Reprod.* 23: 243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human hepatoma cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TR1 cells (Mather *et al.*, *Annals N.Y. Acad. Sci.* 383: 44-68 (1982)); MRC 5 cells or FS4 cells.

[00152] Host cells are transformed or transfected with the above-described nucleic acids or vectors for specific binding agent or antibody production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. In addition, novel vectors and transfected cell lines with multiple copies of transcription units separated by a selective marker are particularly useful and preferred for the expression of specific binding agents or antibodies.

[00153] The host cells used to produce the specific binding agent or antibody of this invention may be cultured in a variety of media. Commercially available media such as

Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham *et al.*, *Meth. Enz.* 58: 44 (1979), Barnes *et al.*, *Anal. Biochem.* 102: 255 (1980), U.S. Pat. No. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO90103430; WO 87/00195; or U.S. Pat. Re. No. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GentamycinTM drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan. The expression vectors, pDC323 and pDC324 as described in U.S. Patent Application No. 20030082735, containing the appropriate respective light chain and heavy chain pair were transfected into the CS9 host cell line.

[00154] Upon culturing the host cells, the specific binding agent or antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the specific binding agent or antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration.

[00155] The specific binding agent or antibody composition can be purified using, for example, hydroxyl apatite chromatography, cation or anion exchange chromatography, or preferably affinity chromatography, using the antigen of interest or protein A or protein G as an affinity ligand. Protein A can be used to purify specific binding agents or antibodies that are based on human $\gamma 1$, $\gamma 2$, or $\gamma 4$ heavy chains (Lindmark *et al.*, *J. Immunol. Meth.* 62: 1-13 (1983)). Protein G is recommended for all mouse isotypes and for human $\gamma 3$ (Guss *et al.*, *EMBO J.* 5: 1567-1575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrene-divinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the specific binding agent or

antibody comprises a C_H3 domain, the Bakerbond ABXTM resin (J. T. Baker, Phillipsburg, N.J.) is useful for purification. Other techniques for protein purification such as ethanol precipitation, Reverse Phase HPLC, chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also possible depending on the specific binding agent or antibody to be recovered.

[00156] *Chimeric and Humanized Antibodies*: Since chimeric or humanized antibodies are less immunogenic in humans than the parental mouse monoclonal antibodies, they can be used for the treatment of humans with far less risk of anaphylaxis. Thus, these antibodies are preferred in therapeutic applications that involve *in vivo* administration to a human.

[00157] Chimeric monoclonal antibodies, in which the variable Ig domains of a rodent monoclonal antibody are fused to human constant Ig domains, can be generated using standard procedures known in the art (See Morrison, S. L., *et al.* (1984) Chimeric Human Antibody Molecules; Mouse Antigen Binding Domains with Human Constant Region Domains, *Proc. Natl. Acad. Sci. USA* 81, 6841-6855; and, Boulianne, G. L., *et al.*, *Nature* 312, 643-646. (1984)). Although some chimeric monoclonal antibodies have proved less immunogenic in humans, the rodent variable Ig domains can still lead to a significant human anti-rodent response.

[00158] Humanized antibodies may be achieved by a variety of methods including, for example: (1) grafting the non-human complementarity determining regions (CDRs) onto a human framework and constant region (a process referred to in the art as humanizing through "CDR grafting"), or, alternatively, (2) transplanting the entire non-human variable domains, but "cloaking" them with a human-like surface by replacement of surface residues (a process referred to in the art as "veneering"). These methods are disclosed in, e.g., Jones *et al.*, *Nature* 321:522 525 (1986); Morrison *et al.*, *Proc. Natl. Acad. Sci., U.S.A.*, 81:6851 6855 (1984); Morrison and Oi, *Adv. Immunol.*, 44:65 92 (1988); Verhoeyer *et al.*, *Science* 239:1534 1536 (1988); Padlan, *Molec. Immun.* 28:489 498 (1991); Padlan, *Molec. Immunol.* 31(3):169 217 (1994); and Kettleborough, C. A. *et al.*, *Protein Eng.* 4(7):773 83 (1991) each of which is incorporated herein by reference.

[00159] In particular, a rodent antibody on repeated *in vivo* administration in man either alone or as a conjugate will bring about an immune response in the recipient against the rodent antibody; the so-called HAMA response (Human Anti Mouse Antibody). The HAMA response may limit the effectiveness of the pharmaceutical if repeated dosing is required. The immunogenicity of the antibody may be reduced by chemical modification of the

antibody with a hydrophilic polymer such as polyethylene glycol or by using the methods of genetic engineering to make the antibody binding structure more human like.

[00160] CDR grafting involves introducing one or more of the six CDRs from the mouse heavy and light chain variable Ig domains into the appropriate framework regions of a human variable Ig domain. This technique (Riechmann, L., *et al.*, *Nature* 332, 323 (1988)), utilizes the conserved framework regions (FR1-FR4) as a scaffold to support the CDR loops which are the primary contacts with antigen. A significant disadvantage of CDR grafting, however, is that it can result in a humanized antibody that has a substantially lower binding affinity than the original mouse antibody, because amino acids of the framework regions can contribute to antigen binding, and because amino acids of the CDR loops can influence the association of the two variable Ig domains. To maintain the affinity of the humanized monoclonal antibody, the CDR grafting technique can be improved by choosing human framework regions that most closely resemble the framework regions of the original mouse antibody, and by site-directed mutagenesis of single amino acids within the framework or CDRs aided by computer modeling of the antigen binding site (e.g., Co, M. S., *et al.* (1994), *J. Immunol.* 152, 2968-2976).

[00161] One method of humanizing antibodies comprises aligning the non-human heavy and light chain sequences to human heavy and light chain sequences, selecting and replacing the non-human framework with a human framework based on such alignment, molecular modeling to predict the conformation of the humanized sequence and comparing to the conformation of the parent antibody. This process is followed by repeated back mutation of residues in the CDR region which disturb the structure of the CDRs until the predicted conformation of the humanized sequence model closely approximates the conformation of the non-human CDRs of the parent non-human antibody. Such humanized antibodies may be further derivatized to facilitate uptake and clearance, e.g., via Ashwell receptors (See, e.g., U.S. Pat. Nos. 5,530,101 and 5,585,089).

[00162] A number of humanizations of mouse monoclonal antibodies by rational design have been reported (See, for example, 20020091240 published Jul. 11, 2002, WO 92/11018 and U.S. Pat. No., 5,693,762, U.S. Pat. No. 5,766,866).

[00163] Human engineering of antibodies has also been described in, e.g., Studnicka *et al.* U.S. Pat. No. 5,766,886; Studnicka *et al.* *Protein Engineering* 7: 805-814 (1994).

[00164] *Production of Antibody Variants:* Amino acid sequence variants of the desired specific binding agent or antibody may be prepared by introducing appropriate nucleotide

changes into the encoding DNA, or by peptide synthesis. Such variants include, for example, deletions and/or insertions and/or substitutions of residues within the amino acid sequences of the specific binding agents or antibodies. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the specific binding agent or humanized or variant antibody, such as changing the number or position of glycosylation sites.

[00165] Nucleic acid molecules encoding amino acid sequence variants of the specific binding agent or antibody are prepared by a variety of methods known in the art. Such methods include oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the specific binding agent or antibody.

[00166] A useful method for identification of certain residues or regions of the specific binding agent or antibody that are preferred locations for mutagenesis is called "alanine scanning mutagenesis," as described by Cunningham and Wells *Science*, 244:1081-1085 (1989). Here, a residue or group of target residues are identified (e.g., charged residues such as Arg, Asp, His, Lys, and Glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with antigen. Those amino acid locations demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at, or for, the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation per se need not be predetermined. For example, to analyze the performance of a mutation at a given site, ala scanning or random mutagenesis is conducted at the target codon or region and the expressed variants are screened for the desired activity.

[00167] Ordinarily, amino acid sequence variants of the specific binding agent or antibody will have an amino acid sequence having at least 60% amino acid sequence identity with the original specific binding agent or antibody (murine or humanized) amino acid sequences of either the heavy or the light chain, or at least 65%, or at least 70%, or at least 75% or at least 80% identity, more preferably at least 85% identity, even more preferably at least 90% identity, and most preferably at least 95% identity, including for example, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, and 100%. Identity or homology with respect to this sequence is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the

original sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. None of N-terminal, C-terminal, or internal extensions, deletions, or insertions into the specific binding agent or antibody sequence shall be construed as affecting sequence identity or homology. Thus, sequence identity can be determined by standard methods that are commonly used to compare the similarity in position of the amino acids of two polypeptides. Using a computer program such as BLAST or FASTA two polypeptides are aligned for optimal matching of their respective amino acids (either along the full length of one or both sequences, or along a pre-determined portion of one or both sequences). The programs provide a default opening penalty and a default gap penalty, and a scoring matrix such as PAM 250 [a standard scoring matrix; see Dayhoff *et al.*, in Atlas of protein Sequence and Structure, vol. 5, supp. 3 (1978)] can be used in conjunction with the computer program. For example, the percent identity can then be calculated as: the total number of identical matches multiplied by 100 and then divided by the sum of the length of the longer sequence within the matched span and the number of gaps introduced into the longer sequences in order to align the two sequences.

