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(54) **IMMUNE RESPONSE ASSESSMENT METHOD**

VERFAHREN ZUR BEURTEILUNG VON IMMUNREAKTIONEN

PROCÉDÉ D'ÉVALUATION DE RÉPONSE IMMUNITAIRE

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Description

TECHNICAL FIELD OF INVENTION

[0001] This application relates to methods for the identification of immunomodulatory peptides, and more particularly, to a novel method for the identification of antigenic peptides by multiplex analysis of the immune response.

BACKGROUND OF THE INVENTION

[0002] The immune system provides protection against microbial insult. It is by its very essence a complex system as it must be able to recognize and adapt to ever-changing threats while limiting collateral damage to self tissue. It is a consequence of this complexity that immune dysfunction, both hyper- and hypo-activity, are at the root of many human diseases. The adaptive immune system (composed of B cells and T cells) recognizes antigens in the form of molecular patterns, such as peptides, glycopeptides, phosphopeptides and lipids, bound to and presented in the context of specialized classes of antigen-presenting molecules. The antigen-derived fragments, for example, peptides that are recognized by the T-cells, are referred to as epitopes. It is widely accepted that the recognition by a T-cell of its specific epitope is the central event in adaptive immune function.

[0003] These specialized antigen-presenting molecules include: i) the classic human leukocyte antigen (HLA) proteins which are highly polymorphic and can be separated into two major classes, Class I and Class II; ii) the non-classic group that is encoded by the HLA E, F, and G subloci (Braud VM, Curr Opin Immunol. 1999 Feb;11(1):100-8), and iii) the CD1 family of antigen-presenting molecules (Bendelac A, Science. 1995 Jul 14;269(5221):185-6). HLA Class I molecules are encoded by HLA A, B and C loci and present 8-10 amino acid (AA) peptides to CD8+ cytotoxic T cells (Adams EJ, Immunol Rev. 2001 Oct;183:41-64). HLA Class II molecules present 12-16 AA peptides to CD4+ helper T cells and are encoded by the HLA DR, DP and DQ loci (Korman AJ, Immunol Rev. 1985 Jul;85:45-86). T-cells recognize antigens only in the context of antigen-presenting molecules, and only after appropriate antigen processing and presentation. For HLA dependent antigen presentation, the antigen processing usually consists of proteolytic fragmentation of the protein, resulting in polypeptides that fit into the groove of the HLA molecule and this peptide/HLA complex is subsequently presented on the cell surface of the antigen-presenting cell to the T-cell (Rock, K, Adv Immunol. 2002;80:1-70, Cresswell P, Curr Biol. 1994 Jun 1;4(6):541-3). The non-classic group is encoded by the HLA E, F, and G subloci and is characterized by a low degree of polymorphism and an ability to bind and present a restricted set of peptides to immune effector cells. Recognition of peptides presented by non-classic HLA molecules has been demonstrated to be important in inducing immune tolerance in areas such as the maternal fetal interface and/or tumor immunity. The other important group among these specialized molecules is the CD1 family, which is involved in the presentation of lipids such as bacterial glycolipids to specialized effector cells in the immune system.

[0004] Three major types of T cell immunity may follow antigen recognition: Type-1 (Th1), Type-2 (Th2), and Regulatory (T-Regulatory). Type-1 immunity is important for the clearance of intracellular pathogens and anti-tumor immunity, and is primarily characterized by the expression of IFN gamma (IFN-γ). Type-2 immunity is critical for the clearance of extracellular pathogens and is primarily characterized by the expression of cytokines such as IL-4, IL-5, IL-9 and IL-13. T-Regulatory type of immunity, mediated by cytokines such as IL-10 or TGF-beta, is essential for the generation and maintenance of self-tolerance. These three types of response are not restricted to the classical alpha/beta TCR+ CD8+ T cells and can also be mediated by gamma/delta TCR+ CD8+ T cells as well as natural killer (NK) cells and NK T cells. Thus, recognition of specific antigenic epitopes by any of these cell types leads to their activation and distinctive, specialized response. The quality of an immune response may therefore be described by observing the coordinated expression of cytokine patterns (Type-1, Type-2, and T-Regulatory).

