A CELL MEDIATED IMMUNE RESPONSE ASSAY WITH ENHANCED SENSITIVITY

FILING DATA

[0001] This application is associated with and claims priority from United States Provisional Patent Application No. 61/502,811, filed on 29 June 2011, entitled "A cell mediated immune response assay with enhanced sensitivity", the entire contents of which, are incorporated herein by reference.

FIELD

[0002] This disclosure relates generally to the field of immunological-based diagnostic assays including an assay to measure cell-mediated immunoresponsiveness. The present disclosure teaches diagnosis of a subject's exposure to an antigen based on cell-mediated immunoresponsiveness with enhanced sensitivity. The assay contemplated herein is capable of integration into standard pathology architecture to provide a diagnostic reporting system and to facilitate point of care clinical management.

BACKGROUND

[0003] Bibliographic details of the publications referred to by author in this specification are collected alphabetically at the end of the description.

[0004] Reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in any country.

[0005] Immunological-based diagnostic assays are important tools in detecting a variety of disease conditions. The effectiveness of these types of assays lies in part in the specificity

of components within the immune system. Notwithstanding this specificity, immunological-based diagnostics are not necessarily always sensitive enough to detect low grade infection or the presence of a persistent low level infection or in subjects with active or latent infectious disease states. There is a need to develop diagnostic assays with enhanced sensitivity in relation to cell-mediated immunoresponsiveness.

[0006] One form of immunological-based diagnostic assay involves the stimulation of T-cells within antigens or mitogens in either isolated cell culture or in whole blood culture followed by the detection of effector molecules such as cytokines produced by the stimulated T-cells (also referred to as effector T-cells). The effector molecules are generally detected using techniques such as enzyme immunoassays, multiplex bead analysis, ELISpot and flow cytometry. Such assays are useful for detecting disease-specific T-cell responses. An example of a T-cell assay is QuantiFERON (Registered Trademark; Cellestis Limited). Another assay employs 15mer peptide antigens to stimulate T-cells. However, peptides of this length, whilst capable of being detected by $CD4^+$ T-cells, are too long to be detected by $CD8^+$ T-cells.

[0007] The ability to quickly assess cell-mediated immunity and with a high degree of sensitivity is of clinical importance. This is particularly the case with immune system compromised patients. A clinician needs to have an appreciation of the development of a disease state and its effect on the host's immune system.

[0008] There is a need, however, to improve the sensitivity of assays of cell-mediated immunoresponsiveness in a subject.

SUMMARY

[0009] Enabled herein is a method for detecting a cell-mediated immune response in a subject, the method comprising incubating lymphocytes from the subject with peptides derived from a protein antigen, the peptides comprising a combination of a set of peptides each about 7 to 14 amino acids in length and a set of peptides greater than 15 amino acids in length which encompasses all or part of the protein antigen, and then screening for levels of effector molecules produced by activated lymphocytes.

[0010] By "about 7 to 14 amino acids" means 7, 8, 9, 10, 11, 12, 13 or 14 amino acids. This is considered herein a first set of peptides. By "greater than 15 amino acids" means from 16 to the entire length of the protein antigen including from 16 to 50 amino acids. This is considered a second set of peptides. The present method is not to be limited to which set of peptides is referred to as first or second. Each set comprises from at least one peptide to a series of over lapping peptides.

[0011] The co-incubation of the 7 to 14 amino acid peptides and the greater than 15 amino acid peptides derived from the protein antigen with the lymphocytes results in a more sensitive assay, enabling earlier detection of lymphocyte stimulation than would otherwise be possible. The increased sensitivity includes at least a 10% increased detection of effector molecules compared to co-incubation with a single peptide in the 7 to 14 amino acid range or >15 amino acid range derived from the antigen or the whole antigen itself. The ability to increase the sensitivity of a cell-mediated immune response assay also enables less sensitive means of detection of effector molecules. Furthermore, the magnitude of the cell-mediated immune response detected in the assay presently disclosed can be correlated to the disease state, progression and/or severity. Hence, the present disclosure teaches an assay of a cell-mediate immunoresponsiveness in a subject.

[0012] Without limiting the present invention to any one theory or mode of action, the two sets of peptides, the 7 to 14mer peptides and >15mer peptides enables detection by both $CD4^+$ and $CD8^+$ T-cells. The $CD4^+$ T-cells recognize the >15 mer peptides and $CD8^+$ T-

cells recognize the 7 to 14 mer peptides. These peptides may be referred to herein as "$CD4^+$ peptides" (>15 mer peptides) or "$CD8^+$ peptides" (7 to 14 mer peptides).

[0013] A method for measuring cell-mediated immune response activity in a subject is therefore provided herein, the method comprising contacting lymphocytes from the subject with at least two sets of peptides, a first set comprising one or more peptides of from about 7 to 14 amino acid residues in length and a second set comprising one or more peptides of from 16 amino acids or greater which encompass all or part of a protein antigen and measuring the presence or elevation in the level of an immune effector molecule from immune cells wherein the presence or level of the immune effector molecule is indicative of the level of cell-mediated responsiveness of the subject to the antigen.

[0014] Usefully, the subject is a human and the sample is undiluted whole blood. Alternatively, the sample is whole blood which comprises from about 10% to 100% by volume of the sample to be assayed or comprises from about 50% to 100% by volume of the sample to be assayed or comprises from about 80% to 100% by volume of the sample to be assayed. The sample volume may be in microliter or milliliter amounts such as from 0.5µl to 5ml. Conveniently, the whole blood is collected in a tube comprising heparin and the immune effector molecule is IFN-γ. Generally, the immune effectors are detected with antibodies specific for same such as using ELISA or an ELISpot.

[0015] The subject may have an infection by a pathogenic agent selected from *Mycobacterium* species such as *Mycobacterium tuberculosis* or tuberculosis (TB), *Staphylococcus* species, *Streptococcus* species, *Borrelia* species, *Escherichia coli*, *Salmonella* species, *Clostridium* species, *Shigella* species, *Proteus* species, *Bacillus* species, Herpes virus, Hepatitis B or C virus and Human immune deficiency virus (HIV) or a disease resulting therefrom.

[0016] The subject may alternatively have a disease condition selected from Celiac's disease, autoimmune diabetes, alopecia areata, ankylosing spondylitis, antiphospholipid syndrome, autoimmune Addison's disease multiple sclerosis, autoimmune disease of the

adrenal gland, autoimmune hemolytic anemia, autoimmune hepatitis, autoimmune oophoritis and orchitis, Behcet's disease, bullous pemphigoid, cardiomyopathy, celiac sprue-dermatitis, chronic fatigue syndrome (CFIDS), chronic inflammatory demyelinating, chronic inflammatory polyneuropathy, Churg-Strauss syndrome, cicatricial pemphigoid, crest syndrome, cold agglutinin disease, Crohn's disease, dermatitis herpetiformis, discoid lupus, essential mixed cryoglobulinemia, fibromyalgia, glomerulonephritis, Grave's disease, Guillain-Barre, Hashimoto's thyroiditis, idiopathic pulmonary fibrosis, idiopathic thrombocytopenia purpura (ITP), IgA nephropathy, insulin dependent diabetes (Type I), lichen planus, lupus, Meniere's disease, mixed connective tissue disease, multiple sclerosis, myasthenia gravis, myocarditis, pemphigus vulgaris, pernicious anemia, polyarteritis nodosa, polychondritis, polyglancular syndromes, polymyalgia rheumatica, polymyositis and dermatomyositis, primary agammaglobulinemia, primary biliary cirrhosis, psoriasis, Raynaud's phenomenon, Reiter's syndrome, rheumatic fever, rheumatoid arrthritis, sarcoidosis, scleroderma, Sjogren's syndrome, stiff-man syndrome, systemic lupus erythematosus, Takayasu arteritis, temporal arteritis/giant cell arteritis, ulcerative colitis, uveitis, vasculitis, vitiligo and inflammatory bowel disease.

[0017] The subject may alternatively have a cancer selected from ABL1 protooncogene, AIDS related cancers, acoustic neuroma, acute lymphocytic leukaemia, acute myeloid leukaemia, adenocystic carcinoma, adrenocortical cancer, agnogenic myeloid metaplasia, alopecia, alveolar soft-part sarcoma, anal cancer, angiosarcoma, aplastic anaemia, astrocytoma, ataxia-telangiectasia, basal cell carcinoma (skin), bladder cancer, bone cancers, bowel cancer, brain stem glioma, brain and CNS tumors, breast cancer, CNS tumors, carcinoid tumors, cervical cancer, childhood brain tumors, childhood cancer, childhood leukaemia, childhood soft tissue sarcoma, chondrosarcoma, choriocarcinoma, chronic lymphocytic leukaemia, chronic myeloid leukaemia, colorectal cancers, cutaneous T-Cell lymphoma, dermatofibrosarcoma-protuberans, desmoplastic-small-round-cell-tumor, ductal carcinoma, endocrine cancers, endometrial cancer, ependymoma, esophageal cancer, Ewing's sarcoma, extra-hepatic bile duct cancer, eye cancer, eye: melanoma, retinoblastoma, fallopian tube cancer, fanconi anemia, fibrosarcoma, gall bladder cancer, gastric cancer, gastrointestinal cancers, gastrointestinal-carcinoid-tumor, genitourinary

cancers, germ cell tumors, gestational-trophoblastic-disease, glioma, gynaecological cancers, hematological malignancies, hairy cell leukaemia, head and neck cancer, hepatocellular cancer, hereditary breast cancer, histiocytosis, Hodgkin's disease, human papillomavirus, hydatidiform mole, hypercalcemia, hypopharynx cancer, intraocular melanoma, islet cell cancer, Kaposi's sarcoma, kidney cancer, Langerhan's-cell-histiocytosis, laryngeal cancer, leiomyosarcoma, leukemia, Li-Fraumeni syndrome, lip cancer, liposarcoma, liver cancer, lung cancer, lymphedema, lymphoma, Hodgkin's lymphoma, non-Hodgkin's lymphoma, male breast cancer, malignant-rhabdoid-tumor-of-kidney, medulloblastoma, melanoma, merkel cell cancer, mesothelioma, metastatic cancer, mouth cancer, multiple endocrine neoplasia, mycosis fungoides, myelodysplastic syndromes, myeloma, myeloproliferative disorders, nasal cancer, nasopharyngeal cancer, nephroblastoma, neuroblastoma, neurofibromatosis, nijmegen breakage syndrome, non-melanoma skin cancer, non-small-cell-lung-cancer-(NSCLC), ocular cancers, oesophageal cancer, oral cavity cancer, oropharynx cancer, osteosarcoma, ostomy ovarian cancer, pancreas cancer, paranasal cancer, parathyroid cancer, parotid gland cancer, penile cancer, peripheral-neuroectodermal-tumors, pituitary cancer, polycythemia vera, prostate cancer, rare-cancers-and-associated-disorders, renal cell carcinoma, retinoblastoma, rhabdomyosarcoma, Rothmund-Thomson syndrome, salivary gland cancer, sarcoma, schwannoma, Sezary syndrome, skin cancer, small cell lung cancer (SCLC), small intestine cancer, soft tissue sarcoma, spinal cord tumors, squamous-cell-carcinoma-(skin), stomach cancer, synovial sarcoma, testicular cancer, thymus cancer, thyroid cancer, transitional-cell-cancer-(bladder), transitional-cell-cancer-(renal-pelvis-/-ureter), trophoblastic cancer, urethral cancer, urinary system cancer, uroplakins, uterine sarcoma, uterus cancer, vaginal cancer, vulva cancer, Waldenstrom's-macroglobulinemia and Wilms' tumor.