[00168] *Insertions:* Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intra-sequence insertions of single or multiple amino acid residues. Examples of terminal insertions include a specific binding agent or antibody with an N-terminal methionyl residue or the specific binding agent or antibody (including antibody fragment) fused to an epitope tag or a salvage receptor epitope. Other insertional variants of the specific binding agent or antibody molecule include the fusion to a polypeptide which increases the serum half-life of the specific binding agent or antibody, e.g. at the N-terminus or C-terminus.

[00169] Examples of epitope tags include the flu HA tag polypeptide and its antibody 12CA5 [Field *et al.*, *Mol. Cell. Biol.* 8: 2159-2165 (1988)]; the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto [Evan *et al.*, *Mol. Cell. Biol.* 5(12): 3610-3616 (1985)]; and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody [Paborsky *et al.*, *Protein Engineering* 3(6): 547-553 (1990)]. Other exemplary tags are a poly-histidine sequence, generally around six histidine residues, that permits isolation of a compound so labeled using nickel chelation. Other labels and tags, such as the FLAGTM tag (Eastman Kodak, Rochester, N.Y.) are well known and routinely used in the art.

[00170] The term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (e.g., IgG₁, IgG₂, IgG₃, or IgG₄) that is responsible for increasing the *in vivo* serum half-life of the IgG molecule.

[00171] *Substitutions:* Another type of variant is an amino acid substitution variant. These variants have at least one amino acid residue in the specific binding agent or antibody molecule removed and a different residue inserted in its place. Substitutional mutagenesis within any of the hypervariable or CDR regions or framework regions is contemplated. Exemplary residue substitutions comprise: Ala (A) val; leu; ile val Arg (R) lys; gln; asn lys Asn (N) gln; his; asp, lys; gln arg Asp (D) glu; asn glu Cys (C) ser; ala ser Gln (Q) asn; glu asn Glu (E) asp; gln asp Gly (G) ala His (H) asn; gln; lys; arg Ile (I) leu; val; met; ala; leu phe; norleucine Leu (L) norleucine; ile; val; ile met; ala; phe Lys (K) arg; gln; asn arg Met (M) leu; phe; ile leu Phe (F) leu; val; ile; ala; tyr Pro (P) ala Ser (S) thr Thr (T) ser ser Trp (W) tyr; phe tyr Tyr (Y) trp; phe; thr; ser phe Val (V) ile; leu; met; phe; leu ala; norleucine

[00172] Substantial modifications in the biological properties of the specific binding agent or antibody are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties: (1) hydrophobic: norleucine, met, ala, val, leu, ile; (2) neutral hydrophilic: cys, ser, thr; (3) acidic: asp, glu; (4) basic: asn, gln, his, lys, arg; (5) residues that influence chain orientation: gly, pro; and (6) aromatic: trp, tyr, phe.

[00173] Conservative substitutions involve replacing an amino acid with another member of its class. Non-conservative substitutions involve replacing a member of one of these classes with a member of another class.

[00174] Any cysteine residue not involved in maintaining the proper conformation of the specific binding agent or humanized or variant antibody also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond(s) may be added to the specific binding agent or antibody to improve its stability (particularly where the antibody is an antibody fragment such as an Fv fragment).

[00175] *Affinity Maturation:* Affinity maturation involves preparing and screening specific binding agent or antibody variants that have mutations (deletions, insertions or substitutions)

within the CDRs of a parent specific binding agent or antibody and selecting variants that have improved biological properties such as binding affinity relative to the parent specific binding agent or antibody. A convenient way for generating such substitutional variants is affinity maturation using phage display. Briefly, several hypervariable region sites (e.g. 6-7 sites) are mutated to generate all possible amino substitutions at each site. The specific binding agent or antibody variants thus generated may be displayed in a monovalent fashion from filamentous phage particles as fusions to the gene III product of M13 packaged within each particle. The phage-displayed variants are then screened for their biological activity (e.g. binding affinity).

[00176] Alanine scanning mutagenesis can be performed to identify hypervariable region residues that contribute significantly to antigen binding. Alternatively, or in addition, it may be beneficial to analyze a crystal structure of the antigen-antibody complex to identify contact points between the specific binding agent or antibody and the antigen. Such contact residues and neighboring residues are candidates for substitution according to the techniques elaborated herein. Once such variants are generated, the panel of variants is subjected to screening as described herein and specific binding agents or antibodies with superior properties in one or more relevant assays may be selected for further development.

[00177] Techniques utilizing gene shuffling and directed evolution may also be used to prepare and screen specific binding agent or antibody variants for desired activity. For example, Jerumtus *et al.*, *Proc Nat'l Acad Sci USA*. 2001 Jan. 2; 98(1):75-80 reports that tailored *in vitro* selection strategies based on ribosome display were combined with *in vitro* diversification by DNA shuffling to evolve either the off-rate or thermodynamic stability of single-chain Fv antibody fragments (scFvs); Fermer *et al.*, *Tumor Biol*. 2004 Jan.-Apr.; 25(1-2):7-13 reports that use of phage display in combination with DNA shuffling raised affinity by almost three orders of magnitude.

[00178] *Altered Glycosylation:* Specific binding agent or antibody variants can also be produced that have a modified glycosylation pattern relative to the parent specific binding agent or antibody, for example, deleting one or more carbohydrate moieties found in the specific binding agent or antibody, and/or adding one or more glycosylation sites that are not present in the specific binding agent or antibody.

[00179] Glycosylation of polypeptides including antibodies is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-

threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. The presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. Thus, N-linked glycosylation sites may be added to a specific binding agent or antibody by altering the amino acid sequence such that it contains one or more of these tripeptide sequences. O-linked glycosylation refers to the attachment of one of the sugars N-acetylglucosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used. O-linked glycosylation sites may be added to a specific binding agent or antibody by inserting or substituting one or more serine or threonine residues to the sequence of the original specific binding agent or antibody.

[00180] *Other Modifications:* Cysteine residue(s) may be removed or introduced in the Fc region, thereby eliminating or increasing interchain disulfide bond formation in this region. The homodimeric specific binding agent or antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron *et al.*, *J. Exp Med.* 176: 1191-1195 (1992) and Shopes, B. *J. Immunol.* 148: 2918-2922 (1992). Homodimeric specific binding agents or antibodies may also be prepared using heterobifunctional cross-linkers as described in Wolff *et al.*, *Cancer Research* 53: 2560-2565 (1993). Alternatively, a specific binding agent or antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson *et al.*, *Anti-Cancer Drug Design* 3: 219-230 (1989).

[00181] It has been shown that sequences within the CDR can cause an antibody to bind to MHC Class II and trigger an unwanted helper T-cell response. A conservative substitution can allow the specific binding agent or antibody to retain binding activity yet reduce its ability to trigger an unwanted T-cell response.

[00182] It is also contemplated that one or more of the N-terminal 20 amino acids of the heavy or light chain are removed.

[00183] Modifications to increase serum half-life also may be desirable, for example, by incorporation of or addition of a salvage receptor binding epitope (e.g., by mutation of the appropriate region or by incorporating the epitope into a peptide tag that is then fused to the specific binding agent or antibody at either end or in the middle, e.g., by DNA or peptide

synthesis) (see, e.g., WO96/32478) or adding molecules such as PEG or other water soluble polymers, including polysaccharide polymers.

[00184] The salvage receptor binding epitope preferably constitutes a region wherein any one or more amino acid residues from one or two loops of a Fc domain are transferred to an analogous position of the specific binding agent or antibody or fragment. Even more preferably, three or more residues from one or two loops of the Fc domain are transferred. Still more preferred, the epitope is taken from the C_{H2} domain of the Fc region (e.g., of an IgG) and transferred to the C_{H1}, C_{H3}, or V_H region, or more than one such region, of the specific binding agent or antibody. Alternatively, the epitope is taken from the C_{H2} domain of the Fc region and transferred to the C_L region or V_L region, or both, of the specific binding agent or antibody fragment. See also International applications WO 97/34631 and WO 96/32478 which describe Fc variants and their interaction with the salvage receptor.

[00185] Other sites of the constant region have been identified that are responsible for complement dependent cytotoxicity (CDC), such as the C1q binding site and/or the antibody-dependent cellular cytotoxicity (ADCC) [see, e.g., *Mol. Immunol.* 29 (5): 633-9 (1992); Shields *et al.*, *J. Biol. Chem.*, 276(9):6591-6604 (2001), incorporated by reference herein in its entirety]. Mutation of residues within Fc receptor binding sites can result in altered (i.e. increased or decreased) effector function, such as altered ADCC or CDC activity, or altered half-life. As described above, potential mutations include insertion, deletion or substitution of one or more residues, including substitution with alanine, a conservative substitution, a non-conservative substitution, or replacement with a corresponding amino acid residue at the same position from a different subclass (e.g. replacing an IgG1 residue with a corresponding IgG2 residue at that position).

