METHODS OF DETECTING SIGNATURES OF DISEASE OR CONDITIONS IN BODILY FLUIDS

RELATED U.S. APPLICATIONS

[01] This application claims the benefit of the filing date of U.S. Provisional Patent Application Nos. 61/073,434, filed on June 18, 2008 and 61/022,033, filed on January 18, 2008, each of which is hereby incorporated herein by reference in its entirety for all purposes.

FIELD

[02] The present invention relates to methods of identifying markers of conditions such as gender of a fetus or disease such as tumor genomic, proteomic, metabolomic, glycomic, glycoproteomic, lipidomic and/or lipoproteomic signatures in cells obtained from bodily fluids of a patient.

BACKGROUND

[03] Tumors originate from normal cells upon the accumulation of genetic and epigenetic alterations. This multi-step process involves multiple genetic alterations that lead to the progressive transformation of normal cells to a malignant phenotype. These alterations are comprised of irreversible changes in DNA sequence (e.g., mutations, deletions, translocations) and lead to the activation of oncogenes, inactivation of tumor suppressor genes, and fusion of genes. The stochastic nature of these events confers genetic heterogeneity that gives the transformed cells molecular fingerprints (e.g., one or more cellular components such as DNA, RNA, protein, lipid, carbohydrate, and the like) indicative of cancer that give them unique phenotypes. Consequently, unique gene set hallmarks/signatures are known to be expressed by various tumors (Perou et al. (2000) *Nature* 406:747; Lobenhoferet al. (2001) *Health Perspect.* 109:881; van't Veer et al. (2002) *Nature* 415:530 (2002); Liotta and Kohn (2003) *Nat. Genet.* 33:10; Ginos et al.(2004) *Cancer Res.* 64:55; Liu (2005) *Proc. Natl. Acad. Sci. USA* 102:3531; Grigoriadis et al. (2006) *Breast Cancer Research* 8:R56).

[04] Both primary and metastatic tumors can lie silent and undetected for years. However, these dormant and occult tumors, as well as previously diagnosed primary and metastatic solid tumors, shed daily into the circulation approximately one-to-six million cells per gram of tumor. A large proportion of these circulating tumor cells, known as CTCs, undergo apoptosis and die, whereas distinct cell populations may develop into metastatic disease. Tumor cell apoptotic bodies, DNA, nucleosomes, RNA, and proteins are also found in the blood of cancer patients. Holmgren et al., *Blood* **93**, 3956 (1999). Efforts have been made to investigate whether signatures of tumors can be identified and whether they can be used to detect or monitor cancer. See, Ransohoff, *Nature Reviews Cancer* **5**, 142 (2005) and McLerran et al., *Clin. Chem*. **54**, 44 (2008).

[05] DNA can be easily transfected into various eukaryotic cells, i.e., once it is internalized into the cytoplasm of cells, it is able to integrate its genes into the genome of the host cell. For example, neutrophils and macrophages can be rapidly and very efficiently (50%–90%) transfected. Passage of DNA from prokaryotic to eukaryotic cells has also been demonstrated and is believed to occur from eukaryotic to eukaryotic cells. DNA released from tumor cells has a high transforming activity. Adding supernatant medium from cultured tumor cells to normal cells results in the appearance of as many transformed foci as those occurring after a transfection with a cloned *ras* gene administered as a calcium precipitate. Furthermore, when healthy rats were injected with plasma from tumor-bearing rats (therefore containing tumor DNA) the tumor marker gene was found in the DNA of their lung cells, i.e., tumor genes have been transcribed in lung cells.

[06] Leukocytes begin as pluripotent hematopoietic stem cells in the bone marrow and develop along either the myeloid lineage (monocytes, macrophages, neutrophils, eosinophils, and basophils) or the lymphoid lineage (T and B lymphocytes and natural killer cells). The major function of the myeloid lineage cells (e.g., neutrophils and macrophages) is the phagocytosis of infectious organisms, live unwanted damaged cells, senescent and dead cells (apoptotic and necrotic), as well as the clearing of cellular debris. Phagocytes from healthy animals do not replicate and are diploid, i.e.,

have a DNA index of one. On average, each cell contains <10 ng DNA, <20 ng RNA, and <300 ng of protein.

[07] Distinct gene expression patterns of variation, e.g., those associated with cell type, gender, age, interindividual differences and the like, have been recognized in WBCs of healthy donors. For example, a "lymphocyte-associated" cluster has 55 unique genes. In neutrophils, significant variability in the expression of 52 unique gene clusters has also been reported. The genes in this cluster can be grouped into three increasingly specific families: (i) those ubiquitously expressed in many types of circulating immune cells; (ii) those expressed by cells of the myeloid lineage; and (iii) those specific to granulocytes.

[08] The lifetime of various WBC subpopulations varies from a few days (e.g., neutrophils) to several months (e.g., macrophages). Like other cell types, leukocytes age and eventually die. During their aging process, human blood- and tissue-derived phagocytes (e.g., neutrophils) exhibit all the classic markers of programmed cell death (i.e., apoptosis), including caspase activation, pyknotic nuclei, and chromatin fragmentation. These cells also display a number of "eat-me" flags (e.g., phosphatidylserine, sugars) on the extracellular surfaces of their plasma membranes. Consequently, dying and dead cells and subcellular fragments thereof are cleared from tissues and blood by other phagocytic cells.

[09] The apoptosis of phagocytes is accelerated following their activation. For example, following the engulfment of *S. aureus* by neutrophils, phosphatidylserine is externalized on their plasma membranes, thereby leading to their rapid phagocytosis by macrophages. Activated monocytes have also been shown to bind various tumor-cell lines with elevated levels of phosphatidylserine.

[10] Circulating phagocytic cells are known to engulf live and dead CTCs and fragments thereof, a process that leads to an increase in the DNA (and other cellular constituent) contents of the phagocytosing cell. For example, apoptotic tumor cells have been shown to be phagocytosed by macrophages and dendritic cells. Consequent to such phagocytic activity, blood macrophages obtained from prostate cancer patients have been shown to contain intracellularly much higher levels of prostate-specific antigen

(PSA) than macrophages obtained from patients with benign prostate conditions. See, Herwig et al., *Clinical Prostate Cancer* **3**, 184 (2004) and Herwig et al., *Prostate* **62** 290 (2005). This is believed to be a consequence of phagocytosing tumor cells. Fetal stem cells, nucleated erythrocytes, fetal lymphocytes, as well as significant amounts of cell-free fetal nucleic acids are known to circulate in maternal blood. See Cheung et al., *Nat. Genet.* **14**, 264 (1996).

[11] It has also been shown that when apoptotic bodies (membrane-encapsulated cell fragments) derived from human Burkitt's lymphoma cells are cultured with human monocytes (phagocytic) or vascular smooth muscle cells (non-phagocytic), the monocytes show a high percentage of Epstein-Barr virus (EBV)-specific, tumor-gene-positive cells, whereas smooth muscle cells exhibit approximately 0.01% frequency of uptake and expression.

[12] Methods are needed that enable the early diagnosis of the presence of disease (e.g., tumors) in individuals, e.g., individuals who are not known to have the disease or who have recurrent disease. One object of the present invention is to facilitate the detection of disease-specific (e.g., tumor-specific) markers, e.g., proteins, RNA, DNA, carbohydrates and/or lipids and the like within subpopulations of white blood cells (WBCs) in an animal, including a human.

SUMMARY

[13] Embodiments of the present invention are based on the use of phagocytes to determine the presence or absence of markers associated with certain diseases or conditions. According to certain embodiments of the present invention, phagocytes incorporate cells and/or fragments and/or components thereof circulating in blood that are characteristic of a particular disease or condition. The contents of the phagocytes provide a marker profile for the disease or condition, for example through DNA and/or proteins content in the cell or through DNA or protein expression by the cell. Comparison of DNA expression profiles of phagocytic and non-phagocytic WBC lead to the detection of tumor specific, disease specific or condition specific DNA signatures within phagocytic cells that were either not expressed or under-expressed in the non-phagocytic cell. Likewise, protein expression profiles of phagocytic and

non-phagocytic WBC lead to the detection of tumor specific, disease specific or condition specific protein signatures within phagocytic cells that were either not expressed or under-expressed in the non-phagocytic cell. Accordingly, in certain embodiments, the methods of the present invention identify the presence of solid tumors (e.g., primary and metastatic lesions) in an individual suspected of having cancer and/or identify the presence of cancer prior to the manifestation of pathologic signs and symptoms and detect disease recurrence. According to other embodiments, the methods of the present invention diagnose certain diseases or other conditions by identifying specific signatures from blood or other bodily fluid.

[14] The present invention is based in part on the discovery that blood cell components, such as phagocytic cells and non-phagocytic cells, of an individual are ideally suited for the facile identification and differentiation of tumor specific and normal, non-specific signatures and therefore the elimination of the inequality of baseline consequent to intrinsic interindividual (e.g., age, gender, ethnic background, health status) and temporal variations in gene expressions.

[15] In certain exemplary embodiments, methods for the identification of tumor- and/or other disease-specific signatures within the WBCs (obtained from the blood or other bodily fluids, e.g., urine, stool, saliva, lymph, cerebrospinal fluid and the like) of an individual suspected of having cancer and/or one or more other diseases or disorders or conditions are provided. Embodiments of the present invention provide patient specific results and are not dependent on population-derived average signature profiles and values obtained from "healthy" controls, i.e., the baseline/background signature(s) is/are specific to the genomic, proteomic, metabolomic, glycomic, glycoproteomic, lipidomic, and/or lipoproteomic profile(s) of the individual being evaluated. Embodiments of the present invention provide a personalized predisposition to, screening, diagnosis, and monitoring of disease.

[16] In certain embodiments and with reference to Figure 1, the present invention is based on the ability of phagocytic cells to engulf and ingest viable, dying and dead cells (e.g., apoptotic cells, necrotic cells), microorganisms (e.g., bacteria (e.g., Rickettsia), viruses, fungi, yeast, protozoa and the like) subcellular particles and/or fragments thereof (cajal bodies, cell membrane, centrioles, centrosomes, gems, golgi apparatus,

lysosomes, mitochondria, nuclear membrane, nuclei, nucleolus, paraspeckles, promyelocytic leukemia bodies (PML bodies), ribosomes, rough endoplasmic reticulum, smooth endoplasmic reticulum, vacuoles, vesicles, microvesicles, and the like), and cellular debris, e.g. chromosomes, DNA (nuclear and mitochondrial), exons, genes, introns, proteins, prions, carbohydrate-binding proteins, glycoproteins, lipoproteins, RNA, microRNA, lipids, apoptotic bodies, nuclei, microvesicles, exosomes, nucleosomes, polymorphic interphase karyosomal associations (PIKA), splicing spreckles, and the like), and the absence of these characteristics in non-phagocytic cells. Accordingly, the analysis of DNA (nuclear, mitochondrial), RNA, microRNA, protein, prions, carbohydrate binding proteins, glycoproteins, lipids, lipoproteins, apoptotic bodies, nuclei, microvesicles, exosomes and/or nucleosomes and/or expression profiles of phagocytic WBCs and their comparison with those from non-phagocytic cells obtained from the blood or other bodily fluids of the same donor provides an identification of tumor- and/or disease-specific signatures within the phagocytic cells (patient-specific signal) that are either not expressed or significantly differentially expressed in the non-phagocytic cells (patient-specific noise). Since both phagocytic and non-phagocytic cells arise from the same pluripotent stem cell within the bone marrow, subtraction of the non-tumor-associated/induced signature profile (identified in the non-phagocytic cells) from the signatures found in the phagocytic cells allows the identification of tumor- and/or disease-specific signatures in the sample of the particular patient as shown in Figure 2. According to certain other embodiments, cellular debris in bodily fluids is internalized by entosis (cell absorption), endocytosis and pinocytosis.

[17] According to one embodiment of the present invention and with reference to Figure 3, a blood sample is obtained from an individual with the blood sample including both phagocytic and non-phagocytic cells (e.g., WBCs). Phagocytic cells(s) (e.g., neutrophils, monocytes, macrophages dendritic cells, foam cells) are then separated from non-phagocytic (e.g., T cells, B cells, null cells, basophils) cell(s) by various methods known to those of skill in the art. According to the present invention, the phenotype of WBCs is altered by the phagocytosis of live/dying/dead CTCs (and subcellular fragments thereof) and/or tumor- and/or disease-specific DNA, RNA, protein, carbohydrate and/or lipid present in blood. Phagocytosis leads to the

internalization of these tumor and/or disease signatures into the phagocytosing cell and possibly the integration of tumor-cell DNA with its tumor-specific somatic mutations (or other disease-related mutations) into the normal phagocytic cell DNA (i.e., its transfection of the chromosomes of the target cell). The subsequent transcription of the "transfected" DNA of phagocytic cells into RNA and the translation of the latter into proteins produces a phenotype different from non-phagocytic WBCs.

[18] Therefore, comparison using genomic, proteomic, metabolomic, glycomic, glycoproteomic, lipidomic and/or lipoproteomic methods known to those of skill in the art of the DNA, RNA, protein, and/or lipid expression profiles of phagocytic and non-phagocytic WBCs (as shown in Figure 3) obtained from an individual with cancer (and/or one or more other diseases) is used to identify tumor-specific (and/or disease-specific and/or condition specific) signature(s) and/or profile(s) selectively in the phagocytic cells which confirm the presence of occult tumor(s) (or other diseases or conditions) in the individual. According to the present invention, the subtraction of the DNA, RNA, protein, carbohydrate and/or lipid profiles of phagocytic cells from non-phagocytic cells provides a method for the identification (e.g., after genomic, proteomic, metabolomic, glycomic, glycoproteomic, lipidomic and/or lipoproteomic analyses) of tumor-specific (and/or disease-specific) signatures in a blood sample (and/or other biological sample) of a particular patient and signify the presence of occult tumor(s) and/or other disease as shown in Figure 2.

[19] In certain exemplary embodiments, phagocytic and non-phagocytic cells (e.g., obtained from the blood or one or more other biological samples (e.g., urine, stool, saliva, lymph, cerebrospinal fluid and the like), are separated. Since the phagocytosis of CTCs (and subcellular fragments thereof) by phagocytic WBC leads to the internalization of the tumor cells into the cytoplasm of phagocytic cells, the quantity of DNA, RNA, protein, carbohydrate and/or lipid within phagocytic cells will be higher than that of non-phagocytic cells. Therefore, comparison of the quantity and profile of these components between the phagocytic and non-phagocytic cells is used as an indication of the presence of cancer.

[20] In certain exemplary embodiments, a method for diagnosing the presence of a cancer cell in an individual is provided. The method includes the steps of obtaining a first expression profile from a blood phagocytic cell from an individual, obtaining a second expression profile from a blood non-phagocytic cell from the individual, comparing the first and second expression profiles, identifying differential expression of one or more markers specific to the first expression profile, and relating the differential expression of the one or more markers specific to the first expression profile to the presence of a cancer cell in the individual.

[21] In certain exemplary embodiments, a method for identifying a tumor-specific signature in an individual having cancer is provided. The method includes the steps of obtaining a first expression profile from a blood phagocytic cell from an individual having cancer, obtaining a second expression profile from a blood non-phagocytic cell from the individual having cancer, comparing the first and second expression profiles, identifying differential expression of two or more markers specific to the first expression profile, and relating the differential expression of the two or more markers specific to a tumor-specific signature in the individual having cancer.

