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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
25 February 2010 (25.02.2010)

PCT

(10) International Publication Number
WO 2010/022227 A1

(51) International Patent Classification:
G01N 33/574 (2006.01)

(21) International Application Number:
PCT/US2009/054443

(22) International Filing Date:
20 August 2009 (20.08.2009)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/090,512 20 August 2008 (20.08.2008) US
61/227,283 21 July 2009 (21.07.2009) US

(71) Applicant *(for all designated States except US)*: **SCHERING CORPORATION** [US/US]; 2000 Galloping Hill Road, Kenilworth, New Jersey 07033 (US).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: **WU, Lingling** [CN/US]; 1594 Mariposa Avenue, Palo Alto, California 94306 (US).

(74) Agent: **MOHAN-PETERSON, Sheela**; Schering Corporation, Patent Dept. K-6-1 1990, 2000 Galloping Hill Road, Kenilworth, New Jersey 07033 (US).

(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).

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

— *with international search report (Art. 21(3))*

WO 2010/022227 A1

(54) **Title**: METHODS FOR MONITORING IL-10 THERAPY

(57) **Abstract**: The present invention provides biomarkers to monitor IL-10 therapy. In particular biomarkers are provided to assess target engagement and efficacy of tumor treatment.

METHODS FOR MONITORING IL-10 THERAPY

FIELD OF THE INVENTION

[0001] The present invention provides methods for monitoring IL-10 therapy. In particular, the present invention provides biomarkers to assess IL-10 target engagement and efficacy of tumor treatment.

BACKGROUND OF THE INVENTION

[0002] Cancers and tumors can be controlled or eradicated by the immune system. The immune system includes several types of lymphoid and myeloid cells, e.g., monocytes, macrophages, dendritic cells (DCs), eosinophils, T cells, B cells, and neutrophils. These lymphoid and myeloid cells produce secreted signaling proteins known as cytokines. The cytokines include, e.g., interleukin-10 (IL-10), interferon-gamma (IFNγ), IL-12, and IL-23. Immune response includes inflammation, i.e., the accumulation of immune cells systemically or in a particular location of the body. In response to an infective agent or foreign substance, immune cells secrete cytokines which, in turn, modulate immune cell proliferation, development, differentiation, or migration. Excessive immune response can produce pathological consequences, such as autoimmune disorders, whereas impaired immune response may result in cancer. Anti-tumor response by the immune system includes innate immunity, e.g., as mediated by macrophages, NK cells, and neutrophils, and adaptive immunity, e.g., as mediated by antigen presenting cells (APCs), T cells, and B cells (see, e.g., Abbas, *et al.* (eds.) (2000) *Cellular and Molecular Immunology*, W.B. Saunders Co., Philadelphia, PA; Oppenheim and Feldmann (eds.) (2001) *Cytokine Reference*, Academic Press, San Diego, CA; von Andrian and Mackay (2000) *New Engl. J. Med.* 343:1020-1034; Davidson and Diamond (2001) *New Engl. J. Med.* 345:340-350).

[0003] Initially known as cytokine synthesis inhibitor factor or CSIF, interleukin-10 (IL-10) is a potent immunomodulator of hematopoietic cells, particularly immune cells. Cells such as activated Th2 cells, B cells, keratinocytes, monocytes and macrophages produce

IL-10. *See, e.g.*, Moore et al., *Annu. Rev. Immunol.* 11:165 (1993). IL-10 inhibits activation and effector functions of a number of cells that include T cells, monocytes and macrophages. In particular, IL-10 inhibits cytokine synthesis, including that of IL-1, IFN-γ, and TNF, by cells such as Th1 cells, natural killer cells, monocytes, and macrophages. *See, e.g.*, Fiorentino et al., (1989) *J. Exp. Med.*, 170:2081-2095; Fiorentino et al., (1991) *J. Immunol.* 146:3444; Hsu et al., (1992) *Int. Immunol.* 4:563; Hsu et al., (1992) *Int. Immunol.* 4:563; D'Andrea et al., (1993) *J. Exp. Med.* 178:1041; de Waal Malefyt et al., (1991) *J. Exp. Med.* 174:915; Fiorentino et al., (1991) *J. Immunol.* 147:3815.

[0004] Methods of modulating immune response have been used in the treatment of cancers, e.g., melanoma. These methods include treatment either with cytokines such as IL-2, IL-10, IL-12, tumor necrosis factor-alpha (TNFalpha), IFNγ, granulocyte macrophage-colony stimulating factor (GM-CSF), and transforming growth factor (TGF), or with cytokine antagonists (e.g., antibodies). Interleukin-10 was first characterized as a cytokine synthesis inhibitory factor (CSIF; see, e.g., Fiorentino, et al (1989) *J. Exp. Med.* 170:2081-2095). IL-10 is a pleiotropic cytokine produced by T cells, B cells, monocytes, that can function as both an immunosuppressant and immunostimulant (see, e.g., Groux, et al. (1998) *J. Immunol.* 160:3188-3193; and Hagenbaugh, et al. (1997) *J. Exp. Med.* 185:2101-2110).

[0005] Animal models suggest that IL-10 can induce NK-cell activation and facilitate target-cell destruction in a dose-dependent manner (see, e.g., Zheng, et al. (1996) *J. Exp. Med.* 184:579-584; Kundu, et al. (1996) *J. Natl. Cancer Inst.* 88:536-541). Further studies indicate that the presence of IL-10 in the tumor microenvironment correlates with better patient survival (see, e.g., Lu, et al. (2004) *J. Clin. Oncol.* 22:4575-4583). IL-10 induces cytotoxic activity of CD8 T-cells, antibody production of B-cell and suppresses macrophage activity and tumor promoting inflammation (see, Chen and Zlotnik (1991) J. Immunol. 147:528-534; Groux, et al. (1999) J. Immunol. 162:1723-1729; and Bergman, et al. (1996) J. Immunol. 157:231-238). The regulation of CD8 cells is dose dependent, wherein higher doses induce stronger cytotoxic responses.

[0006] As the level of IL-10 in the microenvironment is critical to induce cytotoxic activity, a need exists to monitor IL-10 levels (both native and engineered and/or linked IL-10 proteins) in the serum of patients. The present invention fulfills this need by providing biomarkers to assess IL-10 receptor engagement as well IL-10 efficacy levels, thereby providing information to monitor IL-10 therapies.

SUMMARY OF THE INVENTION

[0007] The present invention is based, in part, upon the discovery of a set of biomarkers that have increased expression upon IL-10 target engagement, and a set if biomarkers that have increased expression correlating with tumor treatment efficacy.

[0008] The present invention provides a method for monitoring efficacy of an IL-10 therapeutic agent in treatment of a subject for a tumor or cancer, said method comprising: a) obtaining a baseline biological sample from said subject prior to administering a dose of the IL-10 therapeutic agent; b) measuring the level of at least one biomarker in the baseline biological sample by gene expression analysis or immunoassay, c) administering the IL-10 therapeutic agent to the subject; d) obtaining from the subject at least one subsequent biological sample; e) measuring the level of the biomarker in the subsequent sample by gene expression analysis or immunoassay; f) comparing the level of the biomarker in the subsequent sample with the level of the biomarker in the baseline biological sample; g) and correlating a change in the level of the biomarker with treatment efficacy, wherein an increase of at least one biomarker in the subsequent biological sample indicates an effective dose of the IL-10 therapeutic agent. In certain embodiments, the subject is human and the tumor or cancer is from an organ selected from the group consisting of skin, colon, thyroid, ovarian, lung, pancreas and brain. In yet a further embodiment the tumor is selected from the group consisting of: colon cancer, ovarian cancer, breast cancer, melanoma, lung cancer, glioblastoma, and leukemia. In another embodiment, the IL-10 therapeutic agent is selected from the group consisting of native IL-10, pegylated IL-10 (PEG-IL-10), and IL-10 conjugated to an Fc portion of an immunoglobulin (IL-10-Ig). The biomarker is selected from the group consisting of IP-10 and MIG. In certain embodiments, the increase in IP-10 or MIG expression in the subsequent biological sample is at least 2 fold to at least 19 fold.
In further embodiments, the gene expression analysis is selected from the group consisting of Northern blotting, quantitative PCR, SAGE, and DNA microarray, the immunassay selected from the group consisting of ELISA, RIA, Western blot, luminescent immunoassay, flourescent immunoassay.

The present invention also provides a method for monitoring target engagement following administration of an IL-10 therapeutic agent to treat a subject for a tumor or cancer, said method comprising: a) obtaining a baseline biological sample from said subject prior to administering a dose of the IL-10 therapeutic agent; b) measuring the level of at least one biomarker in the baseline biological sample by gene expression or immunoassay, c) administering the IL-10 therapeutic agent to the subject; d) obtaining from the subject at least one subsequent biological sample; e) measuring the level of the biomarker in the subsequent sample by gene expression analysis or immunoassay; f) comparing the level of the biomarker in the subsequent sample with the level of the biomarker in the baseline biological sample; and g) correlating a change in the level of the biomarker target engagement, wherein an increase of at least one biomarker in the subsequent biological sample indicates target engagement. In certain embodiments, the subject is human. The tumor or cancer is from an organ selected from the group consisting of skin, colon, thyroid, ovarian, lung, pancreas, and brain. In further embodiments, the tumor is selected from the group consisting of: colon cancer, ovarian cancer, breast cancer, melanoma, lung cancer, glioblastoma, and leukemia. In other embodiments, the IL-10 therapeutic agent is selected from the group consisting of native IL-10, pegylated IL-10 (PEG-IL-10), and IL-10 conjugated to an Fc portion of an immunoglobulin (IL-10-Ig). The biomarker is chosen from the group consisting of MCP-1 and phosphorylation of STAT3. In further embodiments, the gene expression analysis is selected from the group consisting of Northern blotting, quantitative PCR, SAGE, and DNA microarray, the immunoassay is selected from the group consisting of ELISA, RIA, Western blot, luminescent immunoassay, flourescent immunoassay.