[00186] *Human Antibodies:* Human antibodies can also be produced using transgenic animals that have no endogenous immunoglobulin production and are engineered to contain human immunoglobulin loci. For example, WO 98/24893 discloses transgenic animals having a human Ig locus wherein the animals do not produce functional endogenous immunoglobulins due to the inactivation of endogenous heavy and light chain loci. WO 91/741 also discloses transgenic non-primate mammalian hosts capable of mounting an immune response to an immunogen, wherein the antibodies have primate constant and/or variable regions, and wherein the endogenous immunoglobulin encoding loci are substituted or inactivated. WO 96/30498 discloses the use of the Cre/Lox system to modify the immunoglobulin locus in a mammal, such as to replace all or a portion of the constant or

variable region to form a modified antibody molecule. WO 94/02602 discloses non-human mammalian hosts having inactivated endogenous Ig loci and functional human Ig loci. U.S. Pat. No. 5,939,598 discloses methods of making transgenic mice in which the mice lack endogenous heavy chains, and express an exogenous immunoglobulin locus comprising one or more xenogeneic constant regions.

[00187] Using a transgenic animal described above, for example, an immune response can be produced to a selected antigenic molecule, and antibody producing cells can be removed from the animal and used to produce hybridomas that secrete human monoclonal antibodies. Immunization protocols, adjuvants, and the like are known in the art, and are used in immunization of, for example, a transgenic mouse as described in WO 96/33735. The monoclonal antibodies can be tested for the ability to inhibit or neutralize the biological activity or physiological effect of the corresponding protein. WO 96/33735 discloses that monoclonal antibodies against IL-8, derived from immune cells of transgenic mice immunized with IL-8, blocked IL-8 induced functions of neutrophils. Human monoclonal antibodies with specificity for the antigen used to immunize transgenic animals are also disclosed in WO 96/34096 and U.S. patent application no. 20030194404; and U.S. patent application no. 20030031667). See also Jakobovits *et al.*, *Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits *et al.*, *Nature*, 362:255-258 (1993); Bruggermann *et al.*, *Year in Immuno.*, 7:33 (1993); and U.S. Pat. No. 5,591,669, U.S. Pat. No. 5,589,369, U.S. Pat. No. 5,545,807; and U.S. Patent Application No. 20020199213. U.S. Patent Application No. and 20030092125 describes methods for biasing the immune response of an animal to the desired epitope. Human antibodies may also be generated by *in vitro* activated B cells (see U.S. Pat. Nos. 5,567,610 and 5,229,275).

[00188] *Production by Phage Display Techniques:* The development of technologies for making repertoires of recombinant human antibody genes, and the display of the encoded antibody fragments on the surface of filamentous bacteriophage, has provided another means for making human antibodies directly. The antibodies produced by phage technology are produced as antigen binding fragments-usually Fv or Fab fragments-in bacteria and thus lack effector functions. Effector functions can be introduced by one of two strategies: The fragments can be engineered either into complete antibodies for expression in mammalian cells, or into bispecific antibody fragments with a second binding site capable of triggering an effector function.

[00189] Typically, the Fd fragment (V_H - C_H1) and light chain (V_L - C_L) of antibodies are separately cloned by PCR and recombined randomly in combinatorial phage display libraries, which can then be selected for binding to a particular antigen. The antibody fragments are expressed on the phage surface, and selection of Fv or Fab (and therefore the phage containing the DNA encoding the antibody fragment) by antigen binding is accomplished through several rounds of antigen binding and re-amplification, a procedure termed panning. Antibody fragments specific for the antigen are enriched and finally isolated.

[00190] In 1994, an approach for the humanization of antibodies, called "guided selection", was described. Guided selection utilizes the power of the phage display technique for the humanization of mouse monoclonal antibody (See Jespers, L. S., *et al.*, *BioTechnology* 12, 899-903 (1994)). For this, the Fd fragment of the mouse monoclonal antibody can be displayed in combination with a human light chain library, and the resulting hybrid Fab library may then be selected with antigen. The mouse Fd fragment thereby provides a template to guide the selection. Subsequently, the selected human light chains are combined with a human Fd fragment library. Selection of the resulting library yields entirely human Fab.

[00191] A variety of procedures have been described for deriving human antibodies from phage-display libraries (See, for example, Hoogenboom *et al.*, *J. Mol. Biol.*, 227:381 (1991); Marks *et al.*, *J. Mol. Biol.*, 222:581-597 (1991); U.S. Pat. Nos. 5,565,332 and 5,573,905; Clackson, T., and Wells, J. A., *TIBTECH* 12, 173-184 (1994)). In particular, *in vitro* selection and evolution of antibodies derived from phage display libraries has become a powerful tool (See Burton, D. R., and Barbas III, C. F., *Adv. Immunol.* 57, 191-280 (1994); and, Winter, G., *et al.*, *Annu. Rev. Immunol.* 12, 433-455 (1994); U.S. patent application no. 20020004215 and WO92/01047; U.S. patent application no. 20030190317 published Oct. 9, 2003 and U.S. Pat. No. 6,054,287; U.S. Pat. No. 5,877,293.

[00192] Watkins, "Screening of Phage-Expressed Antibody Libraries by Capture Lift," *Methods in Molecular Biology, Antibody Phage Display: Methods and Protocols* 178: 187-193, and U.S. patent application no. 200120030044772 published Mar. 6, 2003 describes methods for screening phage-expressed antibody libraries or other binding molecules by capture lift, a method involving immobilization of the candidate binding molecules on a solid support.

[00193] *Other Covalent Modifications:* Covalent modifications of the specific binding agent or antibody are also included within the scope of this invention. They may be made by chemical synthesis or by enzymatic or chemical cleavage of the specific binding agent or

antibody, if applicable. Other types of covalent modifications can be introduced into the specific binding agent or antibody by reacting targeted amino acid residues with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues.

[00194] Cysteiny l residues most commonly are reacted with α -haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteiny l residues also are derivatized by reaction with bromotrifluoroacetone, α -bromo- β -(5-imidazolyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

[00195] Histidyl residues are derivatized by reaction with diethylpyrocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0.

[00196] Lysiny l and amino-terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysiny l residues. Other suitable reagents for derivatizing α -amino-containing residues include imidoesters such as methyl picolinimide, pyridoxal phosphate, pyridoxal, chloroborohydride, trinitrobenzenesulfonic acid, O-methylisourea, 2,4-pentanedione, and transaminase-catalyzed reaction with glyoxylate.

[00197] Arginy l residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

[00198] The specific modification of tyrosyl residues may be made, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidazole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Tyrosyl residues are iodinated using ^{125}I or ^{131}I to prepare labeled proteins for use in radioimmunoassay.

[00199] Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R-N-CN-R'), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl)carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

[00200] Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues, respectively. These residues are deamidated under neutral or basic conditions. The deamidated form of these residues falls within the scope of this invention.

[00201] Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the .alpha.-amino groups of lysine, arginine, and histidine side chains (T. E. Creighton, *Proteins: Structure and Molecular Properties*, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)), acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

[00202] Another type of covalent modification involves chemically or enzymatically coupling glycosides to the specific binding agent or antibody. These procedures are advantageous in that they do not require production of the specific binding agent or antibody in a host cell that has glycosylation capabilities for N- or O-linked glycosylation. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO87/05330, and in Aplin and Wriston, *CRC Crit. Rev. Biochem.*, pp. 259-306 (1981).

[00203] Removal of any carbohydrate moieties present on the specific binding agent or antibody may be accomplished chemically or enzymatically. Chemical deglycosylation requires exposure of the specific binding agent or antibody to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the specific binding agent or antibody intact. Chemical deglycosylation is described by Hakimuddin, *et al. Arch. Biochem. Biophys.* 259: 52 (1987) and by Edge *et al. Anal. Biochem.*, 118: 131 (1981). Enzymatic cleavage of carbohydrate

moieties on a specific binding agent or antibody can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura *et al. Meth. Enzymol.* 138: 350 (1987).

[00204] Another type of covalent modification of the specific binding agent or antibody comprises linking the specific binding agent or antibody to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, polyoxyethylated polyols, polyoxyethylated sorbitol, polyoxyethylated glucose, polyoxyethylated glycerol, polyoxyalkylenes, or polysaccharide polymers such as dextran. Such methods are known in the art, see, e.g. U.S. Pat. Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192, 4,179,337, 4,766,106, 4,179,337, 4,495,285, 4,609,546 or EP 315 456.