[0005] Activation of appropriate T-cell response during the course of an immune reaction often involves dominance of one specialized response over the others. This selective activation of an appropriate specialized T-cell response is a critical determinant for disease outcome. A striking example of this is demonstrated in patient response in leprosy, wherein a Type-1 response to infection is characterized by protective immunity and a Type-2 response often leads to fatal disease. In another dramatic example regarding tumor immunity, tolerogenic epitopes leading to an inappropriate shift toward a T-Regulatory response have been shown to result in subsequent tumor progression in humans, while activation of Type-1 responses by tumor antigen epitopes are associated with the inhibition of tumor growth. Furthermore, an inappropriate shift away from a T-Regulatory response may lead to the development of autoimmune disease if self antigen is presented. The specific clinical picture of autoimmune disease would depend on the dominant response: either Type-1, as in the case of Type-1 diabetes, or Type-2, as in the case of allergy.

[0006] As the activation of these specialized T-cell responses is dependent on presentation of specific epitopes to them, it would be advantageous therefore to identify these peptide epitopes. By identifying these specialized T-cell epitopes early on in the disease state, the opportunity arises to enhance appropriate T-cell responses or eliminate inappropriate T-cell responses, and thereby shift the balance of the immune response to improve disease outcome. A

methodology for quantitative identification and qualitative characterization of these antigenic T-cell epitopes is an important basis for vaccine design as well as diagnostic and therapeutic approaches to infectious, autoimmune and neoplastic diseases.

[0007] The WO 2004/005925 A2 describes a method of diagnosing in an individual recent exposure to an antigen, being e.g. a pathogen or a vaccine. The method comprises determining in vitro whether T-cells of the individual recognise a protein from said agent.

[0008] The WO 99/36568 relates to the analysis of cytokines in a T-cell in order to determine a T-cell response to a peptide within 6 hours of exposure of the T-cell to the peptide.

[0009] Haselden et al., Journal of Allergy and Clinical Immunology, 108 (3), 2001: 349-356, disclose allergic reactions mediated by peripheral blood mononuclear cells.

[0010] Holen et al., Clinical and Experimental Allergy, 31(6), 2001: 952-964, discloses epitopes of the chicken ovomucoid which induce T-cell responses.

[0011] Early et al., Cytometry 50(5), 2002: 239-242 relates to multiplex immunoassays for human cytokines.

[0012] Karlsson et al., Journal of Immunology Methods 283(1-2), 2003: 141-153 relates to ELISPOT and cytokine flow cytometry for determining antigen-specific T-cells.

SUMMARY OF THE INVENTION

[0013] The present invention includes methods for identifying one or more immunomodulatory peptides by culturing isolated immune cells from a subject with a cluster of several overlapping peptides from an overlapping peptide library; analyzing simultaneously a supernatant of a culture medium for multiple parameters of immune reactivity comprising the specificity and cytokine response of cytokines and chemokines, which allow identification of Type-1, Type-2 and T-Regulatory cell phenotypes to the cluster of several overlapping peptides; culturing isolated immune cells from the subject with individual peptides from the cluster of overlapping peptides and analyzing simultaneously the supernatant of the culture medium for said cytokines and chemokines; and determining the type of immune response based on the coordinated cytokine expression in response to the individual peptides in the culture medium. For example, the reactive epitopes may be selected from the immunomodulatory peptides in a pool of peptides. The method may also include providing peptide specific effector cells from that specific individual.