[22] In certain exemplary embodiments, a method for diagnosing the presence of a cancer cell in an individual including the steps of obtaining a first expression profile from a blood phagocytic cell from an individual and obtaining a second expression profile from a blood non-phagocytic cell from the individual is provided. The method includes the steps of comparing the first and second expression profiles, identifying the presence of a circulating tumor cell or subcellular fragment thereof specific to the first expression profile, and relating the presence of a circulating tumor cell or subcellular fragment thereof to the presence of a cancer cell in the individual. In certain aspects, an increase in the quantity of a marker in the first expression profile relative to the second expression profile indicates the presence of a circulating tumor cell or subcellular fragment thereof.

[23] In certain exemplary embodiments and with reference to Figures 4-6, a method for diagnosing the presence of a cancer cell in an individual including the steps of isolating a population of phagocytic cells from an individual and separating 2n phagocytic cells from >2n phagocytic cells is provided. The method includes the

steps of obtaining a first expression profile from the 2n phagocytic cells, obtaining a second expression profile from the >2n phagocytic cells, comparing the first and second expression profiles, and identifying differential expression of one or more markers specific to the first expression profile. The method also includes the step of relating the differential expression of the one or more markers specific to the first expression profile to the presence of a cancer cell in the individual.

[24] In certain aspects of the methods described herein, markers include DNA, RNA, microRNA (e.g., DNA or RNA corresponding to cancer gene, oncogene, a tumor suppressor gene or any combination of these), protein (e.g., a protein or polypeptide encoded by a cancer gene, oncogene, a tumor suppressor gene or any combination of these), lipid, carbohydrate and/or any combination of these. In certain aspects, a blood phagocytic cell is a neutrophil, a macrophage, a monocyte, a dendritic cell, an eosinophil, a foam cell or any combination of these. In certain aspects, a blood non-phagocytic cell is a T cell, a B cell, a null cell, a basophil or any combination thereof. In other aspects, a blood phagocytic cell and a blood non-phagocytic cell are isolated from whole blood using methods known to those skilled in the art, such as antibodies. In still other aspects, a blood phagocytic cell and a blood non-phagocytic cell are isolated from a population of white blood cells using methods know to those of skill in the art such as fluorescence activated cell sorting (FACS). In other aspects, the blood phagocytic cell and the blood non-phagocytic cell are separated using a ligand that binds to a molecular receptor expressed on the plasma membranes of WBC populations. In yet other aspects, the blood phagocytic cell and the blood non-phagocytic cell are separated by one or methods including filtration, gradient-based centrifugation, elution, microfluidics and the like. In certain aspects, an individual has one or more of occult (e.g., dormant, undiagnosed, hidden or concealed) cancer, previously diagnosed primary cancer and metastatic cancer. In certain aspects, a method includes the step of relating the presence of one or more markers to efficacy of a cancer therapy.

[25] In certain exemplary embodiments, the above described methods are applied to detect, identify or diagnose the presence of an infectious agent or disease other than cancer by comparing expression profiles of phagocytic and nonphagocytic cells to determine

differential expression of markers characteristics of the infectious agent or disease other than cancer. In yet another aspect, one or more of the methods described herein are used to detect the DNA, RNA, protein, carbohydrate and/or lipid profiles of pathogens (e.g., viruses, bacteria, rickettsia, protozoans, helminthes, fungi, yeasts and the like) and other diseases or pathologies (e.g., Alzheimer's, dementia, heart failure, atherosclerosis, arthritis, genetic disorders, bone diseases, gastrointestinal diseases, prion diseases, and infectious diseases).

[26] In certain aspects of the methods described herein, markers include pathogen DNA, pathogen RNA, pathogen protein, pathogen polypeptide, pathogen lipid and any combination of these. In certain aspects, an infectious agent is a virus, a bacterium, a fungus, a parasite, an infectious protein and any combination of these. In certain aspects, a method includes the step of relating the presence of one or more markers to the efficacy of a pathogen therapy.

[27] The methods and compositions described herein, therefore, enable the facile identification of tumor specific signatures in the blood sample of a patient, without depending on population-derived average signature profiles and values obtained from "healthy" controls. Specifically, the methods and compositions described herein can easily and economically: (i) identify tumor (primary and metastatic lesions) presence in an individual prior to the manifestation of pathologic signs and symptoms; (ii) identify tumor (primary and metastatic lesions) presence in an individual suspected of having cancer; and/or (iii) detect tumor (primary and metastatic lesions) recurrence in an individual undergoing/following various treatments.

[28] Accordingly, the methods and compositions described herein (i) enable the noninvasive screening of cancer; (ii) allow the diagnosis of tumors, especially at the earliest time points; (iii) move meaningful intervention(s) to a much earlier point in the path of tumor progression, thereby forestalling the development of metastatic disease; (iv) monitor the early response to routine or experimental treatment(s); (v) predict response to routine or experimental treatment(s); (vi) facilitate the selection of effective treatment by allowing rapid identification of ineffective treatments whose side effects might not be balanced by expected benefits; (vii) minimize patient inconvcnicncc and incapacitation; (viii) allow tumor dctcction, diagnosis, and

treatment to be closely coupled (e.g., personalization of anticancer therapy); (ix) provide for prediction and early detection of tumor type and staging; (x) provide for therapy selection; (xi) determine whether a tumor is metastatic or not; (xii) provide methods for the monitoring of diseases; and (xiii) methods for the prognosis of diseases.

BRIEF DESCRIPTION OF THE DRAWINGS

[29] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee. The foregoing and other features and advantages of the present invention will be more fully understood from the following detailed description of illustrative embodiments taken in conjunction with the accompanying drawings in which:

[30] ***Figure 1*** schematically depicts a proposed pathway leading to acquisition of tumor-specific DNA, RNA, protein and/or lipid signatures by phagocytes following engulfment of live CTCs, apoptotic CTCs, fragmented CTCs, tumor DNA, RNA, proteins, and lipids released by viable and/or apoptotic tumor cells. Note that only phagocytic cells (and not non-phagocytic cells) acquire tumor signatures.

[31] ***Figure 2*** schematically depicts an analytical method used in the identification of cancer signatures expressed in/by phagocytic cells of patients with ovarian cancer (OC).

[32] ***Figure 3*** schematically depicts a general flowchart of one embodiment of a method of the invention.

[33] ***Figure 4*** schematically depicts a proposed pathway leading to acquisition of tumor-specific DNA, RNA, protein and lipid signatures by blood phagocytes following engulfment of live CTCs, apoptotic CTCs, fragmented CTCs, tumor DNA, RNA, proteins and lipids released by viable and/or apoptotic tumor cells. Note that DNA contents of phagocytes following phagocytosis is >2n.

[34] ***Figure 5*** schematically depicts analytical approaches used in the identification of breast cancer (BC) signatures in BC-bearing animals.

[35] ***Figure 6*** schematically depicts a general flowchart of another embodiment of a method of the invention.

[36] ***Figure 7*** depicts gel electrophoresis analysis of total RNA isolated from LNCaP and LLC1 cells.

[37] ***Figure 8*** lists the yield and quality of RNA obtained from mouse white blood cells (WBCs).

[38] ***Figures 9A-9D*** depict arrays showing seven up-regulated (≥2 fold), cancer related genes detected in neutrophils from LNCaP (human prostate cancer) tumor-bearing nude mice. (A) LNCaP tumor. (B) Neutrophils obtained from nude mice bearing LNCaP tumors (N_T). (C) T cells obtained from nude mice bearing LNCaP tumors (T_T). (D) Neutrophils obtained from non-tumor-bearing nude mice (N_N). Circled signatures expressed in tumor cells (A) and in neutrophils from tumor-bearing mice (B), and minimally expressed in neutrophils from non-tumor-bearing mice (D), and in non-phagocytic T cells (C). Expression in N_T was ≥2-fold than that in N_N and T_T.

[39] ***Figures 10A-10D*** depict arrays showing three up-regulated, cancer related genes detected in macrophages from LNCaP (human prostate cancer) tumor-bearing nude mice. (A) LNCaP tumor. (B) macrophages obtained from nude mice bearing LNCaP tumors (M_T). (C) T cells obtained from nude mice bearing LNCaP tumors (T_T). (D) macrophages obtained from non-tumor-bearing nude mice (M_N). Circled signatures expressed in tumor cells (A) and in macrophages from tumor-bearing mice (B), and minimally expressed in macrophages from non-tumor-bearing mice (D), and in non-phagocytic T cells (C). Expression in M_T was ≥2-fold than that in M_N and T_T.

[40] ***Figures 11A-11D*** depict arrays showing four up-regulated (≥2 fold), cancer related genes detected in neutrophils from LS174T (human colon cancer) tumor-bearing nude mice. (A) LS174T tumor. (B) Neutrophils obtained from nude mice bearing LS174T tumors (N_T). (C) T cells obtained from nude mice bearing LS174T tumors (T_T). (D) Neutrophils obtained from non-tumor-bearing nude mice (N_N). Circled signatures

expressed in tumor cells (A) and in neutrophils from tumor-bearing mice (B), and minimally expressed in neutrophils from non-tumor-bearing mice (D), and in non-phagocytic T cells (C). Expression was N_T is ≥2-fold than that in N_N and T_T.

[41] ***Figures 12A-12D*** depict arrays showing three up-regulated (≥2 fold), cancer related genes detected in macrophages from LS174T (human colon cancer) tumor-bearing nude mice. (A) LS174T tumor. (B) Macrophages obtained from nude mice bearing LS174T tumors (M_T). (C) T cells obtained from nude mice bearing LS174T tumors (T_T). (D) Macrophages obtained from non-tumor-bearing nude mice (M_N). Circled signatures expressed in tumor cells (A) and in macrophages from tumor-bearing mice (B), and minimally expressed in macrophages from non-tumor-bearing mice (D), and in non-phagocytic T cells (C). Expression in M_T is ≥2-fold than that in M_N and T_T.

[42] ***Figures 13A-13D*** depict arrays showing five up-regulated (≥2 fold), cancer related genes detected in neutrophils from LLC1 (mouse metastatic lung cancer) tumor-bearing C57/Bl mice. (A) LLC1 tumor. (B) Neutrophils obtained from C57/Bl mice bearing LLC1 tumors (N_T). (C) T cells obtained from C57/Bl mice bearing LLC1 tumors (T_T). (D) Neutrophils obtained from non-tumor-bearing C57/Bl mice (N_N). Circled signatures expressed in tumor cells (A) and in neutrophils from tumor-bearing mice (B), and minimally expressed in neutrophils from non-tumor-bearing mice (D), and in non-phagocytic T cells (C). Expression in N_T was ≥2-fold than that in N_N and T_T.

[43] ***Figures 14A-14D*** depict arrays showing two up-regulated (≥2 fold), cancer related genes detected in macrophages from LLC1 (mouse metastatic lung cancer) tumor-bearing C57/Bl mice. (A) LLC1 tumor. (B) Macrophages obtained from C57/Bl mice bearing LLC1 tumors (M_T). (C) T cells obtained from C57/Bl mice bearing LLC1 tumors (T_T). (D) Macrophages obtained from non-tumor-bearing C57/Bl mice (M_N). Circled signatures expressed in tumor cells (A) and in neutrophils from tumor-bearing mice (B), and minimally expressed in neutrophils from non-tumor-bearing mice (D), and in non-phagocytic T cells (C). Expression in M_T was ≥2-fold than that in M_N and T_T.

[44] ***Figure 15A-15D*** depict arrays showing two up-regulated (≥2 fold), cancer related genes detected in neutrophils from B16F10 (mouse metastatic melanoma) tumor bearing C57/Bl mice. (A) B16F10 tumor. (B) Neutrophils obtained from C57/Bl mice bearing B16F10 tumors (N_T). (C) T cells obtained from C57/Bl mice-bearing B16F10 tumors (T_T). (D) Neutrophils obtained from non-tumor-bearing C57/Bl mice (N_N). Circled signatures expressed in tumor cells (A) and in neutrophils from tumor-bearing mice (B), and minimally expressed in neutrophils from non-tumor-bearing mice (D), and in non-phagocytic T cells (C). Expression in N_T was ≥2-fold than that in N_N and T_T.

[45] ***Figure 16A-16D*** depict arrays showing one up-regulated (≥2 fold), cancer related genes detected in macrophages from B16F10 (mouse metastatic melanoma) tumor-bearing C57/Bl mice. (A) B16F10 tumor. (B) Macrophages obtained from C57/Bl mice bearing B16F10 tumors (M_T). (C) T cells obtained from C57/Bl mice bearing B16F10 tumors (T_T). (D: Macrophages obtained from non-tumor-bearing C57/Bl mice (M_N). Circled signatures expressed in tumor cells (A) and in macrophages from tumor-bearing mice (B), and minimally expressed in macrophages from non-tumor-bearing mice (D), and in non-phagocytic T cells (C). Expression in M_T was ≥2-fold than that in M_N and T_T.

[46] ***Figure 17A-17D*** depict arrays showing five up-regulated (≥2 fold), cancer related genes detected in neutrophils from patient with head and neck cancer (squamous cell carcinoma). (A) Normal tissue (skin) biopsy. (B) Tumor tissue biopsy. (C) Neutrophils obtained from patient blood (N_T). (D) T cells obtained from patient blood (T_T). Circled signatures expressed in tumor cells (B) and in neutrophils from patient blood (C), and minimally expressed or not expressed in normal skin (A) or non-phagocytic T cells (D). Expression in N_T was ≥2-fold than that in T_T and skin.

[47] ***Figure 18A-18B*** depict arrays showing 23 up-regulated (≥2 fold), cancer related genes detected in macrophages from patient with ovarian cancer (adenocarcinoma). (A) Macrophages obtained from patient blood (M_T). (B) T cells obtained from patient blood (T_T). Circled signatures expressed in macrophages from patient (A) and minimally expressed in non-phagocytic T cells (B). Expression in M_T was ≥2-fold than that in T_T.

[48] ***Figure 19*** depicts a method used to identify tumor signatures in phagocytic cells. In this example, expression intensities of cancer associated genes in macrophages from tumor-bearing animals (M_T) were quantified compared to those from T cells from the same animals (T_T) and those overexpressed by >2-fold identified. Next, the intensities of all expressed genes in M_T were quantified and compared to those in macrophages obtained from non-tumor bearing animals (M_{NT}) and the genes overexpressed >2-fold were identified. The genes common to both lists were selected and compared to those expressed by the same tumor (shaded area).

[49] ***Figures 20A-20D*** depict gene expression intensity comparisons in (A) macrophages obtained from nude mice bearing LNCaP human prostate tumors (M_{LNCaP}) and T cells from the same animals (T $cells_{LNCaP}$), (B) M_{LNCaP} and macrophages obtained from non-tumor-bearing mice ($M_{non\text{-}tumor}$), (C) neutrophils obtained from nude mice bearing LNCaP human prostate tumors (N_{LNCaP}) and T cells from the same animals (T $cells_{LNCaP}$), and (D) N_{LNCaP} and macrophages obtained from non-tumor-bearing mice ($N_{non\text{-}tumor}$). Genes in red were overexpressed >2 fold; those in green were under-expressed >2 fold.

[50] ***Figure 21*** lists expression of cancer-related genes within phagocytic neutrophils (N) and macrophages (M).

[51] ***Figure 22*** lists cancer-related genes upregulated (>2-fold) in phagocytic macrophages of a patient with ovarian cancer in comparison to non-phagocytic T cells.

[52] ***Figure 23*** depicts SDS gel (10%) electrophoresis of protein sample (5.9 μg) obtained from mouse WBC.