[0018] The subject may alternatively be exposed to a protein toxicant.

[0019] In the above aspects, the antigen is a protein derived from the pathogenic agent associated with the disease condition or cancer or is a toxicant.

[0020] A method is also provided of allowing a user to determine the status of cell-mediated immunoresponsiveness of a subject, the method including:

(a) receiving data in the form of levels or concentrations of an immune effector molecule which, relative to a control, provide a correlation as to the state of cell-mediated immunoresponsiveness in a subject, *via* a communications network, the immune effector molecule measured after exposure of lymphocytes to at least two sets of peptides, a first set comprising one or more peptides of from about 7 to 14 amino acid residues in length and a second set comprising one or more peptides of from 16 amino acid residues or greater which peptides encompass all or part of a protein antigen;

(b) processing the data *via* univariate or multivariate analysis to provide an immunoresponsiveness value;

(c) determining the status of the subject in accordance with the results of the immunoresponsiveness value in comparison with predetermined values; and

(d) transferring an indication of the status of the subject to the user *via* the communications network.

[0021] In an embodiment, the tuberculosis antigen is CFP10, ESAT-6, TB7.7 or TB37.6. In an embodiment, the subject is infected with HIV. In an embodiment, the lymphocytes are contacted with a combination of $CD4^+$ and $CD8^+$ peptides.

BRIEF DESCRIPTION OF THE FIGURES

[0022] Figure 1 is a graphical representation in the form of a histogram showing mean responses to QFN-TB or QFN-TB plus one of the three pools in all evaluable subjects (*n=41*). Mean values with standard error of the mean are shown. A significant increase in the response was observed with the addition of all of the peptide pools ($P<0.001$) [Friedman test with a Dunn's multiple comparison test]. The QFN-TB assay contained $CD4^+$ peptides and a pool of 10 mer peptides ($CD8^+$ peptides).

[0023] Figure 2 is a graphical representation of IFN-γ responses in a QFT-CMV assay comprising 16 mer peptides for CMV pp65 antigen ($CD4^+$ peptides) using a Nil tube with no antigen CMV or 16 mer $CD4^+$ peptides alone; and combined CMV $CD4^+$ + $CD8^+$ peptide; and using mitogen as a control.

DETAILED DESCRIPTION

[0024] Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or method step or group of elements or integers or method steps but not the exclusion of any other element or integer or method step or group of elements or integers or method steps.

[0025] As used in the subject specification, the singular forms "a", "an" and "the" include plural aspects unless the context clearly dictates otherwise. Thus, for example, reference to "a T-cell" includes a single T-cell, as well as two or more T-cells; reference to "an antigen" includes a single antigen, as well as two or more antigens; reference to "the disclosure" includes single or multiple aspects taught by the present disclosure; and so forth. Aspects taught herein are encompassed by the term "invention". All aspects of the invention are enabled within the width of the claims. The terms "T-cells" and "T-lymphocytes" are used interchangeably herein. An "immune cell" includes a lymphocyte such as a T-cell.

[0026] Reference to an "agent", "reagent", "molecule" and "compound" includes single entities and combinations of two or more of such entities. A "combination" also includes multi-part such as a two-part composition where the agents are provided separately and used or dispensed separately or admixed together prior to dispensation. For example, a multi-part assay pack may have a series of overlapping peptides from about 7 to 14 amino acid residues in length and/or greater than 15 amino acid residues in length which encompass all or part of a protein antigen against which a cell-mediated immune response is to be measured. Hence, this aspect of the present disclosure includes agents dried and loose or immobilized to a compartment wall or solid support in an assay pack.

[0027] The present disclosure contemplates sets of peptides. The term "set" may be replaced by other terms such as "pool", "group", "series", "collection" and the like without departing from the method instantly disclosed. Each set comprises at least one peptide and includes in an embodiment a series of overlapping peptides. Hence, a first set may contain

a series of overlapping peptides of from 7 to 14 amino acid residues in length. These peptides are recognized by $CD4^+$ T-cells, ($CD4^+$ peptides). A second set may contain a series of overlapping peptides of greater than 15 amino acid residues in length. These peptides are recognized by $CD8^+$ T-cells ($CD8^+$ peptides) Both sets of peptides encompasses the entire length of or part of a protein antigen. Furthermore, the peptides do not necessarily have to be overlapping or may overlap by a single amino acid or multiple amino acids. The peptides includes pods of peptides which encompass from 80-100% of a protein antigen. From "80-100%" means 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%.

[0028] Reference to a series of overlapping peptides from about 7 to 14 amino acid residues in length which encompass all or part of a protein antigen means a peptide of from about 7 amino acid residues in length to a maximum of 14 amino acid residues which in total span from every amino acid residues which in total span amino acid residues to up to 6 amino acid residues of a protein antigen from its N-terminal end to its C-terminal end or part thereof. Hence, if the length of a given peptide is x amino acid residues in length wherein x is from about 7 to 14, then the extent of overlap between two consecutive peptides is from x-1 to x-6. In an embodiment, the overlap of each consecutive peptide is x-1. A series of overlapping peptides of greater than 15 amino acid residues in length also spans all or part of a protein antigen wherein each peptide is at least 16 amino acid residues in length or up to the length of the full protein antigen. In an embodiment, a peptide of greater than 15 amino acid residues in length is from 16 to 50 amino acids such as 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 amino acid residues. As indicated above, there is no necessity for the peptides to overlap provided there is at least one set of one or more 7 to 14 amino acid peptides and another set of at least one >15mer peptides.

[0029] The present disclosure includes the case where each peptide in the series is the same length (i.e. x). However, the series of peptides may comprise a mixture of x_1, x_2, $x_j...x_i$ peptides where each of x_i peptides is from about 7 to 14 amino acid residues in length or greater than 15 amino acid residues in length.

[0030] Enabled herein is a method for detecting a cell-mediated immune response in a subject, the method comprising incubating lymphocytes from the subject with at least two sets of peptides, a first set comprising at least one peptide of from about 7 to 14 amino acid residues in length and a second set comprising at least one peptide of from 16 amino acid residues or greater which peptides encompass all or part of a protein antigen and then screening for levels of effector molecules produced by activated lymphocytes.

[0031] Lymphocytes are activated by co-incubation with at least two sets of peptides, a first set comprising at least one peptide of from about 7 to 14 amino acid residues in length and a second set comprising at least one peptide of from 16 amino acid residues or greater which peptides encompass all or part of a protein antigen.

[0032] The present disclosure teaches augmentation of production of effector molecules from lymphocytes exposed to at least two sets of peptides, a first set comprising at least one peptide of from about 7 to 14 amino acid residues in length and a second set comprising at least one peptide of from 16 amino acid residues or greater which peptides encompass all or part of a protein antigen. Such lymphocytes are "activated" or "stimulated" lymphocytes. The augmentation occurs by exposing the cells to at least two sets of peptides, a first set comprising at least one peptide of from about 7 to 14 amino acid residues in length and a second set comprising at least one peptide of from 16 amino acid residues or greater which peptides encompass all or part of a protein antigen. The level of the response is greater than in the presence of whole antigen or a peptide derived from the antigen which is less than 7 amino acids or greater than 14 amino acids. This enables a more sensitive assay in order to assess the cell-mediated immune responsiveness of a subject. The present disclosure, therefore, enables an assay to detect, assess or otherwise monitor a cell-mediated response in a subject by measuring the presence or level of effector molecules from T-cells stimulated by at least two sets of peptides, a first set comprising at least one peptide of from about 7 to 14 amino acid residues in length and a second set comprising at least one peptide of from 16 amino acid residues or greater which peptides encompass all or part of a protein antigen. The assay also enables earlier

detection of cell-mediated responsiveness. In an embodiment, the assay taught therein enhances the sensitivity of a cell-mediated assay which may enable less sensitive detection assays to be employed. Furthermore, the extent or magnitude of the cell-mediated immune response is proposed to be reflective or informative of the state, progression and/or severity of a disease condition. For example, the magnitude of the response may determine if a subject has a latent or active or acute infection or disease condition.

[0033] Conveniently, the $CD4^+$ and/or $CD8^+$ peptides are divided into separate pools of peptides.

[0034] Without limiting the present invention to any one theory or mode of action, at least two sets of peptides enables both $CD4^+$ and $CD8^+$ epitopes to be stimulated. The peptides may be referred to herein as " $CD4^+$ peptides" (>15 mer peptides) or " $CD8^+$ peptides" (7 to 14 mer peptides).

[0035] An additional agent may also be added to the incubation mixture such as to modulate the activity of regulatory T-cells (T-reg cells). The latter encompasses inhibiting the suppressor function of T-reg cells. Agents which modulate T-reg cells encompassed herein include a CD25 ligand; a sense or antisense oligonucleotide to genetic material encoding JAK1 or TYK2; a neutralizing antibody; a CpG containing oligonucleotide; an oligonucleotide acting as a toll-like receptor (TLR) modulating agent; and other TLR modulating agents.

[0036] In a particular embodiment, the T-reg cells are immune response suppressor cells the activity of which is inhibited.

[0037] A "CpG molecule" means an oligonucleotide comprising a CpG sequence or motif.

[0038] The present disclosure provides a means to determine the responsiveness of cell-mediated immunity in a subject and, in turn, teaches the determination of whether a disease condition or an agent induces or is associated with immunosuppression. The method also

enables diagnosis of infectious diseases, pathological conditions, determination of the level of immunocompetence and assessing of immune cell responsiveness to endogenous or exogenous agents as well as assessing exposure to protein toxicants. The assay also enables screening of subjects previously exposed to a particular antigen, such as an antigen associated with a disease, infection or contaminant.

[0039] Accordingly, an aspect taught herein contemplates a method for measuring cell-mediated immune response activity in a subject, the method comprising contacting lymphocytes from the subject with at least two sets of peptides, a first set comprising at least one peptide of from about 7 to 14 amino acid residues in length and a second set comprising at least one peptide of from 16 amino acid residues or greater which peptides encompass all or part of a protein antigen and measuring the level of an immune effector molecule produced by immune cells wherein the level of the immune effector molecule is indicative of the level of cell-mediated immunoresponsiveness of the subject.

[0040] Another aspect contemplated herein is a method for measuring cell-mediated immune response activity in a subject, the method comprising contacting lymphocytes from the subject with at least two sets of peptides, a first set comprising at least one peptide of from about 7 to 14 amino acid residues in length and a second set comprising at least one peptide of from 16 amino acid residues or greater which peptides encompass all or part of a protein antigen and measuring the elevation in the level of an immune effector molecule from immune cells wherein the level of the immune effector molecule is indicative of the level of cell-mediated responsiveness of the subject wherein the level of responsiveness is indicative of the presence or absence or level or stage of a disease or condition selected from the list comprising an infection by a pathogenic agent, an autoimmune disease, a cancer, an inflammatory condition and exposure to a toxic proteinaceous agent.

[0041] Yet another aspect enabled herein is a method for measuring cell-mediated immune response activity in a subject, the method comprising contacting lymphocytes from the subject with at least two sets of peptides, a first set comprising at least one peptide of from

about 7 to 14 amino acid residues in length and a second set comprising at least one peptide of from 16 amino acid residues or greater which peptides encompass all or part of a protein antigen and measuring the elevation in the level of an immune effector molecule from immune cells wherein the level of the immune effector molecule is indicative of the level of cell-mediated responsiveness and is indicative of the presence or absence or level or stage of a disease or condition selected from the list comprising an infection by a pathogenic agent, an autoimmune disease, a cancer, an inflammatory condition and exposure to a toxic proteinaceous agent.