DETAILED DESCRIPTION

[0009] As used herein, including the appended claims, the singular forms of words such as "a," "an," and "the," include their corresponding plural references unless the context clearly dictates otherwise.

[0010] All references cited herein are incorporated by reference to the same extent as if each individual publication, patent application, or patent, was specifically and individually indicated to be incorporated by reference.

I. Definitions.

[0011] "Activity" of a molecule may describe or refer to the binding of the molecule to a ligand or to a receptor, to catalytic activity, to the ability to stimulate gene expression, to antigenic activity, to the modulation of activities of other molecules, and the like. "Activity" of a molecule may also refer to activity in modulating or maintaining cell-to-cell interactions, e.g., adhesion, or activity in maintaining a structure of a cell, e.g., cell membranes or cytoskeleton. "Activity" may also mean specific activity, e.g., [catalytic activity]/[mg protein], or [immunological activity]/[mg protein], or the like.

[0012] "Activation," "stimulation," and "treatment," as it applies to cells or to receptors, may have the same meaning, e.g., activation, stimulation, or treatment of a cell or receptor with a ligand, unless indicated otherwise by the context or explicitly. "Ligand" encompasses natural and synthetic ligands, e.g., cytokines, cytokine variants, analogues, muteins, and binding compositions derived from antibodies. "Ligand" also encompasses small molecules, e.g., peptide mimetics of cytokines and peptide mimetics of antibodies. "Activation" can refer to cell activation as regulated by internal mechanisms as well as by external or environmental factors. "Response," e.g., of a cell, tissue, organ, or organism, encompasses a change in biochemical or physiological behavior, e.g., concentration, density, adhesion, or migration within a biological compartment, rate of gene expression, or state of differentiation, where the change is correlated with activation, stimulation, or treatment, or with internal mechanisms such as genetic programming.

[0013] "Activity" of a molecule may describe or refer to the binding of the molecule to a ligand or to a receptor, to catalytic activity; to the ability to stimulate gene expression or cell signaling, differentiation, or maturation; to antigenic activity, to the modulation of

activities of other molecules, and the like. "Activity" of a molecule may also refer to activity in modulating or maintaining cell-to-cell interactions, e.g., adhesion, or activity in maintaining a structure of a cell, e.g., cell membranes or cytoskeleton. "Activity" can also mean specific activity, e.g., [catalytic activity]/[mg protein], or [immunological activity]/[mg protein], concentration in a biological compartment, or the like. "Proliferative activity" encompasses an activity that promotes, that is necessary for, or that is specifically associated with, e.g., normal cell division, as well as cancer, tumors, dysplasia, cell transformation, metastasis, and angiogenesis.

[0014] "Administration" and "treatment," as it applies to an animal, human, experimental subject, cell, tissue, organ, or biological fluid, refers to contact of an exogenous pharmaceutical, therapeutic, diagnostic agent, compound, or composition to the animal, human, subject, cell, tissue, organ, or biological fluid. "Administration" and "treatment" can refer, e.g., to therapeutic, placebo, pharmacokinetic, diagnostic, research, and experimental methods. "Treatment of a cell" encompasses contact of a reagent to the cell, as well as contact of a reagent to a fluid, where the fluid is in contact with the cell. "Administration" and "treatment" also means *in vitro* and *ex vivo* treatments, e.g., of a cell, by a reagent, diagnostic, binding composition, or by another cell. "Treatment," as it applies to a human, veterinary, or research subject, refers to therapeutic treatment, prophylactic or preventative measures, to research and diagnostic applications. "Treatment" as it applies to a human, veterinary, or research subject, or cell, tissue, or organ, encompasses contact of PEG-IL-10 to a human or animal subject, a cell, tissue, physiological compartment, or physiological fluid. "Treatment of a cell" also encompasses situations where PEG-IL-10 contacts IL-10 receptor (heterodimer of IL-10R1 and IL-10R2) e.g., in the fluid phase or colloidal phase, as well as situations where an IL-10 agonist or antagonist contacts a fluid, e.g., where the fluid is in contact with a cell or receptor, but where it has not been demonstrated that the agonist or antagonist directly contacts the cell or receptor.

[0015] "Effective amount" encompasses an amount sufficient to ameliorate or prevent a symptom or sign of the medical condition. Effective amount also means an amount sufficient to allow or facilitate diagnosis. An effective amount for a particular patient or veterinary subject may vary depending on factors such as the condition being treated, the overall health of the patient, the method route and dose of administration and the severity of side effects (see, e.g., U.S. Pat. No. 5,888,530 issued to Netti, *et al.*). An effective amount

can be the maximal dose or dosing protocol that avoids significant side effects or toxic effects. The effect will result in an improvement of a diagnostic measure or parameter by at least 5%, usually by at least 10%, more usually at least 20%, most usually at least 30%, preferably at least 40%, more preferably at least 50%, most preferably at least 60%, ideally at least 70%, more ideally at least 80%, and most ideally at least 90%, where 100% is defined as the diagnostic parameter shown by a normal subject (see, e.g., Maynard, *et al.* (1996) *A Handbook of SOPs for Good Clinical Practice*, Interpharm Press, Boca Raton, FL; Dent (2001) *Good Laboratory and Good Clinical Practice*, Urch Publ., London, UK). An effective amount of PEG-IL-10 would be an amount sufficient to reduce a tumor volume, inhibit tumor growth, prevent metastasis, or increase CD8+ T cell infiltration in to the tumor site.

[0016] "Exogenous" refers to substances that are produced outside an organism, cell, or human body, depending on the context. "Endogenous" refers to substances that are produced within a cell, organism, or human body, depending on the context.

[0017] "Specifically" or "selectively" binds, when referring to a ligand/receptor, antibody/antigen, or other binding pair, indicates a binding reaction which is determinative of the presence of the protein in a heterogeneous population of proteins and other biologics. Thus, under designated conditions, a specified ligand binds to a particular receptor and does not bind in a significant amount to other proteins present in the sample. The antibody, or binding composition derived from the antigen-binding site of an antibody, of the contemplated method binds to its antigen, or a variant or mutein thereof, with an affinity that is at least two fold greater, preferably at least ten times greater, more preferably at least 20-times greater, and most preferably at least 100-times greater than the affinity with any other antibody, or binding composition derived thereof. In a preferred embodiment the antibody will have an affinity that is greater than about 10^9 liters/mol, as determined, e.g., by Scatchard analysis (Munsen, *et al.* (1980) *Analyt. Biochem.* 107:220-239).

[0018] "Interleukin-10" or "IL-10", as used herein, whether conjugated to a polyethylene glycol, or in a non-conjugated form, is a protein comprising two subunits noncovalently joined to form a homodimer. As used herein, unless otherwise indicated "interleukin-10" and "IL-10" can refer to human or mouse IL-10 (Genbank Accession Nos. NP_000563; M37897; or US 6,217,857) which are also referred to as "hIL-10" or "mIL-10".

[0019] "Pegylated IL-10" or "PEG-IL-10" is an IL-10 molecule having one or more polyethylene glycol molecules covalently attached to one or more than one amino acid residue of the IL-10 protein via a linker, such that the attachment is stable. The terms "monopegylated IL-10" and "mono-PEG-IL-10", mean that one polyethylene glycol molecule is covalently attached to a single amino acid residue on one subunit of the IL-10 dimer via a linker. The average molecular weight of the PEG moiety is preferably between about 5,000 and about 50,000 daltons. The method or site of PEG attachment to IL-10 is not critical, but preferably the pegylation does not alter, or only minimally alters, the activity of the biologically active molecule. Preferably, the increase in half-life is greater than any decrease in biological activity. For PEG-IL-10, biological activity is typically measured by assessing the levels of inflammatory cytokines (e.g., TNFα, IFNγ) in the serum of subjects challenged with a bacterial antigen (lipopolysaccharide, LPS) and treated with PEG-IL-10, as described in US 7,052,686.

[0020] "IL-10 therapeutic agent" as used herein, includes native IL-10 or IL-10 conjugated to heterologous conjugates. Heterologous conjugates can be the Fc portion of an immunoglobulin molecule, serum albumin, or other known proteins or peptides that increase IL-10 serum half life (e.g., superoxide dismutase (SOD)). Other conjugates include polyethylene glycol, (PEG), oligosaccharides, etc.

[0021] As used herein, "serum half-life", abbreviated "$t_{1/2}$", means elimination half-life, i.e., the time at which the serum concentration of an agent has reached one-half its initial or maximum value. The term "increased serum half-life" used herein in reference to a synthetic agent means that the synthetic agent is cleared at a slower rate than either the non-synthetic, endogenous agent or the recombinantly produced version thereof.

[0022] The term "biomarker" as used in the present application refers generally to a molecule, including a gene, protein, carbohydrate structure, or glycolipid, the expression of which in biological sample can be detected by standard methods (or methods disclosed herein) and is indicative of IL-10 target engagement or IL-10 efficacy in the treatment of tumors. Optionally, the expression of such a biomarker is determined to be higher than that observed for a control sample. Optionally, for example, the expression of such a biomarker will be determined using a gene expression microarray, quantitative PCR or immunohistochemistry (IHC) assay.

[0023] By "subject" or "patient" is meant any single subject for which therapy is desired, including humans. Also intended to be included as a subject are any subjects involved in clinical research trials not showing any clinical sign of disease, or subjects involved in epidemiological studies, or subjects used as controls.