[53] ***Figure 24*** depicts Western blot analysis of TAG-72 and PSA expression in T cells and monocytes/macrophages (M/M) obtained from tumor-bearing mice, illustrating the presence of signatures in phagocytic cells only.

DETAILED DESCRIPTION OF CERTAIN EMBODIMENTS

[54] Embodiments of the present invention are directed to a method of providing a patient-specific expression profile of markers associated with diseases, infectious agents and

bodily conditions based on the cellular content and/or expression profiles of phagocytic cells. According to one aspect of the present invention, the cellular contents and/or expression profiles of phagocytic cells is compared to known markers for a particular disease state or condition to detect and/or diagnose the particular disease state or condition. According to an additional aspect of the present invention, the cellular content and/or expression profile of phagocytic cells is compared to the cellular content and/or expression profile of non-phagocytic cells from the blood of a single patient. Subtracting the cellular content and/or expression profile from non-phagocytic cells from that of phagocytic cells creates a cellular content and/or expression profile representative of only the disease state of the individual.

[55] According to an additional embodiment of the present invention, a phagocytic cell population from an individual is obtained and the cellular content and/or expression profile of phagocytic cells from the population where the DNA content is greater than 2n is compared with the cellular content and/or expression profile of phagocytic cells from the same population where the DNA content is 2n. According to a still additional embodiment of the present invention, a phagocytic cell population from an individual is obtained and the expression profile of phagocytic cells from the population where the RNA, protein, carbohydrate and/or lipid content is larger than normal and have a DNA index greater than 1 is compared with the expression profile of phagocytic cells from the same population where the RNA, protein, carbohydrate and/or lipid content is normal and/or have a DNA index of 1.

[56] Such a patient specific expression profile eliminates the dependence on a population-derived average signature profile for a particular disease or infectious agent, which may introduce error into the detection or diagnosis of a particular disease in the individual. Such a patient specific expression profile for a disease state of the present invention allows detection, diagnosis and treatment to be personalized to the individual.

[57] With reference to Figures 1-3 and according to certain embodiments of the present invention, the gene expression profiles of phagocytic and non-phagocytic WBCs obtained from mice bearing approximately three week old human subcutaneous (s.c.) tumors (prostatc LNCaP adcnocarcinoma or LS174T colon adcnocarcinoma) or

mouse tumors (B16F10 metastatic melanoma, administered intravenously, or LLC1 lung cancer, injected s.c.), were compared. The results demonstrated that neutrophils and macrophages obtained from these tumor-bearing mice express various oncogenes and other cancer-related gene signatures that are also expressed in each of the respective tumors. See Figures 9-16 and 19-21. These cancer-related genes and oncogenes (e.g., *ERBB2*, *Jun*, *Fos*, etc.) are not expressed or are minimally expressed by (i) non-phagocytic T cells isolated from tumor-bearing mice, and (ii) neutrophils and macrophages obtained from non-tumor-bearing mice. Furthermore, only the phagocytic cells from tumor-bearing mice were found to express tumor-specific proteins. See Figures 23 and 24. CTCs and/or tumor-specific DNA and/or proteins in the blood of the mice were phagocytosed and some of the tumor-cell DNA, with its tumor-specific mutations and genes, was integrated, likely by transfection (without intending to be bound by theory), into normal phagocyte DNA, transcribed into RNA, and translated into protein.

[58] With reference to Figures 1-3 and according to certain exemplary embodiments of the present invention, the gene expression profiles of phagocytic and non-phagocytic WBCs obtained from patients with head and neck tumors or with ovarian cancer were also compared. The results demonstrated that neutrophils and macrophages obtained from these patients express various oncogenes and other cancer-related gene signatures that are also expressed in each of the respective tumors. See Figures 17-18 and 22. These cancer-related genes and oncogenes were not expressed or were minimally expressed by non-phagocytic T cells isolated from the same individual patient. CTCs and/or tumor-specific DNA and RNA in the blood of the patient were phagocytosed and some of the tumor-cell DNA and/or RNA, with its tumor-specific mutations and genes, was integrated, likely through transfection (without intending to be bound by theory), into normal phagocyte DNA, transcribed into RNA, and translated into protein.

[59] With reference to Figures 4-6 and according to certain exemplary embodiments, the quantitative analysis of DNA (nuclear and/or mitochondrial), RNA, microRNA, protein, and/or lipid expression profiles of phagocytic cells (e.g., macrophages) obtained from the blood or one or more other biological samples (e.g., urine, stool,

saliva, lymph, cerebrospinal fluid and the like) whose (1) DNA content is >2n ($P_{n>2}$), or (2) RNA, protein, carbohydrate and/or lipid content is larger than normal, i.e., cells that have phagocytosed CTCs and/or their subcellular fragments or DNA/RNA/lipids (i.e., tumor-specific signatures or other disease-specific signatures) and/or have a DNA index greater than one, and their comparison with the *same* phagocytic cell population (e.g., macrophages) whose (1) DNA content is 2n ($P_{n=2}$), or (2) RNA, protein, carbohydrate and/or lipid content is normal, i.e., cells that have not phagocytosed CTCs and/or their subcellular fragments and have a DNA index of one, provides a method to detect tumor-specific (or other disease-specific) signatures within the $P_{n>2}$ cells (patient-specific signal) that are either not expressed or minimally expressed in the $P_{n=2}$ cells (patient-specific noise). With reference to Figure 6, the subtraction of the DNA, RNA, protein, and/or lipid profiles of $P_{n=2}$ from those of $P_{n>2}$ as shown in Figure 5 provides a method to identify (e.g., after one or more genomic, proteomic, metabolomic, glycomic, glycoproteomic, lipidomic and/or lipoproteomic analyses) tumor-specific (and/or disease-specific and/or condition specific) signatures in a blood sample (or one or more other biological samples such as, e.g., other bodily fluids) of an animal and/or a human with cancer (and/or disease and or bodily conditions) and signify the presence of occult tumor(s) and/or other disease and/or other conditions. Unlike the methods described above in which the genomic, proteomic, metabolomic, glycomic, glycoproteomic, lipidomic and/or lipoproteomic profiles of phagocytic cells are compared with those of non-phagocytic cells, the major advantages of this analytic detection method according to the present invention are: (i) it utilizes a single phagocytic cell subpopulation as a source of the "tumor-specific" (e.g., $P_{n>2}$ macrophage) and "normal-non-specific" (e.g., $P_{n=2}$ macrophage) signatures, i.e., both share the same baseline genotype; and (ii) the signature-acquiring cells (e.g., $P_{n>2}$ neutrophil) are not diluted with those that have not phagocytosed, and therefore have not acquired, dead CTCs and/or fragments thereof (e.g., $P_{n=2}$ neutrophils).

[60] With reference to Figures 4-6 and according to certain exemplary embodiments, the quantitative analysis of phagocytic cells (e.g., macrophages) obtained from the blood or one or more other biological samples or bodily fluid (e.g., urine, stool, saliva, lymph, cerebrospinal fluid and the like) whose intracellular content consequent to

phagocytosis or internalization of other live, dying, or dead (e.g., apoptotic or necrotic) cells, apoptotic bodies, nuclei, microvesicles, exosomes, nucleosomes, mitochondria, endoplasmic reticulum, and the like, is greater than that of the same phagocytic cell population (e.g., macrophages) with normal intracellular contents (P_{NIC}), i.e., cells that have not phagocytosed any of the above mentioned cells and/cellular debris (patient-specific noise), provides a method to detect tumor-specific (or other disease-specific or other condition specific) signatures within the phagocytes with increased intracellular content (P_{IIC}) that are either not expressed or minimally expressed in the phagocytes with a normal intracellular contents (patient-specific noise). With reference to Figure 6, the subtraction of the DNA, RNA, protein, and/or lipid profiles of P_{NIC} from those of P_{IIC} as shown in Figure 5 provides a method to identify (e.g., after one or more genomic, proteomic, metabolomic, glycomic, glycoproteomic, lipidomic, and/or lipoproteomic analyses) tumor-specific (and/or disease-specific) signatures in a blood sample (or one or more other biological samples such as, e.g., other bodily fluids) of an animal with cancer or other disease and signify the presence of occult tumor(s) and/or other disease. Unlike the methods described above in which the genomic, proteomic, metabolomic, glycomic, glycoproteomic, lipidomic, and/or lipoproteomic profiles of phagocytic cells are compared with those of non-phagocytic cells, the major advantages of this analytic detection method according to the present invention are: (i) it utilizes a single phagocytic cell subpopulation as a source of the "disease-specific" (e.g., P_{IIC} macrophage) and "normal-non-specific" (e.g., P_{NIC} macrophage) signatures, i.e., both share the same baseline genotype; and (ii) the signature-acquiring cells (e.g., P_{IIC} neutrophil) are not diluted with those that have not phagocytosed, and therefore have not acquired, dead CTCs and/or fragments thereof (e.g., P_{NIC} neutrophils).

[61] The methods described herein (i) have high specificity, sensitivity, and accuracy and should enable the detection of tumor-specific (and/or other disease-specific) and normal-nonspecific signatures present within a blood sample (or other biological sample such as, e.g., a bodily fluid); and (ii) eliminate the "inequality of baseline" that is known to occur among individuals due to intrinsic (e.g., age, gender, ethnic background, health status and the like) and temporal variations in gene expression. Accordingly, in certain aspects, the invention provides non-invasive assays for the

early detection of occult primary and metastatic tumors (and/or one or more other diseases or conditions) in patients, i.e., before the disease can be diagnosed by conventional imaging techniques (e.g., PET, MRI, CT and the like), and, therefore, provide a foundation for improved decision-making relative to the needs and strategies for intervention, prevention, and treatment of individuals with cancer.

[62] As used herein, the term "tumor specific marker" is intended to include, but is not limited to, one or more cellular components such as one or more DNA sequences, one or more RNA sequences, one or more proteins, one or more polypeptides, one or more lipids and the like. In certain aspects, a tumor specific marker is present in one or more WBCs such as, for example, a neutrophil, a macrophage and/or a dendritic cell.

[63] As used herein, the term "cancer related genes" refers to genes such as, for example, cancer genes, oncogenes and/or tumor suppressor genes, that have altered expression (e.g., increased expression or decreased expression when compared to a non-cancerous cell) in a cancerous cell (e.g., a WBC such as, for example, a macrophage, a neutrophil, a T cell or the like). Many cancer related genes are known in the art. Cancer related genes include for example, but are not limited to, *ERBB2*, *JUN*, *RB1*, *SUPP1*, *MDM2*, *MAP2K1*, *MMP2*, *PDGFB*, *PLAUR*, *FGR*, *MYCL1*, *BLYM*, *NRAS1*, *PE1*, *SKI*, *TRK*, *ABL2*, *MYCN*, *RAB1*, *REL*, *RALB*, *LCO*, *ERBB4*, *RAF1*, *ECT2*, *KIT*, *FGF5*, *GRO1*, *GRO2*, *GRO3*, *FMS*, *PIM*, *KRAS1P*, *FYN*, *MYB*, *ROS1*, *MAS1*, *RALA*, *MYCLK1*, *GLI3*, *ARAF2*, *MET*, *BRAF*, *MOS*, *LYN*, *MYBL1*, *MYC*, *OVC*, *VAV2*, *BMI1*, *RET*, *HRAS*, *SPI1*, *RELA*, *SEA*, *EMS1*, *ETS1*, *KRAS2*, *ERBB3*, *GLI*, *FLT*, *BRCA2*, *RB1*, *FOS*, *AKT1*, *ELK2*, *FES*, *MAF*, *TP53*, *CRK*, *ERBA1*, *NF1*, *EVI2*, *ERBBB2*, *INT4*, *BRCA1*, *YES1*, *JUND*, *JUNB*, *MEL*, *LPSA*, *VAV1*, *AKT2*, *FOSB*, *RRAS*, *HKR1*, *HKR2*, *ERBAL2*, *SRC*, *MYBL2*, *ETS2*, *ERG*, *ARAF1*, *YUASA*, *HS2*, *INT3*, *SNO*, *RMYC*, *BMYC*, *HRASP*, *TC21*, *TIM*, *PTI-1*, *JAK*, one or members of the *CEA* family (see, e.g., Zhou et al. (2001) *Gene* 264:105), *PSA*, *MUC-16* and the like.

[64] As used herein, the term "cancer" refers to various types of malignant neoplasms, most of which can invade surrounding tissues, and may metastasize to different sites (see, for example, *PDR Medical Dictionary,* 1st edition (1995), incorporated herein by reference in its entirety for all purposes). The terms "neoplasm" and "tumor" refer

to an abnormal tissue that grows by cellular proliferation more rapidly than normal and continues to grow after the stimuli that initiated proliferation is removed. *Id.* Such abnormal tissue shows partial or complete lack of structural organization and functional coordination with the normal tissue which may be either benign (i.e., benign tumor) or malignant (i.e., malignant tumor).

[65] Examples of general categories of cancer include, but are not limited to, carcinomas (i.e., malignant tumors derived from epithelial cells such as, for example, common forms of breast, prostate, lung and colon cancer), sarcomas (i.e., malignant tumors derived from connective tissue or mesenchymal cells), lymphomas (i.e., malignancies derived from hematopoietic cells), leukemias (i.e., malignancies derived from hematopoietic cells), germ cell tumors (i.e., tumors derived from totipotent cells. In adults most often found in the testicle or ovary; in fetuses, babies and young children, most often found on the body midline, particularly at the tip of the tailbone), blastic tumors (i.e., a typically malignant tumor which resembles an immature or embryonic tissue) and the like.

[66] Examples of the types of neoplasms intended to be encompassed by the present invention include but are not limited to those neoplasms associated with cancers of neural tissue, blood forming tissue, breast, skin, bone, prostate, ovaries, uterus, cervix, liver, lung, brain, larynx, gallbladder, pancreas, rectum, parathyroid, thyroid, adrenal gland, immune system, head and neck, colon, stomach, bronchi, and/or kidneys.

[67] In certain exemplary embodiments, one or more methods and/or compositions described herein are applied to detect, identify and/or diagnose disorders associated with the presence of fetal chromosomal abnormalities (e.g., Down's syndrome, autism and related autism spectrum disorders (including, but not limited to, Asperger's syndrome and pervasive developmental disorder-not otherwise specified), sickle cell anemia, thalassemia and the like) consequent to the presence of fetal cells and DNA within maternal blood. Screening and diagnosing of one or more of these disorders can be performed using the methods and/or compositions described herein to detect one or more chromosomal markers, e.g., DNA and RNA, and the like, within maternal blood phagocytic cells.

[68] In certain exemplary embodiments, one or more methods and/or compositions described herein can be applied to test the gender of a fetus within a pregnant woman by detecting the presence of fetus-derived proteomic, lipidomic, and/or genomic signatures within blood of the pregnant woman, as fetal stem cells, nucleated erythrocytes, fetal lymphocytes, as well as significant amounts of cell-free fetal nucleic acids are known to circulate in maternal blood. According to the methods described herein, the cellular content and/or expression profile of phagocytic cells is compared to the cellular content and/or expression profile of non-phagocytic cells from the blood of a pregnant woman. Subtracting the cellular content and/or expression profile from non-phagocytic cells from that of phagocytic cells creates a cellular content and/or expression profile representative of the gender of the fetus being carried by the pregnant woman.