[0042] Still another aspect taught by the present disclosure is an assay to detect the presence, absence, level or stage of a disease or condition in a subject, the method comprising contacting lymphocytes from the subject with at least two sets of peptides, a first set comprising at least one peptide of from about 7 to 14 amino acid residues in length and a second set comprising at least one peptide of from 16 amino acid residues or greater which peptides encompass all or part of a protein antigen and measuring the elevation in the level of an immune effector molecule from immune cells wherein the level of the immune effector molecule is indicative of the disease or condition.

[0043] The present disclosure further contemplates a method for determining whether an agent induces immunosuppression in a subject, the method comprising contacting lymphocytes from the subject after exposure to the agent with at least two sets of peptides, a first set comprising at least one peptide of from about 7 to 14 amino acid residues in length and a second set comprising at least one peptide of from 16 amino acid residues or greater which peptides encompass all or part of a protein antigen and measuring the presence and level of an effector molecule from the lymphocytes wherein the level of the effector molecule determines the level of immunosuppression induced by the agent.

[0044] In accordance with this aspect, the agent may be a medicament or an environmental toxicant.

[0045] In an embodiment, the lymphocytes are comprised within a blood sample. In an

embodiment, the blood sample is co-stimulated with at least two sets of peptides, a first set comprising at least one peptide of from about 7 to 14 amino acid residues in length and a second set comprising at least one peptide of from 16 amino acid residues or greater which peptides encompass all or part of a protein antigen.

[0046] A use is also provided for at least two sets of peptides, a first set comprising at least one peptide of from about 7 to 14 amino acid residues in length and a second set comprising at least one peptide of from 16 amino acid residues or greater which peptides encompass all or part of a protein antigen in the manufacture of a diagnostic assay of cell-mediated immune responsiveness by the method of incubating lymphocytes with a limiting amount of the agonist and detecting the presence or elevation in an effector molecule.

[0047] In another embodiment, taught herein is a method for detecting whether a disease condition is inducing immunosuppression in a subject the method comprising contacting lymphocytes from the subject with a disease condition with at least two sets of peptides, a first set comprising at least one peptide of from about 7 to 14 amino acid residues in length and a second set comprising at least one peptide of from 16 amino acid residues or greater which peptides encompass all or part of a protein antigen and measuring the presence or level of an immune effector molecule from the lymphocytes wherein the level of the immune effector molecule is indicative of the extent of immunosuppression induced or associated with the disease condition.

[0048] A use is also provided for at least two sets of peptides, a first set comprising at least one peptide of from about 7 to 14 amino acid residues in length and a second set comprising at least one peptide of from 16 amino acid residues or greater which peptides encompass all or part of a protein antigen in the manufacture of a diagnostic assay of cell-mediated immune responsiveness. Generally, the method comprising incubating lymphocytes with at least two sets of peptides, a first set comprising at least one peptide of from about 7 to 14 amino acid residues in length and a second set comprising at least one peptide of from 16 amino acid residues or greater which peptides encompass all or part of a protein antigen.

[0049] This use includes the use for detecting or monitoring the presence, absence, level or stage of a disease or condition such as an infection by a pathogenic agent, an autoimmune disease, a cancer, an inflammatory condition and/or exposure to a medicament or a toxic proteinaceous agent such as an environmental toxicant. Measuring "an immune effector molecule" includes measuring one or more different types of molecules.

[0050] The present disclosure further enables a method for measuring cell-mediated immune response activity in a subject, the method comprising contacting a regulatory T-cell from the subject with an agent selected from (i) an inhibitor of suppressor regulatory T-cells; and (ii) an activator of immune augmenting cells or a subset thereof; and further contacting T-cells with at least two sets of peptides, a first set comprising at least one peptide of from about 7 to 14 amino acid residues in length and a second set comprising at least one peptide of from 16 amino acid residues or greater which peptides encompass all or part of a protein antigen and measuring the elevation in the level of an immune effector molecule from immune cells wherein the level of the immune effector molecule is indicative of the level of cell-mediated responsiveness of the subject.

[0051] Examples of inhibitors or modulators of T-reg function include CD25 ligands such as but not limited to a polyclonal or monoclonal antibody to CD25 or an antigen-binding fragment thereof, humanized or deimmunized polyclonal or monoclonal antibodies to CD25 or a recombinant or synthetic form of the polyclonal or monoclonal antibodies. Other examples of agents include sense or antisense nucleic and molecules directed to the mRNA or DNA (i.e. genetic material) encoding Janus Tyrosine Kinase 1 (JAK1) or Tyrosine Kinase 2 (TYK2) or small molecule inhibitors of JAK1 or TYK2 proteins. Reference to "small molecules" includes immunoglobulin new antigen receptors (IgNARs) as described in International Patent Publication No. WO 2005/118629. Yet still further examples of suitable agents stimulating agents such as CpG molecules which act *via* Toll-like receptors (TLRs) and/or other mechanisms. Hence, CpG containing oligonucleotides and an oligonucleotide acting as a TLR modulating agent also form part of the present disclosure.

[0052] A single type of agent may be used or two or more types of agents may be employed to modulate T-reg cells. For example, the assay may be conducted with a CD25 ligand and a JAK1/TYK2 sense or antisense oligonucleotide; a CD25 ligand and a TLR modulating agent; a JAK1/TYK2 sense or antisense oligonucleotide and a TLR modulating agent; or a CD25 ligand, a JAK1/TYK2 sense or antisense oligonucleotide and a TLR modulating agent. Alternatively, just one type of agent is employed. In another alternative, a CpG comprising oligonucleotide and a TLR modulating agent is used.

[0053] Reference to a "subject" includes a human or non-human species including primates, livestock animals (e.g. sheep, cows, pigs, horses, donkey, goats), laboratory test animals (e.g. mice, rats, rabbits, guinea pigs, hamsters), companion animals (e.g. dogs, cats), avian species (e.g. poultry birds, aviary birds), reptiles and amphibians. The present subject matter has applicability in human medicine as well as having livestock and veterinary and wild-life applications which includes the horse, dog and camel racing industries. For example, the assay of the present disclosure may be routinely carried out on horses before and/or after heavy exertion (such as a race) to screen for evidence of exercise-induced pulmonary hemorrhage (EIPH). All horses exhibit some form of EIPH to some degree during exercise. However, sub-clinical forms of EIPH can be hard to detect.

[0054] Reference to a "human" includes particular populations of humans such as pediatric, elderly and infirmed populations of humans as well as particular cohorts or populations of humans of a particular ethnicity.

[0055] In another embodiment, the subject is a human and the cell-mediated immune response assay is used in screening for responsiveness to pathogenic microorganisms, viruses and parasites, potential for development or monitoring autoimmune conditions, Celiac's disease, monitoring a subject's response to oncological challenge and for determining the presence of any immunodeficiency or immunosuppression. The latter may occur, for example, due to certain medicaments including various chemotherapeutic agents. Alternatively, exposure to environmental proteinaceous toxicants and pollutants.

[0056] The immune effector molecules may be any of a range of molecules which are produced in response to cell activation or stimulation by an antigen. Although an interferon (IFN) such as IFN-γ is a particularly useful immune effector molecule, others include a range of cytokines such as interleukins (IL), e.g. IL-2, IL-4, IL-6, IL-6 (CXCL8), IL-10, IL-12, IL-13, IL-16 (LCF) or IL-17, IL-1α (IL-1F1), IL-1β (IL-1F2), IL-1rα (IL-1F3), Tumor Necrosis Factor alpha (TNF-α), Transforming Growth Factor beta (TGF-β), a Colony Stimulating Factor (CSF) such as Granulocyte (G)-CSF or Granulocyte Macrophage (GM)-CSF, complement component 5a (C5a), Groα (CXCL1), sICAM-1 (CD54), IP-10 (CXCL10), I-TAC (CXCL11), MCP-1 (CCL2), MIF (GIF), MIP-1α (CCL3), MIP-1β (CCL4), RANTES (CCL5) or MIG (CXCL9).

[0057] The present disclosure also enables a method for measuring cell-mediated immune response activity in a subject, the method comprising contacting lymphocytes from the subject with at least two sets of peptides, a first set comprising at least one peptide of from about 7 to 14 amino acid residues in length and a second set comprising at least one peptide of from 16 amino acid residues or greater which peptides encompass all or part of a protein antigen and measuring the level of an immune effector molecule from immune cells wherein the level of the immune effector molecule is indicative of the level of cell-mediated responsiveness of the subject.

[0058] The assay taught herein enables detection of the presence or absence or level or stage of a disease or condition in a subject such as infection by a pathogenic agent, an autoimmune disease, cancer, exposure to an inflammatory agent exposure to a medicament, exposure to a toxic proteinaceous agent and immunodeficiency or immunosuppression such as induced by a disease condition.

[0059] In an embodiment, the sample collected from the subject is generally deposited into a blood collection tube. A blood collection tube includes a blood draw tube or other similar vessel. Conveniently, when the sample is whole blood, the blood collection tube is heparinized. Alternatively, heparin is added to the tube after the blood is collected.

Notwithstanding that whole blood is particularly contemplated and a most convenient sample, the present disclosure extends to other samples containing immune cells such as lymph fluid, cerebral fluid, tissue fluid and respiratory fluid including nasal and pulmonary fluid as well as samples having undergone cell depletion. Reference to "whole blood" includes whole blood which has not been diluted such as with tissue culture, medium, reagents, excipients, etc. In one embodiment, the term "whole blood" includes an assay sample (i.e. reaction mixture) comprising at least 10% by volume whole blood. The term "at least 10% by volume" includes blood volumes of 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 and 100% by volume of total assay volume of the reaction mixture. Additional agents may be added such as culture media, enzymes, excipients antigen and the like without departing from the sample comprising "whole blood".

[0060] Blood volumes may be from about 0.5µl to 200ml. Examples include 0.5µl, 1, 5µl, 10µl, 20µl, 50µl, 100µl, 500µl, 1ml, 5ml, 10ml, and 20ml. The present disclosure also enables the use of acoustic microstreaming to improve the mixing of components in the assay. Acoustic microstreaming is disclosed in International Patent Application No. PCT/AU01/00420 and in Petkovic-Duran *et al.* (2009) *Biotechniques* 47:827-834.

[0061] Hence, contemplated herein is a method of mixing one or more lymphocytes and at least two sets of peptides, a first set comprising at least one peptide of from about 7 to 14 amino acid residues in length and a second set comprising at least one peptide of from 16 amino acid residues or greater which peptides encompass all or part of a protein antigen in a vessel, the method comprising providing from about 0.5µl to 150µl of fluid comprising the components in the vessel so as to establish a discontinuity in acoustic impedance and applying an acoustic signal to cause mixing within the fluid. A second acoustic signal may also be applied, the first and second signals having respective frequencies each selected from about 1Hz to about 20,000Hz in an alternating manner to effect chaotic mixing within the fluid.

[0062] The use of blood collection tubes is compatible with standard automated laboratory systems and these are amenable to analysis in large-scale and random access sampling. Blood collection tubes also minimize handling costs and reduce laboratory exposure to whole blood and plasma and, hence, reduce the risk of laboratory personnel from contracting a pathogenic agent such as HIV or Hepatitis B virus (HBV) or Hepatitis C virus (HCV).