[0024] The term "mammal" as used herein refers to any mammal classified as a mammal, including humans, cows, horses, dogs and cats. In a preferred embodiment of the invention, the mammal is a human.

[0025] By "biological sample" is meant a collection of similar cells obtained from a tissue of a subject or patient. The source of the sample may be solid tissue as from a fresh, frozen and/or preserved organ or tissue sample or biopsy or aspirate; blood or any blood constituents (e.g., serum); bodily fluids such as cerebral spinal fluid, amniotic fluid, peritoneal fluid, or interstitial fluid; cells from any time in gestation or development of the subject. The sample may also be primary or cultured cells or cell lines. Optionally, the sample is obtained from a primary or metastatic tumor. The tissue sample may contain compounds which are not naturally intermixed with the tissue in nature such as preservatives, anticoagulants, buffers, fixatives, nutrients, antibiotics, or the like.

[0026] By "correlate" or "correlating" is meant comparing, in any way, the performance and/or results of a first analysis or protocol with the performance and/or results of a second analysis or protocol. For example, one may use the results of a first analysis or protocol in carrying out a second protocols and/or one may use the results of a first analysis or protocol to determine whether a second analysis or protocol should be performed. With respect to various embodiments herein, one may use the results of an analytical assay such as mRNA expression or an immunoassay to determine whether a dosage of IL-10 engages the receptor and/or is efficacious in reducing the size of a tumor.

[0027] By "nucleic acid" is meant to include any DNA or RNA. For example, chromosomal, mitochondrial, viral and/or bacterial nucleic acid present in tissue sample. The term "nucleic acid" encompasses either or both strands of a double stranded nucleic acid molecule and includes any fragment or portion of an intact nucleic acid molecule.

[0028] By "gene" is meant any nucleic acid sequence or portion thereof with a functional role in encoding or transcribing a protein or regulating other gene expression. The gene may consist of all the nucleic acids responsible for encoding a functional protein or only a portion of the nucleic acids responsible for encoding or expressing a protein. The nucleic

acid sequence may contain a genetic abnormality within exons, introns, initiation or termination regions, promoter sequences, other regulatory sequences or unique adjacent regions to the gene.

[0029] The term "gene expression analysis" is the measurement of mRNA in a sample. Gene expression analysis can be performed by methodologies that include, but are not limited to, Northern blots, PCR, including real-time or quantitative PCR, serial analysis of gene expression (SAGE), DNA microarrays, etc.

[0030] The term "immunoassay" refers to assays combining immunological and chemical methods to detect protein levels in a sample, including biological samples. Immunoassays include ELISA, RIA, Western blots, luminescent assays, fluorescent assays, etc.

[0031] The word "label" when used herein refers to a compound or composition which is conjugated or fused directly or indirectly to a reagent such as a nucleic acid probe or an antibody and facilitates detection of the reagent to which it is conjugated or fused. The label may itself be detectable (e.g., radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable.

[0032] The term "antibody" herein is used in the broadest sense and specifically covers intact monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g. bispecific antibodies) formed from at least two intact antibodies, and antibody fragments so long as they exhibit the desired biological activity.

[0033] "Antibody fragments" comprise a portion of an intact antibody, preferably comprising the antigen-binding or variable region thereof. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

[0034] "Native antibodies" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its

other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light-chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light chain and heavy chain variable domains.

[0035] The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called hypervariable or complementary determining regions both in the light chain and the heavy chain variable domains. The more highly conserved portions of variable domains are called the framework regions (FRs). The variable domains of native heavy and light chains each comprise four FRs, largely adopting a β-sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the β-sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., *Sequences of Proteins of immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cell-mediated cytotoxicity (ADCC).

[0036] Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-binding sites and is still capable of cross-linking antigen.

[0037] "Fv" is the minimum antibody fragment which contains a complete antigen-recognition and antigen-binding site. This region consists of a dimer of one heavy chain and one light chain variable domain in tight, non-covalent association. It is in this configuration that the three hypervariable regions of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer Collectively, the six hypervariable regions confer antigen-binding specificity to the antibody. However, even a single variable domain

(or half of an Fv comprising only three hypervariable regions specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

[0038] The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear at least one free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

[0039] The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

[0040] Depending on the amino acid sequence of the constant domain of their heavy chains, antibodies can be assigned to different classes. There are five major classes of intact antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of antibodies are called α, λ, ε, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

[0041] "Single-chain Fv" or "scFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv see Plueckthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

[0042] The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) in the same polypeptide chain (V_H-V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-

binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., (1993) *Proc. Natl. Acad. Sci. USA*, 90:6444-6448.

[0043] The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., (1975) *Nature*, 256:495, or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567) The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al., (1991) *Nature*, 352:624-628 and Marks et al., (1991) *J. Mol. Biol.*, 222:581-597, for example.

[0044] The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; Morrison et al., (1984) *Proc. Natl. Acad. Sci. USA*, 81:6851-6855). Chimeric antibodies of interest herein include "primatized" antibodies comprising variable domain antigen-binding sequences derived from a non-human primate (e.g. Old World Monkey, such as baboon, rhesus or cynomolgus monkey) and human constant region sequences (U.S. Pat. No. 5,693,780).

[0045] "Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., (1986) *Nature* 321:522-525; Riechmann et al., (1988) *Nature* 332:323-329; and Presta, (1992) *Curr. Op. Struct. Biol.* 2:593-596.

[0046] The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region comprises amino acid residues from a "complementarity determining region" or "CDR" (e.g. residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat et al., *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)) and/or those residues from a "hypervariable loop" (e.g. residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Chothia and Lesk *J. Mol. Biol.* 196:901-917 (1987)). "Framework" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined.

[0047] An antibody "which binds" an antigen of interest is one capable of binding that antigen with sufficient affinity and/or avidity such that the antibody is useful as a therapeutic or diagnostic agent for targeting a cell expressing the antigen.

[0048] For the purposes herein, "immunotherapy" will refer to a method of treating a mammal (preferably a human patient) with an antibody, wherein the antibody may be an unconjugated or "naked" antibody, or the antibody may be conjugated or fused with heterologous molecule(s) or agent(s), such as one or more cytotoxic agent(s), thereby generating an "immunoconjugate".

[0049] An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

[0050] The expression "effective amount" refers to an amount of an agent (e.g. IL-10) which is effective for preventing, ameliorating or treating the disease or condition in question.

[0051] The terms "treating", "treatment" and "therapy" as used herein refer to curative therapy, prophylactic therapy, and preventative therapy. Consecutive treatment or administration refers to treatment on at least a daily basis without interruption in treatment by one or more days. Intermittent treatment or administration, or treatment or administration in an intermittent fashion, refers to treatment that is not consecutive, but rather cyclic in nature.

[0052] The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, chemokines, and traditional polypeptide hormones. In particular, chemokines such as gamma-interferon-induced monokine (MIG; also known as CXCL9), interferon inducible cytokine, IP-10 (also known as CXCL10), and monocytes chemoattractant protein-1 (MCP-1).

Table 1: Biomarker Accession Numbers

Chemokine	GenBank Accession Number
Human MIG	AAA39706
Mouse MIG	NP_032625
Human IP-10	NP_001556
Mouse IP-10	AAA75249
Human MCP-1	AAB29926
Mouse MCP-5	Q62401

[0053] "STAT3" or "signal transducer and activator of transcription 3" as used herein is a biomarker indicative of IL-10 target engagement or engagement with the IL-10R complex. STAT3 is a factor that is phosphorylated upon binding of IL-10 to the IL-10R complex.

[0054] The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g., I^{131}, I^{125}, Y^{90} and Re^{186}), chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

[0055] A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclosphosphamide (CYTOXAN™); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphoramide and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CBI-TMI); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine,

chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics such as the enediyne antibiotics (e.g. calicheamicin, especially calicheamicin gamma1I and calicheamicin phi1I, see, e.g., Agnew, Chem. Intl. Ed. Engl., 33:183-186 (1994); dynemicin, including dynemicin A; bisphosphonates, such as clodronate; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antibiotic chromomophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, caminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (Adriamycin™) (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; losoxantrone; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK®; razoxane; rhizoxin; sizofuran; spirogermanium; tenuazonic acid; triaziquone; 2, 2′,2″-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, e.g. paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, N.J.) and doxetaxel (TAXOTERE®, Rhône-Poulenc Rorer, Antony, France); chlorambucil; gemcitabine (Gemzar™); 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide;

mitoxantrone; vincristine; vinorelbine (Navelbine™); novantrone; teniposide; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoids such as retinoic acid; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition are anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens and selective estrogen receptor modulators (SERMs), including, for example, tamoxifen (including Nolvadex™), raloxifene, droloxifene, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and toremifene (Fareston); aromatase inhibitors that inhibit the enzyme aromatase, which regulates estrogen production in the adrenal glands, such as, for example, 4(5)-imidazoles, aminoglutethimide, megestrol acetate (Megace™), exemestane, formestane, fadrozole, vorozole (Rivisor™), letrozole (Femara™), and anastrozole (Arimidex™); and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

[0056] A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell, especially cancer cell over expressing any of the genes identified herein, either in vitro or in vivo. Thus, the growth inhibitory agent is one which significantly reduces the percentage of cells over expressing such genes in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine) taxol, and topo II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in *The Molecular Basis of Cancer*, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogens, and antineoplastic drugs" by Murakami et al. (WB Saunders: Philadelphia, 1995).