[69] In certain exemplary embodiments, one or more methods and/or compositions described herein can be used to detect, identify and/or diagnose disorders associated with the presence of proteomic and/or genomic myocyte signatures within blood of subjects having or at risk of developing cardiac disease (e.g., myocardial infarction, chronic heart failure, ischemic heart disease, cardiovascular death and the like) by detecting the presence of dying/dead myocytes and/or fragments thereof (e.g., DNA, proteins and the like). Screening and diagnosing of one or more of these disorders is performed using methods and/or compositions described herein to detect one or more markers, e.g., DNA and RNA, protein and the like, within blood phagocytic cells.

[70] In certain exemplary embodiments, one or more methods and/or compositions described herein can be used to detect, identify and/or diagnose disorders associated with the presence of proteomic, lipidomic, and/or genomic signatures within blood of subjects having or at risk of developing atherosclerosis consequent to coronary artery narrowing, abdominal aortic aneurism, and the like. Screening and diagnosing of these disorders can performed using the methods and/or compositions described herein to detect one or more markers, e.g., DNA, RNA, protein and the like, within blood phagocytic cells.

[71] Biopsy-confirmed rejection, one method for diagnosis of allograft rejection, is invasive and subject to sampling errors. Therefore, the development of noninvasive

assays that detect molecular biomarkers for diagnosing and managing transplanted organ rejection is useful in management of transplant recipients by (a) detecting a pre-rejection profile that will allow therapeutic interventions before rejection causes graft dysfunction, (b) improving the sensitivity and specificity of rejection diagnosis, (c) developing new classification systems for rejection that will improve prognosis, and (d) providing information for designing individualized immunosuppressive regimens that could prevent rejection while minimizing drug toxicity.

[72] Accordingly, in certain exemplary embodiments, one or more methods and/or compositions described herein can be used to detect, identify or diagnose disorders associated with the presence of proteomic, lipidomic, and genomic signatures within blood of subjects having undergone organ transplants by detecting one or more markers, e.g., DNA, RNA, protein or the like, within blood phagocytic cells.

[73] Mitochondrial diseases result from failures of the mitochondria. Cell injury and even cell death follow. Diseases of the mitochondria appear to cause the most damage to cells of the brain, heart, liver, skeletal muscles, kidney and the endocrine and respiratory systems as well as diabetes, respiratory complications, seizures, Alzheimer's disease, visual/hearing problems, lactic acidosis, developmental delays, susceptibility to infection, and cancer.

[74] Accordingly, in certain exemplary embodiments, one or more methods and/or compositions described herein can be used to screen, diagnose and/or detect mitochondrial disease, by detecting one or more genomic, mitochondria-associated DNA markers within blood phagocytic cells.

[75] In certain exemplary embodiments, one or more methods and/or compositions described herein can be used to screen, diagnose and/or detect Alzheimer's disease and/or dementia by detecting one or more markers, e.g., DNA, RNA, protein and the like, within blood phagocytic cells.

[76] Systemic lupus erythematosus (SLE) is a complex autoimmune disorder that affects various organs and systems. Accordingly, in certain exemplary embodiments, one or more methods and/or compositions described herein can be used to screen, diagnose

and/or detect SLE by detecting one or more markers, e.g., DNA, RNA, lipids, protein and the like, within blood phagocytic cells.

[77] In certain exemplary embodiments, one or more methods and/or compositions described herein can be used to screen and/or detect genomic and/or proteomic signatures useful in the development of therapeutic and/or imaging molecules by detecting one or more markers, e.g., DNA, RNA, protein and the like, within blood phagocytic cells.

[78] In certain exemplary embodiments, one or more methods and/or compositions described herein can be used to screen, diagnose and/or detect alteration in genomic, proteomic and/or lipidomic signatures useful in detection of diseases and pathologies consequent to one or more external or internal insults (e.g., dirty bomb exposure, radiation exposure, chemical exposure, radiotherapy, radiopharmaceutical administration, therapeutic molecule exposure, radon exposure, asbestos exposure, pollution exposure and the like) by detecting one or more markers, e.g., DNA, RNA, protein, lipid and the like, within blood phagocytic cells.

[79] As used herein, the term "organism" includes, but is not limited to, a human individual, a non-human primate, a cow, a horse, a sheep, a goat, a pig, a dog, a cat, a rabbit, a mouse, a rat, a gerbil, a frog, a toad and a transgenic species thereof. The term "organism" further includes pathogenic organisms, including, but not limited to, a pathogen such as a parasite, a yeast cell, a yeast tetrad, a yeast colony, a bacterium, a bacterial colony, a virion, a virosome, a virus-like particle and/or cultures of any of these, and the like.

[80] In certain exemplary embodiments, the assays described herein can be used for the detection of an infectious agent and/or the diagnosis of a disorder associated with an infection of a cell, tissue, organ or the like by an infectious agent. In certain aspects, detection of an infectious agent and/or the diagnosis of a disorder associated with an infection is performed using the methods and/or compositions described herein to detect one or more infectious agent markers, e.g., DNA, RNA, proteins, lipids and the like, from one or more infectious agents.

[81] As used herein, the term "infectious agent" includes, but is not limited to, pathogenic organisms such as viruses, bacteria, fungi, parasites, infectious proteins and the like.

[82] Viruses include, but are not limited to, DNA or RNA animal viruses. As used herein, RNA viruses include, but are not limited to, virus families such as *Picornaviridae* (e.g., polioviruses), *Reoviridae* (e.g., rotaviruses), *Togaviridae* (e.g., encephalitis viruses, yellow fever virus, rubella virus), *Orthomyxoviridae* (e.g., influenza viruses), *Paramyxoviridae* (e.g., respiratory syncytial virus, measles virus, mumps virus, parainfluenza virus), *Rhabdoviridae* (e.g., rabies virus), *Coronaviridae*, *Bunyaviridae*, *Flaviviridae*, *Filoviridae*, *Arenaviridae*, *Bunyaviridae* and *Retroviridae* (e.g., human T cell lymphotropic viruses (HTLV), human immunodeficiency viruses (HIV)). As used herein, DNA viruses include, but are not limited to, virus families such as *Papovaviridae* (e.g., papilloma viruses), *Adenoviridae* (e.g., adenovirus), *Herpesviridae* (e.g., herpes simplex viruses), and *Poxviridae* (e.g., variola viruses).

[83] Bacteria include, but are not limited to, gram positive bacteria, gram negative bacteria, acid-fast bacteria and the like.

[84] As used herein, gram positive bacteria include, but are not limited to, *Actinomedurae*, *Actinomyces israelii*, *Bacillus anthracis*, *Bacillus cereus*, *Clostridium botulinum*, *Clostridium difficile*, *Clostridium perfringens*, *Clostridium tetani*, *Corynebacterium*, *Enterococcus faecalis*, *Listeria monocytogenes*, *Nocardia*, *Propionibacterium acnes*, *Staphylococcus aureus*, *Staphylococcus epiderm*, *Streptococcus mutans*, *Streptococcus pneumoniae* and the like.

[85] As used herein, gram negative bacteria include, but are not limited to, *Afipia felis*, *Bacteriodes*, *Bartonella bacilliformis*, *Bortadella pertussis*, *Borrelia burgdorferi*, *Borrelia recurrentis*, *Brucella*, *Calymmatobacterium granulomatis*, *Campylobacter*, *Escherichia coli*, *Francisella tularensis*, *Gardnerella vaginalis*, *Haemophilius aegyptius*, *Haemophilius ducreyi*, *Haemophilius influenziae*, *Heliobacter pylori*, *Legionella pneumophila*, *Leptospira interrogans*, *Neisseria meningitidia*, *Porphyromonas gingivalis*, *Providencia sturti*, *Pseudomonas aeruginosa*, *Salmonella enteridis*, *Salmonella typhi*, *Serratia marcescens*, *Shigella boydii*, *Streptobacillus*

moniliformis, *Streptococcus pyogenes*, *Treponema pallidum*, *Vibrio cholerae*, *Yersinia enterocolitica*, *Yersinia pestis* and the like.

[86] As used herein, acid-fast bacteria include, but are not limited to, *Myobacterium avium*, *Myobacterium leprae*, *Myobacterium tuberculosis* and the like.

[87] As used herein, other bacteria not falling into the other three categories include, but are not limited to, *Bartonella henseiae*, *Chlamydia psittaci*, *Chlamydia trachomatis*, *Coxiella burnetii*, *Mycoplasma pneumoniae*, *Rickettsia akari*, *Rickettsia prowazekii*, *Rickettsia rickettsii*, *Rickettsia tsutsugamushi*, *Rickettsia typhi*, *Ureaplasma urealyticum*, *Diplococcus pneumoniae*, *Ehrlichia chafensis*, *Enterococcus faecium*, *Meningococci* and the like.

[88] As used herein, fungi include, but are not limited to, *Aspergilli*, *Candidae*, *Candida albicans*, *Coccidioides immitis*, *Cryptococci*, and combinations thereof.

[89] As used herein, parasitic microbes include, but are not limited to, *Balantidium coli*, *Cryptosporidium parvum*, *Cyclospora cayatanensis*, Encephalitozoa, *Entamoeba histolytica*, *Enterocytozoon bieneusi*, *Giardia lamblia*, Leishmaniae, Plasmodii, *Toxoplasma gondii*, *Trypanosomae*, trapezoidal amoeba and the like.

[90] As used herein, parasites include worms (e.g., helminthes), particularly parasitic worms including, but not limited to, Nematoda (roundworms, e.g., whipworms, hookworms, pinworms, ascarids, filarids and the like), Cestoda (e.g., tapeworms)

[91] As used herein, infectious proteins include prions. Disorders caused by prions include, but are not limited to, human disorders such as Creutzfeldt-Jakob disease (CJD) (including, e.g., iatrogenic Creutzfeldt-Jakob disease (iCJD), variant Creutzfeldt-Jakob disease (vCJD), familial Creutzfeldt-Jakob disease (fCJD), and sporadic Creutzfeldt-Jakob disease (sCJD)), Gerstmann-Sträussler-Scheinker syndrome (GSS), fatal familial insomnia (fFI), sporadic fatal insomnia (sFI), kuru, and the like, as well as disorders in animals such as scrapie (sheep and goats), bovine spongiform encephalopathy (BSE) (cattle), transmissible mink encephalopathy (TME) (mink), chronic wasting disease (CWD) (elk, mule deer), feline spongiform

encephalopathy (cats), exotic ungulate encephalopathy (EUE) (nyala, oryx, greater kudu), spongiform encephalopathy of the ostrich and the like.

[92] In certain exemplary embodiments, methods of detecting markers such as nucleic acid sequences (e.g., DNA, RNA and the like), proteins, polypeptides, lipids polysaccharides and the like in a biological sample are provided. As used herein, the term "nucleic acid" is intended to include DNA molecules (e.g., cDNA or genomic DNA) and RNA molecules (e.g., mRNA) and analogs of the DNA or RNA generated using nucleotide analogs. The nucleic acid molecule can be single-stranded or double-stranded.

[93] As used herein, the term "amino acid" includes organic compounds containing both a basic amino group and an acidic carboxyl group. Included within this term are natural amino acids (e.g., L-amino acids), modified and unusual amino acids (e.g., D-amino acids and β-amino acids), as well as amino acids which are known to occur biologically in free or combined form but usually do not occur in proteins. Natural protein occurring amino acids include alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tyrosine, tryptophan, proline, and valine. Natural non-protein amino acids include arginosuccinic acid, citrulline, cysteine sulfinic acid, 3,4-dihydroxyphenylalanine, homocysteine, homoserine, ornithine, 3-monoiodotyrosine, 3,5-diiodotryosine, 3,5,5,-triiodothyronine, and 3,3',5,5'-tetraiodothyronine. Modified or unusual amino acids include D-amino acids, hydroxylysine, 4-hydroxyproline, N-Cbz-protected amino acids, 2,4-diaminobutyric acid, homoarginine, norleucine, N-methylaminobutyric acid, naphthylalanine, phenylglycine, .alpha.-phenylproline, tert-leucine, 4-aminocyclohexylalanine, N-methyl-norleucine, 3,4-dehydroproline, N,N-dimethylaminoglycine, N-methylaminoglycine, 4-aminopiperidine-4-carboxylic acid, 6-aminocaproic acid, trans-4-(aminomethyl)-cyclohexanecarboxylic acid, 2-, 3-, and 4-(aminomethyl)-benzoic acid, 1-aminocyclopentanecarboxylic acid, 1-aminocyclopropanecarboxylic acid, and 2-benzyl-5-aminopentanoic acid.

[94] As used herein, the term "peptide" includes compounds that consist of two or more amino acids that are linked by means of a peptide bond. Peptides may have a

molecular weight of less than 10,000 Daltons, less than 5,000 Daltons, or less than 2,500 Daltons. The term "peptide" also includes compounds containing both peptide and non-peptide components, such as pseudopeptide or peptidomimetic residues or other non-amino acid components. Such compounds containing both peptide and non-peptide components may also be referred to as a "peptide analog."

[95] As used herein, the term "protein" includes compounds that consist of amino acids arranged in a linear chain and joined together by peptide bonds between the carboxyl and amino groups of adjacent amino acid residues.

[96] As used herein, the term "lipid" includes synthetic or naturally-occurring compounds which are generally amphipathic and biocompatible. Lipids typically comprise a hydrophilic component and a hydrophobic component. Exemplary lipids include, but are not limited to fatty acids, neutral fats, phosphatides, glycolipids and the like. As used herein, the term "lipid composition" refers to a composition which comprises a lipid compound, typically in an aqueous medium. Exemplary lipid compositions include, but are not limited to, suspensions, emulsions, vesicle compositions and the like.

[97] An exemplary method for detecting the presence or absence of a polypeptide or nucleic acid corresponding to a marker of the invention in a biological sample involves obtaining a biological sample (e.g., a bodily fluid sample (e.g., blood) and/or tumor sample) from a test subject and contacting the biological sample with a compound or an agent capable of detecting one or more markers (e.g., DNA, RNA, protein, polypeptide, carbohydrate, lipid or the like).

[98] Detection methods described herein can be used to detect one or more markers (e.g., DNA, RNA, protein, polypeptide, carbohydrate, lipid or the like) in a biological sample *in vitro* as well as *in vivo*. For example, *in vitro* techniques for detection of mRNA include Northern hybridizations and *in situ* hybridizations. *In vitro* techniques for detection of a polypeptide corresponding to a marker of the invention include enzyme linked immunosorbent assays (ELISAs), Western blots, immunoprecipitations and immunofluorescence. *In vitro* techniques for detection of genomic DNA include Southern hybridizations. Furthermore, *in vivo* techniques for

detection of a polypeptide corresponding to a marker of the invention include introducing into a subject a labeled antibody directed against the polypeptide. For example, the antibody can be labeled with a radioactive marker whose presence and location in a subject can be detected by standard imaging techniques.

[99] Methods for analyzing lipid content in a biological sample are known in the art (See, e.g., Kang et al. (1992) *Biochim. Biophys. Acta.* 1128:267; Weylandt et al. (1996) *Lipids* 31:977; J. Schiller et al. (1999) *Anal. Biochem.* 267:46; Kang et al. (2001) *Proc. Natl. Acad. Sci. USA* 98:4050; Schiller et al. (2004) *Prog. Lipid Res.* 43:499). An exemplary method of lipid analysis is to extract lipids from a biological sample (e.g. using chloroform:methanol (2:1, vol:vol) containing 0.005% butylated hydroxytoluene (BHT, as an antioxidant)), prepare fatty acid methyl esters were (e.g., 14% BF3-methanol reagent), and quantifying the fatty acid methyl esters are quantified (e.g., by HPLC, TLC, by gas chromatography-mass spectroscopy using commercially available gas chromatographs, mass spectrometers, and/or combination gas chromatograph/mass spectrometers). Fatty acid mass is determined by comparing areas of various analyzed fatty acids to that of a fixed concentration of internal standard.