[0014] Disclosed is a method for identifying one or more immunomodulatory fragments by: isolating immune cells from the subject; culturing the immune cells in the presence of one or more fragments from the processed peptide library; and simultaneously analyzing the culture for multiple parameters of immune specificity and cytokine response to the fragments, wherein an immunomodulatory fragment is identified by the presence of immune reactivity toward the fragment. Using this method, multiple peptide fragment-specific effector cells may be isolated and even purified, e.g., into a mix or clonal population. Also disclosed is a method for identifying the type of immune reactivity expressed by a subject towards an antigenic material by isolating and culturing one or more immune cells from a subject in the presence of one or more antigenic fragments; identifying the cytokines produced by the immune cells; and determining the type of immune reactivity based on the coordinated cytokine expression in response to the antigenic material. Antigenic material may include an antigen, a peptide, a microbe, a cell, and mixtures of combinations thereof. The antigenic protein may include peptides with known or unknown amino acid sequences, from known or unknown proteins and/or protein fragments, or even an overlapping peptide library taken from a known protein or peptides fractionated by size prior to use in the method and subsequent characterization. In some embodiment, the antigenic material may includes multiple antigenic materials and/or peptides that are provided in different ratios to drive the type of immune response, e.g., Th1 v. Th2 or Tc1 v. Tc2 or mixtures and combinations thereof.

[0015] The present invention also includes a method for assessing the immune status of a subject by identifying a series of immunomodulatory peptides as mentioned above, wherein the type of immune reactivity is indicative of the subject's immune status. For example, the method may assesses the degree of immunosuppression of the subject, the degree of hyperactivation of the immune system of the subject, the T-cell repertoire for the subject and even the type of immune reactivity, which may be indicative of the subject's risk of immune-related diseases. Non-limiting examples of immune related diseases that may be monitored using the present invention include: infectious diseases, autoimmune diseases, autoinflammatory disease, cancer, chronic inflammation and allergies.

[0016] Another use for the present invention is in a method for predicting a subject's response to therapy by determining the epitope-specific immune status of the subject as an indicator of treatment outcome. The therapy may be an immunotherapy such as vaccination, passive immunotherapy and adoptive cell transfer. Another example is a method for predicting a subject's disease course and clinical outcome by culturing one or more immune cells from the subject in the presence of a series of peptides from an overlapping peptide library taken from an antigenic protein of interest and analyzing the culture for cell proliferation and cytokine production profile to determine the type of immune reactivity to the immunomodulatory molecule, wherein the cytokine production profile is a marker of disease severity and status of disease progression or regression.

[0017] Also disclosed is a method of tailoring a therapeutic intervention for a subject by identifying one or more immunomodulatory molecule for the subject based on an analysis of multiple parameters of immune reactivity including an identification of the cytokines produced; and preparing an epitope-specific immune vaccine comprising the one or more immunomodulatory molecules. The vaccine may include, e.g., one or more antigens that: enhance or reduce a specific type of immune response in the subject; one or more antigens that enhance or reduce epitope-specific cytokine production in the subject; one or more antigens that enhance or reduce epitope-specific cytokine production in the subject; and/or one or more antigens that enhance or reduce antibody production or enhance cell mediated immunity in the subject.

[0018] Yet another disclosed method for immunomonitoring a subject includes immunizing the subject with one or more of the immunomodulatory peptides identified from the library; determining the epitope-specific immune status of the subject by isolating immune cells from the subject after exposure to the one or more immunomodulatory peptides; and comparing the type of immune response prior to and after immunization to determine changes in the subject's epitope-specific immune status.