[0057] The terms "cancer", "cancerous", or "malignant" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, carcinoma, lymphoma, leukemia, blastoma, and sarcoma. More particular examples of such cancers include squamous cell carcinoma, myeloma, small-cell lung cancer, non-small cell lung cancer, glioma, hodgkin's

lymphoma, non-hodgkin's lymphoma, gastrointestinal (tract) cancer, renal cancer, ovarian cancer, liver cancer, lymphoblastic leukemia, lymphocytic leukemia, colorectal cancer, endometrial cancer, kidney cancer, prostate cancer, thyroid cancer, melanoma, chondrosarcoma, neuroblastoma, pancreatic cancer, glioblastoma multiforme, cervical cancer, brain cancer, stomach cancer, bladder cancer, hepatoma, breast cancer, colon carcinoma, and head and neck cancer.

II. General.

[0058] The present invention provides methods of monitoring target engagement and efficacy of IL-10 in the treatment of tumors. In particular, serum levels of MCP-1 and STAT3 are biomarkers of IL-10 target engagement, while serum levels of MIG and IP-10 are biomarkers of efficacy.

A. Sample Collection and Preparation

[0059] The biomarkers can be measured in biological samples from cancer subjects before and after exposure of the subject to a therapeutic drug. Accordingly, samples may be collected from a subject over a period of time. Furthermore, obtaining numerous samples from a subject over a period of time can be used to verify results from earlier detections and/or identify a differential expression as a result of exposure to a therapeutic drug. Generally, biological samples can be collected from a subject via biopsy, but may be collected using other known clinical methods, such as the collection of peripheral blood, including serum, or bone marrow for hematological malignancies, or the collection of peripheral blood mononuclear cells for surrogate assays of target inhibition/biomarker measurement.

[0060] In one embodiment of the invention, the samples are analyzed without additional preparation and/or separation procedures. In another embodiment of the invention, sample preparation and/or separation can involve, without limitation, any of the following procedures, depending on the type of sample collected and/or types of biomarkers searched: removal of high abundance polypeptides; addition of preservatives and calibrants, desalting of samples; concentration of sample substances; protein digestions; and fraction collection. In yet another embodiment of the invention, sample preparation techniques concentrate

information-rich biomarkers and deplete polypeptides or other substances that would carry little or no information such as those that are highly abundant in or native to the tumor.

[0061] In another embodiment of the invention, sample preparation takes place in a manifold or preparation/separation device. Such a preparation/separation device may, for example, be a microfluidics device. In yet another embodiment of the invention, the preparation/separation device interfaces directly or indirectly with a detection device. Such a preparation/separation device may, for example, be a fluidics device.

[0062] In another embodiment of the invention, the removal of undesired polypeptides (e.g., high abundance, uninformative, or undetectable polypeptides) can be achieved using high affinity reagents, high molecular weight filters, column purification, ultracentrifugation and/or electrodialysis. High affinity reagents include antibodies that selectively bind to high abundance polypeptides or reagents that have a specific pH, ionic value, or detergent strength. High molecular weight filters include membranes that separate molecules on the basis of size and molecular weight. Such filters may further employ reverse osmosis, nanofiltration, ultrafiltration and microfiltration.

[0063] Ultracentrifugation constitutes another method for removing undesired polypeptides. Ultracentrifugation is the centrifugation of a sample at about 60,000 rpm while monitoring with an optical system the sedimentation (or lack thereof) of particles. Finally, electrodialysis is an electromembrane process in which ions are transported through ion permeable membranes from one solution to another under the influence of a potential gradient. Since the membranes used in electrodialysis have the ability to selectively transportions having positive or negative charge and reject ions of the opposite charge, electrodialysis is useful for concentration, removal, or separation of electrolytes.

[0064] In another embodiment of the invention, the manifold or microfluidics device performs electrodialysis to remove high molecular weight polypeptides or undesired polypeptides. Electrodialysis can be used first to allow only molecules under approximately 30 kD to pass through into a second chamber. A second membrane with a very small molecular weight (roughly 500 D) allows smaller molecules to egress the second chamber.

[0065] Upon preparation of the samples, biomarkers of interest may be separated in another embodiment of the invention. Separation can take place in the same location as the preparation or in another location. In one embodiment of the invention, separation occurs in the same microfluidics device where preparation occurs, but in a different location on the

device. Samples can be removed from an initial manifold location to a microfluidics device using various means, including an electric field. In another embodiment of the invention, the samples are concentrated during their migration to the microfluidics device using reverse phase beads and an organic solvent elution such as 50% methanol. This elutes the molecules into a channel or a well on a separation device of a microfluidics device.

[0066] Chromatography constitutes another method for separating subsets of substances. Chromatography is based on the differential absorption and elution of different substances. Liquid chromatography (LC), for example, involves the use of fluid carrier over a non-mobile phase. Conventional LC columns have an in inner diameter of roughly 4.6 mm and a flow rate of roughly 1 ml/min. Micro-LC has an inner diameter of roughly 1.0 mm and a flow rate of roughly 40 µl/min. Capillary LC utilizes a capillary with an inner diameter of roughly 300 µm and a flow rate of approximately 5 ul/min. Nano-LC is available with an inner diameter of 50 um-1 mm and flow rates of 200 nl/min. The sensitivity of nano-LC as compared to HPLC is approximately 3700 fold. Other types of chromatography contemplated for additional embodiments of the invention include, without limitation, thin-layer chromatography (TLC), reverse-phase chromatography, high-performance liquid chromatography (HPLC), and gas chromatography (GC).

[0067] In another embodiment of the invention, the samples are separated using capillary electrophoresis separation. This will separate the molecules based on their eletrophoretic mobility at a given pH (or hydrophobicity).

[0068] In another embodiment of the invention, sample preparation and separation are combined using microfluidics technology. A microfluidic device is a device that can transport liquids including various reagents such as analytes and elutions between different locations using microchannel structures.

[0069] A biomarker can be modified before analysis to improve its resolution or to determine its identity. For example, the biomarker may be subject to proteolytic digestion before analysis. Any protease can be used. Proteases, such as trypsin, that are likely to cleave the biomarkers into a discrete number of fragments are particularly useful. The fragments that result from digestion function as a fingerprint for the products, thereby enabling their detection indirectly. This is particularly useful where there are biomarkers with similar molecular masses that might be confused for the product in question. Also, proteolytic fragmentation is useful for high molecular weight products, because smaller products are

more easily resolved by mass spectrometry. In specific embodiments, the proteases occur or naturally exist in the tumor sample.

[0070] To improve detection resolution of the biomarkers, neuraminidase can, for instance, be used to remove terminal sialic acid residues from glycoproteins to improve binding to an anionic adsorbent (e.g., cationic exchange ProteinChip® arrays) and to improve detection resolution. In another example, the biomarkers can be modified by the attachment of a tag of particular molecular weight that specifically bind to molecular markers, further distinguishing them. Optionally, after detecting such modified products, the identity of the products can be further determined by matching the physical and chemical characteristics of the modified products in a protein database (e.g., SwissProt).

[0071] It has been found that proteins frequently exist in a sample in a plurality of different forms characterized by a detectably different mass. These forms can result from either, or both, of pre- and post-translational modification. Pre-translational modified forms include allelic variants, slice variants and RNA editing forms. Post-translationally modified forms include forms resulting from proteolytic cleavage (e.g., fragments of a parent protein), glycosylation, phosphorylation, lipidation, oxidation, methylation, cystinylation, sulphonation and acetylation. Modified forms of any biomarker of this invention also may be used, themselves, as biomarkers in the profiles. In certain cases, the modified forms may exhibit better discriminatory power in diagnosis than the specific forms set forth herein.

[0072] For some of the method embodiments of the invention, it may be helpful to purify the biomarker whose differential presence has been detected by the methods disclosed herein prior to subsequent analysis. Nearly any means known to the art for the purification and separation of small molecular weight substances, e.g., anion or cation exchange chromatography, gas chromatography, liquid chromatography or high pressure liquid chromatography may be used. Methods of selecting suitable separation and purification techniques and means of carrying them out are known in the art (see, e.g., Labadarious et. al., (1984) *J Chromatography* 310:223-231, and references cited therein; and Shahrokhin and Gehrke, (1968) *J. Chromatography* 36:31-41, and Niessen (1998) *J. Chromatography* 794:407-435). To the extent that it is desired to determine the differential presence of a nucleic acid biomarker, the biomarker may be purified using known methods including, slab or capillary gel electrophoresis.

[0073] In another embodiment of the method of the invention, purification of the biomarker comprises fractioning a sample comprising one or more protein products by size-exclusion chromatography and collecting a fraction that includes the one or more products; and/or fractioning a sample comprising the one or more products by anion exchange chromatography and collecting a fraction that includes the one or more products. Fractionation is monitored for purity on normal phase and immobilized nickel arrays. Generating data on immobilized biomarker fractions on an array is accomplished by subjecting the array to laser ionization and detecting intensity of signal for mass/charge ratio; and transforming the data into computer readable form. Preferably, fractions are subjected to gel electrophoresis and correlated with data generated by mass spectrometry. In one aspect, gel bands representative of potential biomarkers are excised and subjected to enzymatic treatment and are applied to biochip arrays for peptide mapping.

B. Detection and Quantitation of Biomarkers

[0074] Any suitable method can be used to detect (a differential presence of) one or more of the biomarkers described herein. Successful practice of the invention can be achieved with one or a combination of methods that can detect and, preferably, quantify the biomarkers. These methods include, without limitation, hybridization-based methods including those employed in biochip arrays, mass spectrometry (e.g., laser desorption/ionization mass spectrometry), fluorescence (e.g. sandwich immunoassay), surface plasmon resonance, ellipsometry and atomic force microscopy. For nucleic acid biomarkers, methods for detection and quantitation include PCR, quantitative PCR, northern blot analysis, southern blot analysis, mass spectrometry and the like.