[100] A general principle of diagnostic and prognostic assays involves preparing a sample or reaction mixture that may contain a marker (e.g., one or more of DNA, RNA, protein, polypeptide, carbohydrate, lipid and the like) and a probe under appropriate conditions and for a time sufficient to allow the marker and probe to interact and bind, thus forming a complex that can be removed and/or detected in the reaction mixture. These assays can be conducted in a variety of ways.

[101] For example, one method to conduct such an assay would involve anchoring the marker or probe onto a solid phase support, also referred to as a substrate, and detecting target marker/probe complexes anchored on the solid phase at the end of the reaction. In one embodiment of such a method, a sample from a subject, which is to be assayed for presence and/or concentration of marker, can be anchored onto a carrier or solid phase support. In another embodiment, the reverse situation is possible, in which the probe can be anchored to a solid phase and a sample from a subject can be allowed to react as an unanchored component of the assay.

[102] There are many established methods for anchoring assay components to a solid phase. These include, without limitation, marker or probe molecules which are immobilized through conjugation of biotin and streptavidin. Such biotinylated assay components can be prepared from biotin-NHS(N-hydroxy-succinimide) using techniques known in the art (e.g., biotinylation kit, Pierce Chemicals, Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). In certain embodiments, the surfaces with immobilized assay components can be prepared in advance and stored.

[103] Other suitable carriers or solid phase supports for such assays include any material capable of binding the class of molecule to which the marker or probe belongs. Well known supports or carriers include, but are not limited to, glass, polystyrene, nylon, polypropylene, nylon, polyethylene, dextran, amylases, natural and modified celluloses, polyacrylamides, gabbros, and magnetite.

[104] In order to conduct assays with the above mentioned approaches, the non-immobilized component is added to the solid phase upon which the second component is anchored. After the reaction is complete, uncomplexed components may be removed (e.g., by washing) under conditions such that any complexes formed will remain immobilized upon the solid phase. The detection of marker/probe complexes anchored to the solid phase can be accomplished in a number of methods outlined herein.

[105] In certain exemplary embodiments, the probe, when it is the unanchored assay component, can be labeled for the purpose of detection and readout of the assay, either directly or indirectly, with detectable labels discussed herein and which are well-known to one skilled in the art.

[106] It is also possible to directly detect marker/probe complex formation without further manipulation or labeling of either component (marker or probe), for example by utilizing the technique of fluorescence energy transfer (see, for example, U.S. Patent Nos. 5,631,169 and 4,868,103). A fluorophore label on the first, 'donor' molecule is selected such that, upon excitation with incident light of appropriate wavelength, its emitted fluorescent energy will be absorbed by a fluorescent label on a second

'acceptor' molecule, which in turn is able to fluoresce due to the absorbed energy. Alternately, the 'donor' protein molecule may simply utilize the natural fluorescent energy of tryptophan residues. Labels are chosen that emit different wavelengths of light, such that the 'acceptor' molecule label may be differentiated from that of the 'donor'. Since the efficiency of energy transfer between the labels is related to the distance separating the molecules, spatial relationships between the molecules can be assessed. In a situation in which binding occurs between the molecules, the fluorescent emission of the 'acceptor' molecule label in the assay should be maximal. An FET binding event can be conveniently measured through standard fluorometric detection means well known in the art (e.g., using a fluorimeter).

[107] In another embodiment, determination of the ability of a probe to recognize a marker can be accomplished without labeling either assay component (probe or marker) by utilizing a technology such as real-time Biomolecular Interaction Analysis (BIA) (see, e.g., Sjolander, S. and Urbaniczky, C., 1991, *Anal. Chem.* 63:2338 2345 and Szabo et al., 1995, *Curr. Opin. Struct. Biol.* 5:699 705). As used herein, "BIA" or "surface plasmon resonance" is a technology for studying biospecific interactions in real time, without labeling any of the interactants (e.g., BIAcore). Changes in the mass at the binding surface (indicative of a binding event) result in alterations of the refractive index of light near the surface (the optical phenomenon of surface plasmon resonance (SPR)), resulting in a detectable signal which can be used as an indication of real-time reactions between biological molecules.

[108] Alternatively, in another embodiment, analogous diagnostic and prognostic assays can be conducted with marker and probe as solutes in a liquid phase. In such an assay, the complexed marker and probe are separated from uncomplexed components by any of a number of standard techniques, including but not limited to: differential centrifugation, chromatography, electrophoresis and immunoprecipitation. In differential centrifugation, marker/probe complexes may be separated from uncomplexed assay components through a series of centrifugal steps, due to the different sedimentation equilibria of complexes based on their different sizes and densities (see, for example, Rivas and Minton (1993) *Trends Biochem. Sci.* 18:284). Standard chromatographic techniques may also be utilized to separate complexed

molecules from uncomplexed ones. For example, gel filtration chromatography separates molecules based on size, and through the utilization of an appropriate gel filtration resin in a column format, for example, the relatively larger complex may be separated from the relatively smaller uncomplexed components. Similarly, the relatively different charge properties of the marker/probe complex as compared to the uncomplexed components may be exploited to differentiate the complex from uncomplexed components, for example through the utilization of ion-exchange chromatography resins. Such resins and chromatographic techniques are well known to one skilled in the art (see, e.g., Heegaard (1998) *J. Mol. Recognit.* 11:141; Hage and Tweed (1997) *J. Chromatogr. B. Biomed. Sci. Appl.* 12:499). Gel electrophoresis may also be employed to separate complexed assay components from unbound components (see, e.g., Ausubel et al., ed., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, 1987 1999). In this technique, protein or nucleic acid complexes are separated based on size or charge, for example. In order to maintain the binding interaction during the electrophoretic process, non-denaturing gel matrix materials and conditions in the absence of reducing agent are typically preferred. Appropriate conditions to the particular assay and components thereof will be well known to one skilled in the art.

[109] In certain exemplary embodiments, the level of mRNA corresponding to the marker can be determined either by *in situ* and/or by *in vitro* formats in a biological sample using methods known in the art. Many expression detection methods use isolated RNA. For *in vitro* methods, any RNA isolation technique that does not select against the isolation of mRNA can be utilized for the purification of RNA from blood cells (see, e.g., Ausubel et al, ed., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York 1987 1999). Additionally, large numbers of cells and/or samples can readily be processed using techniques well known to those of skill in the art, such as, for example, the single-step RNA isolation process of Chomczynski (1989, U.S. Patent No. 4,843,155).

[110] Isolated mRNA can be used in hybridization or amplification assays that include, but are not limited to, Southern or Northern analyses, polymerase chain reaction analyses and probe arrays. In certain exemplary embodiments, a diagnostic method for the

detection of mRNA levels involves contacting the isolated mRNA with a nucleic acid molecule (probe) that can hybridize to the mRNA encoded by the gene being detected. The nucleic acid probe can be, for example, a full-length cDNA, or a portion thereof, such as an oligonucleotide of at least 7, 15, 30, 50, 100, 250 or 500 nucleotides in length and sufficient to specifically hybridize under stringent conditions to an mRNA or genomic DNA encoding a marker of the present invention. Other suitable probes for use in the diagnostic assays of the invention are described herein. Hybridization of an mRNA with the probe indicates that the marker in question is being expressed.

[111] In one format, the mRNA is immobilized on a solid surface and contacted with a probe, for example by running the isolated mRNA on an agarose gel and transferring the mRNA from the gel to a membrane, such as nitrocellulose. In an alternative format, the probe(s) are immobilized on a solid surface and the mRNA is contacted with the probe(s), for example, in a gene chip array. A skilled artisan can readily adapt known mRNA detection methods for use in detecting the level of mRNA encoded by the markers of the present invention.

[112] An alternative method for determining the level of mRNA corresponding to a marker of the present invention in a sample involves the process of nucleic acid amplification, e.g., by rtPCR (the experimental embodiment set forth in U.S. Patent Nos. 4,683,195 and 4,683,202), COLD-PCR (Li et al. (2008) *Nat. Med.* 14:579), ligase chain reaction (Barany, 1991, *Proc. Natl. Acad. Sci. USA*, 88:189), self sustained sequence replication (Guatelli et al., 1990, *Proc. Natl. Acad. Sci. USA* 87:1874), transcriptional amplification system (Kwoh et al. (1989) *Proc. Natl. Acad. Sci. USA* 86:1173), Q-Beta Replicase (Lizardi et al. (1988) *Bio/Technology* 6:1197), rolling circle replication (U.S. Patent No. 5,854,033) or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques well known to those of skill in the art. These detection schemes are especially useful for the detection of nucleic acid molecules if such molecules are present in very low numbers. As used herein, amplification primers are defined as being a pair of nucleic acid molecules that can anneal to 5' or 3' regions of a gene (plus and minus strands, respectively, or vice-versa) and contain a short region in between. In general,

amplification primers are from about 10 to 30 nucleotides in length and flank a region from about 50 to 200 nucleotides in length. Under appropriate conditions and with appropriate reagents, such primers permit the amplification of a nucleic acid molecule comprising the nucleotide sequence flanked by the primers.

[113] For *in situ* methods, mRNA does not need to be isolated from the sample (e.g., a bodily fluid (e.g., blood cells)) prior to detection. In such methods, a cell or tissue sample is prepared/processed using known histological methods. The sample is then immobilized on a support, typically a glass slide, and then contacted with a probe that can hybridize to mRNA that encodes the marker.

[114] As an alternative to making determinations based on the absolute expression level of the marker, determinations may be based on the normalized expression level of the marker. Expression levels are normalized by correcting the absolute expression level of a marker by comparing its expression to the expression of a gene that is not a marker, e.g., a housekeeping gene that is constitutively expressed. Suitable genes for normalization include housekeeping genes such as the actin gene, or epithelial cell-specific genes. This normalization allows the comparison of the expression level in a patient sample from one source to a patient sample from another source, e.g., to compare a phagocytic blood cell from an individual to a non-phagocytic blood cell from the individual.

[115] In another exemplary embodiment, a protein or polypeptide corresponding to a marker is detected. In certain exemplary embodiments, an agent for detecting a polypeptide of the invention is an antibody capable of binding to a polypeptide corresponding to a marker of the invention, such as an antibody with a detectable label. Antibodies can be polyclonal, or more preferably, monoclonal. An intact antibody, or a fragment thereof (e.g., Fab or $F(ab')_2$) can be used. The term "labeled," with respect to the probe or antibody, is intended to encompass direct labeling of the probe or antibody by coupling (i.e., physically linking) a detectable substance to the probe or antibody, as well as indirect labeling of the probe or antibody by reactivity with another reagent that is directly labeled. Examples of indirect labeling include detection of a primary antibody using a fluorescently labeled secondary antibody and

end-labeling of a DNA probe with biotin such that it can be detected with fluorescently labeled streptavidin.

[116] A variety of formats can be employed to determine whether a sample contains a protein that binds to a given antibody. Examples of such formats include, but are not limited to, enzyme immunoassay (EIA), radioimmunoassay (RIA), Western blot analysis, enzyme linked immunoabsorbant assay (ELISA) and the like. A skilled artisan can readily adapt known protein/antibody detection methods for use in determining whether cells (e.g., bodily fluid cells such as blood cells) express a marker of the present invention.

[117] In one format, antibodies, or antibody fragments, can be used in methods such as Western blots or immunofluorescence techniques to detect the expressed proteins. In such uses, it is generally preferable to immobilize either the antibody or proteins on a solid support. Suitable solid phase supports or carriers include any support capable of binding an antigen or an antibody. Well known supports or carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, gabbros, magnetite and the like.

[118] One skilled in the art will know many other suitable carriers for binding antibody or antigen, and will be able to adapt such support for use with the present invention. For example, protein isolated from cells (e.g., bodily fluid cells such as blood cells) can be run on a polyacrylamide gel electrophoresis and immobilized onto a solid phase support such as nitrocellulose. The support can then be washed with suitable buffers followed by treatment with the detectably labeled antibody. The solid phase support can then be washed with the buffer a second time to remove unbound antibody. The amount of bound label on the solid support can then be detected by conventional means.

[119] In certain exemplary embodiments, diagnostic assays are provided. An exemplary method for detecting the presence or absence of a bodily condition, a disease and/or disorder associated with cancer, an infectious agent, and/or another disease in a biological sample involves obtaining a biological sample from a test subject and contacting the biological sample with a compound or an agent capable of detecting

one or more of the markers of a disease and/or disorder associated with cancer, an infectious agent, and/or another disease or condition, e.g., marker nucleic acid (e.g., mRNA, genomic DNA), marker peptide (e.g., polypeptide or protein) or marker lipid encoded by the marker nucleic acid such that the presence of a marker nucleic acid or marker peptide encoded by the nucleic acid is detected in the biological sample. In one embodiment, an agent for detecting marker mRNA or genomic DNA is a labeled nucleic acid probe capable of hybridizing to marker mRNA or genomic DNA. The nucleic acid probe can be, for example, a full-length marker nucleic acid or a portion thereof. Other suitable probes for use in the diagnostic assays of the invention are described herein.

[120] An agent for detecting marker peptide can be an antibody capable of binding to a marker peptide, such as an antibody with a detectable label. Antibodies can be polyclonal or monoclonal. An intact antibody, or a fragment thereof (e.g., Fab or $F(ab')_2$) can be used. The term "labeled," with regard to the probe or antibody, is intended to encompass direct labeling of the probe or antibody by coupling (i.e., physically linking) a detectable substance to the probe or antibody, as well as indirect labeling of the probe or antibody by reactivity with another reagent that is directly labeled. Examples of indirect labeling include detection of a primary antibody using a fluorescently labeled secondary antibody and end-labeling of a DNA probe with biotin such that it can be detected with fluorescently labeled streptavidin.

[121] As used herein, the term "biological sample" is intended to include tissues, cells (e.g., phagocytic cells, non-phagocytic cells, 2n cells, >2n cells and the like) and biological fluids (e.g., whole blood, WBCs and the like) isolated from a subject, as well as tissues, cells (e.g., phagocytic cells, non-phagocytic cells, 2n cells, >2n cells and the like) and bodily fluids (e.g., urine, whole blood, WBCs and the like) present within a subject. That is, the detection method of the invention can be used to detect marker polypeptide, protein, carbohydrate, lipid, oligosaccharide, mRNA, microRNA, genomic DNA and the like in a biological sample *in vitro* as well as *in vivo*. In one embodiment, the biological sample contains proteins, polypeptides, lipids and/or oligosaccharides from the test subject. Alternatively, the biological sample can contain mRNA molecules from the test subject and/or genomic DNA molecules from

the test subject. In one embodiment biological sample is a serum sample, saliva sample or a biopsy sample isolated by conventional means from a subject.

[122] In another embodiment, the methods further involve obtaining a control biological sample from a control subject (e.g., non-phagocytic cell or 2n cell), contacting the control sample (e.g., non-phagocytic cell or 2n cell) with a compound or agent capable of detecting marker polypeptide, protein lipid, oligosaccharide, mRNA, microRNA, genomic DNA and the like is detected in the biological sample, and comparing the presence of marker polypeptide, protein lipid, oligosaccharide, mRNA, genomic DNA and the like in control sample with the presence of marker polypeptide, protein lipid, oligosaccharide, mRNA, genomic DNA and the like in the test sample (e.g., phagocytic cell or >2n cell). Alternatively, the presence of marker polypeptide, protein lipid, oligosaccharide, mRNA, genomic DNA and the like in the test sample (e.g., phagocytic cell or >2n cell) can be compared with information in a database or on a chart to result in detection or diagnosis.