[0019] In operation, the disclosed method for treatment of a subject in need of immunotherapy includes: exposing immune cells from the subject to one or more immunomodulatory peptides to determine the type of immune response triggered by the one or more immunomodulatory peptides; treating the subject with immunomodulatory peptides that affect the type of immune response; and evaluating the type of immune response after treating the subject with one or more immunomodulatory peptides and if necessary modifying the one or more immunomodulatory peptides to change the type of immune response. The assessment of therapeutic efficacy may be used to monitor vaccine therapy, anti-cancer therapy, anti-tumor therapy, organ transplantation, allergy, therapy of autoimmune disease, and therapy of infectious disease. Another method of the present invention includes identifying and characterizing new therapeutic agents for immunotherapy by culturing isolated immune cells in the presence and absence of an immune modulator and one or more peptides from an overlapping peptide library taken from an antigenic protein of interest; simultaneously analyzing the cultures for multiple parameters of immune reactivity such as specificity and cytokine response to epitopes on the peptides; and comparing the immune reactivity when the immune modulator is present to the immune reactivity when the immune modulator is absent, wherein the inhibition of the immune reactivity is indicative of an immunosuppressive therapeutic agent and wherein the enhancement of the immune reactivity is indicative of an adjuvant and may even drive the type of immune response to a Th1, a Th2, a Tc1, a Tc2, and combinations thereof.

[0020] One or more therapeutic agents may be identified by identifying a series of immunomodulatory peptides as described above, wherein the immune cells are cultured in the presence and absence of an immune modulator and one or more peptides from the overlapping peptide library, wherein the inhibition of the immune reactivity is indicative of an immunosuppressive therapeutic agent and wherein the enhancement of the immune reactivity is indicative of an adjuvant. The peptide library can be of an antigenic agent that may be an infectious disease, a cancer, a tumor, an autoimmune disease, an autoinflammatory disease, an arthritis, a diabetes, an allergy, an organ transplantation and a bone marrow transplantation. Vaccine epitopes as therapeutic agents may also be identified by culturing isolated immune cells in the presence of one or more peptides from an overlapping peptide library taken from an antigenic protein of interest; analyzing the culture for multiple parameters of immune reactivity and cytokine production to determine the type of immune reactivity to the peptides on the identification of the cytokines produced as described above. A vaccine may be prepared with one or more peptides having the same identified sequence. The method is flexible enough that the sequence of the one or more peptides may even be determined after isolation. Vaccine epitopes may also be identified by culturing isolated immune cells in the presence of a series of peptides from an overlapping peptide library taken from an antigenic agent and analyzing the culture for multiple parameters of immune reactivity and cytokine production in the culture to determine the type of immune reactivity to the one or more peptides, wherein the vaccine epitopes are characterized by the immune reactivity they elicit to the antigenic agent. Examples of antigenic agent include a virus, a bacteria, a fungi, a protozoan, a parasite or a helminth. The antigenic agent may be a self-antigen. The cell culture may also be analyzed and the profile of one or more T cells, B cells, dendritic cells, monocytes, neutrophils, mast cells and red blood cells determined.

[0021] Compositions herein disclosed include one or more peptides that are selected to modify the type of immune response isolated from a peptide library identified as immune reactive for a specific individual in which reactive peptides for both Th1 and Th2 T cells have been identified. Yet other examples of compositions include one or more peptides that are selected to modify the type of immune response isolated from a peptide library identified as immune reactive for a specific individual in which reactive peptides for both Tc1 and Tc2 T cells have been identified. These compositions may be formulated as vaccines, in dry or liquid form and the like.

BRIEF DESCRIPTION OF THE DRAWINGS

[0022] For a more complete understanding of the features and advantages of the present invention, reference is now made to the detailed description of the invention along with the accompanying figures and in which:

Figure 1 is a graph that depicts the immune response of a normal donor evaluated according the present invention, wherein peripheral blood mononuclear cells (PBMC) were isolated from freshly drawn blood from an HLA-A0201+ healthy volunteer, seeded at 2 x 105 cells/well in triplicates in round-bottom 96-well plates, and incubated with 1 μl of either of HLA-A0201 restricted peptides Flu-MP_{58-66} (GILGFVFTL) or MAGE-$3_{271-279}$ (FLWGPRALV), or peptide diluent (5% DMSO in H_2O); and culture supernatants were harvested 48 h later and evaluated quantitatively for the presence of cytokines and chemokines using Luminex technology;