[0075] Methods may further include, by one or more of electrospray ionization mass spectrometry (ESI-MS), ESI-MS/MS, ESI-MS/$(MS)^n$, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS), desorption/ionization on silicon (DIOS), secondary ion mass spectrometry (SIMS), quadrupole time-of-flight (Q-TOF), atmospheric pressure chemical ionization mass spectrometry (APCI-MS), APCI-MS/MS, APCI-$(MS)^n$, atmospheric pressure photoionization mass spectrometry (APPI-MS), APPI-MS/MS, and APPI-$(MS)_n$, quadrupole mass spectrometry, fourier transform mass spectrometry (FTMS), and ion trap mass spectrometry,

where n is an integer greater than zero. Spectroscopic methods for detecting and quantifying protein biomarkers are known in the art and are described, for example in U.S. Pat. Nos. 5,719,060; 6,225,047; 5,719,060; 6,124,137 and PCT International Publication No. WO 03/64594.

[0076] In another embodiment of the invention, the biomarkers of the invention are measured by a method other than mass spectrometry or other than methods that rely on a measurement of the mass of the biomarker. In one such embodiment that does not rely on mass, the biomarkers of this invention are measured by immunoassay. Immunoassay requires biospecific capture reagents, such as antibodies, to capture the biomarkers. Antibodies can be produced by methods well known in the art, e.g., by immunizing animals with the biomarkers. Biomarkers can be isolated from samples based on their binding characteristics. Alternatively, if the amino acid sequence of a polypeptide biomarker is known, the polypeptide can be synthesized and used to generate antibodies by methods well known in the art.

[0077] This invention contemplates traditional immunoassays including, for example, sandwich immunoassays including ELISA or fluorescence-based immunoassays, as well as other enzyme immunoassays. Nephelometry is an assay done in liquid phase, in which antibodies are in solution. Binding of the antigen to the antibody results in changes in absorbance, which is measured. In the SELDI-based immunoassay, a biospecific capture reagent for the biomarker is attached to the surface of an MS probe, such as a pre-activated ProteinChip array. The biomarker is then specifically captured on the biochip through this reagent, and the captured biomarker is detected by mass spectrometry.

[0078] In one embodiment, the biomarker can be a nucleic acid, wherein the nucleic acid can be detected and/or quantified using methods known in the art. For example, nucleic acid biomarkers may be detected using PCR (disclosed in U.S. Pat. No. 4,683,195, U.S. Pat. No. 4,683,202 and U.S. Pat. No. 4,965,188 and others in detail). In one embodiment, a real time PCR method is used to enable a convenient and reliable quantitative measurement of biomarker nucleic acids having a wide dynamic range. The real time PCR technique includes the method by using a TaqMan probe using ABIPRISM7700.™. (Applied Biosystems) and the method by using LightCycler.™. (Ropche Diagnostics). Particularly in the latter case, in a high rate reaction cycle in which a temperature cycle of PCR is completed for some 10 minutes, a change of an amplified amount of a DNA synthesized for every cycle can be

detected in a real time. DNA detection method of the real time PCR method includes 4 methods using a DNA-binding pigment (intercalator), a hybridization probe (kissing probe), TaqMan probe, or Sunrise Uniprimer (molecular beacon). On the other hand, the expression level of a biomarker gene can be analyzed by using a DNA-binding pigment such as SYBR GreenI. SYBR GreenI is a binding pigment specific to a double strand of the DNA and, when bound to a double strand, an inherent fluorescence intensity is reinforced. By adding SYBR GreenI at the PCR reaction and measuring the fluorescence intensity at the end of each cycle of an elongation reaction, the increase in a PCR product can be detected. For detection of a biomarker gene, similar to normal PCR, a primer is designed by using a commercialized gene analysis software on the basis of a sequence of the biomarker gene. SYBR GreenI detects a nonspecific product and, thus, requires designing an optimal primer. Required designing standards are a length of an oligomer, a base composition of the sequence, a GC content, and a Tm value.

[0079] Detection methods may include use of a microarray/biochip array. Biochip arrays useful in the invention include protein and nucleic acid arrays. One or more biomarkers are captured on the biochip array and subjected to laser ionization to detect the molecular weight of the products. Analysis of the products is, for example, by molecular weight of the one or more biomarkers against a threshold intensity that is normalized against total ion current.

[0080] The biochip surfaces may, for example, be ionic, anionic, hydrophobic; comprised of immobilized nickel or copper ions, comprised of a mixture of positive and negative ions; and/or comprised of one or more antibodies, single or double stranded nucleic acids, proteins, peptides or fragments thereof, amino acid probes, or phage display libraries. Many protein biochips are described in the art. These include, for example, protein biochips produced by Ciphergen Biosystems (Fremont, Calif.), Packard BioScience Company (Meriden Conn.), Zyomyx (Hayward, Calif.) and Phylos (Lexington, Mass.). Examples of such protein biochips are described in the following patents or patent applications: U.S. Pat. No. 6,225,047 (Hutchens and Yip, "Use of retentate chromatography to generate difference maps," May 1, 2001); International publication WO 99/51773 (Kuimelis and Wagner, "Addressable protein arrays," Oct. 14, 1999); U.S. Pat. No. 6,329,209 (Wagner et al., "Arrays of protein-capture agents and methods of use thereof," Dec. 11, 2001) and International publication WO 00/56934 (Englert et al., "Continuous porous matrix arrays," Sep. 28, 2000).

[0081] Biomarkers may be captured with capture reagents immobilized to a solid support, such as a biochip, a multiwell microtiter plate, a resin, or nitrocellulose membranes that are subsequently probed for the presence of proteins. Capture can be on a chromatographic surface or a biospecific surface. For example, a tumor sample containing the biomarkers may be placed on the active surface of a biochip for a sufficient time to allow binding. Then, unbound molecules are washed from the surface using a suitable eluant, such as phosphate buffered saline. In general, the more stringent the eluant, the more tightly the proteins must be bound to be retained after the wash.

[0082] Upon capture on a biochip, analytes can be detected by a variety of detection methods selected from, for example, a gas phase ion spectrometry method, an optical method, an electrochemical method, atomic force microscopy and a radio frequency method. Also of interest is the use of mass spectrometry, for example, SELDI. Optical methods include, for example, detection of fluorescence, luminescence, chemiluminescence, absorbance, reflectance, transmittance, birefringence or refractive index (e.g., surface plasmon resonance, ellipsometry, a resonant mirror method, a grating coupler waveguide method or interferometry). Optical methods include microscopy (both confocal and non-confocal), imaging methods and non-imaging methods. Immunoassays in various formats (e.g., ELISA) are popular methods for detection of analytes captured on a solid phase. Electrochemical methods include voltametry and amperometry methods. Radio frequency methods include multipolar resonance spectroscopy.

C. Qualification of Cancer Status

[0083] The biomarkers of the present invention have a number of uses. For example, identification of an anti-tumor response or monitoring the efficacy of a therapeutic drug in a cancer subject takes into account the amount of the biomarker(s) in a tumor sample before and after exposure of the subject to a therapeutic drug (up or down regulation of the biomarker(s)). The amounts are measured under the same or substantially similar experimental conditions but at different time periods preceding and following treatment. The biomarkers of the invention can also be used to identify an agent useful in the treatment of cancer.

[0084] The detection of a differential presence of a plurality of biomarkers in a tumor sample may improve the indication of therapeutic efficacy of a therapeutic drug in the treatment of a cancer.

[0085] The biomarkers of the present invention may also be used for determining efficacy and/or receptor engagement following therapy with and IL-10 therapeutic agent. The biomarkers can be used alone or in combination with other products. The biomarkers are differentially present in tumor samples of a cancer patient before and after exposure to a therapeutic drug. For example, some of the markers are expressed at an elevated level and/or are present at a higher frequency after treatment, while some of the products are expressed at a decreased level and/or are present at a lower frequency after treatment. Therefore, generating a biomarker profile for a subject would provide useful information regarding cancer status.

[0086] In certain embodiments of the methods of qualifying cancer status, the methods further comprise managing subject treatment based on the status. The invention also provides for such methods where the biomarkers (or specific combination of biomarkers) are measured again after such subject management. In these cases, the methods are used to monitor the status of the cancer, e.g., candidacy for treatment with IL-10 or a modified IL-10 protein, response to IL-10 treatment, remission of the disease or progression of the disease.

[0087] The biomarkers of the present invention can be used to adjust the dosage of therapeutic IL-10 or PEG-IL-10 provided to a subject. For example, MCP-1 serum protein level expression and phosphorylation of STAT3 is indicative of target engagement. To achieve maximal target engagement of IL-10 or PEG-IL-10, the clinician can adjust the dosage such that the expression of MCP-1 and/or phosphorylation STAT3 is optimized. Similarly, the expression of MIG and/or IP-10 is indicative of tumor inhibition efficacy. Again, by optimizing the expression of MIG and/or IP-10 by varying IL-10 or PEG-IL-10 dosage, the clinician can achieve maximal tumor inhibition. Thus, by changing the dosage level in a systematic manner, the optimal level of therapeutic IL-10 or PEG-IL-10 is determined for a subject. Amelioration of symptoms may be monitored concurrently. This method is used to find an optimal dosage level at which to treat a subject.

[0088] In another embodiment of the invention, the output from a detection device can subsequently be processed, stored, and further analyzed or assayed using a bio-informatics system. A bio-informatics system may include one or more of the following,

without limitation: a computer; a plurality of computers connected to a network; a signal processing tool(s); a pattern recognition tool(s); a tool(s) to control flow rate for sample preparation, separation, and detection.