[123] The invention also encompasses kits for detecting the presence of one or more markers associated with cancer and/or an infectious agent in a biological sample. For example, the kit can comprise a labeled compound or agent capable of detecting marker polypeptide, protein lipid, oligosaccharide, mRNA, microRNA, genomic DNA and the like in a biological sample; means for determining the amount of marker in the sample; and means for comparing the amount of marker in the sample with a standard (e.g., a non-phagocytic cell or a 2n cell). The compound or agent can be packaged in a suitable container. The kit can further comprise instructions for using the kit to detect marker peptide or nucleic acid.

[124] In certain exemplary embodiments, prognostic assays are provided. The diagnostic methods described herein can furthermore be utilized to identify subjects having a condition or at risk of developing a disease and/or disorder associated with cancer and/or an infectious agent, or another disorder described herein associated with upregulated (or downregulated) expression of one or more of the markers described herein. For example, the assays described herein, such as the preceding diagnostic assays or the following assays, can be utilized to identify a subject having or at risk of

developing a disease and/or disorder associated with cancer and/or an infectious agent and/or one or more other disorders described herein.

[125] The prognostic assays described herein can be used to determine whether a subject can be administered an agent (e.g., an agonist, antagonist, peptidomimetic, protein, peptide, nucleic acid, small molecule, or other drug candidate) to treat a disease and/or disorder associated with cancer and/or an infectious agent, and/or one or more other disorders described herein associated with one or more of the markers described herein. For example, such methods can be used to determine whether a subject can be effectively treated with an agent for treating, ameliorating or reducing one or more symptoms associated with cancer. Thus, the present invention provides methods for determining whether a subject can be effectively treated with an agent for a disease and/or disorder associated with cancer and/or an infectious agent, and/or one or more other disorders described herein.

[126] The methods of the invention can also be used to detect genetic alterations in a marker gene, thereby determining if a subject with the altered gene is at risk for developing a disease and/or disorder associated with cancer and/or an infectious agent, and/or one or more other disorders described herein characterized by misregulation in a marker protein activity or nucleic acid expression, such as cancer. In certain embodiments, the methods include detecting, in a sample of cells (e.g., bodily fluid cells such as blood cells) from the subject, the presence or absence of a genetic alteration characterized by an alteration affecting the integrity of a gene encoding a marker peptide and/or a marker gene. For example, such genetic alterations can be detected by ascertaining the existence of at least one of: 1) a deletion of one or more nucleotides from one or more marker genes; 2) an addition of one or more nucleotides to one or more marker genes; 3) a substitution of one or more nucleotides of one or more marker genes, 4) a chromosomal rearrangement of one or more marker genes; 5) an alteration in the level of a messenger RNA transcript of one or more marker genes; 6) aberrant modification of one or more marker genes, such as of the methylation pattern of the genomic DNA; 7) the presence of a non-wild type splicing pattern of a messenger RNA transcript of one or more marker genes; 8) a non-wild type level of a one or more marker proteins; 9) allelic loss of one or more marker genes; and 10)

inappropriate post-translational modification of one or more marker proteins. As described herein, there are a large number of assays known in the art which can be used for detecting alterations in one or more marker genes.

[127] In certain embodiments, detection of the alteration involves the use of a probe/primer in a polymerase chain reaction (PCR) (see, e.g., U.S. Patent Nos. 4,683,195, 4,683,202 and 5,854,033), such as real-time PCR, COLD-PCR, anchor PCR, recursive PCR or RACE PCR, or, alternatively, in a ligation chain reaction (LCR) (see, e.g., Landegran et al. (1988) *Science* 241:1077; Prodromou and Pearl (1992) *Protein Eng.* 5:827; and Nakazawa et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:360), the latter of which can be particularly useful for detecting point mutations in a marker gene (see Abravaya et al. (1995) *Nucleic Acids Res.* 23:675). This method can include the steps of collecting a sample of cells from a subject, isolating nucleic acid (e.g., genomic, mRNA or both) from the cells of the sample, contacting the nucleic acid sample with one or more primers which specifically hybridize to a marker gene under conditions such that hybridization and amplification of the marker gene (if present) occurs, and detecting the presence or absence of an amplification product, or detecting the size of the amplification product and comparing the length to a control sample. It is anticipated that PCR and/or LCR may be desirable to use as a preliminary amplification step in conjunction with any of the techniques used for detecting mutations described herein.

[128] Alternative amplification methods include: self sustained sequence replication (Guatelli et al., (1990) *Proc. Natl. Acad. Sci. USA* 87:1874), transcriptional amplification system (Kwoh et al., (1989) *Proc. Natl. Acad. Sci. USA* 86:1173), Q-Beta Replicase (Lizardi et al. (1988) *Bio-Technology* 6:1197), or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques well known to those of skill in the art. These detection schemes are especially useful for the detection of nucleic acid molecules if such molecules are present in very low numbers.

[129] In an alternative embodiment, mutations in one or more marker genes from a sample cell can be identified by alterations in restriction enzyme cleavage patterns. For example, sample and control DNA is isolated, optionally amplified, digested with one

or more restriction endonucleases, and fragment length sizes are determined by gel electrophoresis and compared. Differences in fragment length sizes between sample and control DNA indicates mutations in the sample DNA. Moreover, the use of sequence specific ribozymes (see, for example, U.S. Pat. No. 5,498,531) can be used to score for the presence of specific mutations by development or loss of a ribozyme cleavage site.

[130] In other embodiments, genetic mutations in one or more of the markers described herein can be identified by hybridizing a sample and control nucleic acids, e.g., DNA or RNA, to high density arrays containing hundreds or thousands of oligonucleotides probes (Cronin et al. (1996) *Human Mutation* 7: 244; Kozal et al. (1996) *Nature Medicine* 2:753). For example, genetic mutations in a marker nucleic acid can be identified in two dimensional arrays containing light-generated DNA probes as described in Cronin, M. T. et al. *supra*. Briefly, a first hybridization array of probes can be used to scan through long stretches of DNA in a sample and control to identify base changes between the sequences by making linear arrays of sequential overlapping probes. This step allows the identification of point mutations. This step is followed by a second hybridization array that allows the characterization of specific mutations by using smaller, specialized probe arrays complementary to all variants or mutations detected. Each mutation array is composed of parallel probe sets, one complementary to the wild-type gene and the other complementary to the mutant gene.

[131] In yet another embodiment, any of a variety of sequencing reactions known in the art can be used to directly sequence a marker gene and detect mutations by comparing the sequence of the sample marker gene with the corresponding wild-type (control) sequence. Examples of sequencing reactions include those based on techniques developed by Maxam and Gilbert ((1977) *Proc. Natl. Acad. Sci. USA* 74:560) or Sanger ((1977) *Proc. Natl. Acad. Sci. USA* 74:5463). It is also contemplated that any of a variety of automated sequencing procedures can be utilized when performing the diagnostic assays ((1995) *Biotechniques* 19:448), including sequencing by mass spectrometry (see, e.g., PCT International Publication No. WO 94/16101; Cohen et al.

(1996) *Adv. Chromatogr.* 36:127-162; and Griffin et al. (1993) *Appl. Biochem. Biotechnol.* 38:147).

[132] Other methods for detecting mutations in a marker gene include methods in which protection from cleavage agents is used to detect mismatched bases in RNA/RNA or RNA/DNA heteroduplexes (Myers et al. (1985) *Science* 230:1242). In general, the art technique of "mismatch cleavage" starts by providing heteroduplexes formed by hybridizing (labeled) RNA or DNA containing the wild-type marker sequence with potentially mutant RNA or DNA obtained from a tissue sample. The double-stranded duplexes are treated with an agent which cleaves single-stranded regions of the duplex such as which will exist due to base pair mismatches between the control and sample strands. For instance, RNA/DNA duplexes can be treated with RNase and DNA/DNA hybrids treated with S1 nuclease to enzymatically digesting the mismatched regions. In other embodiments, either DNA/DNA or RNA/DNA duplexes can be treated with hydroxylamine or osmium tetroxide and with piperidine in order to digest mismatched regions. After digestion of the mismatched regions, the resulting material is then separated by size on denaturing polyacrylamide gels to determine the site of mutation. See, for example, Cotton et al. (1988) *Proc. Natl. Acad. Sci.* USA 85:4397; Saleeba et al. (1992) *Methods Enzymol.* 217:286. In one embodiment, the control DNA or RNA can be labeled for detection.

[133] In still another embodiment, the mismatch cleavage reaction employs one or more proteins that recognize mismatched base pairs in double-stranded DNA (so called "DNA mismatch repair" enzymes) in defined systems for detecting and mapping point mutations in marker cDNAs obtained from samples of cells. For example, the mutY enzyme of *E. coli* cleaves A at G/A mismatches and the thymidine DNA glycosylase from HeLa cells cleaves T at G/T mismatches (Hsu et al. (1994) *Carcinogenesis* 15:1657). According to an exemplary embodiment, a probe based on a marker sequence, e.g., a wild-type marker sequence, is hybridized to a cDNA or other DNA product from a test cell(s). The duplex is treated with a DNA mismatch repair enzyme, and the cleavage products, if any, can be detected from electrophoresis protocols or the like. See, for example, U.S. Patent No. 5,459,039.

[134] In other embodiments, alterations in electrophoretic mobility will be used to identify mutations in marker genes. For example, single strand conformation polymorphism (SSCP) may be used to detect differences in electrophoretic mobility between mutant and wild type nucleic acids (Orita et al. (1989) *Proc. Natl. Acad. Sci. USA* 86:2766, see also Cotton (1993) *Mutat. Res.* 285:125; and Hayashi (1992) *Genet. Anal. Tech. Appl.* 9:73). Single-stranded DNA fragments of sample and control marker nucleic acids will be denatured and allowed to renature. The secondary structure of single-stranded nucleic acids varies according to sequence, the resulting alteration in electrophoretic mobility enables the detection of even a single base change. The DNA fragments may be labeled or detected with labeled probes. The sensitivity of the assay may be enhanced by using RNA (rather than DNA), in which the secondary structure is more sensitive to a change in sequence. In one embodiment, the subject method utilizes heteroduplex analysis to separate double stranded heteroduplex molecules on the basis of changes in electrophoretic mobility (Keen et al. (1991) *Trends Genet.* 7:5).

[135] In yet another embodiment the movement of mutant or wild-type fragments in polyacrylamide gels containing a gradient of denaturant is assayed using denaturing gradient gel electrophoresis (DGGE) (Myers et al. (1985) *Nature* 313:495). When DGGE is used as the method of analysis, DNA will be modified to insure that it does not completely denature, for example by adding a GC clamp of approximately 40 bp of high-melting GC-rich DNA by PCR. In a further embodiment, a temperature gradient is used in place of a denaturing gradient to identify differences in the mobility of control and sample DNA (Rosenbaum and Reissner (1987) *Biophys. Chem.* 265:12753).

[136] Examples of other techniques for detecting point mutations include, but are not limited to, selective oligonucleotide hybridization, selective amplification or selective primer extension. For example, oligonucleotide primers may be prepared in which the known mutation is placed centrally and then hybridized to target DNA under conditions which permit hybridization only if a perfect match is found (Saiki et al. (1986) *Nature* 324:163; Saiki et al. (1989) *Proc. Natl. Acad. Sci. USA* 86:6230). Such allele specific oligonucleotides are hybridized to PCR amplified target DNA or a

number of different mutations when the oligonucleotides are attached to the hybridizing membrane and hybridized with labeled target DNA.

[137] Alternatively, allele specific amplification technology which depends on selective PCR amplification may be used in conjunction with the instant invention. Oligonucleotides used as primers for specific amplification may carry the mutation of interest in the center of the molecule (so that amplification depends on differential hybridization) (Gibbs et al. (1989) *Nucl. Acids Res.* 17:2437) or at the extreme 3' end of one primer where, under appropriate conditions, mismatch can prevent, or reduce polymerase extension (Prossner (1993) *Tibtech* 11:238). In addition it may be desirable to introduce a novel restriction site in the region of the mutation to create cleavage-based detection (Gasparini et al. (1992) *Mol. Cell Probes* 6:1). It is anticipated that in certain embodiments amplification may also be performed using *Taq* ligase for amplification (Barany (1991) *Proc. Natl. Acad. Sci. USA* 88:189). In such cases, ligation will occur only if there is a perfect match at the 3' end of the 5' sequence making it possible to detect the presence of a known mutation at a specific site by looking for the presence or absence of amplification.

[138] It is to be understood that the embodiments of the present invention which have been described are merely illustrative of some of the applications of the principles of the present invention. Numerous modifications may be made by those skilled in the art based upon the teachings presented herein without departing from the true spirit and scope of the invention. The contents of all references, patents and published patent applications cited throughout this application are hereby incorporated by reference in their entirety for all purposes.

[139] The following examples are set forth as being representative of the present invention. These examples are not to be construed as limiting the scope of the invention as these and other equivalent embodiments will be apparent in view of the present disclosure, figures, and accompanying claims.

EXAMPLE 1

Representative Method I for the Separation of Phagocytic Cells from Non-Phagocytic Cells and the Analysis of Expression Profiles

[140] 1. With reference to Figures 2 and 3, coat plates with avidin.

[141] 2. Add biotinylated antibody to non-phagocytic blood cell (e.g., T cells) to the wells, incubate for 30 min at RT, wash wells.

[142] 3. Add magnetic beads.

[143] 4. Add WBC blood sample.

[144] 5. Incubate at 37 ^{o}C (30 minutes – 1 hour).

[145] 6. Following phagocytosis of beads by phagocytic cells and binding of avidin-biotin-antibody to non-phagocytic cells, place plate on top of magnet and wash (the phagocytic cells that internalized the magnetic beads and the non-phagocytic cells bound to the antibody will stay; all other cells will be washed away).

[146] 7. Remove magnet and collect phagocytic cells.

[147] 8. Isolate RNA from phagocytic cells (e.g., cells bound to a magnetic bead) and of non-phagocytic cells (e.g., those cells attached to the bottom of the wells via the anti-non-phagocytic cell biotinylated antibody-avidin bound), prepare cDNA, cRNA and use to differentiate genetic profiles (e.g., whole gene arrays and/or cancer gene arrays) of phagocytic and non-phagocytic cells.

[148] 9. Isolate DNA from each cell sample and identify tumor-DNA signatures selectively present in phagocytes (i.e., absent in non-phagocytes); compare the profiles (e.g., whole gene arrays, DNA mutations and/or SNPs obtained in phagocytic and non-phagocytic cells).

[149] 10. Isolate protein from each cell sample, run Western blots using antibodies to known proteins overexpressed by human tumors (e.g., PSA and PSMA in prostate cancer; CEA in colon cancer; and CA125 in ovarian cancer), and compare the profiles

obtained in phagocytic and non-phagocytic cells. Alternatively, use mass spectroscopy to identify the proteins.

[150] 11. Isolate lipids from each cell sample and compare quantity and quality, for example using HPLC.

EXAMPLE 2

Representative Method II for the Separation of Phagocytic Cells from Non-Phagocytic Cells and the Analysis of Expression Profiles

[151] 1. With reference to Figures 2 and 3, lyse RBCs in blood sample.

[152] 2. Cytospin WBC on glass slides.