[0063] Combining the incubation step with the collection tube is particularly efficacious and enhances the sensitivity of the assay as does the optional feature of incubating the cells in the presence of a simple sugar such as dextrose or glucose.

[0064] The cells of the cell-mediated immune system lose the capacity to mount an immune response in whole blood after extended periods following blood draw from the subject, and responses without intervention are often severely reduced or absent 24 hours following blood draw. The reduction of labor and need for specialized plasticware allows cell-mediated immune stimulation with the peptide antigens to be performed at the point of care locations such as physicians' offices, clinics, outpatient facilities and veterinary clinics or on farms. Once antigen stimulation is complete, the requirement for fresh and active cells no longer exists. IFN-γ and other cytokines or immune effector molecules are stable in plasma and, thus, the sample can be stored, or shipped without special conditions or rapid time requirements.

[0065] The incubation step may be from 1 to 50 hours, such as 1 to 40 hours or 8 to 24 hours or a time period in between including 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 hours. A period of 24 hours is particularly convenient.

[0066] The ability to measure cell-mediated immunity is important for assessing a subject's ability to respond to an infection by a pathogenic agent such as a microorganism

or virus or parasite, to mount an autoimmune response such as in autoimmune diabetes or to protect against cancers or other oncological conditions or to detect an inflammatory condition or to detect exposure or sensitivity of a subject to a toxic agent such as beryllium. The assay described herein also enables detection of disease conditions which lead to immunosuppression or immunosuppresion induced by medicaments Consequently, reference to "measuring a cell-mediated immune response in a subject" includes and encompasses immune diagnosis of infectious and autoimmune diseases, a marker for immunocompetence as well as a marker for inflammatory diseases, cancer and toxic agents. Importantly, the combined innate and/or adaptive immune responsiveness is determined. Furthermore, the ability to use small blood volumes enables assays to be readily conducted on, for example, the pediatric, elderly and infirmed populations. The assay herein enables early detection or more sensitive detection of immunoresponsiveness.

[0067] In an embodiment, disease conditions leading to immunosuppression include chronic infection and cancer. Medicaments which can lead to immunosuppression include those used to treat rheumatoid arthritis, cancer and inflammatory bowel disease.

[0068] Pathogenic or infectious agents include bacteria, parasites and viruses. Examples of bacteria include Gram positive and Gram negative microorganisms such as *Mycobacterium* species, *Staphylococcus* species, *Streptococcus* species, *Escherichia coli*, *Salmonella* species, *Clostridium* species, *Shigella* species, *Proteus* species, *Bacillus* species, *Hemophilus* species, *Borrelia* species amongst others. *Mycobacterium tuberculosis* is a particularly useful target as well as conditions arising from infection by *M. tuberculosis* such as tuberculosis (TB). Examples of viruses include Hepatitis virus (Hepatitis B virus and Hepatitis C virus), Herpes virus and Human immune deficiency virus (HIV) as well as diseases resulting therefrom. Parasites include *Plasmodium* species, ringworm, liver parasites and the like. Other pathogenic agents include eukaryotic cells such as yeasts and fungi.

[0069] In an embodiment, the tuberculosis antigen is CFP10, ESAT-6, TB7.7 or TB37.6. In an embodiment, the subject is infected with HIV.

[0070] The present invention is particularly useful for screening for exposure to *M. tuberculosis*. Hence, the present disclosure teaches a method for measuring cell-mediated immune response activity in a subject, the method comprising contacting lymphocytes from the subject with at least two sets of peptides, a first set comprising at least one peptide of from about 7 to 14 amino acid residues in length and a second set comprising at least one peptide of from 16 amino acid residues or greater which peptides encompass all or part of a protein antigen, wherein the antigen is selected from CFP10, ESAT-6, TB7.7 and TB37.6 from *Mycobacterium tuberculosis* and measuring the level of an immune effector molecule produced by immune cells wherein the level of the immune effector molecule is indicative of the level of cell-mediated immunoresponsiveness of the subject to *M. tuberculosis*.

[0071] CFP10 is also known as ESAT-6-like protein eesxB and secreted antigenic protein MTSA-10. ESAT-6 is a six kDa early secretary antigenic target of *M. tuberculosis*. Other suitable target protein antigens for *M. tuberculosis* include TB7.7 and TB37.6.

[0072] Autoimmune diseases contemplated herein for detection include *inter alia* alopecia areata, ankylosing spondylitis, antiphospholipid syndrome, autoimmune Addison's disease multiple sclerosis, autoimmune disease of the adrenal gland, autoimmune hemolytic anemia, autoimmune hepatitis, autoimmune oophoritis and orchitis, Behcet's disease, bullous pemphigoid, cardiomyopathy, celiac sprue-dermatitis, chronic fatigue syndrome (CFIDS), chronic inflammatory demyelinating, chronic inflammatory polyneuropathy, Churg-Strauss syndrome, cicatricial pemphigoid, crest syndrome, cold agglutinin disease, Crohn's disease, dermatitis herpetiformis, discoid lupus, essential mixed cryoglobulinemia, fibromyalgia, glomerulonephritis, Grave's disease, Guillain-Barre, Hashimoto's thyroiditis, idiopathic pulmonary fibrosis, idiopathic thrombocytopenia purpura (ITP), IgA nephropathy, insulin dependent diabetes (Type I), lichen planus, lupus, Meniere's disease, mixed connective tissue disease, multiple sclerosis, myasthenia gravis, myocarditis, pemphigus vulgaris, pernicious anemia, polyarteritis nodosa, polychondritis, polyglancular syndromes, polymyalgia rheumatica, polymyositis and dermatomyositis, primary

agammaglobulinemia, primary biliary cirrhosis, psoriasis, Raynaud's phenomenon, Reiter's syndrome, rheumatic fever, rheumatoid arrthritis, sarcoidosis, scleroderma, Sjogren's syndrome, stiff-man syndrome, systemic lupus erythematosus, Takayasu arteritis, temporal arteritis/gianT-cell arteritis, ulcerative colitis, uveitis, vasculitis and vitiligo.

[0073] It is generally important to assess the potential or actual cell-mediated responsiveness in subjects exposed to these infectious entities. The method of the present disclosure can also be used to detect the presence or absence of these conditions as well as the level or stage of disease process.

[0074] Other disease conditions which can lead to immunosuppression include inflammatory disease conditions.

[0075] Examples of inflammatory disease conditions contemplated by the present disclosure include but are not limited to those disease and disorders which result in a response of redness, swelling, pain, and a feeling of heat in certain areas that is meant to protect tissues affected by injury or disease. Inflammatory diseases which can be treated using the methods of the present disclosure include, without being limited to, acne, angina, arthritis, aspiration pneumonia, disease, empyema, gastroenteritis, inflammation, intestinal flu, NEC, necrotizing enterocolitis, pelvic inflammatory disease, pharyngitis, PID, pleurisy, raw throat, redness, rubor, sore throat, stomach flu and urinary tract infections, chronic inflammatory demyelinating polyneuropathy, chronic inflammatory demyelinating polyradiculoneuropathy, chronic inflammatory demyelinating polyneuropathy, chronic inflammatory demyelinating polyradiculoneuropathy. In terms of non-human applications, the present disclosure extends to detecting EIPH in horses and various conditions in animals such as facial tumor disease in the Tasmanian Devil.

[0076] Cancer therapy also is somewhat dependent on cell-mediated immunity and the cancer itself or drugs used to treat cancer can lead to immunosuppression. Cancers contemplated herein include: a group of diseases and disorders that are characterized by uncontrolled cellular growth (e.g. formation of tumor) without any differentiation of those

cells into specialized and different cells. Such diseases and disorders include ABL1 protooncogene, AIDS related cancers, acoustic neuroma, acute lymphocytic leukaemia, acute myeloid leukaemia, adenocystic carcinoma, adrenocortical cancer, agnogenic myeloid metaplasia, alopecia, alveolar soft-part sarcoma, anal cancer, angiosarcoma, aplastic anaemia, astrocytoma, ataxia-telangiectasia, basal cell carcinoma (skin), bladder cancer, bone cancers, bowel cancer, brain stem glioma, brain and CNS tumors, breast cancer, CNS tumors, carcinoid tumors, cervical cancer, childhood brain tumors, childhood cancer, childhood leukaemia, childhood soft tissue sarcoma, chondrosarcoma, choriocarcinoma, chronic lymphocytic leukaemia, chronic myeloid leukaemia, colorectal cancers, cutaneous T-Cell lymphoma, dermatofibrosarcoma-protuberans, desmoplastic-small-round-cell-tumor, ductal carcinoma, endocrine cancers, endometrial cancer, ependymoma, esophageal cancer, Ewing's sarcoma, extra-hepatic bile duct cancer, eye cancer, eye: melanoma, retinoblastoma, fallopian tube cancer, fanconi anemia, fibrosarcoma, gall bladder cancer, gastric cancer, gastrointestinal cancers, gastrointestinal-carcinoid-tumor, genitourinary cancers, germ cell tumors, gestational-trophoblastic-disease, glioma, gynaecological cancers, hematological malignancies, hairy cell leukaemia, head and neck cancer, hepatocellular cancer, hereditary breast cancer, histiocytosis, Hodgkin's disease, human papillomavirus, hydatidiform mole, hypercalcemia, hypopharynx cancer, intraocular melanoma, islet cell cancer, Kaposi's sarcoma, kidney cancer, Langerhan's-cell-histiocytosis, laryngeal cancer, leiomyosarcoma, leukemia, Li-Fraumeni syndrome, lip cancer, liposarcoma, liver cancer, lung cancer, lymphedema, lymphoma, Hodgkin's lymphoma, non-Hodgkin's lymphoma, male breast cancer, malignant-rhabdoid-tumor-of-kidney, medulloblastoma, melanoma, merkel cell cancer, mesothelioma, metastatic cancer, mouth cancer, multiple endocrine neoplasia, mycosis fungoides, myelodysplastic syndromes, myeloma, myeloproliferative disorders, nasal cancer, nasopharyngeal cancer, nephroblastoma, neuroblastoma, neurofibromatosis, nijmegen breakage syndrome, non-melanoma skin cancer, non-small-cell-lung-cancer-(NSCLC), ocular cancers, oesophageal cancer, oral cavity cancer, oropharynx cancer, osteosarcoma, ostomy ovarian cancer, pancreas cancer, paranasal cancer, parathyroid cancer, parotid gland cancer, penile cancer, peripheral-neuroectodermal-tumors, pituitary cancer, polycythemia vera, prostate cancer, rare-cancers-and-associated-disorders, renal

cell carcinoma, retinoblastoma, rhabdomyosarcoma, Rothmund-Thomson syndrome, salivary gland cancer, sarcoma, schwannoma, Sezary syndrome, skin cancer, small cell lung cancer (SCLC), small intestine cancer, soft tissue sarcoma, spinal cord tumors, squamous-cell-carcinoma-(skin), stomach cancer, synovial sarcoma, testicular cancer, thymus cancer, thyroid cancer, transitional-cell-cancer-(bladder), transitional-cell-cancer-(renal-pelvis-/-ureter), trophoblastic cancer, urethral cancer, urinary system cancer, uroplakins, uterine sarcoma, uterus cancer, vaginal cancer, vulva cancer, Waldenstrom's-macroglobulinemia and Wilms' tumor.