[0089] The data processing utilizes mathematical foundations. In another embodiment of the invention, dynamic programming is used to align a separation axis with a standard separation profile. Intensities may be normalized, for example, by fitting roughly 90% of the intensity values into a standard spectrum. The data sets can then be fitted using wavelets designed for separation and mass spectrometer data. In yet another embodiment of the invention, data processing filters out some of the noise and reduces spectrum dimensionality, potentially allowing for pattern recognition.

[0090] Following data processing, pattern recognition tools can be utilized to identify subtle differences between phenotypic states. Pattern recognition tools are based on a combination of statistical and computer scientific approaches, which provide dimensionality reduction. Such tools are scalable. Data so obtained may be stored on a computer readable medium.

D. Kits

[0091] In one aspect, the invention provides kits for qualifying cancer status in a subject, wherein the kits can be used to detect the differential presence of the biomarkers described herein. For example, the kits can be used to detect a differential presence of any combination of the biomarkers in tumor samples of cancer subjects before and after exposure to a therapeutic drug. The kits of the invention have many applications. For example, the kits can be used to monitor efficacy of a therapeutic drug in a cancer subject. The kits can also be used to identify agents useful in the treatment of cancer.

[0092] In specific embodiments, kits of the invention contain a biomarker, which is optionally isotopically or fluorescently labeled.

[0093] The kits of the invention may include instructions, reagents, testing equipment (test tubes, reaction vessels, needles, syringes, etc.), standards for calibration, and/or equipment. Reagents may include acids, bases, oxidizing agents, and marker species. The instructions provided in a kit according to the invention may be directed to suitable operational parameters in the form of a label or a separate insert.

[0094] The kits may also include an adsorbent, wherein the adsorbent retains one or more biomarkers described herein (polynucleotide or polypeptide), and written instructions for use of the kit for qualification of cancer status in a subject. Such a kit could, for example, comprise: (a) a substrate comprising an adsorbent thereon, wherein the adsorbent is suitable for binding a biomarker, and (b) instructions to detect the biomarker(s) by contacting a tumor sample with the adsorbent and detecting the product(s) retained by the adsorbent. Accordingly, the kit could comprise (a) a DNA probe that specifically binds to a biomarker; and (b) a detection reagent. Such a kit could further comprise an eluant (as an alternative or in combination with instructions) or instructions for making an eluant, wherein the combination of the adsorbent and the eluant allows detection of the biomarker using, for example, gas phase ion spectrometry.

[0095] This invention is further illustrated by the following examples, which should not be construed as limiting. A skilled artisan should readily understand that other similar instruments with equivalent function/specification, either commercially available or user modified, are suitable for practicing the instant invention. Rather, the invention should be construed to include any and all applications provided herein and all equivalent variations within the skill of the ordinary artisan.

EXAMPLES

I. General methods.

[0100] Standard methods in molecular biology are described. Maniatis *et al.* (1982) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; Sambrook and Russell (2001) *Molecular Cloning, 3rd ed.*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; Wu (1993) *Recombinant DNA*, Vol. 217, Academic Press, San Diego, CA. Standard methods also appear in Ausbel *et al.* (2001) *Current Protocols in Molecular Biology, Vols.1-4*, John Wiley and Sons, Inc. New York, NY, which describes cloning in bacterial cells and DNA mutagenesis (Vol. 1), cloning in mammalian cells and yeast (Vol. 2), glycoconjugates and protein expression (Vol. 3), and bioinformatics (Vol. 4).

[0101] Methods for protein purification including immunoprecipitation, chromatography, electrophoresis, centrifugation, and crystallization are described. Coligan *et al.* (2000) *Current Protocols in Protein Science, Vol. 1*, John Wiley and Sons, Inc., New York. Chemical analysis, chemical modification, post-translational modification, production of fusion proteins, glycosylation of proteins are described. *See, e.g.*, Coligan *et al.* (2000) *Current Protocols in Protein Science, Vol. 2*, John Wiley and Sons, Inc., New York; Ausubel *et al.* (2001) *Current Protocols in Molecular Biology, Vol. 3*, John Wiley and Sons, Inc., NY, NY, pp. 16.0.5-16.22.17; Sigma-Aldrich, Co. (2001) *Products for Life Science Research*, St. Louis, MO; pp. 45-89; Amersham Pharmacia Biotech (2001) *BioDirectory*, Piscataway, N.J., pp. 384-391. Production, purification, and fragmentation of polyclonal and monoclonal antibodies are described. Coligan *et al.* (2001) *Current Protcols in Immunology, Vol. 1*, John Wiley and Sons, Inc., New York; Harlow and Lane (1999) *Using Antibodies*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; Harlow and Lane, *supra.* Standard techniques for characterizing ligand/receptor interactions are available. *See, e.g.*, Coligan *et al.* (2001) *Current Protcols in Immunology, Vol. 4*, John Wiley, Inc., New York.

[0102] Methods for flow cytometry, including fluorescence activated cell sorting detection systems (FACS®), are available. *See, e.g.*, Owens *et al.* (1994) *Flow Cytometry Principles for Clinical Laboratory Practice*, John Wiley and Sons, Hoboken, NJ; Givan (2001) *Flow Cytometry, 2nd ed.*; Wiley-Liss, Hoboken, NJ; Shapiro (2003) *Practical Flow Cytometry*, John Wiley and Sons, Hoboken, NJ. Fluorescent reagents suitable for modifying

nucleic acids, including nucleic acid primers and probes, polypeptides, and antibodies, for use, e.g., as diagnostic reagents, are available. Molecular Probes (2003) *Catalogue*, Molecular Probes, Inc., Eugene, OR; Sigma-Aldrich (2003) *Catalogue*, St. Louis, MO.

[0103] Standard methods of histology of the immune system are described. *See, e.g.*, Muller-Harmelink (ed.) (1986) *Human Thymus: Histopathology and Pathology*, Springer Verlag, New York, NY; Hiatt, *et al.* (2000) *Color Atlas of Histology*, Lippincott, Williams, and Wilkins, Phila, PA; Louis, *et al.* (2002) *Basic Histology:Text and Atlas*, McGraw-Hill, New York, NY.

[0104] Software packages and databases for determining, e.g., antigenic fragments, leader sequences, protein folding, functional domains, glycosylation sites, and sequence alignments, are available. *See, e.g.*, GenBank, Vector NTI® Suite (Informax, Inc, Bethesda, MD); GCG Wisconsin Package (Accelrys, Inc., San Diego, CA); DeCypher® (TimeLogic Corp., Crystal Bay, Nevada); Menne *et al.* (2000) *Bioinformatics* 16: 741-742; Menne *et al.* (2000) *Bioinformatics Applications Note* 16:741-742; Wren *et al.* (2002) *Comput. Methods Programs Biomed.* 68:177-181; von Heijne (1983) *Eur. J. Biochem.* 133:17-21; von Heijne (1986) *Nucleic Acids Res.* 14:4683-4690.

II. **Tumor implantation and blood sample collection.**

[0105] Female C57/B6 mice approximately 8 to 10 weeks of age were used. The mouse squamous cell carcinoma line PDV6 was cultured in DMEM with 10% FBS. $1x10^6$ cells were injected subcutaneously in the right flank of each mouse and tumors were monitored regularly. Tumor staging and dosing of IL-10 or control was determined when average tumor size reached approximately 100-150 mm^3 (e.g., at day 10 to day 14 post-implant). Animals were then treated with PEG-IL-10 or vehicle control subcutaneously at designated dosing regimen. At given time points, animals were euthanized in accordance with IACUC recommendations and blood samples were collected.

III. **Serum sample collection and chemokines measurement.**

[0106] Using a serum separator tube to collect the mouse blood samples, the blood was allowed to clot for 30 minutes, then centrifuged for 15 minutes at approximately 1000g to separate the serum. The serum sample was either snap frozen or assayed (e.g., ELISA) immediately. The serum concentrations of MCP-5, MCP-1, IP-10 and MIG were quantified

using the appropriate commercial ELISA kits (R&D Systems) according to the manufacturer's instructions.

[0107] Single dosing of administration of PEG-IL-10 (0.1 mg/kg) induces early elevation of mouse MCP-5 in the serum in the diseased or non-diseased animal. (see, e.g., tables 2 and 3).

Table 2: MCP-5 induction upon PEG-IL-10 treatment of tumor bearing mouse.

Time post-dose (hours)	MCP-5 Serum Concentration (pg/mL)	Fold induction
0	58.7	1.0
0.5	59.4	1.0
1.0	64.5	1.1
2.0	112.2	1.9
4.0	172.2	2.9
9.0	184.2	3.1
16.0	211.8	3.6
24.0	186.8	3.2
32.0	104.7	1.8
38.0	67.1	1.1
48.0	68.2	1.2

Table 3: Mouse MCP-5 induction upon PEG-IL-10 treatment of non-tumor bearing mouse.

Time post-dose (hours)	MCP-5 Serum Concentration (pg/mL)	Fold Induction
0	76.5	1.0
2.0	185.1	1.9
4.0	312.2	2.9
9.0	352.0	3.1
16.0	451.2	3.6
24.0	404.2	3.2

[0108] Mouse MIG and IP-10 were induced later following PEG-IL-10 administration to tumor bearing mice (see, e.g., tables 4 and 5). Increased expression of MIG and IP-10 correlated with reduction of tumor volumes.