Gene Therapy

[00205] Delivery of a therapeutic specific binding agent polypeptide or antibody to appropriate cells can be effected via gene therapy *ex vivo*, *in situ*, or *in vivo* by use of any suitable approach known in the art. For example, for *in vivo* therapy, a nucleic acid encoding the desired specific binding agent or antibody, either alone or in conjunction with a vector, liposome, or precipitate may be injected directly into the subject, and in some embodiments, may be injected at the site where the expression of the specific binding agent or antibody compound is desired. For *ex vivo* treatment, the subject's cells are removed, the nucleic acid is introduced into these cells, and the modified cells are returned to the subject either directly or, for example, encapsulated within porous membranes which are implanted into the patient. See, e.g. U.S. Pat. Nos. 4,892,538 and 5,283,187.

[00206] There are a variety of techniques available for introducing nucleic acids into viable cells. The techniques vary depending upon whether the nucleic acid is transferred into cultured cells *in vitro*, or *in vivo* in the cells of the intended host. Techniques suitable for the transfer of nucleic acid into mammalian cells *in vitro* include the use of liposomes, electroporation, microinjection, cell fusion, chemical treatments, DEAE-dextran, and calcium phosphate precipitation. Other *in vivo* nucleic acid transfer techniques include transfection with viral vectors (such as adenovirus, Herpes simplex I virus, adeno-associated virus or retrovirus) and lipid-based systems. The nucleic acid and transfection agent are optionally associated with a microparticle. Exemplary transfection agents include calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, quaternary ammonium amphiphile DOTMA ((dioleoyloxypropyl)trimethylammonium bromide, commercialized as Lipofectin by GIBCO-BRL)) (Felgner *et al.*, (1987) *Proc. Natl. Acad. Sci.*

USA 84, 7413-7417; Malone *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86 6077-6081); lipophilic glutamate diesters with pendent trimethylammonium heads (Ito *et al.* (1990) *Biochem. Biophys. Acta* 1023, 124-132); the metabolizable parent lipids such as the cationic lipid dioctadecylamido glycylspermine (DOGS, Transfectam, Promega) and dipalmitoylphosphatidyl ethanolamylspermine (DPPES) (J. P. Behr (1986) *Tetrahedron Lett.* 27, 5861-5864; J. P. Behr *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86, 6982-6986); metabolizable quaternary ammonium salts (DOTB, N-(1-[2,3-dioleoyloxy]propyl)-N,N,N-trimethylammonium methylsulfate (DOTAP) (Boehringer Mannheim), polyethyleneimine (PEI), dioleoyl esters, ChoTB, ChoSC, DOSC) (Leventis *et al.* (1990) *Biochim. Inter.* 22, 235-241); 3beta[N--(N',N'-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol), dioleoylphosphatidyl ethanolamine (DOPE)/3beta[N--(N',N'-dimethylaminoethane)-carbamoyl]cholesterol DC-Chol in one to one mixtures (Gao *et al.*, (1991) *Biochim. Biophys. Acta* 1065, 8-14), spermine, spermidine, lipopolyamines (Behr *et al.*, *Bioconjugate Chem.*, 1994, 5: 382-389), lipophilic polylysines (LPLL) (Zhou *et al.*, (1991) *Biochim. Biophys. Acta* 939, 8-18), [(1,1,3,3-tetramethylbutyl)cre-soxy]ethoxy]ethyl]dimethylbenzylammonium hydroxide (DEBDA hydroxide) with excess phosphatidylcholine/cholesterol (Ballas *et al.*, (1988) *Biochim. Biophys. Acta* 939, 8-18), cetyltrimethylammonium bromide (CTAB)/DOPE mixtures (Pinnaduwa *et al.*, (1989) *Biochim. Biophys. Acta* 985, 33-37), lipophilic diester of glutamic acid (TMAG) with DOPE, CTAB, DEBDA, didodecylammonium bromide (DDAB), and stearylamine in admixture with phosphatidylethanolamine (Rose *et al.*, (1991) *Biotechniques* 10, 520-525), DDAB/DOPE (TransfectACE, GIBCO BRL), and oligogalactose bearing lipids. Exemplary transfection enhancer agents that increase the efficiency of transfer include, for example, DEAE-dextran, polybrene, lysosome-disruptive peptide (Ohmori N I *et al.*, *Biochem Biophys Res Commun* Jun. 27, 1997; 23 5(3):726-9), chondroitin-based proteoglycans, sulfated proteoglycans, polyethylenimine, polylysine (Pollard H *et al.* *J Biol Chem*, 1998 273 (13):7507-11), integrin-binding peptide CYGGRGDTP, linear dextran nonasaccharide, glycerol, cholesteryl groups tethered at the 3'-terminal internucleoside link of an oligonucleotide (Letsinger, R. L. 1989 *Proc Natl Acad Sci USA* 86: (17):6553-6), lysophosphatide, lysophosphatidylcholine, lysophosphatidylethanolamine, and 1-oleoyl lysophosphatidylcholine.

[00207] In some situations it may be desirable to deliver the nucleic acid with an agent that directs the nucleic acid-containing vector to target cells. Such "targeting" molecules include antibodies specific for a cell-surface membrane protein on the target cell, or a ligand for a

receptor on the target cell. Where liposomes are employed, proteins which bind to a cell-surface membrane protein associated with endocytosis may be used for targeting and/or to facilitate uptake. Examples of such proteins include capsid proteins and fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, and proteins that target intracellular localization and enhance intracellular half-life. In other embodiments, receptor-mediated endocytosis can be used. Such methods are described, for example, in Wu *et al.*, 1987 or Wagner *et al.*, 1990. For review of the currently known gene marking and gene therapy protocols, see Anderson 1992. See also WO 93/25673 and the references cited therein. For additional reviews of gene therapy technology, see Friedmann, *Science*, 244: 1275-1281 (1989); Anderson, *Nature*, supplement to vol. 392, no 6679, pp. 25-30 (1998); and Miller, *Nature*, 357: 455-460 (1992).

Adjuvants

[00208] In some embodiments, it may be desired to combine administration of a TNFR25 composition with adjuvants. Most adjuvants contain a substance designed to protect the antigen from rapid catabolism, such as aluminum hydroxide or mineral oil, and a stimulator of immune responses, such as *Bordetella pertussis* or *Mycobacterium tuberculosis* derived proteins. Suitable adjuvants are commercially available as, for example, Freund's Incomplete Adjuvant and Complete Adjuvant (Pifco Laboratories, Detroit, Mich.); Merck Adjuvant 65 (Merck and Company, Inc., Rahway, N.J.); aluminum salts such as aluminum hydroxide gel (alum) or aluminum phosphate; salts of calcium, iron or zinc; an insoluble suspension of acylated tyrosine; acylated sugars; cationically or anionically derivatized polysaccharides; polyphosphazenes; biodegradable microspheres; and Quil A.

[00209] Suitable adjuvants also include, but are not limited to, toll-like receptor (TLR) agonists, monophosphoryl lipid A (MPL), synthetic lipid A, lipid A mimetics or analogs, aluminum salts, cytokines, saponins, muramyl dipeptide (MDP) derivatives, CpG oligos, lipopolysaccharide (LPS) of gram-negative bacteria, polyphosphazenes, emulsions, virosomes, cochleates, poly(lactide-co-glycolides) (PLG) microparticles, poloxamer particles, microparticles, and liposomes. Preferably, the adjuvants are not bacterially-derived exotoxins. Preferred adjuvants are those which stimulate a Th1 type response such as 3DMPL or QS21.

[00210] Monophosphoryl Lipid A (MPL), a non-toxic derivative of lipid A from *Salmonella*, is a potent TLR-4 agonist that has been developed as a vaccine adjuvant. In pre-

clinical murine studies intranasal MPL has been shown to enhance secretory, as well as systemic, humoral responses. It has also been proven to be safe and effective as a vaccine adjuvant in clinical studies of greater than 120,000 patients. MPL stimulates the induction of innate immunity through the TLR-4 receptor and is thus capable of eliciting nonspecific immune responses against a wide range of infectious pathogens, including both gram negative and gram positive bacteria, viruses, and parasites. Inclusion of MPL in intranasal formulations should provide rapid induction of innate responses, eliciting nonspecific immune responses from viral challenge while enhancing the specific responses generated by the antigenic components of the vaccine.

[00211] Accordingly, in one embodiment, the present invention provides a composition comprising monophosphoryl lipid A (MPL™) or 3 De-O-acylated monophosphoryl lipid A (3D-MPL™) as an enhancer of adaptive and innate immunity. Chemically 3D-MPL™ is a mixture of 3 De-O-acylated monophosphoryl lipid A with 4, 5 or 6 acylated chains. A preferred form of 3 De-O-acylated monophosphoryl lipid A is disclosed in European Patent 0 689 454 B1 (SmithKline Beecham Biologicals SA), which is incorporated herein by reference. In another embodiment, the present invention provides a composition comprising synthetic lipid A, lipid A mimetics or analogs, such as BioMira's PET Lipid A, or synthetic derivatives designed to function like TLR-4 agonists.