[0077] In the above aspects, the antigen may be derived from the pathogenic agent, be associated with the disease condition or cancer or be the toxicant. Alternatively, the infection, disease condition, cancer or toxicant may suppress cell-mediated immunity in which case any antigen to which the subject has been prior exposed could be employed.

[0078] The detection of the immune effector molecules may be measured at the protein or nucleic acid levels. Consequently, reference to "presence or level" of the immune effector molecule includes direct and indirect data. For example, high levels of cytokine mRNA are indirect data showing increased levels of the cytokine.

[0079] Ligands to the immune effectors are particularly useful in detecting and/or quantitating these molecules. Antibodies to the immune effectors are particularly useful. Techniques for the assays contemplated herein are known in the art and include, for example, radioimmunoassay, sandwich assays, ELISA and ELISpot. Reference to "antibodies" includes parts of antibodies, mammalianized (e.g. humanized) antibodies, deimmunized antibodies, recombinant or synthetic antibodies and hybrid and single chain antibodies. For skin tests, in humans, humanized or deimmunized antibodies are particularly contemplated herein to detect effector molecules.

[0080] Both polyclonal and monoclonal antibodies are obtainable by immunization with the immune effector molecules or antigenic fragments thereof and either type is utilizable for immunoassays. Methods of obtaining both types of sera are well known in the art.

Polyclonal sera are less preferred but are relatively easily prepared by injection of a suitable laboratory animal with an effective amount of the immune effector, or antigenic part thereof, collecting serum from the animal and isolating specific sera by any of the known immunoadsorbent techniques. Although antibodies produced by this method are utilizable in virtually any type of immunoassay, they are generally less favoured because of the potential heterogeneity of the product.

[0081] The use of monoclonal antibodies in an immunoassay is particularly useful because of the ability to produce them in large quantities and the homogeneity of the product. The preparation of hybridoma cell lines for monoclonal antibody production derived by fusing an immortal cell line and lymphocytes sensitized against the immunogenic preparation can be done by techniques which are well known to those who are skilled in the art.

[0082] Another aspect enabled herein, therefore, is a method for detecting an immune effector molecule in a sample comprising lymphocytes from a subject, the method comprising contacting the sample or an aliquot of the sample with an antibody specific for the immune effector molecule or an antigenic fragment thereof for a time and under conditions sufficient for an antibody-effector complex to form, and then detecting the complex wherein the immune effector molecule is generated after incubation of the lymphocytes with at least two sets of peptides, a first set comprising at least one peptide of from about 7 to 14 amino acid residues in length and a second set comprising at least one peptide of from 16 amino acid residues or greater which peptides encompass all or part of a protein antigen.

[0083] A "sample" includes whole blood or a fraction thereof comprising lymphocytes. This method includes micro-arrays, macro-arrays and nano-arrays on planar or spherical solid supports. A micro- or macro-array is useful. A "sample" also includes a small volume sample of from about 0.5µl to 1000µl including 5µl, 10µl, 20µl, 50µl and 100µl as well as larger volumes such as from 1ml to about 200ml such as 1ml, 2ml, 5ml, 10ml or 20ml.

[0084] A wide range of immunoassay techniques are available as can be seen by reference to U.S. Patent Nos. 4,016,043, 4,424,279 and 4,018,653.

[0085] The following is a description of one type of assay. An unlabeled antibody is immobilized on a solid substrate and the sample to be tested for the immune effector molecules (e.g. a cytokine) brought into contact with the bound molecule. After a suitable period of incubation, for a period of time sufficient to allow formation of an antibody-immune effector molecule complex, a second antibody specific to the effector molecule, labeled with a reporter molecule capable of producing a detectable signal, is then added and incubated, allowing time sufficient for the formation of another complex of antibody-effector-labeled antibody. Any unreacted material is washed away, and the presence of the effector molecule is determined by observation of a signal produced by the reporter molecule. The results may either be qualitative, by simple observation of the visible signal, or may be quantitated by comparing with a control sample containing known amounts of antigen. This generalized technique is well known to those skilled in the art as would be any of a number of variations.

[0086] In these assays, a first antibody having specificity for the instant immune effectors is either covalently or passively bound to a solid surface. The solid surface is typically glass or a polymer, the most commonly used polymers being cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene. The solid supports may be in the form of tubes, beads, spheres, discs of microplates, or any other surface suitable for conducting an immunoassay. The binding processes are well known in the art and generally consist of cross-linking covalently binding or physically adsorbing, the polymer-antibody complex is washed in preparation for the test sample. An aliquot of the sample to be tested is then added to the solid phase complex and incubated for a period of time sufficient (e.g. 2-120 minutes or where more convenient, overnight) and under suitable conditions (e.g. for about 20°C to about 40°C) to allow binding of any subunit present in the antibody. Following the incubation period, the antibody subunit solid phase is washed and dried and incubated with a second antibody specific for a portion of the effector molecule. The second antibody is linked to a reporter molecule which is used to indicate

the binding of the second antibody to the effector molecule.

[0087] There are many variations to this assay. One particularly useful variation is a simultaneous assay where all or many of the components are admixed substantially simultaneously. Furthermore, binding of an antibody to a cytokine may be determined by binding of a labeled antibody directed to the first mentioned antibody.

[0088] By "reporter molecule" as used in the present specification, is meant a molecule which, by its chemical nature, provides an analytically identifiable signal which allows the detection of antigen-bound antibody. Detection may be either qualitative or quantitative. The most commonly used reporter molecules in this type of assay are either enzymes, fluorophores or radionuclide containing molecules (i.e. radioisotopes) and chemiluminescent molecules. Examples of suitable fluorophores are provided in Table 1. In the case of an enzyme immunoassay, an enzyme is conjugated to the second antibody, generally by means of glutaraldehyde or periodate. As will be readily recognized, however, a wide variety of different conjugation techniques exist, which are readily available to the skilled artisan. Commonly used enzymes include horseradish peroxidase, glucose oxidase, beta-galactosidase and alkaline phosphatase, amongst others. The substrates to be used with the specific enzymes are generally chosen for the production, upon hydrolysis by the corresponding enzyme, of a detectable color change. Examples of suitable enzymes include alkaline phosphatase and peroxidase. It is also possible to employ fluorogenic substrates, which yield a fluorescent product rather than the chromogenic substrates noted above. In all cases, the enzyme-labeled antibody is added to the first antibody-antigen complex, allowed to bind, and then the excess reagent is washed away. A solution containing the appropriate substrate is then added to the complex of antibody-antigen-antibody. The substrate will react with the enzyme linked to the second antibody, giving a qualitative visual signal, which may be further quantitated, usually spectrophotometrically, to give an indication of the amount of antigen which was present in the sample. Again, the present disclosure extends to a substantially simultaneous assay.

[0089] Alternately, fluorescent compounds, such as fluorescein and rhodamine, may be

chemically coupled to antibodies without altering their binding capacity. When activated by illumination with light of a particular wavelength, the fluorochrome-labeled antibody adsorbs the light energy, inducing a state to excitability in the molecule, followed by emission of the light at a characteristic color visually detectable with a light microscope. The fluorescent labeled antibody is allowed to bind to the first antibody-antigen complex. After washing off the unbound reagent, the remaining tertiary complex is then exposed to the light of the appropriate wavelength the fluorescence observed indicates the presence of the antigen of interest. Immunofluorescene and enzyme immunoassay techniques are both very well established in the art and are particularly preferred for the present method. However, other reporter molecules, such as radioisotope, chemiluminescent or bioluminescent molecules, may also be employed.

[0090] There are a range of other detection systems which may be employed including colloidal gold and all such detection systems are encompassed by the present disclosure.

[0091] The present disclosure also contemplates genetic assays such as involving PCR analysis to detect RNA expression products of a genetic sequence encoding an immune effector.

[0092] In one embodiment, PCR is conducted using pairs of primers, one or both of which are generally labeled with the same or a different reporter molecule capable of giving a distinguishable signal. The use of fluorophores is particularly useful in the practice of the present disclosure. Examples of suitable fluorophores may be selected from the list given in Table 1. Other labels include luminescence and phosphorescence as well as infrared dyes. These dyes or fluorophores may also be used as reporter molecules for antibodies.

Table 1

List of suitable fluorophores

Probe	Ex[1] (nm)	Em[2] (nm)
Reactive and conjugated probes		
Hydroxycoumarin	325	386
Aminocoumarin	350	455
Methoxycoumarin	360	410
Cascade Blue	375; 400	423
Lucifer Yellow	425	528
NBD	466	539
R-Phycoerythrin (PE)	480; 565	578
PE-Cy5 conjugates	480; 565; 650	670
PE-Cy7 conjugates	480; 565; 743	767
APC-Cy7 conjugates	650; 755	767
Red 613	480; 565	613
Fluorescein	495	519
FluorX	494	520
BODIPY-FL	503	512
TRITC	547	574
X-Rhodamine	570	576
Lissamine Rhodamine B	570	590
PerCP	490	675
Texas Red	589	615
Allophycocyanin (APC)	650	660
TruRed	490, 675	695
Alexa Fluor 350	346	445
Alexa Fluor 430	430	545
Alexa Fluor 488	494	517
Alexa Fluor 532	530	555
Alexa Fluor 546	556	573
Alexa Fluor 555	556	573
Alexa Fluor 568	578	603
Alexa Fluor 594	590	617
Alexa Fluor 633	621	639
Alexa Fluor 647	650	688
Alexa Fluor 660	663	690
Alexa Fluor 680	679	702
Alexa Fluor 700	696	719
Alexa Fluor 750	752	779
Cy2	489	506
Cy3	(512); 550	570; (615)
Cy3,5	581	596; (640)
Cy5	(625); 650	670
Cy5,5	675	694

Probe	Ex[1] (nm)	Em[2] (nm)
Cy7	743	767
Nucleic acid probes		
Hoeschst 33342	343	483
DAPI	345	455
Hoechst 33258	345	478
SYTOX Blue	431	480
Chromomycin A3	445	575
Mithramycin	445	575
YOYO-1	491	509
SYTOX Green	504	523
SYTOX Orange	547	570
Ethidium Bormide	493	620
7-AAD	546	647
Acridine Orange	503	530/640
TOTO-1, TO-PRO-1	509	533
Thiazole Orange	510	530
Propidium Iodide (PI)	536	617
TOTO-3, TO-PRO-3	642	661
LDS 751	543; *590*	712; *607*
Fluorescent Proteins		
Y66F	360	508
Y66H	360	442
EBFP	380	440
Wild-type	396, 475	50, 503
GFPuv	385	508
ECFP	434	477
Y66W	436	485
S65A	471	504
S65C	479	507
S65L	484	510
S65T	488	511
EGFP	489	508
EYFP	514	527
DsRed	558	583
Other probes		
Monochlorobimane	380	461
Calcein	496	517

1 Ex: Peak excitation wavelength (nm)

2 Em: Peak emission wavelength (nm)

[0093] Any suitable method of analyzing fluorescence emission is encompassed herein. In this regard, techniques taught herein include but are not restricted to 2-photon and 3-photon time resolved fluorescence spectroscopy as, for example, disclosed by Lakowicz *et al.* (1997) *Biophys. J. 72:*567, fluorescence lifetime imaging as, for example, disclosed by Eriksson *et al.* (1993) *Biophys. J. 2:*64 and fluorescence resonance energy transfer as, for example, disclosed by Youvan *et al.* (1997) *Biotechnology et elia 3:*1-18.

[0094] Luminescence and phosphorescence may result respectively from a suitable luminescent or phosphorescent label as is known in the art. Any optical means of identifying such label may be used in this regard.