Table 4: Mouse MIG induction after administration of PEG-IL-10

Time post-dose (days)	Serum MIG Level (pg/mL)	Fold Induction	Tumor Volume (mm^3)
5	160.0	1.8	151.4
9	1129.3	12.7	140.5
17	1711.2	19.3	52.5
Vehicle Control	88.7	1.0	372.1

Table 5: Mouse IP-10 induction after administration of PEG-IL-10

Time Post-Dose (Days)	Serum IP-10 Level (pg/mL)	Fold induction	Tumor Volume (mm^3)
5	476.7	2.0	151.4
9	1228.8	5.1	140.5
17	1997.3	8.3	52.5
Vehicle Control	242.0	1.0	372.1

IV. **Human PBMC preparation, stimulation, and chemokines measurement.**

[0109] Human peripheral blood mononuclear cells (PBMC) were prepared by Histopaque (Sigma10771) density centrifugation and cultured with RPMI-1640 containing 10% of heat-inactivated fetal bovine serum.

[0110] To investigation of chemokine production, human PBMC were maintained in the presence or absence of human IL-10 or human PEG-IL-10. After 24h and 48h, the supernatants were collected. The concentrations of MCP-1, IP-10 and MIG were quantified using commercial ELISA kits (R&D Systems) according to the manufacturer's instructions.

Table 6: Induction of human MCP-1 from unprimed PBMC treated with varying concentrations of PEG-IL-10.

IL-10 Concentration (ng/ml)	MCP-1(pg/ml)	fold induction
0	4178.3	1.0
0.1	6976.1	1.7
1	19636.8	4.7
10	30334.9	7.3
100	34573.6	8.3
1000	26845.9	6.4

Table 7: Induction of human MIG from unprimed PBMC treated with varying concentrations of PEG-IL-10.

IL-10 Concentration (ng/ml)	MIG Level (pg/ml)	Fold Induction
0	4098.1	1.0
0.1	6993.2	1.7
1.0	11869.5	2.9
10.0	9484.0	2.3
100.0	8357.5	2.0
1000.0	9408.0	2.3

Table 8: Induction of human IP-10 from unprimed PBMC with varying concentrations of PEG-IL-10

IL-10 Concentration (ng/ml)	IP-10 Level (pg/ml)	Fold Induction
0	7117.2	1.0
0.1	27110.6	3.8
1.0	58219.4	8.2
10.0	64606.9	9.1
100.0	65836.6	9.3
1000.0	69620.5	9.8

From the above, PEG-IL-10 induces mouse MCP-5 in the sera early after administration to tumor bearing and naïve mice. The human homolog of MCP-5, MCP-1 (see, e.g., Sarafi et al. (1997) J. Exp. Med.185:99-109), is induced in a dose dependent manner, upon addition of PEG-IL-10 to unprimed PBMC cultures. Therefore MCP-1 can be used as a marker for IL-10 target engagement.

[0100] Further induction of IP-10 and MIG appear to occur later following administration of PEG-IL-10 to tumor bearing mice, and was correlated to tumor volume reduction. Therefore IP-10 and MIG are markers for efficacy.

V. **Human PBMC cell lysate preparation for STAT3 phosphorylation assay**

[0101] To assess STAT3 phosphorylation levels, human PBMC were maintained in the presence or absence of human IL-10 or human PEG-IL-10. After 24h and 48h, the cells were harvested by centrifugation at 500g at 4°C for 5 minutes. The pellet was washed once with cold PBS and subsequently incubated with complete lysis buffer (150 mM NaCl, 20 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100) containing protease (Protease Inhibitor Set III, EMD Biosciences, Cat. No. 539134) and phosphatase inhibitors (Phosphatase Inhibitors I and II, Sigma, Cat. Nos. P2850 and P5726, respectively) on ice, for 20 minutes. After removal of cell debris by centrifugation, the cell lysate was snap frozen for a STAT3 phosphorylation assay

VI. **STAT3 phosphorylation assay**

[0102] The STAT3 phosphorylation assay employed standard ELISA format to assess PEG-IL-10 engagement with its target receptor. 96 well flat bottom immunoplates were coated with 100 µl/well of STAT3 specific capture antibody (MAB1799; R&D Systems) at a concentration of 1 µg/ml. 80 µl of cell lysate, described above, was added to each well of the ELISA plate. Plates were incubated with shaking at room temperature. Plates were washed six times with 1X wash buffer (50 mM Tris, pH 7.5, 0.15 M NaCl, 0.02% Tween-20; Meso Scale Discovery, Cat. No. R61TX-2). Detection antibody (Cell Signaling, Cat. No. 9145) was diluted to a final concentration of 1:167, and 100 µl was added to each well. Plates were incubated with shaking at room temperature for 2 hours. Plates were again washed six time with 1X wash buffer. A secondary antibody (anti-rabbit HRP conjugate, Pierce, Cat. No. 31460) was diluted 1:5000, and 100 µl was added to each well. Plates were incubated and washed as above. Plates were read in an ELISA plate reader at an absorbance of 450-650 nm.

Table 9: STAT3 Phosphorylation with varying concentrations of PEG-IL-10

IL-10 concentration (ng/mL)	OD (450-650 nm) of phosphorylated STAT3	Fold induction
0	0.794	1.00
0.1	0.698	0.88
1.0	0.911	1.15
10.0	1.094	1.38
100.0	1.303	1.64
1000.0	1.157	1.46

[0103] STAT3 phosphorylation is induced upon administration of PEG-IL-10, and therefore can be used as a biomarker for target engagement.

VII. **Rapid modulations of MCP-5 and its receptor CCR2 mRNA expression by PEG-mIL-10 in tumor tissue.**

[0104] 4T1 tumor-bearing mice received 1mpk peg-IL-10 subcuteously. Tumor tissue was harvested at 0.5, 1, 2, 6, 12, 24 and 36 hours post dose. Total RNA was extracted and mRNA expression level was assessed by Taqman real-time PCR. Table 10 shows the mRNA expression of MCP-5 and its receptor CCR2 pooled sample of three individual animals at each time point. MCP-1 was used as a negative control. Each sample was normalized to unbiquitin.

Table 10: mRNA expression of MCP-1, MCP-5 and CCR2 in tumor tissues of PEG-IL-10 treated mice

Time(hour)	MCP-1	MCP-5	CCR2
0	21.85	84.09	126.24
0.5	38.68	128.61	259.59
1	29.7	112.9	172.88
2	42.58	176.2	192.91
6	32.9	109.31	166.02
12	43.84	137.45	162.25
24	29.37	145.32	111.03
36	33.17	136.42	112.7

VIII. **IL-10 Induced MCP-5 Production is Dependent on IL-10Rβ**

[0105] C57BL/6 mice or IL-10Rβ knockout mice were treated with 0.25 mg/kg of PEG-mIL-10, and the sera were collected at 0, 4, and 8 hours post treatment. The serum concentration of MCP-5 (pg/mL) was assessed by using a commercial ELISA kit for R&D System. The induction of MCP-5 was inhibited in the IL-10Rβ deficient mouse compared to that of in wild-type mouse. There were five mice in the wild-type group and seven mice in the IL-10Rβ knockout group.

Table 11: MCP-5 protein expression in wild-type (WT) and IL-10Rβ KO mice following PEG-IL-10 treament (non-tumor bearing mice).

	0h	4h	8h
IL-10Rβ KO-1	74.145	76.92	117.47
IL-10Rβ KO-2	58.698	51.57	59.82
IL-10Rβ KO-3	71.036	59.23	79.87
IL-10Rβ KO-4	61.767	60.7	69.55
IL-10Rβ KO-5	81.647	86.66	92.28
IL-10Rβ KO-6		66.3	100.56
IL-10Rβ KO-7		77.51	96.12
WT-1	74.768	166.81	243.6
WT-2	119.923	183.12	219.36
WT-3	48.949	176.46	259.38
WT-4	55.639	147.89	236.82
WT-5	119.275	240.83	266.55

IX. MCP-5 Levels in PDV6-IL-10Rβ Knockout Mice Treated with IL-10 Minicircle DNA

[0106] C57BL/6 mice or IL-10Rβ knockout mice were treated with 6ug of mIL-10 minicircle DNA or control minicircle DNA (hATT) (see, e.g., US Patent Application 2004/0214329), at day 24 after PDV6 tumor implantation, and sera were collected 24 days after treatment. The serum concentration of MCP-5 was assessed by using a commercial ELISA kit from R&D Systems using manufacturer's instructions. Table 12 shows that induction of MCP-5 was inhibited in the IL-10Rβ deficient mouse compared to the wild-type mouse.

Table 12: MCP-5 serum levels following hydrodynamic delivery of IL-10 or control minicircle ("MC") DNA at day 24 post tumor implantation

IL-10Rβ KO-hATT-MC	IL-10Rβ KO-IL-10 MC	WT-hAAT-MC	WT-IL-10-MC
157.179	153.427	151.377	505.697
147.886	146.976		
139.712	143.141		
155.105	175.748		

Table 13 shows the relative tumor size at various time points following delivery of IL-10 or control minicircle ("MC") DNA to IL-10Rβ KO or wild type mice.

Days	hAAT-MC-IL10Rβ KO (mm^3)(N=9)	IL-10MC- IL10Rβ KO (mm^3)(N=6)	IL-10MC-WT (mm^3)(N=5)	hAAT MC-WT (mm^3) (N=5)
14			172.6	158.3
17			211.6	165.1
18	178.3	135.4		
21			272.3	255.0
22	283.6	238.5		
27			400.5	403.8
28	498.6	440.6		
30			431.7	484.9
31	617.4	528.3		
36	832.6	770.2	238.6	632.5
41	1158.9	952.4	128.5	774.3
45	1369.2	1204.4	87.6	876.3
48	1369.7	1126.8	45.2	926.5

X. **IL-10 induced MIG elevation is dependent on T cell presence, but MCP-5 induction is not dependent on T cell presence**

[0107] RAG2 knockout mice or wild type C57BL6 mice were treated with 6 μgs of mIL-10 MC or control hAAT MC at day 12 after PDV6 tumor implantation. Sera were collected 24 days after treatment. The serum concentrations of MCP-5 and MIG were assessed by using a commercial ELISA kits from R&D Systems using manufacturer's instructions. Table 14 shows that MCP-5 concentration is elevated in the T cell deficient RAG mice treated with IL-10 MC DNA. Thus MCP-5 expression is not dependent on the presence of T cells.