Figure 2 is a graph that depicts another example of immune response to HLA-A0201 restricted peptides Flu-MP_{58-66} (GILGFVFTL) in a normal donor according the present invention. PBMCs were. isolated from freshly drawn blood from another HLA-A0201+ healthy volunteer, seeded at 5 x 10^5 cells/well in triplicates in round-bottom 96-well plates, and incubated with 10 μg/ml of either of HLA-A0201 restricted peptides Flu-MP_{58-66} (GILGFVFTL) or MART-1_{27-35} (AAGIGILTV), or peptide diluent (5% DMSO in H_2O); and culture supernatants were harvested 48 h later and evaluated quantitatively for the presence of cytokines and chemokines using Luminex technology;

Figure 3A-3D are graphs that depict the immune response of 2 melanoma patients as evaluated according the present invention;

Figure 4A-4C are graphs that depict the immune response of a melanoma patient as evaluated according the present invention;

Figure 5 is a graph that depicts the kinetics of the immune response of a normal donors evaluated according the present invention;

Figure 6 depicts the scheme of epitope focusing according to the present invention in which overlapping peptide libraries were split into clusters of peptides (5-10 peptides/cluster) a cluster analysis conducted, followed by epitope focusing;

Figure 7 is a graph that depicts the immune response of a melanoma patient as evaluated according the present invention;

Figure 8 is a graph that depicts the immune response of a melanoma patient as evaluated according the present invention;

Figure 9 is a graph that depicts the immune response of melanoma patients as evaluated according the present invention;

Figure 10 is a graph that depicts the immune response of a melanoma patient as evaluated according the present invention;

Figure 11 is a graph that depicts the immune response of a melanoma patient as evaluated according the present invention;

Figures 12A and 12B are graphs that depict the immune response of a melanoma patient as evaluated according the present invention in which PBMCs obtained before DC vaccination and post 8 DC vaccination from a melanoma patient (same as Figure 10) were stimulated with single 15-mer MART-1 peptide or diluent;

Figure 13 is a graph that depicts the immune response of a melanoma patient as evaluated according the present invention;

Figure 14 is a graph that depicts the immune response of a melanoma patient as evaluated according the present invention;

Figure 15 is a graph that depicts the immune response of a melanoma patient as evaluated according the present invention;

Figures 16A-D are graphs that depicts the immune response of a melanoma patient as evaluated according the present invention;

Figure 17 is a graph that depicts the immune response of a melanoma patient as evaluated according the present invention;

Figure 18 is a graph that depicts the immune response of melanoma patients as evaluated according the present invention;

Figure 19 is a graph that depicts the immune response of a melanoma patient as evaluated according the present invention;

Figure 20 is a graph that depicts IL-2 production from CD4+ T cells upon stimulation with the identified 15-mer peptide as shown in Figure 19;

Figures 21 A-B are graphs that depict the immune response of a melanoma patient as evaluated according the present invention with PBMCs obtained from the same melanoma patient shown in Figure 19;

Figures 22 A-C are graphs that depict the immune response of a melanoma patient as evaluated according the present invention;

Figure 23 is a graph that depicts the immune response of a melanoma patient as evaluated according the present invention;

Figure 24 is a graph that depicts IL-5 production from CD4+ T cells upon stimulation with the identified 15-mer peptide as shown in Figure 23;

Figure 25 is a graph that depicts the immune response of melanoma patients as evaluated according the present invention;

Figure 26 is a graph that depicts the immune response of three melanoma patients as evaluated according the present invention;

Figure 27 depicts a summary of new epitopes for CD4+ and CD8+ T cells within 4 melanoma antigens, i.e. gp100, MART-1, NY-ESO1, and TRP-1, which are identified with EPIMAX methodology;

Figure 28 depicts the immune response of three melanoma patients as evaluated according the present invention;