[00212] The term "effective adjuvant amount" or "effective amount of adjuvant" will be well understood by those skilled in the art, and includes an amount of one or more adjuvants which is capable of stimulating the immune response to an administered composition or antigen (in this case TNFR25 composition) i.e., an amount that increases the immune response of an administered antigen composition, as measured in terms of the IgA levels in the nasal washings, serum IgG or IgM levels, or B and T-Cell proliferation. Suitably effective increases in immunoglobulin levels include by more than 5%, preferably by more than 25%, and in particular by more than 50%, as compared to the same antigen composition without any adjuvant.

[00213] When administered, the therapeutic compositions of the present invention are administered in pharmaceutically acceptable preparations. Such preparations may routinely contain pharmaceutically acceptable concentrations of salt, buffering agents, preservatives, compatible carriers, supplementary immune potentiating agents such as adjuvants and cytokines and optionally other therapeutic agents.

[00214] The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients. The term "physiologically acceptable" refers to a non-toxic material that is compatible with a biological system such as a cell, cell culture, tissue, or organism. The characteristics of the carrier will depend on the route of administration. Physiologically and pharmaceutically acceptable carriers include diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials which are well known in the art.

[00215] The therapeutics of the invention can be administered by any conventional route, including injection or by gradual infusion over time. The administration may, for example, be oral, intravenous, intraperitoneal, intramuscular, intracavity, subcutaneous, or transdermal. When antibodies are used therapeutically, a preferred route of administration is by pulmonary aerosol. Techniques for preparing aerosol delivery systems containing antibodies are well known to those of skill in the art. Generally, such systems should utilize components which will not significantly impair the biological properties of the antibodies, such as the paratope binding capacity (see, for example, Sciarra and Cutie, "Aerosols," in Remington's Pharmaceutical Sciences, 18th edition, 1990, pp 1694-1712). Those of skill in the art can readily determine the various parameters and conditions for producing antibody aerosols without resort to undue experimentation. When using antisense preparations of the invention, slow intravenous administration is preferred.

[00216] Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

[00217] The preparations of the invention are administered in effective amounts. An effective amount is that amount of a pharmaceutical preparation that alone, or together with further doses, stimulates the desired response. In the case of treating cancer, the desired response is inhibiting the progression of the cancer. This may involve only slowing the

progression of the disease temporarily, although more preferably, it involves halting the progression of the disease permanently. In the case of stimulating an immune response, the desired response is an increase in antibodies or T lymphocytes which are specific for the immunogen(s) employed. These responses can be monitored by routine methods or can be monitored according to diagnostic methods of the invention discussed herein.

[00218] Where it is desired to stimulate an immune response using a therapeutic composition of the invention, this may involve the stimulation of a humoral antibody response resulting in an increase in antibody titer in serum, a clonal expansion of cytotoxic lymphocytes, or some other desirable immunologic response. It is believed that doses of TNFR25 compositions ranging from one nanogram/kilogram to 100 milligrams/kilogram, depending upon the mode of administration, would be effective. The preferred range is believed to be between 500 nanograms and 500 micrograms per kilogram. The absolute amount will depend upon a variety of factors, including the material selected for administration, whether the administration is in single or multiple doses, and individual patient parameters including age, physical condition, size, weight, and the stage of the disease. These factors are well known to those of ordinary skill in the art and can be addressed with no more than routine experimentation.

[00219] The following examples are offered by way of illustration, not by way of limitation. While specific examples have been provided, the above description is illustrative and not restrictive. Any one or more of the features of the previously described embodiments can be combined in any manner with one or more features of any other embodiments in the present invention. Furthermore, many variations of the invention will become apparent to those skilled in the art upon review of the specification.

[00220] All publications and patent documents cited in this application are incorporated by reference in pertinent part for all purposes to the same extent as if each individual publication or patent document were so individually denoted. By their citation of various references in this document, Applicants do not admit any particular reference is "prior art" to their invention.

EXAMPLES

[00221] The following examples serve to illustrate the invention without limiting it thereby. It will be understood that variations and modifications can be made without departing from the spirit and scope of the invention.

[00222] Embodiments of the invention may be practiced without the theoretical aspects presented. Moreover, the theoretical aspects are presented with the understanding that Applicants do not seek to be bound by the theory presented.

[00223] While various embodiments of the present invention have been described above, it should be understood that they have been presented by way of example only, and not limitation. Numerous changes to the disclosed embodiments can be made in accordance with the disclosure herein without departing from the spirit or scope of the invention. Thus, the breadth and scope of the present invention should not be limited by any of the above described embodiments.

Example 1: Agonistic TNFR25 antibody 4C12 enhances CD8 CTL expansion mediated by Gp96-Ig vaccines.

[00224] C57Bl/6 mice received 1 million gfp-marked OT-1 (ovalbumin specific, TCR transgenic CD8 T cells) by i.v. adoptive transfer which establish a 0.2% frequency in the recipient mouse (Figure 1). Two days later the mice are immunized i.p. with 2 million allogeneic 3T3-ova-gp96-Ig secreting 200ng gp96-Ig in 24 hours. Agonistic 4C12 anti TNFR25 antibody (50µg i.p.) or control IgG is given on day 1 and 3 after immunization and mice are analyzed on day 5 after immunization by determining the frequency of gfp-OT-I cells in the CD8 gate by flow cytometry. The data show that TNFR25 agonists in conjunction with gp96-vaccines enhance expansion of cytotoxic CD8⁺ CTL in peripheral tissues and in the intestinal mucosa.

Example 2: TNFR25 agonists enhance expansion of HIV-specific CD8 CTL in response to gp96-Ig vaccination.

[00225] HEK-293 cells were transfected with gp96-Ig and HIV-gag. One million 293-HIV-gag-gp96-Ig cells secrete 1µg gp96-Ig in 24hours. To measure HIV responses in mice HLA A2 transgenic mice were immunized with 1 million 293-HIV-gag-gp96-Ig. 1 day later the mice received 100µg 4C12 or control IgG and 3 days after immunization 40µg of 4C12 or IgG. Mice were analyzed on day 5 by staining lymphocytes from various sites with HLA A2-gag tetramers. The data show that TNFR25 agonists are powerful enhancers of CD8 CTL activation and Expansion induced by gp96-Ig or other vaccines. This procedure can serve to generate a new class of HIV and cancer vaccines.

Other Embodiments

[00226] It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention. Other aspects, advantages, and modifications are within the scope of the following claims.

[00227] All references cited herein, are incorporated herein by reference. Although the invention has been illustrated and described with respect to one or more implementations, equivalent alterations and modifications will occur to others skilled in the art upon the reading and understanding of this specification and the annexed drawings. In addition, while a particular feature of the invention may have been disclosed with respect to only one of several implementations, such feature may be combined with one or more other features of the other implementations as may be desired and advantageous for any given or particular application.

[00228] The Abstract will allow the reader to quickly ascertain the nature of the technical disclosure. It is submitted with the understanding that it will not be used to interpret or limit the scope or meaning of the following claims.

What is claimed is:

1. A composition comprising a TNFR25 agonist as an immune modulator.
2. The composition of claim 1, wherein the TNFR25 agonist comprises antibodies, ligands, aptamers, oligonucleotides, polynucleotides, peptides, proteins, organic molecules or inorganic molecules.
3. The composition of claim 1, wherein the TNFR25 agonist is an antibody specific for TNFR25.
4. The composition of claim 1 further comprising the TNFR25 agonist and a vaccine.
5. A method of modulating an antigen specific immune response *in vivo*, comprising:
administering to a patient, a therapeutically effective amount of an agent and an agonistic anti-TNFR25 antibody; and,
modulating an antigen specific immune response *in vivo*.
6. The method of claim 5, wherein the agonistic anti-TNFR25 antibody up-regulates an agent-induced immune response as compared to the agent-induced immune response in the absence of the agonistic anti-TNFR25 antibody.
7. The method of claim 5, wherein the anti-TNFR25 is administered to a patient prior to, concurrently with, or after administration of the agent.
8. The method of claim 7, wherein the agent induces an antigen specific immune response.
9. The method of claim 8, wherein anti-TNFR25 antibody decreases or prevents generation of antigen specific Treg cells.
10. The method of claim 8, wherein the agent is a vaccine.

11. The method of claim 5, wherein the agent is a tumor vaccine and administration of the anti-TNFR25 antibody increases an anti-tumor immune response as compared to the anti-tumor immune response in the absence of the anti-TNFR25 antibody.
12. The method of claim 5, wherein the agent is an anti-HIV vaccine and administration of the anti-TNFR25 antibody enhances an anti-HIV immune response as compared to the anti-HIV immune response in the absence of the anti-TNFR25 antibody.
13. The method of claim 12, wherein the anti-HIV vaccine comprises HIV-gp96-Ig.
14. The method of claim 5, wherein the anti-TNFR25 antibody enhances antigen specific immune responses to diseases or disorders comprising tumors, infectious disease organisms, parasites or fungus.
15. A method of enhancing an antigen specific immune response comprising:
 - obtaining a sample from a patient;
 - separating immune cells from the sample;
 - culturing the immune cells with a TNFR25 agonist and/or antigen;
 - expanding the immune cells and re-introducing said cells to a patient; and,enhancing the antigen specific immune response.
16. The method of claim 15, wherein the immune cells comprise antigen presenting cells, T cells, B cells and natural killer cells.
17. The method of claim 15, wherein the TNFR25 agonist is an anti-TNFR25 antibody.
18. The method of claim 15, wherein the immune cells are cultured with antigen, prior to, concurrently with or after contact with the TNFR25 agonist.