Table 14: MCP-5 concentration at day 24 post HDD delivery of MC DNA

RAG-hATT-MC(pg/ml)	RAG-IL-10 MC(pg/ml)	WT-hAAT-MC(pg/ml)	WT-IL-10-MC(pg/ml)
150.291	441.509	151.377	505.697
202.182	569.123		
120.956	802.545		
138.694	708.3		
236.278	545.757		
112.088	527.78		
145.153	387.34		
161.355	542.245		
110.127	585.585		
178.526	445.937		
129.271	560.268		
146.522			

[0108] Table 15 shows that MIG expression is inhibited in RAG mice given IL-10 MC DNA, thus induction of MIG expression is dependent on the presence of T cells.

Table 15: MIG concentration at day 24 post HDD delivery of MC DNA

RAG-hATT-MC(pg/ml)	RAG-IL-10 MC(pg/ml)	WT-IL-10-MC(pg/ml)
31.313	25	1103.912
37.173	14.387	737.771
25	22.932	1828.632
25	5.384	
25	31.313	
15.636	9.317	
5.384	25	
22.969	45.28	
5.384	16.843	
9.317	5.384	
25	13.139	
2.633		

[0109] Table 16 shows that tumor growth is not dependent upon the presence of T cells.

Table 16: Average tumor size through day 40 post MC DNA delivery in RAG mice

Day	hAAT MC-RAG(mm^3)(**N=11**)	IL-10 MC-RAG(mm^3)(**N=11**)
11	89.4	117.4
17	203.8	220.9
21	285.7	329.5
26	475.5	488.6
29	534.8	519.8
33	638.9	736.7
36	742.6	816.4
40	915.1	1086.3

WHAT IS CLAIMED IS:

1. A method for monitoring efficacy of an IL-10 therapeutic agent in treatment of a subject for a tumor or cancer, said method comprising:

a) obtaining a baseline biological sample from the subject prior to administering a dose of the IL-10 therapeutic agent;

b) measuring the level of at least one biomarker in the baseline biological sample by gene expression analysis or immunoassay,

c) administering the IL-10 therapeutic agent to the subject;

d) obtaining from the subject at least one subsequent biological sample;

e) measuring the level of the biomarker in the subsequent sample by gene expression analysis or immunoassay;

f) comparing the level of at least one biomarker in the subsequent sample with the level of at least one biomarker in the baseline biological sample, wherein an increase of at least one biomarker in the subsequent biological sample indicates an effective dose of the IL-10 therapeutic agent.

2. The method of claim 1, wherein the subject is human.

3. The method of claim 1, wherein the tumor or cancer is from an organ selected from the group consisting of skin, colon, thyroid, ovarian, lung, pancreas and brain.

4. The method of claim 1 wherein the tumor is selected from the group consisting of: colon cancer, ovarian cancer, breast cancer, melanoma, lung cancer, glioblastoma, and leukemia.

5. The method of claim 1, wherein the IL-10 therapeutic agent is selected from the group consisting of native IL-10, pegylated IL-10 (PEG-IL-10), and IL-10 conjugated to an Fc portion of an immunoglobulin (IL-10-Ig).

6. The method of claim 1, wherein the biomarker is selected from the group consisting of IP-10 and MIG.

7. The method of claim 6, wherein the increase in IP-10 or MIG expression in the subsequent biological sample is at least 2 fold to at least 19 fold.

8. The method of claim 1, wherein the gene expression analysis is selected from the group consisting of Northern blotting, quantitative PCR, SAGE, and DNA microarray.

9. The method of claim 1 wherein the immunoassay is selected from the group consisting of ELISA, RIA, Western blot, luminescent immunoassay, fluorescent immunoassay.

10. A method for monitoring target engagement following administration of an IL-10 therapeutic agent to treat a subject for a tumor or cancer, said method comprising:

a) obtaining a baseline biological sample from the subject prior to administering a dose of the IL-10 therapeutic agent;

b) measuring the level of at least one biomarker in the baseline biological sample by gene expression analysis or immunoassay,

c) administering the IL-10 therapeutic agent to the subject;

d) obtaining from the subject at least one subsequent biological sample;

e) measuring the level of the biomarker in the subsequent sample by gene expression analysis or immunoassay;

f) comparing the level of at least one biomarker in the subsequent biological sample with the level of at least one biomarker in the baseline biological sample, wherein an increase of at least one biomarker in the subsequent biological sample indicates target engagement.

11. The method of claim 10, wherein the subject is human.

12. The method of claim 10, wherein the tumor or cancer is from an organ selected from the group consisting of skin, colon, thyroid, ovarian, lung, pancreas, and brain.

13. The method of claim 10 wherein the tumor is selected from the group consisting of: colon cancer, ovarian cancer, breast cancer, melanoma, lung cancer, glioblastoma, and leukemia.

14. The method of claim 10, wherein the IL-10 therapeutic agent is selected from the group consisting of native IL-10, pegylated IL-10 (PEG-IL-10), and IL-10 conjugated to an Fc portion of an immunoglobulin (IL-10-Ig).

15. The method of claim 10, wherein the biomarker is chosen from the group consisting of MCP-1 and phosphorylation of STAT3.

16. The method of claim 10, wherein the gene expression analysis is selected from the group consisting of Northern blotting, quantitative PCR, SAGE, and DNA microarray.

17. The method of claim 10, wherein the immunoassay is selected from the group consisting of ELISA, RIA, Western blot, luminescent immunoassay, fluorescent immunoassay.

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2009/054443

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/574

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)
G01N

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DEHQANZADA ZA ET AL: "Assessing serum cytokine profiles in breast cancer patients receiving a HER2/neu vaccine using Luminex technology" ONCOLOGY REPORTS, vol. 17, no. 3, 1 March 2007 (2007-03-01), pages 687-694, XP002553959 abstract; figures 1-3; tables I-III	1-17
Y	WO 96/01318 A1 (STEENO RES GROUP AS [DK]; GROENHOEJ LARSEN CHRISTIAN [DK]; GESSER BORB) 18 January 1996 (1996-01-18) page 9 - page 11; claims 39-46	1-17
Y	WO 2007/123722 A2 (BAYER PHARMACEUTICALS CORP [US]; WILHELM SCOTT [US]) 1 November 2007 (2007-11-01) claims 1-18	1-17

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* 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 document 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	Date of mailing of the international search report
5 November 2009	18/11/2009

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Schmidt, Harald

2

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2005/083440 A2 (UNIV YALE [US]; MOR GIL G [US]; WARD DAVID C [US]; BRAY-WARD PATRICIA) 9 September 2005 (2005-09-09) claims 1-45	1-17
Y	US 2007/010483 A1 (IIAN YARON [IL] ET AL) 11 January 2007 (2007-01-11) paragraph [0025] - paragraph [0026]; claims 1-36	1-17
Y	POGGI A ET AL: "Relationship between clinical/hematological response and increase of plasmacells in the bone marrow of patients with chronic myelogenous leukemia imatinib mesylate treatment" BLOOD, vol. 110, no. 11, part 2, 4552, 16 November 2007 (2007-11-16), pages 209B-210B, XP008114508 abstract	1-17
T	DÖCKE WD ET AL: "Comprehensive biomarker monitoring in cytokine therapy: heterogenous, time-dependent, and persisting immune effects of interleukin-10 application in psoriasis" JOURNAL OF LEUKOCYTE BIOLOGY, vol. 85, 1 March 2009 (2009-03-01), pages 582-593, XP008114459 the whole document	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2009/054443

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9601318 A1	18-01-1996	AP 690 A	16-10-1998
		AT 195003 T	15-08-2000
		AU 686816 B2	12-02-1998
		AU 2612195 A	25-01-1996
		BR 9508243 A	21-10-1997
		CA 2194444 A1	18-01-1996
		CN 1159830 A	17-09-1997
		CZ 9700014 A3	14-05-1997
		DE 69518156 D1	31-08-2000
		DE 69518156 T2	11-01-2001
		DK 0769054 T3	13-11-2000
		EE 9700029 A	15-08-1997
		EP 0769054 A1	23-04-1997
		ES 2148525 T3	16-10-2000
		FI 970009 A	04-03-1997
		GR 3034628 T3	31-01-2001
		HK 1012416 A1	01-12-2000
		HU 76673 A2	28-10-1997
		JP 10502249 T	03-03-1998
		NO 970020 A	05-03-1997
		NZ 287406 A	28-07-1998
		PL 319429 A1	04-08-1997
		PT 769054 E	30-11-2000
		RO 118758 B1	30-10-2003
		RU 2218348 C2	10-12-2003
		SK 161496 A3	06-08-1997
		US 6168791 B1	02-01-2001
		US 6159937 A	12-12-2000
WO 2007123722 A2	01-11-2007	CA 2648106 A1	01-11-2007
		EP 2002264 A2	17-12-2008
		JP 2009532029 T	10-09-2009
WO 2005083440 A2	09-09-2005	AU 2005217375 A1	09-09-2005
		CA 2557438 A1	09-09-2005
		EP 1723428 A2	22-11-2006
		EP 2026071 A2	18-02-2009
		JP 2007523346 T	16-08-2007
US 2007010483 A1	11-01-2007	NONE	