Figures 29A-D are graphs that depict the immune response of a melanoma patients as evaluated according the present invention;

Figure 30 includes graphs that depict the immune response of a melanoma patient as evaluated according the present invention;

Figure 31 is a graph that depicts the immune response of melanoma patients as evaluated according the present invention;

Figure 32 is a graph that depicts the immune response of melanoma patients as evaluated according the present invention;

Figure 33 includes graphs that depict the immune response of three normal, healthy volunteers as evaluated according the present invention;

Figure 34 includes graphs that depict the immune response of three normal, healthy volunteers as evaluated according the present invention;

Figure 35 includes graphs that depict the immune response of normal healthy volunteers as evaluated according the present invention;

Figure 36 are graphs that depicts the immune response of normal healthy volunteers as evaluated according the present invention from frozen and thawed PBMCs;

Figure 37 are graphs that depicts the immune response of normal healthy volunteers as evaluated according the present invention from frozen and thawed PBMCs;

Figure 38A and 38B are graphs that depict the immune response of normal healthy volunteers as evaluated according the present invention;

Figure 39A and 39B are graphs that depict the immune response of normal healthy volunteers against the peptides identified in the EPIMAX assay and demonstrate that EPIMAX allows for the identification of epitope for functional Flu-MP specific CD4+ T cells;

Figure 40 are graphs that depict the immune response of a normal healthy volunteer as evaluated according the present invention and shows the identification of novel epitopes for specific CD4+ T cells;

Figure 41 A and B are graphs that depict the immune response of normal healthy volunteers against the peptides identified in the EPIMAX assay;

Figure 42 are graphs that depict the immune response of a melanoma patient as evaluated according the present invention and the antigenic epitopes for the patient, as both the peptides contain amino acid mismatches and show the ability to identify allogeneic-antigen specific T cells in a setting of organ transplantation;

Figure 43 is a graph that depicts the immune response of a type-1 diabetic patient as evaluated according to the present invention;

Figures 44A-44B depict the immune response of a melanoma patient as evaluated according the present invention, which allows the identification of various types of immune responses;

Figure 45 is a graph that depicts the immune response of five normal, healthy volunteers as evaluated according the present invention, which allows the identification of various types of immune responses by non-T cells;

Figure 46 includes two graphs that depict the immune response in a melanoma patient as evaluated according to the present invention, which allows the identification of various types of immune responses by non-T cells;

Figure 47 is a graph that depicts the immune response in a melanoma patient as evaluated according to the present invention, which allows the identification of various types of immune responses by non-T cells;

Figure 48 includes four graphs that depict the immune response in a melanoma patient as evaluated according to the present invention, which allows the identification of various types of immune responses by non-T cells; and

Figure 49 includes two graphs that depict the immune response in a melanoma patient as evaluated according to the present invention and demonstrates the identification of various types of immune responses by non-T cells.

DETAILED DESCRIPTION OF THE INVENTION

[0023] While the making and using of various embodiments of the present invention are discussed in detail below, it should be appreciated that the present invention provides many applicable inventive concepts that can be embodied in a wide variety of specific contexts. The specific embodiments discussed herein are merely illustrative of specific ways to make and use the invention and do not delimit the scope of the invention.

[0024] To facilitate the understanding of this invention, a number of terms are defined below. Terms defined herein have meanings as commonly understood by a person of ordinary skill in the areas relevant to the present invention. Terms such as "a", "an" and "the" are not intended to refer to only a singular entity, but include the general class of which a specific example may be used for illustration. The terminology herein is used to describe specific embodiments of the invention, but their usage does not delimit the invention, except as outlined in the claims.