[0095] Infrared radiation may result from a suitable infrared dye. Exemplary infrared dyes that may be employed in the present disclosure include but are not limited to those disclosed in Lewis *et al.* (1999) *Dyes Pigm. 42(2):*197, Tawa *et al. Mater. Res. Soc. Symp. Proc.488* [Electrical, Optical and Magnetic Properties of Organic Solid-State Materials IV], 885-890, Daneshvar *et al.* (1999) *J. Immunol. Methods 226(1-2):*119-128, Rapaport *et al.* (1999) *Appl. Phys. Lett. 74(3):*329-331 and Durig *et al.* (1993) *J. Raman Spectrosc. 24(5):*281-285. Any suitable infrared spectroscopic method may be employed to interrogate the infrared dye. For instance, fourier transform infrared spectroscopy as, for example, described by Rahman *et al.* (1998) *J. Org. Chem. 63:*6196 may be used in this regard.

[0096] Suitably, electromagnetic scattering may result from diffraction, reflection, polarization or refraction of the incident electromagnetic radiation including light and X-rays. Such scattering can be used to quantitate the level of mRNA or level of protein.

[0097] Flow cytometry is particularly useful in analyzing fluorophore emission.

[0098] As is known in the art, flow cytometry is a high throughput technique which involves rapidly analyzing the physical and chemical characteristics of particles (e.g.

labeled mRNA, DNA or proteins) as they pass through the path of one or more laser beams while suspended in a fluid stream. As each particle intercepts the laser beam, the scattered light and fluorescent light emitted by each cell or particle is detected and recorded using any suitable tracking algorithm as, for example, described hereunder.

[0099] A modern flow cytometer is able to perform these tasks up to 100,000 cells/particles s^{-1}. Through the use of an optical array of filters and dichroic mirrors, different wavelengths of fluorescent light can be separated and simultaneously detected. In addition, a number of lasers with different excitation wavelengths may be used. Hence, a variety of fluorophores can be used to target and examine, for example, different immune effectors within a sample or immune effectors from multiple subjects.

[0100] Suitable flow cytometers which may be used in the methods of the present disclosure include those which measure five to nine optical parameters (see Table 2) using a single excitation laser, commonly an argon ion air-cooled laser operating at 15 mW on its 488 nm spectral line. More advanced flow cytometers are capable of using multiple excitation lasers such as a HeNe laser (633 nm) or a HeCd laser (325 nm) in addition to the argon ion laser (488 or 514 nm).

Table 2

Exemplary optical parameters which may be measured by a flow cytometer.

Parameter	Acronym	Detection angle form incident laser beam	Wavelength (nm)
Forward scattered light	FS	2-5°	488*
Side scattered light	SS	90°	488*
"Green" fluorescence	FL1	90°	510-540†
"Yellow" fluorescence	FL2	90°	560-580†
"Red" fluorescence	FL3	90°	>650#

* using a 488 nm excitation laser

† width of bandpass filter

\# longpass filter

[0101] For example, Biggs *et al.* (1999) *Cytometry 36:*36-45 have constructed an 11-parameter flow cytometer using three excitation lasers and have demonstrated the use of nine distinguishable fluorophores in addition to forward and side scatter measurements for purposes of immunophenotyping (i.e. classifying) particles. Selection of parameters can be adequately used depends heavily on the extinction coefficients, quantum yields and amount of spectral overlap between all fluorophores (Malemed *et al.* (1990) *"Flow cytometry and sorting"*, 2nd Ed., New York, Wiley-Liss). It will be understood that the present disclosure is not restricted to any particular flow cytometer or any particular set of parameters. In this regard, the disclosure also contemplates use in place of a conventional flow cytometer, a microfabricated flow cytometer as, for example, disclosed by Fu *et al.* (1999) *Nature Biotechnology 17:*1109-1111.

[0102] The assay enabled herein may be automated or semi-automated for high throughput screening or for screening for a number of immune effectors from the one subject. The automation is conveniently controlled by computer software.

[0103] The present disclosure further contemplates therefore web-based and non-web-based systems where data on the cell-mediated immunoresponsiveness of a subject are provided by a client server or other architecture platform to a central processor which analyses and compares to a control and optionally considers other information such as patient age, sex, weight and other medical conditions and then provides a report, such as, for example, a risk factor for disease severity or progression or status or an index of probability of disease development. A business method is therefore also provided whereby blood is collected in transportable tubes which is then analyzed for cell-mediated immunoresponsiveness at a defined location and the results then sent in the form of an electronic report *via* a client server or other architecture platform to a clinical care provider.

[0104] Hence, knowledge-based computer software and hardware also form part of the present disclosure. This facilitates clinical care to ascertain whether a disease condition

including infection, cancer of inflammation or a medicament or toxicant is inducing or is associated with immunosuppression.

[0105] The assays enabled by the instant disclosure may be used in existing or newly developed knowledge-based architecture or platforms associated with pathology services. For example, results from the assays are transmitted *via* a communications network (e.g. the internet) or telephone connection to a processing system in which an algorithm is stored and used to generate a predicted posterior probability value which translates to the index of cell-mediated immunoresponsiveness or immunosuppression which is then forwarded to an end user in the form of a diagnostic or predictive report. This report may also form the basis of clinical care management and personalized medicine.

[0106] The assay may, therefore, be in the form of a kit or computer-based system which comprises the reagents necessary to detect the concentration of the immune effector molecule following exposure of lymphocytes to at least two sets of peptides, a first set comprising at least one peptide of from about 7 to 14 amino acid residues in length and a second set comprising at least one peptide of from 16 amino acid residues or greater which peptides encompass all or part of a protein antigen and the computer hardware and/or software to facilitate determination and transmission of reports to a clinician.

[0107] For example, the present disclosure contemplates a method of allowing a user to determine the status of cell-mediated immunoresponsiveness of a subject, the method including:

(a) receiving data in the form of levels or concentrations of an immune effector molecule which, relative to a control, provide a correlation as the state of cell-mediated immunoresponsiveness in a subject, *via* a communications network, the immune effector molecule measured after exposure of lymphocytes to at least two sets of peptides, a first set comprising at least one peptide of from about 7 to 14 amino acid residues in length and a second set comprising at least one peptide of from 16 amino acid residues or greater which peptides encompass all or part of a protein antigen;

(b) processing the subject data *via* univariate or multivariate analysis to provide

an immunoresponsiveness value;

(c) determining the status of the subject in accordance with the results of the immunoresponsiveness value in comparison with predetermined values; and

(d) transferring an indication of the status of the subject to the user *via* the communications network.

[0108] Reference to the "univariate" or "multivariate" analysis includes an algorithm which performs the univariate or multivariate analysis function.

[0109] Conveniently, the method generally further includes:

(a) having the user determine the data using a remote end station; and

(b) transferring the data from the end station to the base station *via* the communications network.

[0110] The base station can include first and second processing systems, in which case the method can include:

(a) transferring the data to the first processing system;

(b) transferring the data to the second processing system; and

(c) causing the first processing system to perform the univariate or multivariate analysis function to generate the cell-mediated immunoresponsiveness value.

[0111] The method may also include:

(a) transferring the results of the univariate or multivariate analysis function to the first processing system; and

(b) causing the first processing system to determine the status of the subject.

[0112] In this case, the method also includes at least one of:

(a) transferring the data between the communications network and the first processing system through a first firewall; and

(b) transferring the data between the first and the second processing systems through a second firewall.

[0113] The second processing system may be coupled to a database adapted to store predetermined data and/or the univariate or multivariate analysis function, the method including:

(a) querying the database to obtain at least selected predetermined data or access to the univariate or multivariate analysis function from the database; and

(b) comparing the selected predetermined data to the subject data or generating a predicted probability index of a level of cellular immunoresponsiveness or immunosuppression.

[0114] The second processing system can be coupled to a database, the method including storing the data in the database.

[0115] The method can also include causing the base station to:

(a) determine payment information, the payment information representing the provision of payment by the user; and

(b) perform the comparison in response to the determination of the payment information.

[0116] The present disclosure also provides a base station for determining the status of a subject with respect to cell-mediated immunoresponsiveness or immunosuppression, the base station including:

(a) a store method;

(b) a processing system, the processing system being adapted to:

(c) receive subject data from the user *via* a communications network, the data including levels of immune effector molecule wherein the level of the effector molecule relative to a control provides a correlation to the state of cell-mediated immunoresponsiveness wherein the immune effector molecule is determined after exposure of lymphocytes to at least two sets of peptides, a first set comprising at least one peptide of from about 7 to 14 amino acid residues in length and/or a second set comprising

at least one peptide of from 16 amino acid residues or greater which peptides encompass all or part of a protein antigen;

(d) performing an algorithmic function including comparing the data to predetermined data;

(e) determining the status of the subject in accordance with the results of the algorithmic function including the comparison; and

(c) output an indication of the status of the subject to the user *via* the communications network.

[0117] The processing system can be adapted to receive data from a remote end station adapted to determine the data.

[0118] The processing system may include:

(a) a first processing system adapted to:

(i) receive the data; and

(ii) determine the status of the subject in accordance with the results of the univariate or multivariate analysis function including comparing the data; and

(b) a second processing system adapted to:

(i) receive the data from the processing system;

(ii) perform the univariate or multivariate analysis function including the comparison; and

(iii) transfer the results to the first processing system.

[0119] The processing system can be coupled to a database, the processing system being adapted to store the data in the database.

[0120] In accordance with this embodiment, levels of the immune effector molecule may be screened alone or in combination with other biomarkers or disease indicators. An "altered" level means an increase or elevation or a decrease or reduction in the concentrations of the immune effector molecule.

[0121] The determination of the concentrations or levels of the immune effector molecule enables establishment of a diagnostic rule based on the concentrations relative to controls. Alternatively, the diagnostic rule is based on the application of a statistical and machine learning algorithm. Such an algorithm uses relationships between effector molecule and disease status observed in training data (with known disease or cell-mediated immunoresponsiveness status) to infer relationships which are then used to predict the status of subjects with unknown status. An algorithm can be employed which provides an index of probability that a subject has a certain level of cell-mediated immunoresponsiveness and/or a disease condition. The algorithm performs a univariate or multivariate analysis function.

[0122] Hence, the present disclosure provides a diagnostic rule based on the application of statistical and machine learning algorithms. Such an algorithm uses the relationships between immune effector molecule and level of cell-mediated immunoresponsiveness or immunosuppression observed in training data (with known immune status) to infer relationships which are then used to predict the status of patients with unknown immune status. Practitioners skilled in the art of data analysis recognize that many different forms of inferring relationships in the training data may be used without materially changing the present disclosure.

[0123] The present disclosure further contemplates the use of a knowledge base of training data comprising levels of immune effector molecule from a subject with a known cell-mediated immunoresponsiveness level to generate an algorithm which, upon input of a second knowledge base of data comprising levels of the same immune effector molecule from a subject with an unknown immunoresponsiveness level, provides an index of probability that predicts the nature of the cell-mediated immunoresponsiveness.

[0124] The term "training data" includes knowledge of levels of immune effector molecule relative to a control wherein the immune effector molecule is determined after exposure of lymphocytes at least two sets of peptides, a first set comprising at least one peptide of from about 7 to 14 amino acid residues in length and a second set comprising at least one

peptide of from 16 amino acid residues or greater which peptides encompass all or part of a protein antigen. A "control" includes a comparison to levels of immune effector molecule in a subject with "normal" immunoresponsiveness or may be a statistically determined level based on trials.