[153] 3. Fix cells in acetone/methanol (-20 ^{o}C for 5 minutes).

[154] 4. Stain with hematoxylin and eosin stain and anti-T cell antibody.

[155] 5. Isolate T cells (non-phagocytic) and macrophages (phagocytic) using laser capture microscopy (LCM).

[156] 6. Isolate RNA from phagocytic cells and of non-phagocytic cells, prepare cDNA, cRNA and use to differentiate genetic profiles (e.g., whole gene arrays and/or cancer gene arrays) of phagocytic and non-phagocytic cells.

[157] 7. Isolate DNA from each cell sample, run DNA arrays, and compare the profiles (e.g., whole gene arrays, DNA mutations and/or SNPs) obtained in phagocytic and non-phagocytic cells.

[158] 8. Isolate protein from each cell sample, run Western blots using antibodies to known proteins overexpressed by human tumors (e.g., PSA and PSMA in prostate cancer; CEA in colon cancer; and CA125 in ovarian cancer), and compare the profiles obtained in phagocytic and non-phagocytic cells. Alternatively, use mass spectroscopy to identify the proteins.

[159] 9. Isolate lipids from each cell sample and compare quantity and quality, for example using HPLC.

EXAMPLE 3

Representative Method III for the Separation of Phagocytic Cells from Non-Phagocytic Cells and the Analysis of Expression Profiles

[160] 1. With reference to Figures 2 and 3, lyse RBC from a blood sample.

[161] 2. Use magnetic antibody-conjugated beads to isolate non-phagocytic (e.g., T cells) and phagocytic cells (e.g., neutrophils and/or macrophages and/or monocytes) from whole blood.

[162] 3. Isolate RNA from each cell sample, prepare cDNA, cRNA and use to differentiate genetic profiles (e.g., cancer gene array) of phagocytic and non-phagocytic cells.

[163] 4. Isolate DNA from each cell sample, run DNA arrays, and compare the profiles obtained in phagocytic and non-phagocytic cells.

[164] 5. Isolate protein from each cell sample, run Western blots using antibodies to known proteins overexpressed by human tumors (e.g., PSA and PSMA in prostate cancer; CEA in colon cancer; and CA125 in ovarian cancer), and compare the profiles obtained in phagocytic and non-phagocytic cells. Alternatively, use mass spectroscopy to identify the proteins.

[165] 6. Isolate lipids from each cell sample and compare quantity and quality, for example using HPLC.

EXAMPLE 4

Representative Method IV for the Separation of Phagocytic Cells from Non-Phagocytic Cells and the Analysis of Expression Profiles

[166] 1. With reference to Figures 2 and 3, stain WBC with fluorescent antibodies specific against a particular cell subpopulation (e.g., neutrophils, macrophages, monocytes, T cells and the like).

[167] 2. Sort the cells (e.g., by FACS).

[168] 3. Isolate RNA from each cell sample, prepare cDNA, cRNA and use to differentiate genetic profiles (e.g., cancer gene array) of phagocytic and non-phagocytic cells.

[169] 4. Isolate DNA from each cell sample, run DNA arrays, and compare the profiles obtained in phagocytic and non-phagocytic cells.

[170] 5. Isolate protein from each cell sample, run Western blots using antibodies to known proteins overexpressed by human tumors (e.g., PSA and PSMA in prostate cancer; CEA in colon cancer; and CA125 in ovarian cancer), and compare the profiles obtained in phagocytic and non-phagocytic cells. Alternatively, use mass spectroscopy to identify the proteins.

[171] 6. Isolate lipids from each cell sample and compare quantity and quality, for example using HPLC.

EXAMPLE 5

Representative Method V for the Separation of Phagocytic Cells from Non-Phagocytic Cells and the Analysis of Expression Profiles

[172] 1. With reference to Figures 5 and 6, stain WBC with fluorescent antibodies to each cell subpopulation (e.g., neutrophils, macrophages, monocytes, and T cells), and then stain with DNA dye (e.g., propidium iodide).

[173] 2. Sort the cells (FACS) into T cells, neutrophils (2n), neutrophils (>2n), macrophages (2n), macrophages (>2n), monocytes (2n), and monocytes (>2n).

[174] 3. Isolate RNA from T cells, neutrophils (>2n), macrophages (>2n), and monocytes (>2n). Then prepare cDNA, cRNA and use to differentiate genetic profiles (e.g., cancer gene array) of phagocytic and non-phagocytic cells.

[175] 4. Isolate DNA from T cells, neutrophils (>2n), macrophages (>2n), and monocytes (>2n). Run DNA arrays and compare the profiles obtained in phagocytic and non-phagocytic cells.

[176] 5. Isolate protein from T cells, neutrophils (>2n), macrophages (>2n), and monocytes (>2n). Run Western blots using antibodies to known proteins overexpressed by human tumors (e.g., PSA and PSMA in prostate cancer; CEA in colon cancer; and CA125 in ovarian cancer), and compare the profiles obtained in phagocytic and non-phagocytic cells. Alternatively, use mass spectroscopy to identify the proteins.

[177] 6. Isolate lipids from T cells, neutrophils (>2n), macrophages (>2n), and monocytes (>2n). Compare quantity and quality of lipids, for example using HPLC

EXAMPLE 6

Representative Method VI for the Separation of Phagocytic Cells and the Analysis of Expression Profiles

[178] 1. With reference to figures 5 and 6, stain WBC with fluorescent antibodies specific against one or more phagocytic cells (e.g., neutrophils, macrophages, or monocytes) and then stain with DNA-binding dye (e.g., propidium iodide).

[179] 2. Sort the cells (FACS) into 2n and >2n phagocytes.

[180] 3. Isolate RNA from each of the 2n and >2n phagocytes. Prepare cDNA, cRNA and use to differentiate genetic profiles (e.g., cancer gene array) of 2n-phagocytic and >2n-phagocytic cells.

[181] 4. Isolate DNA from each of the 2n and >2n phagocytes. Run DNA arrays and compare the profiles obtained from 2n-phagocytic and >2n-phagocytic cells.

[182] 5. Isolate protein from each of the 2n and >2n phagocytes. Run Western blots using antibodies to known proteins overexpressed by human tumors (e.g., PSA and PSMA in prostate cancer; CEA in colon cancer; and CA125 in ovarian cancer), and compare the profiles obtained from 2n-phagocytic and >2n-phagocytic cells.

[183] 6. Isolate lipids from each of the 2n and >2n phagocytes. Compare quantity and quality of lipids, for example using HPLC.

EXAMPLE 7

Detection of Tumor-Specific Gene Signatures in Phagocytes Obtained from Tumor-Bearing Mice

[184] According to embodiments of the present invention, methods are provided to differentiate between "normal non-specific noise" and "tumor-specific" and/or "disease-specific" signatures in blood or other bodily fluids. The gene-expression profiles of blood monocytes/macrophages and neutrophils from tumor-bearing mice

were compared with that of non-phagocytic T cells from the same donor mice to identify tumor-specific signatures within the phagocytic cells that were either not expressed or significantly differentially expressed in non-phagocytic cells from the same tumor-bearing mice and from non-tumor-bearing animals.

Human Prostate LNCaP Cancer Cells

[185] Athymic nude mice (n = 5) were injected subcutaneously (s.c.) with human prostate LNCaP cancer cells. Twenty-seven days later (tumor size = ~0.4 cm), the mice were bled by cardiac puncture (~1 mL/mouse) into EDTA-containing tubes that were then centrifuged. The buffy coat was isolated and washed, and neutrophils, macrophages, and T cells were separated using, respectively, anti-mouse neutrophil-, macrophage-, and T cell-immunomagnetic DynaBeads. RNA was isolated from each cell sample (Triazol®). The RNA quality was determined as shown in Figure 3. The RNA yield is shown in Figure 20. cDNA and biotinylated cRNA (cRNA-B) were prepared. Finally, the cRNA-B samples were incubated with cancer-gene human microarrays (Oligo GEArray® Human Cancer PathwayFinder Microarray – OHS-033 – SuperArray Bioscience). Following hybridization, the membranes were washed and stained with avidin–alkaline phosphatase, and the genes were detected using chemiluminescence (X-ray film).

Human LS174T Colon Adenocarcinoma Tumors, LLC1 Carcinoma Cells, B16F10 Mouse Melanoma Cells

[186] Similar experiments were carried out with cells isolated from athymic nude mice (n = 5) injected s.c. with human LS174T colon adenocarcinoma tumors (tumor size = ~0.3 cm), C57Bl mice (n = 5) injected s.c. with Lewis lung mouse LLC1 carcinoma cells (tumor size = ~0.6 cm), and C57Bl mice (n = 5) injected intravenously 22 days earlier with 10^6 B16F10 mouse melanoma cells (when the tumor cells were of mouse origin, the cRNA-B samples were hybridized with the Oligo GEArray® Mouse Cancer PathwayFinder Microarray – OMM-033 – SuperArray Bioscience). RNA was also isolated from exponentially growing LS174T, LLC1, B16F10, and LNCaP cells in culture and from neutrophils, macrophages and T cells isolated from non-tumor-bearing C57Bl and nude mice, and their cancer-related gene profiles were determined.

[187] According to the data obtained from these experiments and shown in Figures 9-17, neutrophils and macrophages – obtained from mice injected with human prostate or colon tumor cells and from mice bearing mouse lung cancer or melanoma – have various cancer-related gene signatures that are also found in their respective tumor cells. These cancer-related genes were not expressed or were minimally expressed by (i) non-phagocytic T cells isolated from tumor-bearing mice; and (ii) phagocytic neutrophils and macrophages obtained from non-tumor-bearing mice.

[188] For example, neutrophils isolated from the blood of nude mice bearing LNCaP human prostate cancer cells expressed several human tumor gene signatures (Human Cancer PathwayFinder Microarray) that were also expressed in LNCaP cells (compare profiles of arrays in Figures 9A and 9B). These genes were either not expressed or minimally expressed in T cells obtained from tumor-bearing mice or neutrophils isolated from normal mice (see profiles in Figures 9C and 9D). Similarly, neutrophils isolated from the blood of mice bearing LLC1 mouse lung cancer cells expressed several mouse tumor gene signatures (Mouse Cancer PathwayFinder Microarray) that were expressed in LLC1 cells (compare profiles of arrays in Figures 13A and 13B).

[189] These genes were either not expressed or minimally expressed in T cells obtained from tumor-bearing mice or neutrophils isolated from normal mice (see profiles shown in Figures 13C and 13D). Finally, the arrays were scanned, the intensity of each gene quantified using the software provided by the company, and those genes overexpressed selectively by phagocytic cells identified as shown in Figures 19 and 20. Figures 21 and 22 list the gene signatures acquired and differentially exhibited by the phagocytic WBCs of tumor-bearing mice. As shown in Figure 21, many oncogenes (genes depicted in red, e.g., *ERBB2* and *Jun*) were detected and often they were expressed simultaneously in macrophages and neutrophils.

[190] C57BI mice (n = 5) were injected subcutaneously with 1E6 Lewis lung mouse carcinoma cells (LLCI). Twenty days later, the mice were anesthetized and bled by cardiac puncture (approximately 1 mL/mouse) into an EDTA-containing tube. Following centrifugation at 2,000 rpm for 5 minutes at room temperature, the buffy coat was transferred to a tube and washed with PBS.

[191] Anti-mouse macrophage/monocyte rat IgG antibodies (monocyte/macrophage marker - F4/80 - IgG2b from AbD Serotec, Raleigh, NC) were incubated (room temperature for 30 minutes) with anti-rat IgG antibody magnetic beads (DYNABEAD® sheep anti-rat IgG from INVITROGEN™, Carlsbad, CA). The anti-macrophage/monocyte beads were then washed in PBS and stored on ice.

[192] Anti-mouse neutrophil rat IgG (Neutrophil Marker NIMP-R14 - IgG2a - Santa Cruz Biotechnology, Santa Cruz, CA) was incubated (room temperature for 30 minutes) with anti-rat IgG antibody magnetic beads (DYNABEAD® sheep anti-rat IgG - INVITROGEN™), washed in PBS, and stored on ice.

[193] DYNABEAD® mouse Pan T (Thy1.2) beads (INVITROGEN™) were also washed in PBS and stored on ice.

[194] Mouse blood macrophages and monocytes were isolated from the WBC suspension prepared above using the anti-macrophage/monocyte beads. In essence, the beads were added to the WBC sample and following their incubation (4 °C for 30 minutes), the macrophage-bound beads were isolated using a magnet and washed with PBS three times and stored on ice.

[195] Mouse T cells were then isolated from the remaining WBC. Briefly, the anti-mouse T cell beads were added to the WBC suspension, the samples incubated (4 °C for 30 minutes), the T cell-bound beads were isolated using a magnet, washed with PBS, and stored on ice.

[196] Finally, mouse neutrophils were isolated from the remaining WBC sample. The anti-mouse neutrophil magnetic beads were added to the cells and the samples were incubated (4 °C for 30 minutes). The neutrophil-bound beads were isolated using a magnet, washed with PBS, and stored on ice.

[197] RNA was then isolated from each sample (using TRIZOL®, INVITROGEN™, Carlsbad, CA). The RNA quality was determined as shown in Figure 7. The RNA yield is shown in Figure 8. Next, cDNA (biotinylated) were prepared and incubated (60°C overnight) with cancer-gene human microarrays (OLIGO GEARRAY® Human Cancer PathwayFinder Microarray OMM-033, SuperArray Bioscience, Frederick,

MD). Following hybridization, the membranes were washed and stained with avidin-alkaline phosphatase and the genes detected using chemiluminescence (X-ray film).

Human LS175T Colon Adenocarcinoma Tumors, LLC1 Carcinoma and B16F10 Mouse Melanoma Cells

[198] Similar experiments were carried out with cells isolated from athymic nude mice (n = 5) injected s.c. with human LS174T colon adenocarcinoma tumors (tumor size = ~0.3 cm), C57/Bl mice (n = 5) injected s.c. with Lewis lung mouse LLC1 carcinoma cells (tumor size = ~0.6 cm), and C57Bl mice (n = 5) injected intravenously 22 days earlier with 10^6 B16F10 mouse melanoma cells (when the tumor cells were of mouse origin, the cRNA-B samples were hybridized with the Oligo GEARRAY® Mouse Cancer PathwayFinder Microarray – OMM-033 – SuperArray Bioscience (when the tumors were of human origin, the Oligo GEArray® Human Cancer PathwayFinder Microarray – OHS-033 – was used). RNA was also isolated from exponentially growing LS174T, LLC1, B16F10, and LNCaP cells in culture and from neutrophils, macrophages and T cells isolated from non-tumor-bearing C57Bl and nude mice, and their cancer-related gene profiles were determined.

[199] According to the data obtained from these experiments and shown in Figures 9A-9D, 10A-10D, 11A-11D, 12A-12D, 13A-13D, 14A-14D, 15A-15D, 16A-16D, neutrophils and macrophages (obtained from mice injected with human prostate or colon tumor cells and from mice bearing mouse lung cancer or melanoma) had various cancer-related gene signatures that were also found in their respective tumor cells (Figure 21). These cancer-related genes were not expressed or were minimally expressed by (i) non-phagocytic T cells isolated from tumor-bearing mice; and (ii) phagocytic neutrophils and macrophages obtained from non-tumor-bearing mice.