19. The method of claim 15, wherein the administration of an anti-TNFR25 antibody enhances an immune response to a specific antigen as compared to the antigen specific immune response in the absence of the anti-TNFR25 antibody.
20. The method of claim 15, wherein administration of the anti-TNFR25 antibody inhibits generation of antigen specific Treg cells induced by CD103⁺ dendritic cells.
21. The method of claim 15, wherein a specific antigen comprises at least one of: viral antigen(s), tumor antigen(s), parasitic antigen(s), bacterial antigen(s), protozoan antigen(s) or combinations thereof.
22. A method of preventing or treating cancer, comprising:
administering to a patient in need thereof, a tumor antigen, an anti-TNFR25 antibody;
and,
preventing or treating cancer.
23. The method of claim 22, wherein a tumor antigen is derived from a patient tumor comprising at least one of: tumor cell, tumor cell membranes, tumor proteins, tumor nucleic acids or combinations thereof.
24. The method of claim 22, wherein the tumor antigen comprises a tumor vaccine.
25. The method of claim 22, wherein the anti-TNFR25 antibody is administered in conjunction, prior to or after administration of a tumor antigen or vaccine.
26. A method of prevent or treating a disease caused by a biological agent, comprising:
administering to a patient in need thereof, a vaccine or a biological agent antigen, an anti-TNFR25 antibody; and,
prevent or treating a disease caused by a biological agent.

27. The method of claim 26, wherein a biological agent antigen comprising at least one of: viral antigen(s), tumor antigen(s), parasitic antigen(s), bacterial antigen(s), protozoan antigen(s) or combinations thereof.
28. The method of claim 26, wherein a biological agent antigen comprises a viral vaccine.
29. The method of claim 28, wherein the viral vaccine is an HIV vaccine.
30. The method of claim 26, wherein the anti-TNFR25 antibody is administered in conjunction, prior to or after administration of a biological agent antigen or vaccine.
31. A method of enhancing an antigen specific immune response *in vivo*, comprising:
administering to a patient in need thereof, an antigen, a TNFR25 agonist; and,
enhancing the antigen specific immune response *in vivo*.
32. The method of claim 31, wherein an antigen specific immune response activates immune cells comprising: antigen presenting cells, T cells, B cells and natural killer cells.
33. The method of claim 31, wherein the TNFR25 agonist is an anti-TNFR25 antibody.
34. The method of claim 31, wherein the administration of an anti-TNFR25 antibody enhances an immune response to a specific antigen as compared to the antigen specific immune response in the absence of the anti-TNFR25 antibody.
35. The method of claim 31, wherein administration of the anti-TNFR25 antibody inhibits generation of antigen specific Treg cells induced by CD103⁺ dendritic cells.
36. The method of claim 31, wherein a specific antigen comprises at least one of: viral antigen(s), tumor antigen(s), parasitic antigen(s), bacterial antigen(s), protozoan antigen(s) or combinations thereof.