[0025] The present invention includes methods that are capable of assessing the antigen specific immune responses, without respect to the type of response induced. As used herein, the term "type of immune response induced" or "type of immune response," is used to describe the activation or regulation of an immune cell response via Th1, Th2, Tc1, Tc2 and combinations thereof or other T cell-based adaptive immunity regardless of the Major Histocompatibility Complex (MHC) presenting the peptide or antigen, whereby the T cell that recognizes and responds to the specific peptide or antigen in the context of MHC and its effect on the same cell, adjacent cells or cell that interact with that particular T cell

or its effect on the cell presenting the peptide or antigen. The skilled immunologist will recognize that terminology may be overlapping, that is, it is common to refer to a protein that has been processed and loaded by an antigen presenting cell into a peptide, antigen or epitope, which is presented in the context of a particular MHC glycoprotein, that is, Class I or Class II.

[0026] It will be clear as used in context herein that the terms, "peptide," "antigen" or "epitope," are used in reference to the presentation of immune modulation by T cells after presentation by an antigen presenting cell. As used herein, the term "peptide" is used to describe a string of amino acids.

[0027] As used herein, the terms "antigen," "immunogen," "epitope" and "antigenic determinant," are used interchangeably to describe those peptides that modulate an immune response. As used herein the term, "modulate" is used to describe the activation, regulation, anergization or even suppression of an immune response as measured by, e.g., the proliferation of cell, cytokine or immunokine secretion, interactions between cells, apoptosis and the like. For example, a T cell response may be "modulated" in which certain cytokine or immunokines are released by the T cell that are capable of activating a certain type of response, e.g., antibody production; and concurrently reducing another type of response, e.g., the activation of cytotoxic T cells or NK cells.

[0028] As used herein, the term "immunomodulatory" is used to describe the effect that a particular antigen, immunogen, epitope or antigenic determinant has on effect cells, that is, cell that are activated in situ or even downstream in an effector cascade.

[0029] The present inventors recognized that many tools for prediction and characterization of antigenic epitopes have been previously developed. For example, computer modeling of peptide-major histocompatibility complex (MHC) interactions, based largely on X-ray crystal structures data of MHC peptide complexes, is often used to predict potential epitopes within proteins. Though this methodology has benefited greatly from the introduction of new prediction algorithms, it is, however, dependent on the size of the structure database and may therefore be less efficient at predicting MHC complexes not represented in that data set. This method provides a means of identifying antigenic epitopes but cannot be used to characterize the specific response elicited by those epitopes. (Flower DR, Novartis Found Symp. 2003;254:102-20; discussion 120-5, 216-22, 250-2).

[0030] Elution of peptides from MHC followed by mass spectrometry sequencing has been used successfully to identify antigenic epitopes (Hunt DF, Science. 1992 Mar 6;255(5049):1261-3). Although a powerful methodology for identification, peptide elution is expensive, slow, and requires a large sample size, making it impractical for use in a clinical setting. This method also provides a means of identifying antigenic epitopes but cannot be used to characterize the specific response elicited by those epitopes.

[0031] Overlapping peptide libraries have been used to identify antigenic epitopes in pathogen associated proteins (Martin R, Methods. 2003 Mar;29(3):236-47). Peptide library screening uses biological assays as readouts for reactivity and can, therefore, be used to assess the magnitude of response to the epitope. By using overlapping libraries, all potential Class I and Class II epitopes can be identified. To date, the biological assays used to evaluate potential antigenic epitopes using peptide libraries include the following methodologies: intracytoplasmic cytokines, ELISA, and cell proliferation (Martin R, Methods. 2003 Mar;29(3):236-47). Reactivity to a given peptide within the library in any of these assays is an indication that an antigenic epitope is present; however, none of these methods give a clear indication as to the quality of the response to that epitope, e.g., antigenic, allergenic, tolerogenic or any other specific response.

[0032] Combinations of known technologies useful for the prediction and characterization of antigenic epitopes have also been reported. Examples of combination assays are given below.