[200] For example, neutrophils isolated from the blood of nude mice bearing LNCaP human prostate cancer cells expressed seven human tumor gene signatures (Human Cancer PathwayFinder Microarray) that were also expressed in LNCaP cells (compare profiles of arrays in Figures 9A and 9B). These genes were either not expressed or minimally expressed in T cells obtained from tumor-bearing mice or neutrophils isolated from normal mice (see profiles in Figures 9C and 9D). Finally, the arrays

were scanned, the intensity of each gene quantified using the software provided by the company, and those genes overexpressed selectively by phagocytic cells identified as shown in Figures 19 and 20. Figures 21 and 22 list the gene signatures acquired and differentially exhibited by the phagocytic WBCs of tumor-bearing mice. As shown in Figure 21, many oncogenes (e.g., *ERBB2* and *Jun*) were detected and often they were expressed simultaneously in macrophages and neutrophils (shown by the genes highlighted in green).

EXAMPLE 8

Detection of Tumor-Specific Gene Signatures in Phagocytes Obtained from Cancer Patients

[201] According to certain embodiments of the present invention, the gene-expression profiles of blood monocytes/macrophages and neutrophils from cancer patients were compared with that of non-phagocytic T cells from the same donor individuals to identify tumor-specific signatures within the phagocytic cells that were either not expressed or significantly differentially expressed in non-phagocytic cells.

Patients with Head and Neck Tumors

[202] Ten milliliters of venous blood was obtained (into an EDTA-containing tube) from patients known to have squamous cell carcinoma of the neck and scheduled for surgery. Following centrifugation at 2,000 rpm for 5 minutes at room temperature, the buffy coat was transferred to a tube and washed with PBS.

[203] The cells were separated employing T cell-, neutrophil-, and macrophage/monocyte-rat anti-human immunomagnetic DynaBeads® from INVITROGEN™, Carlsbad, CA. In essence, the beads were added consecutively to the WBC sample and following individual 4 °C, 30 minute incubations, the T cells-, neutrophils-, and macrophages/monocytes-bound beads were isolated using a magnet and washed with PBS three times.

[204] RNA was then isolated from each sample (using TRIZOL®, INVITROGEN™, Carlsbad, CA). The RNA quantity and quality was determined and cDNA and biotinylated cRNA (cRNA-B) were prepared. Finally, the cRNA-B samples were incubated (60°C overnight) with cancer-gene human microarrays (Oligo GEArray®

Human Cancer PathwayFinder Microarray – OHS-033 – SuperArray Bioscience, Frederick, MD). Following hybridization, the membranes were washed and stained with avidin–alkaline phosphatase, and the genes were detected using chemiluminescence (X-ray film).

[205] According to the data obtained from these experiments, neutrophils and macrophages (obtained from head and neck cancer patients) had various cancer-related gene signatures that were also found in their respective tumor cells. These cancer-related genes were not expressed or were minimally expressed by non-phagocytic T cells.

[206] For example, neutrophils isolated from the blood of one such patient expressed four human tumor gene signatures (Human Cancer PathwayFinder Microarray) that were also expressed in the tumor biopsy obtained from the same patient (compare profiles of arrays in **Figures 17B and 17C**). These genes were either not expressed or minimally expressed in normal skin biopsy and in T cells isolated from the same blood sample (see profiles in **Figures 17A and 17D**, respectively). Finally, the arrays were scanned, the intensity of each gene quantified using the software provided by the company, and the following genes that were overexpressed (>2-fold) selectively by phagocytic cells were identified: E26 viral oncogene homolog (ETS2), HIV-1 Tat interactive protein (HTAT1P2), IL8 (neutrophil activation and chemotaxis), Jun oncogene (JUN), and matrix metalloproteinase 9 (MMP9).

Ovarian Cancer Patients

[207] Similar experiments were carried out with cells isolated from a patient with ovarian cancer. According to the data obtained from these experiments, neutrophils and macrophages (obtained from the diseased woman) expressed many cancer-related genes that were not expressed or were minimally expressed by non-phagocytic T cells.

[208] For example, macrophages isolated from the blood of the ovarian cancer patient expressed 23 human tumor gene signatures (Human Cancer PathwayFinder Microarray) that were either not expressed or minimally expressed in T cells isolated from the same blood sample (compare profiles in Figures 18A and 18B). Finally, the arrays were scanned, the intensity of each gene quantified using the software provided

by the company, and the intensities of each cancer-related gene in each cell type determined. The list of 23 cancer-related genes differentially upregulated/overexpressed in macrophages as well as the macrophage-to-T cell intensity ratios are both shown in Figure 22. Note that a total of five oncogenes were detected (shown in red in Figure 21).

EXAMPLE 9

Detection of Tumor-Specific Protein Signatures in Phagocytes Obtained from Mice Bearing Human Prostate LNCaP Tumors and Human Colon LS174T Tumors

[209] A protein purification kit (Norgen, Incorporated, Product # 23500) was used to isolate and purify proteins from mouse WBCs, T cells, and macrophages. The assay was very simple and fast (approximately 30 minutes) and the isolated proteins, which were of high quality and excellent yield (117.6 ± 10.60 μg per 4 mL blood, $n = 5$), could be used in a number of downstream applications, such as SDS-PAGE analysis as shown in Figure 23 and Western blots.

[210] Protein samples were isolated from phagocytic (monocytes/macrophages) and non-phagocytic (T-lymphocytes) cells obtained from mice bearing LNCaP and LS174T tumors were selected for these studies since the former cell line expresses PSA (Denmeade et al. (2001) *Prostate* 48:1; Lin et al. (2001) *J. Urol.* 166:1943) and the latter exhibits a tumor-specific glycoprotein (TAG-72), a high molecular weight mucin (Colcher et al. (1981) *Proc. Natl. Acad. Sci. USA* 78:3199); Colcher et al. (1984) *Cancer Res.* 44:5744; Kassis et al. (1996) *J. Nucl. Med.* 37:343. Western blot analysis was carried out with 16 μg of the purified protein samples. In essence, each sample was mixed with two volumes of SDS loading buffer and run on 10% SDS-PAGE along with unstained precision plus protein standards (Biorad) in Tris-glycine-SDS buffer (pH 8.4) at 200 volts. The proteins were transferred to a nitrocellulose membrane (overnight at 4°C) using a Mini Trans-Blot (Biorad) apparatus and a transfer buffer containing 25 mM Tris, pH 8.4, 192 mM glycine, and 20% methanol. The membrane was blocked with 5% nonfat dry milk (60 min at room temperature (RT)) and incubated (1 hour, RT) with either B72.3, a mouse monoclonal antibody against human TAG-72, or ER-PR8, a mouse monoclonal antibody against human

PSA. The blots were washed and then incubated with Immun-Star Goat Anti Mouse–HRP conjugate (Biorad), a secondary antibody specific to mouse IgG, and developed by incubation (5 min, RT) with a 1:1 mixture of luminol solution and peroxide buffer (Biorad), followed by autoradiography.

[211] The data clearly indicated that phagocytic cells from LNCaP tumor-bearing mice were positive for PSA, whereas this protein could not be detected in non-phagocytic T cells from the same animals as shown in Figure 24. Similarly, TAG-72 was expressed by monocytes/macrophages obtained from LS174T tumor-bearing mice and was completely absent in T cells from the same animals. These findings demonstrate the "acquisition" and expression of tumor-specific protein signatures by phagocytic cells.

[212] While these data are specific to animals with cancer and phagocytic and non-phagocytic cells obtained from the blood of mice, the described methods are also useful in humans and in the diagnosis and/or detection of one or more other disorders and/or diseases and with phagocytic and non-phagocytic cells obtained from other bodily fluids.

EXAMPLE 10

Profiling Experiments

Isolation of Blood Phagocytic Cells

[213] A sample of blood is obtained from a patient. The blood (~5 mL) will be transferred to a 50-mL tube containing 50 µL 0.5 M EDTA (final EDTA concentration = ~4.8 mM). The tube will be vortexed gently and 25 mL RBC Lysis Buffer (Norgen, Incorporated) will be added. The tube will be vortexed gently again, incubated at room temperature until the color of the solution changes to bright red (3–5 min), and centrifuged at 2,000 rpm for 3 min. Following careful aspiration of the supernatant, the WBCs will be washed with 40 mL Ca/Mg-free 0.1 M PBS (containing 2% FBS, 2 mM EDTA, and 20 mM glucose), and the cells (10^6/mL) will then be incubated (30 min, 4 °C, in the dark) with a cell-staining solution containing (i) the DNA, viable cell-permeable stain Hoechst 33342 (4 µg/mL; Em = 483 nm), (ii) the anti-human monocytes/macrophages monoclonal antibody (Alexa Fluor® 647-conjugate; Em =

668 nm), which recognizes the human F4/80 antigen expressed by circulating monocytes/macrophages, and (iii) the anti-human neutrophil monoclonal antibody (RPE-conjugate; Em = 578 nm), which recognizes human circulating neutrophils. The cells will then be washed and sorted (BD FACSAria) into neutrophils ($N_{n=2}$), neutrophils ($N_{n>2}$), monocytes/macrophages ($M/M_{n=2}$), and monocytes/macrophages ($M/M_{n>2}$).

Gene Profiling

[214] Human whole-genome gene profiling will be performed. For RNA samples obtained from human tumor cells or neutrophils ($N_{n=2}$, $N_{n>2}$) and monocytes/macrophages ($M/M_{n=2}$, $M/M_{n>2}$), the GeneChip® Human Genome U133 Plus 2.0 Array by Affymetrix, Incorporated will be used. This array analyzes the expression level of over 47,000 transcripts and variants, including 38,500 well-characterized human genes. In general, the extracted RNA will be used to determine the expression profiles of human genes using the above-mentioned array. To ensure array reproducibility, each sample will be profiled in triplicate and the experiment repeated once. The microarray data will be filtered for cancer-induction-related genes as described below and validated using quantitative real-time, reverse transcriptase, polymerase chain reaction (RT-PCR).

Upregulation/Downregulation of Cancer-Induction-Related Genes

[215] RNA will be isolated using Triazol (Invitrogen, Incorporated) and purified using the cartridges provided in the kit. The RNA quality and quantity will be assessed with the Bioanalyzer 2100 (Agilent Technologies, Incorporated, Palo Alto, CA) and Degradometer software version 1.41 (Worldwide Web: dnaarrays.org). These experimental results will help in distinguishing the molecular pathways perturbed consequent to the presence of tumors.

Analysis of Microarray Experiments

[216] The analysis of the large scale/high throughput molecular expression data generated will rely heavily on the ability to (i) identify genes differentially expressed in phagocytic cells with a DNA content >2, (ii) annotate the identified genes, and (iii)

assign the annotated genes to those specifically expressed by a specific tumors. Statistical analysis of the microarray data can be done, for example, using the dChip package which easily accommodates this type of gene list construction in its "Analysis/Compare Samples" menu. When using Affymetrix GeneChips, one or more Gene Chips and associated methods will be applied to ascertain the quality of the raw microarray data (Gautier et al. (2004) *Bioinformatics* **20**:307). Furthermore, various background correction and normalization procedures will be utilized to arrive at an optimal protocol for normalization and summarization of the probe sets (to produce expression values) (Huber et al. (2002) *Bioinformatics* **18(Suppl. 1)**:S96; Wu et al. (2004) *Journal of the American Statistical Association* **99**:909; Seo and Hoffman (2006) *BioMed Central Bioinformatics* **7**:395). In a two-step filtration approach, we will compare the gene profiles of $P_{n=2}$ to those of $P_{n>2}$ and construct a list of expressed genes and then compare these genes to the tumor-specific genes identified for each tumor cell line – post filtration of $P_{n=2}$ gene profile as shown in Figure 5. For example, (i) blood will be obtained from breast cancer patients; (ii) neutrophils (n>2 and n=2) will be isolated and their gene profiles determined in triplicate; (iii) the mean (from the 3 samples) of each identified gene and its respective standard error (SE) will be calculated for each group ($N_{n>2}$ and $N_{n=2}$); (iv) the gene expression profiles of the two groups will then be compared and a list (L-1) of expressed genes identified on the basis of an *absolute* ≥2-fold log change ($N_{n>2}/N_{n=2}$), according to the Welch modified two-sample t-test; (v) the gene expression profiles of $N_{n=2}$ and that of breast cancer (obtained from tumor and normal breast tissue biopsies) will be compared and a list (L-2) of expressed genes identified; and (vi) breast-cancer-specific gene signatures that have been acquired/expressed by $N_{n>2}$ will be identified by comparing the genes in L-1 and L-2 ("Analysis/Compare Samples/Combine Comparisons," dChip) and filtering common genes.

Protein Profiling

[217] Fifty to one hundred micrograms of the total protein from each type of cells will be denatured and reduced with tris-(2-carboxyethyl)phosphinetrypsin (1 mM) and 0.02% sodium dodecyl sulfate at 60 °C for 1 hour. Cysteines are subsequently blocked and total protein is digested with trypsin at 37 °C for 12–16 hours. The resulting peptides

will be iTRAQ-labeled (with tags 113-119 and 121) for 1 hour (4-plex or 8-plex depending on the number of cell types to be compared). Following labeling, the separately tagged samples are combined and injected into an Agilent 1200 Series HPLC system equipped with a strong cation exchange column (Applied Biosystems 4.6 x 100 Porous). The 96 collected fractions are then pooled into 14 fractions, and each fraction is injected into the LC Packings Ultimate HPLC System for a second round of fractionation under reverse-phase conditions (LC Packings 15 cm x 75 μm analytical column). The reverse-phase fractions are spotted directly onto the target plate using an LC Packings Probot and are analyzed with mass spectrometry (Applied Biosystems 4800 Plus Proteomics Analyzer). Following data acquisition, the spectra are processed using the ProteinPilot software package (Applied Biosystems MDS Sciex), and the individual proteins in each of the cell types with their relative expression levels are identified using the ProteinPilot™ software (the analysis and identification of cancer-associated proteomic signatures will be similar to that outlined in Figure 5 for the genomic signatures).

What is claimed:

1. An in vitro method for diagnosing the presence of a cancer cell in an individual comprising the steps of:

comparing a first expression profile from blood phagocytic cells having a DNA content of 2n from an individual and a second expression profile from blood phagocytic cells having a DNA content of greater than 2n of the same individual;

identifying differential expression of one or more markers specific to the first expression profile; and

relating the differential expression of the one or more markers specific to the first expression profile to the presence of a cancer cell in the individual.

2. The method of claim 1, wherein the one or more markers are selected from the group consisting of DNA, RNA, protein, and combinations thereof.

3. The method of claim 1, wherein the one or more markers are selected from the group consisting of DNA, RNA, microRNA and combinations thereof corresponding to two or more cancer genes, oncogenes, tumor suppressor genes or combinations thereof.

4. The method of claim 1, wherein the one or more markers are proteins or polypeptides encoded by two or more cancer genes, oncogenes, tumor suppressor genes or any combination thereof.

5. The method of claim 1, wherein the blood phagocytic cell is selected from the group consisting of a neutrophil, a macrophage, a monocyte, a dendritic cell, a foam cell and any combination thereof.

6. The method of claim 1, wherein the blood phagocytic cell is isolated from whole blood, urine, stool, saliva, lymph or cerebrospinal fluid.

7. The method of claim 6, wherein the blood phagocytic cell is isolated using antibodies.

8. The method of claim 6, wherein the blood phagocytic cell is separated using one or more methods selected from the group consisting of fluorescence activated cell sorting, filtration, gradient-based centrifugation, elution and microfluidics.

9. The method of claim 6, wherein the blood phagocytic cell is separated using a ligand that binds to a molecular receptor expressed on the plasma membranes of WBC populations.