[0125] Hence, the term "training data" includes levels of an immune effector molecule.

[0126] The levels or concentrations of the immune effector molecule provide the input test data referred to herein as a "second knowledge base of data". The second knowledge base of data either is considered relative to a control or is fed into an algorithm generated by a "first knowledge base of data" which comprise information of the levels of an immune effector in a subject with a known immunological status. The second knowledge base of data is from a subject of unknown status with respect to cell mediated immunoresponsiveness. The output of the algorithm or the comparison to a control is a probability or risk factor, referred to herein as "an index of probability", of a subject having a certain level of immunoresponsiveness or immunosuppressive.

[0127] Data generated from the levels of immune effector molecule are input data. The input of data comprising the immune effector levels is compared with a control or is put into the algorithm which provides a risk value of the likelihood that the subject has, for example, an immunosuppressive condition. A treatment regime can also be monitored to ascertain the presence of any immunosuppression. A level of immunosuppression may increase the risk of a subject getting a secondary infection or having a relapse (e.g. during cancer therapy or treatment of a pathogenic infection).

[0128] As described above, methods for diagnosing an immunoresponsiveness or immunosuppressive condition by determining the extent to which a subject can mount an innate and/or adaptive immune response *via* a level of an immune effector molecule provides a second knowledge base data in an algorithm generated with first knowledge base data or levels of the same effector molecule in subjects with a known immune status. Also provided are methods of detecting immunoresponsiveness comprising determining

the presence and/or velocity of an immune effector molecule following stimulation of the innate and/or adaptive immune system in a subject's sample. By "velocity" it is meant the change in the concentration of the effector molecule in a subject's sample over time.

[0129] As indicated above, the term "sample" as used herein means any sample containing one or more lymphocytes including, but not limited to, whole blood, a whole blood fraction, tissue extracts and freshly harvested cells.

[0130] The method of the subject disclosure may be used in the diagnosis and staging of a disease. The present disclosure may also be used to monitor the progression of a condition and to monitor whether a particular treatment is effective or not. In particular, the method can be used to monitor immunosuppression following surgery, cancer therapy or other or medication or exposure to toxicants.

[0131] In an embodiment, the subject disclosure contemplates a method for monitoring for immunosuppression in a subject, comprising:

(a) providing a sample from a subject;

(b) determining the level of an immune effector molecule following stimulation by at least two sets of peptides, a first set comprising at least one peptide of from about 7 to 14 amino acid residues in length and a second set comprising at least one peptide of from 16 amino acid residues or greater which peptides encompass all or part of a protein antigen;

wherein the level of the immune effector relative to a control provides a correlation to the state of cell-mediated immunoresponsiveness and subjecting the levels to an algorithm to provide an index of probability of the subject having a certain level of immunoresponsiveness; and

(c) repeating steps (a) and (b) at a later point in time and comparing the result of step (b) with the result of step (c) wherein a difference in the index of probability is indicative of the progression of the condition in the subject.

[0132] Reference to an "algorithm" or "algorithmic functions" as outlined above includes

the performance of a univariate or multivariate analysis function. A range of different architectures and platforms may be implemented in addition to those described above. It will be appreciated that any form of architecture suitable for implementing the present disclosure may be used. However, one beneficial technique is the use of distributed architectures. In particular, a number of end stations may be provided at respective geographical locations. This can increase the efficiency of the system by reducing data bandwidth costs and requirements, as well as ensuring that if one base station becomes congested or a fault occurs, other end stations could take over. This also allows load sharing or the like, to ensure access to the system is available at all times.

[0133] In this case, it would be necessary to ensure that the base station contains the same information and signature such that different end stations can be used.

[0134] It will also be appreciated that in one example, the end stations can be hand-held devices, such as PDAs, mobile phones, or the like, which are capable of transferring the subject data to the base station *via* a communications network such as the Internet, and receiving the reports.

[0135] In the above aspects, the term "data" means the levels or concentrations of the immune effector following stimulation by a series of overlapping peptides from about 7 to 14 amino acid residues in length which encompass the entire length of a protein antigen. The "communications network" includes the internet and mobile telephone network and telephone land line. When a server is used, it is generally a client server or more particularly a simple object application protocol (SOAP).

[0136] One aspect of the present disclosure includes experiments that demonstrate the cell-mediated immune responsiveness of a subject by measuring responsiveness to at least two sets of peptides, a first set comprising at least one peptide of from about 7 to 14 amino acid residues in length and a second set comprising at least one peptide of from 16 amino acid residues or greater which peptides encompass all or part of a protein antigen. In an embodiment, one or more samples such as a sample of peripheral blood, of enriched white

cell fraction of blood or bronchoalveolar lavage may be obtained from a subject having or suspected of development of a particular disease (e.g. autoimmune disease, infection to a pathogenic agent or exposure to a proteinaceous toxicant) and the immune responsiveness measured by determination of effector molecules from effector T-cells (e.g. $CD4^+$ T-cells and $CD8^+$ T-cells).

[0137] The immunobinding methods include methods for detecting or quantifying the amount of a reactive component in a sample, which methods require the detection or quantitation of any immune complexes formed during the binding process. Here, one would obtain a sample suspected of containing a cytokine following stimulation of lymphocytes by at least two sets of peptides, a first set comprising at least one peptide of from about 7 to 14 amino acid residues in length and a second set comprising at least one peptide of from 16 amino acid residues or greater which peptides encompass all or part of a protein antigen and contacting the sample with an antibody and then detecting or quantifying the amount of immune complexes formed under the specific conditions.

[0138] Contacting the chosen biological sample with the antibody under conditions effective and for a period of time sufficient to allow the formation of immune complexes (primary immune complexes) is generally a matter of adding the composition to the sample and incubating the mixture for a period of time long enough for the antibodies to form immune complexes with, i.e. to bind to, any effector molecules present. After this time, the sample-antibody composition, such as a tissue section, ELISA plate, ELISpot, dot blot or Western blot, will generally be washed to remove any non-specifically bound antibody species, allowing only those antibodies specifically bound within the primary immune complexes to be detected.

[0139] In a particular embodiment, the present disclosure teaches a method for detecting the presence, absence, level or stage of a disease or condition in a human subject, the method comprising contacting whole blood, which comprises at least 10% of the total volume in a reaction mixture, with at least two sets of peptides, a first set comprising at least one peptide of from about 7 to 14 amino acid residues in length and a second set

comprising at least one peptide of from 16 amino acid residues or greater which peptides encompass all or part of a protein antigen and measuring the presence or elevation in the level of an immune effector molecule from T-cells wherein the presence or level of the immune effector molecule is indicative of the disease or condition.

[0140] In a further embodiment, the present disclosure enables kits for use with the methods described above. In one embodiment, an immunodetection kit is contemplated. In another embodiment, a kit for analysis of a sample from a subject having or suspected of developing a metal or chemically-induced disease is contemplated. In a more particular embodiment, a kit for analysis of a sample from a subject having or suspected of developing a disease is contemplated. In an embodiment, a kit is for assessing the cell-mediated immune responsiveness of a subject before or after a disease state has developed or before or after a subject is given a medicament or is exposed to a toxicant or pollutant. If an antigen is also employed, the kit may also comprise a particular antigen.

[0141] The immunodetection reagents of the kit may take any one of a variety of forms, including those detectable labels that are associated with or linked to the given antibody or antigen, and detectable labels that are associated with or attached to a secondary binding ligand. Exemplary secondary ligands are those secondary antibodies that have binding affinity for the first antibody or antigen, and secondary antibodies that have binding affinity for a human antibody.

[0142] Further suitable immunodetection reagents for use in the present kits include the two-component reagent that comprises a secondary antibody that has binding affinity for the first antibody or antigen, along with a third antibody that has binding affinity for the second antibody, the third antibody being linked to a detectable label.

[0143] The kits may further comprise a suitably aliquoted composition of antigen or effector molecule, whether labeled or unlabeled, as may be used to prepare a standard curve for a detection assay.

[0144] The kits may contain antibody-label conjugates either in fully conjugated form, in the form of intermediates, or as separate moieties to be conjugated by the user of the kit. The components of the kits may be packaged either in aqueous media or in lyophilized form.

[0145] The container means of any of the kits generally includes at least one vial, test tube, flask, bottle, syringe or other container means, into which the testing agent, the antibody or antigen may be placed, and generally, suitably aliquoted. Where a second or third binding ligand or additional component is provided, the kit will also generally contain a second, third or other additional container into which this ligand or component may be placed. The kits taught by the present disclosure also typically include a means for containing the antibody, peptides derived from an antigen and any other reagent containers in close confinement for commercial sale. Such containers may include injection or blow-molded plastic containers into which the desired vials are retained.

[0146] Also contemplated herein is an improved assay to detect a cell-mediated immune response or the level thereof in a subject, the assay comprising incubating lymphocytes from the subject with an antigen and detecting for the presence of or elevation in effector molecules, the improvement comprising incubating the lymphocytes with at least two sets of peptides, a first set comprising at least one peptide of from about 7 to 14 amino acid residues in length and a second set comprising at least one peptide of from 16 amino acid residues or greater which peptides encompass all or part of a protein antigen.

[0147] The present disclosure further provides a method of treatment of a subject having a pathogenic infection, an autoimmune disorder or cancer or a propensity for developing such a condition or disorder, the method comprising contacting a source of lymphocytes from the subject with at least two sets of peptides, a first set comprising at least one peptide of from about 7 to 14 amino acid residues in length and a second set comprising at least one peptide of from 16 amino acid residues or greater which peptides encompass all or part of a protein antigen and measuring the presence or elevation in the level of an immune effector molecule from T-cells wherein the presence or level of the immune

effector molecule is indicative of the level of cell-mediated responsiveness of the subject which is indicative of the presence, absence, level or state of the condition or disorder and then treating the condition or disorder.

[0148] Aspects taught herein are further described by the following non-limiting Examples.

EXAMPLE 1

Development of Assay

[0149] Heparinized blood samples are collected into Li-Hep Vacuette [Registered Trade Mark] tubes (Greiner Bio-one, Germany).

[0150] Aliquots of the blood samples were incubated with at least two sets of peptides, a first set comprising at least one peptide of from about 7 to 14 amino acid residues in length (recognizing $CD8^+$ T-cells) and a second set comprising at least one peptide of from 16 amino acid residues or greater which peptides (recognizing $CD4^+$ T-cells) encompass all or part of a protein antigen, wherein the antigen is selected from *M. tuberculosis* CFP10, ESAT-6, TB7.7 and TB37.6.

[0151] In some experiments, glucose is added at various concentrations to the blood before initiation of incubation.

[0152] Stimulated blood samples were incubated for 1 to 48 hours including 16-24 hours in the presence of the antigen peptides at 37°C, after which plasma was harvested from above the settled blood cells. The amount of IFN-γ present in each plasma sample was then quantified using the Quantiferon-TB [Registered Trade Mark] ELISA (Cellestis Limited, Australia) as per the manufacturer's instructions. Sample IFN-γ was alternatively quantified using the more sensitive Quantiferon-TB Gold [Registered Trade Mark] ELISA (Cellestis Limited, Australia) as per the manufacturer's instructions.

[0153] ELISA optical density values for IFN-γ standards run on each ELISA plate were used to construct a standard curve from which the amount of IFN-γ present in each of the test plasma samples was converted to IU/mL values.