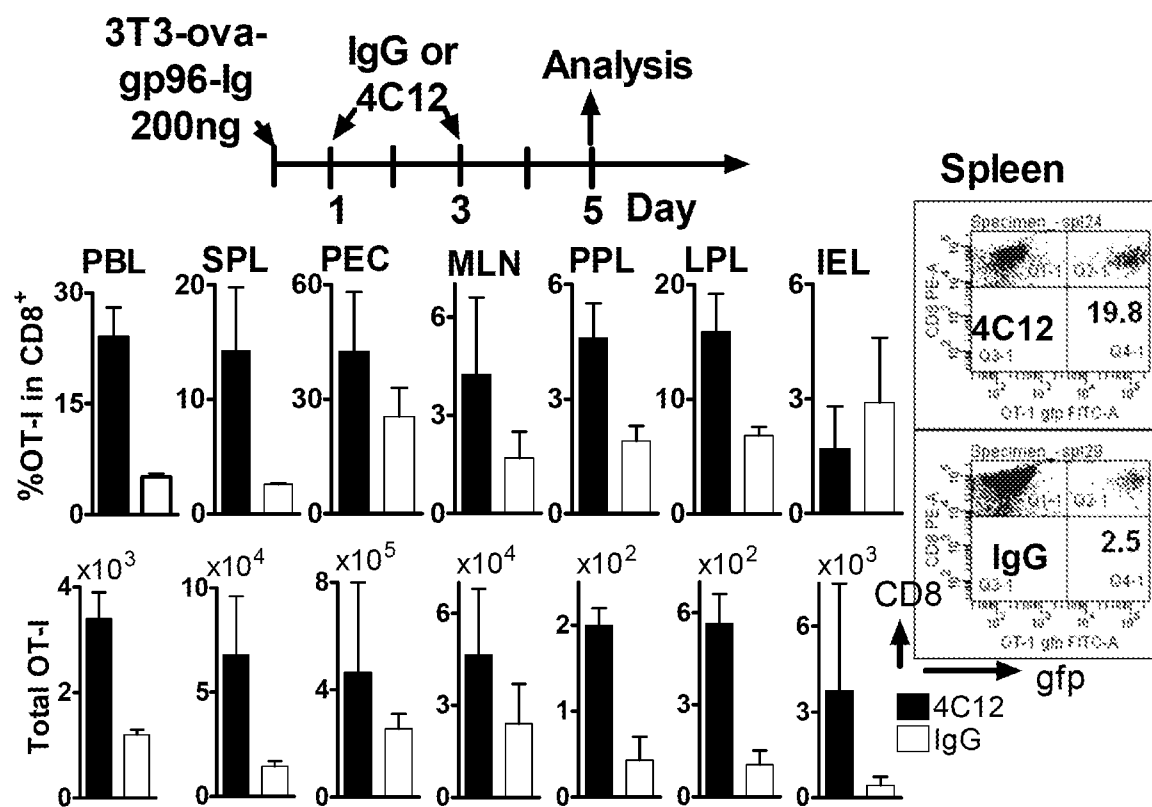


FIGURE 1

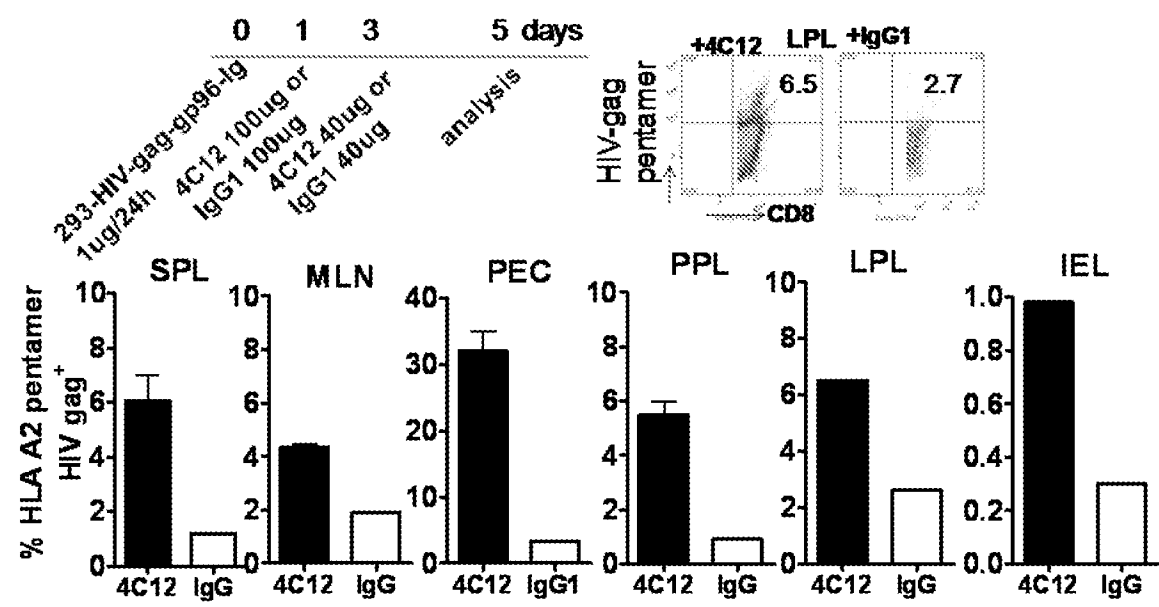


FIGURE 2

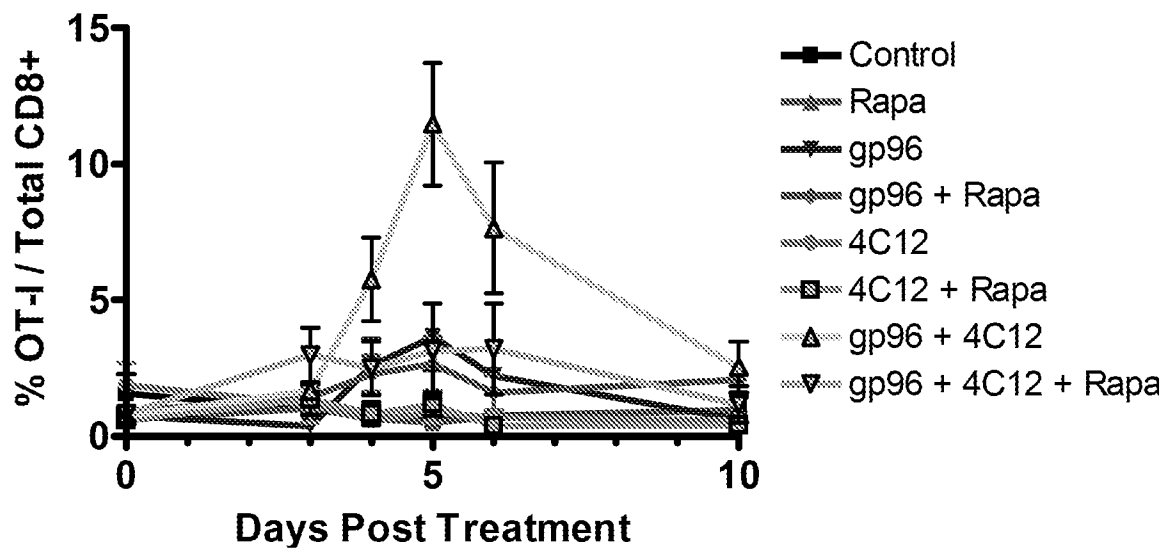


FIGURE 3A

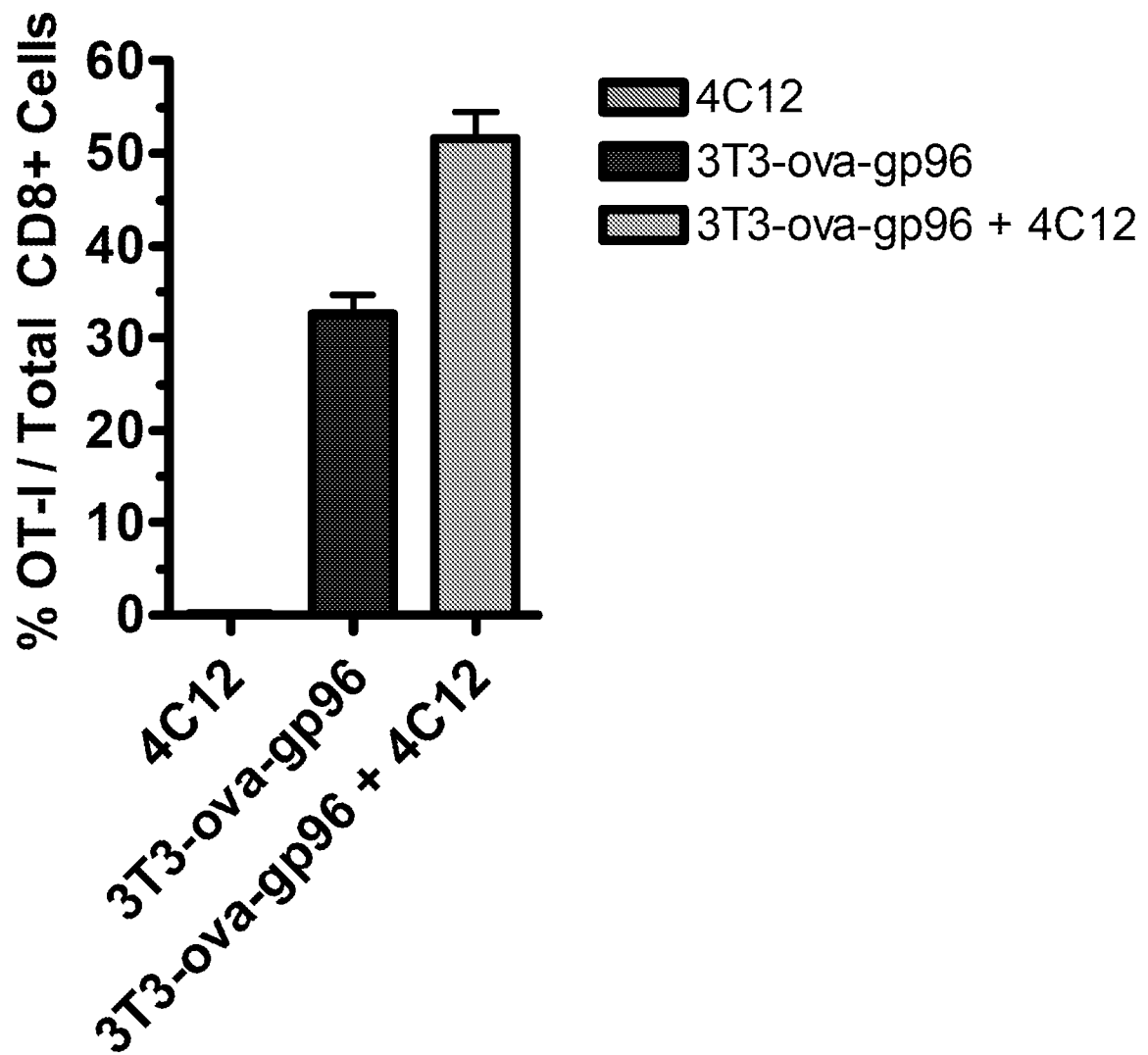


FIGURE 3B

5 / 7

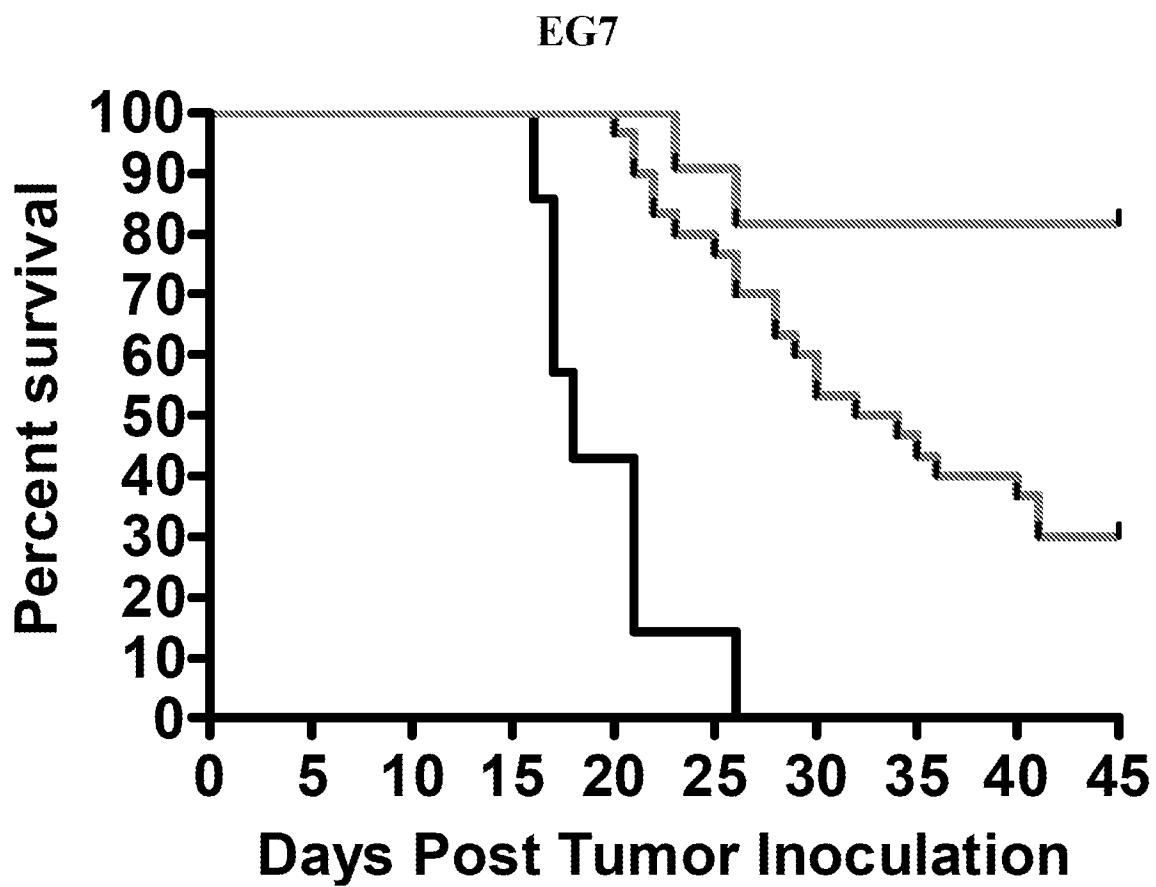


FIGURE 4A

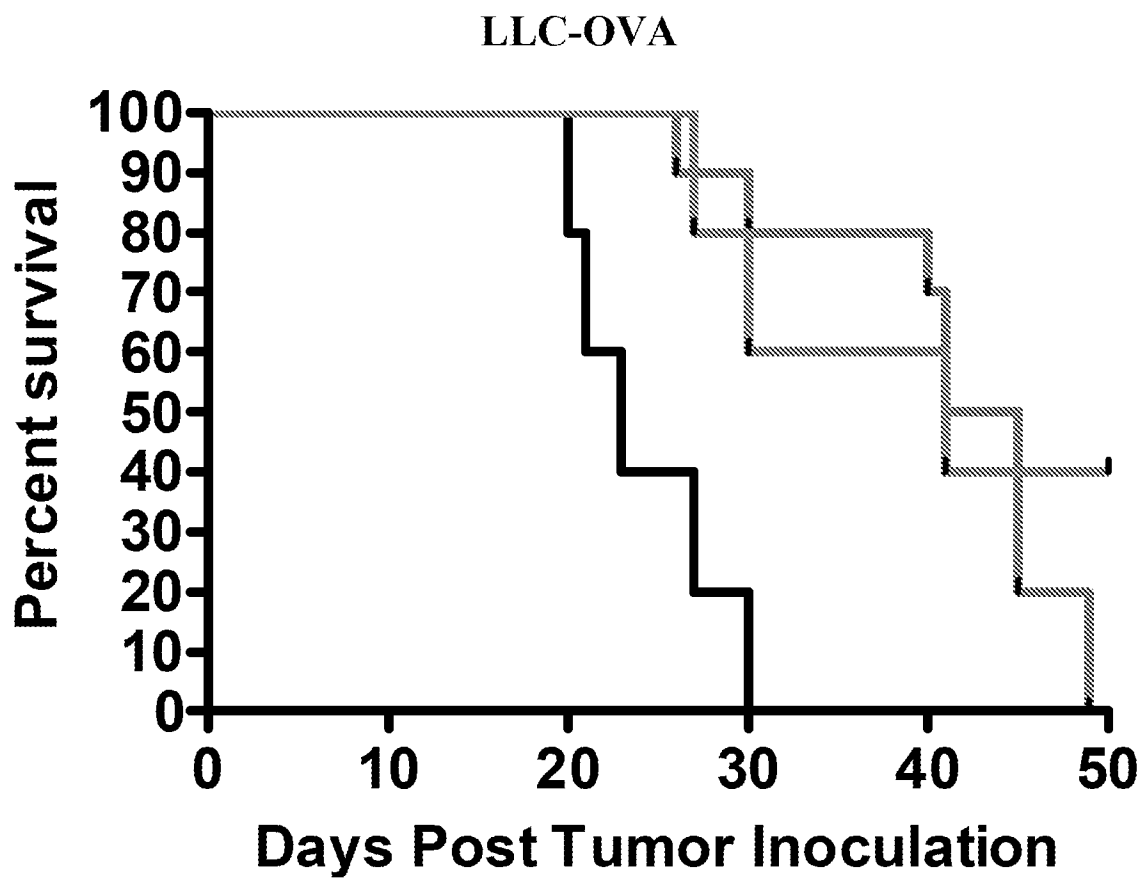


FIGURE 4B

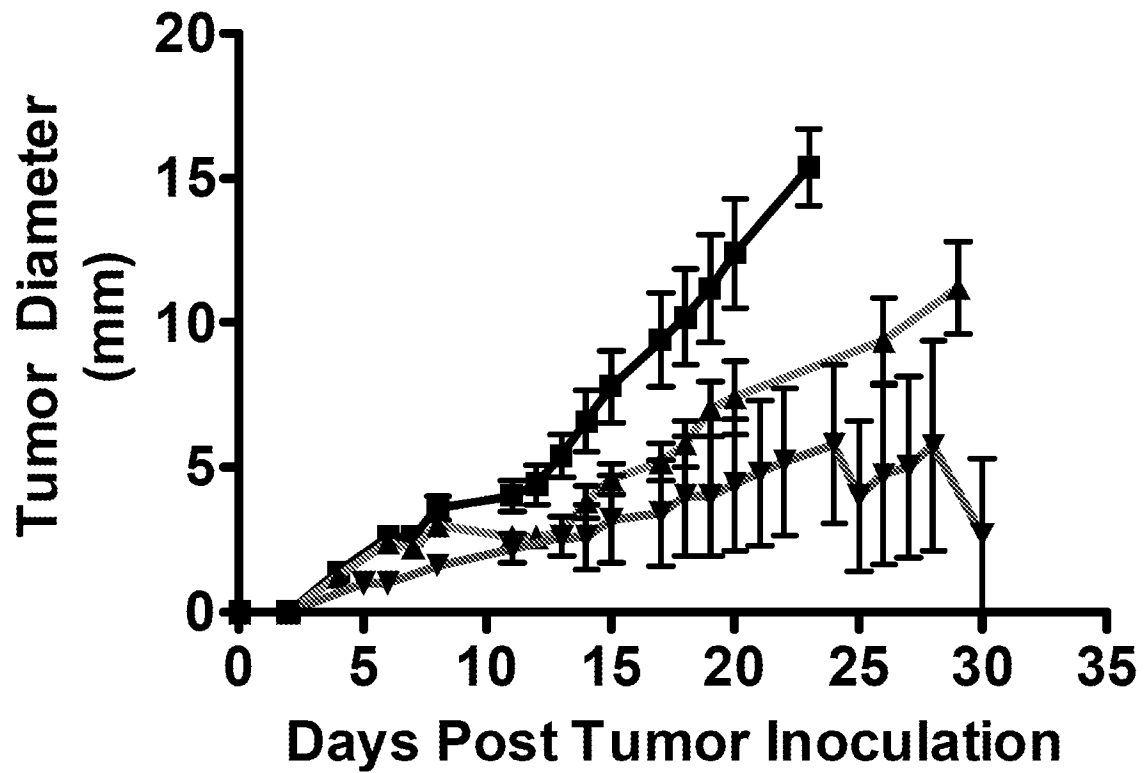
3-MCA INDUCED SARCOMA (MCAS)

FIGURE 4C

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 09/68966

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 39/395 (2010.01)

USPC - 424/143.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) - A61K 39/395 (2010.01)

USPC - 424/143.1; 424/143.1, 424/144.1, 424/154.1; 514/2, 514/12, 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

IPC(8) - A61K 39/395 (2010.01) - see keyword below

USPC - 424/143.1; 424/143.1, 424/144.1, 424/154.1; 514/2, 514/12, 514/44 - see keyword below

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PubWEST(USPT,PGPB,EPAB,JPAB); Medline, Google

Search terms: Tumor Necrosis Factor Receptor 25, TNFR25, Death receptor 3, DR3, angonist, immune modulator, antibody, ligands, aptamer, oligonucleotide, polynucleotide, peptide, protein, organic, small molecule, inorganic, vaccine, immunological, stimulate

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2007/0128184 A1 (PODACK et al.) 07 June 2007 (07.06.2007), Abstract, para [0015], [0021], [0054], [0085], [0094], [0095], [0097], [0144], and [0150]	1-4
A	WO 2007/027751 A2 (PODACK et al.) 08 March 2007 (08.03.2007) abstract	1-4
A	FANG et al. Essential role of TNF receptor superfamily 25 (TNFRSF25) in the development of allergic lung inflammation. J. Exp. Med. May 2008, Vol. 205, No. 5, pp 1037-1048, especially the abstract and pg 1038, col 2, para 2	1-4

☐ Further documents are listed in the continuation of Box C.


* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

23 April 2010 (23.04.2010)

Date of mailing of the international search report

10 MAY 2010

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-3201

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 09/68966

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-4 are drawn to a composition.

Group II: Claims 5-14 are drawn to a method of modulating an antigen specific immune response in vivo comprising administering an agent and an agonistic anti-TNFR25 antibody.

Group III: Claims 15-21 are drawn to a method of enhancing an antigen specific immune response comprising culturing immune cells from a patient with a TNFR25 agonist and reintroducing the cells.

*****Continued in the extra sheet*****

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-4

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 09/68966

Continuation of:

Box No III (unity of invention is lacking)

Group IV: Claims 22-36 are drawn to a method of preventing or treating cancer and a method of prevent or treating a disease caused by a biological agent, which both comprise administering a TNFR25 agonist antibody with an antigen.

The groups listed above do not relate to a single general inventive concept under PCT Rule 13.1 because under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons.

The technical features that link the listed groups are a composition comprising a TNFR25 agonist as an immune modulator and a method of enhancing an antigen specific immune response by administering a TNFR25 agonist such as an agonistic anti-TNFR25 antibody. However, this does not represent an improvement over the prior art of Podack et al. (WO 2007/027751 A2) who teach such a composition and method (abstract; pg 4, ln 8-10; and pg 5, ln 9-11).

Accordingly, unity of invention is lacking.