EXAMPLE 2

Boosting responses in the QuantiFERON-TB tubes by the addition of $CD8^+$ TB-specific peptides to the $CD4^+$ peptides

[0154] These studies were carried out in a group of patients with a clinically confirmed active tuberculosis infection. Patients were tested with the current QuantiFERON-TB In-Tube diagnostic test. The QuantiFERON-TB tube contains peptide pools specific for $CD4^+$ T-cells (> 15 mer peptides). Patients were also tested using a modified tube in which pools of peptides specific for $CD8^+$ T-cells had been added (10 mer peptides). These additional peptides were tested as a complete pool of 91 peptides, and as two smaller pools. These pools were also added to QuantiFERON-Nil tubes as controls to assess background responses to these peptides alone.

[0155] In the patients with active TB disease, adding the $CD8^+$ peptides to the QuantiFERON-TB tubes resulted in a 10% increase in sensitivity compared to the current QuantiFERON-TB test.

EXAMPLE 3

Diagnosis of TB using CF10 peptide pools

[0156] The aim of this Example was to test whether TB antigens, designed to be recognized by $CD8^+$ T-cells (10 mer peptides), are able to induce the production of detectable levels of IFN-γ in blood from patients with an active TB infection. It was proposed that the use of MHC class I-restricted peptides (termed "$CD8^+$ peptides") alone, or in conjunction with the current peptides, would improve the sensitivity of the diagnosis of TB. This is particularly relevant in HIV-infected individuals who have reduced numbers of $CD4^+$ T-cells.

[0157] A total of 91 peptides, each 10 amino acids in length, covering the entire length of the CFP10 *Mtb*protein were pooled together in three pools. Pool 1 contained all 91 peptides, Pool 2 contained peptides covering the first half of the CFP10 protein (peptides 1-45) and Pool 3 contained peptides covering the second half of the CFP10 protein (peptides 46-91). Pools were tested alone (added to a Nil tube) or in combination with the current QFT-TB Gold antigen tube.

[0158] In total, 63 patients were recruited. Of these, 50 patients tested positive to the QuantiFERON-TB Gold In-tube test. 31 patients were recruited who had active TB disease confirmed by culture (or clinical symptoms in one case) and 19 patients recruited were TB suspects.

Patient information:

TB disease: 31 (one not confirmed by culture)

TB suspects: 19

HHC: 13

Total # of patients: 63

[0159] The results are shown in Table 3 through 7 and in Figure 1 and show the qualitative results for peptide pools tested in combination with a QFT-TB Gold antigen tube.

Table 3

Patients with TB disease (n=31):

	QFT-TB Gold	Pool 1	Pool 2	Pool 3
Indeterminate	0	0	0	0
Negative	3	0	1	0
Positive	28	31	30	31
Sensitivity	*90%*	*100%*	*97%*	*100%*

Table 4

TB suspects (n=19):

	QFT-TB Gold	Pool 1	Pool 2	Pool 3
Indeterminate	0	0	0	0
Negative	9	7	6	7
Positive	10	12	13	12

Table 5

HHC Patients (n=3):

	QFT-TB Gold	Pool 1	Pool 2	Pool 3
Indeterminate	0	0	0	0
Negative	0	0	0	0
Positive	3	3	3	3

[0160] A quantitative analysis was performed to examine the effects of adding the pools of $CD8^+$ peptides to existing tubes of $CD4^+$ peptides, with regard to boosting the IFN-γ response, in comparison to the QFT-TB Gold antigen tube alone. A boost in the IFN-γ response was defined as an increase > 1.5 fold of the QFN TB result. Evaluable patients exclude those with a response that converted/reverted or where not all plasma samples were quantifiable. Figure 1 indicates the mean IFN-γ response to the QFT-TB alone or in combination with each pool.

Table 6

Evaluable Patients with TB disease (n=24):

	Pool 1	Pool 2	Pool 3
Boost (>1.5x QFN TB result)	17	20	19
No boost	6	4	5
% Boost (*of evaluable patients*)	74%	83%	79%

Table 7

Evaluable TB suspects (n=6):

	Pool 1	Pool 2	Pool 3
Boost (>1.5x QFN TB result)	0	0	0
No boost	6	6	6
% Boost (*of evaluable patients*)	0%	0%	0%

[0161] These data indicate that the addition of the $CD8^+$ peptides to the current QFT-TB Gold In Tube assay (which contains $CD4^+$ peptides) results in a 10% increase in assay sensitivity. Furthermore, the data indicate that an increase in response magnitude ("boost") of greater than 1.5-fold resulting from the addition of the CD8 peptides is only apparent when the assay is performed on patient samples derived from subjects with confirmed active TB-disease. These data indicate that the addition of CD8 peptides to the current QFT-TB Gold In Tube assay differentiates between subjects with active versus latent TB disease.

EXAMPLE 4

Effect of CFP10 CD8$^+$ peptides on assay specificity

[0162] The aim of this Example was to investigate if the addition of the CFP10 CD8$^+$ peptides to the QFT-TB Gold tube results in a reduction in the assay specificity. Therefore, peptide pools were used in conjunction with the QFT-TB Gold In Tube assay (which contain CD4$^+$ peptides) in a population of healthy control donors recruited from a country with low TB incidence (Melbourne, Australia).

[0163] A total of 91 peptides, each 10 amino acids in length, covering the entire length of the CFP10 *Mtb*protein were pooled together in three pools. Pool 1 contained peptides covering the first half of the CFP10 protein (peptides 1-45), Pool 2 contained peptides covering the second half of the CFP10 protein (peptides 46-91) and Pool 3 contained all 91 peptides.

[0164] In total, 92 subjects were recruited. Of these, 3 subjects tested positive to the QuantiFERON-TB Gold In Tube test. No QFT-TB negative subjects showed a response to any of the peptide pools. The results are shown in Tables 8 and 9.

Table 8

	QFT-TB Gold	Pool 1	Pool 2	Pool 3
Indeterminate	0	0	0	0
Negative	89	89	89	89
Positive	3	3	3	3

[0165] Of the 3 QFT-TB positive donors, no boost in the IFN-γ response was observed with the addition of the peptide pools (increase > 1.5 fold of the QFN TB result).

Table 9

	Pool 1	Pool 2	Pool 3
Boost (>1.5x QFN TB result)	0	0	0
No boost	3	3	3
% Boost (of evaluable patients)	0%	0%	0%

[0166] These data indicate that the addition of the $CD8^+$ peptides to the current QFT-TB Gold In Tube assay does not negatively impact the assay specificity.

EXAMPLE 5

Combination of CMV 16 mer $CD4^+$ with peptide $CD8^+$ peptides in a QFT-CMV assay

[0167] This Example investigated whether if the addition of the $CD8^+$ peptides enhanced the response in a QFT-CMV assay using a 16 mer peptide from the CMV antigen pp65.

[0168] Blood from three healthy donors, with a positive CMV serology result, was used in the QFT-CMV assay with the addition of 1) a Nil tube containing 1μg/ml (final concentration) of the 16-mer peptide, and 2) a QFT-CMV tube with the addition of 1μg/ml (final concentration) of the 16-mer peptide. The assay was performed according to the manufacturer's instructions.

[0169] 0/3 donors responded to the 16-mer peptide alone. 3/3 donors responded to the 16 mer + $CD8^+$-peptides in the QFT-CMV assay. The results are shown graphically in figure 1 below.

[0170] No response to the 16 mer peptide was observed in any donor. All donors showed a positive response to the 16 mer peptide in combination with the $CD8^+$ peptides in the QFT-CMV assay.

[0171] Those skilled in the art will appreciate that aspects of the subject matter described. It is to be understood that the disclosure encompasses all such variations and modifications. The disclosure also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of the steps or features.

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CLAIMS:

1. A method for measuring cell-mediated immune response activity in a subject, said method comprising co-incubation of a sample of whole blood containing lymphocytes from the subject with a first and second set of peptides, wherein the whole blood comprises from 50% to 100% by volume of the sample to be assayed and wherein the first set comprises peptides of from 7 to 14 amino acid residues in length which are recognised by $CD8^+$ lymphocytes and the second set comprises peptides of from 16 amino acid residues or greater which are recognised by $CD4^+$ lymphocytes which peptides encompass all or part of a protein antigen and measuring the presence or elevation in the level of an immune effector molecule from immune cells wherein the presence or level of the immune effector molecule is indicative of the level of cell-mediated responsiveness of the subject.

2. The method of Claim 1 wherein the subject is a human.

3. The method of Claim 1 wherein the sample is undiluted whole blood.

4. The method of Claim 1 or 3 wherein the whole blood is collected in a tube comprising heparin.

5. The method of Claim 1 wherein the immune effector molecule is a cytokine.

6. The method of Claim 5 wherein the cytokine is IFN-γ.

7. The method of Claim 1 wherein the immune effector molecule is detected with antibodies specific for same.

8. The method of Claim 7 wherein the immune effector molecule is detected using ELISA.

9. The method of Claim 8 wherein the immune effector molecule is detected using ELISpot.

10. The method of Claim 1 wherein the subject has an infection by a pathogenic agent selected from *Mycobacterium* species, *Staphylococcus* species, *Streptococcus* species, *Borrelia* species, *Escherichia coli*, *Salmonella* species, *Clostridium* species, *Shigella* species, *Proteus* species, *Bacillus* species, Herpes virus, Hepatitis B or C virus and Human immune deficiency virus (HIV) or a disease resulting therefrom.

11. The method of Claim 10 wherein the infection is by *Mycobacterium tuberculosis* or tuberculosis (TB).

12. The method of Claim 11 wherein the antigen is selected from CFP10, ESAT-6, TB7.7 and TB37.6.

13. The method of Claim 1 wherein the subject has Celiac's disease.

14. The method of Claim 1 wherein the subject has autoimmune diabetes.

15. The method of Claim 1 wherein the subject has cancer.

16. The method of Claim 1 wherein the subject was exposed to a proteinaceous toxicant.

17. The method of any one of Claims 1 to 16 wherein the magnitude of the cell-mediated immune response correlates with the state, progression and/or severity of a disease condition.

18. Use of at least two sets of peptides, a first set comprising peptides of from 7 to 14 amino acid residues in length which are recognised by $CD8^+$ lymphocytes and a second set comprising peptides of from 16 amino acid residues or greater which are recognised by $CD4^+$ lymphocytes which peptides encompass all or part of a protein antigen in the manufacture of a diagnostic assay of cell-mediated immune responsiveness by the method of co-incubation of the peptides with lymphocytes and detecting the presence or elevation in effector molecules.

19. A method of allowing a user to determine the status of cell-mediated immunoresponsiveness of a subject, the method including:

(a) receiving data in the form of levels or concentrations of an immune effector molecule which relative to a control provide a correlation to the state of cell-mediated immunoresponsiveness from the user *via* a communications network, the immune effector molecule measured after co-incubation of a sample of whole blood containing lymphocytes from the subject with a first and second set of peptides, wherein the whole blood comprises from 50% to 100% by volume of the sample to be assayed and wherein the first set comprises peptides of from 7 to 14 amino acid residues in length which are recognised by $CD8^+$ lymphocytes and the second set comprises peptides of from 16 amino acid residues or greater which are recognised by $CD4^+$ lymphocytes which peptides encompass all or part of a protein antigen;

(b) processing the subject data *via* univariate or multivariate analysis to provide an immunoresponsiveness value;

(c) determining the status of the subject in accordance with the results of the immunoresponsiveness value in comparison with predetermined values; and

(d) transferring an indication of the status of the subject to the user *via* the communications network.