[0033] Peptide libraries and ELISPOT. The ELISPOT technique uses a surface bound capture antibody to bind cytokines secreted from cells cultured on the plate. A second labeled antibody is used to quantitate the number of cytokine secreting cells. By incubating cells with overlapping peptide libraries and assessing cytokine production by ELISPOT, antigenic epitopes have been isolated (Geginat G, J Immunol, 2001 Feb 1;166(3):1877- 84). This methodology is limited, however, in that for any given sample, the production of only a few cytokines can be measured. This inability to measure the full range of cytokines and chemokines produced in response to epitope recognition makes any characterization of the immune response (i.e., Type-1, Type-2, or T-Regulatory) extremely difficult.

[0034] Peptide libraries and ELISA. The ELISA technique uses a surface bound capture antibody to bind soluble cytokines secreted from cells. A second labeled antibody is used to quantitate the concentration of secreted cytokine when compared to a standard curve. By incubating cells with overlapping peptide libraries and assessing cytokine production by ELISA, antigenic epitopes have been isolated. This methodology is limited, however, in that for any given sample the production of only a few cytokines can be measured. This inability to measure the full range of cytokines and chemokines produced in response to epitope recognition makes any characterization of the response (i.e., Type-1, Type-2, or T-Regulatory) extremely difficult. Furthermore, assessment of a single cytokine by ELISA requires a significant volume of at least 100 microliters of biological fluid and/or culture supernatant.

[0035] Peptide libraries and cellular proliferation assays. The cellular proliferation assay uses radionucleotide incorporation or fluorescent dye dilution to monitor cell division in response to stimulus. By incubating cells with overlapping peptide libraries and assessing cell division by cellular proliferation assay, antigenic epitopes have been isolated (Mutch

D, J Acquir Immune Defic Syndr. 1994 Sep;7(9):879-90). This methodology is limited, however, in that although cell division may be an indication of the magnitude of antigenic response, this assay gives no information as to the quality of the response (i.e., Type-1, Type-2, or T-Regulatory). It should also be noted that some antigen-specific responses are not associated with rapid cellular proliferation such as T-Regulatory responses.

[0036] Peptide libraries and cytotoxic lymphocyte assays (CTL). The cytotoxic lymphocyte assay monitors the release of radionucleotide or fluorescent dye from antigen loaded (including but not limited to cDNA, virus and phage expression libraries, whole protein, protein fragments, peptide, modified peptide and lipids) target cells as a measure of epitope recognition and function of cytolytic cells. By incubating cells with overlapping peptide libraries and assessing cellular cytotoxicity by cytotoxic lymphocyte assay, antigenic epitopes have been isolated. Although cellular cytotoxicity may be an indication of the magnitude of a Type-1 antigenic response, this methodology is of limited use when assessing responses other than Type-1 (i.e., Type-2 or T-Regulatory). Characterization of antigen-specific cytotoxicity using CTL assays is often plagued with low sensitivity due to reporter release from target cells. It should also be noted that this methodology requires extensive cell culture manipulation and therefore, cannot be considered high throughput.

[0037] Peptide libraries and intracytoplasmic cytokine staining. The intracytoplasmic cytokine staining technique uses standard fluorescent staining procedures and anti-cytokine antibodies to measure the level of intracellular cytokines produced in response to an antigenic stimulus. Measurements of cytokine production and quantitation of cytokine-producing cells can then be accomplished by standard cytometric, microscopic or spectrophotometric techniques. By incubating cells with overlapping peptide libraries and assessing cytokine production by intracytoplasmic staining, antigenic epitopes have been isolated (Karlsson AC, J Immunol Methods. 2003 Dec;283(1-2):141-53). This methodology is limited, however, in that for any given sample, the production of only a few cytokines can be measured. This inability to measure the full range of cytokines and chemokines produced in response to epitope recognition makes any characterization of the response (i.e., Type-1, Type-2, T-Regulatory) extremely difficult. This methodology is further limited in that it requires a large numbers of cells for the analysis and is destructive to analyzed cells.

[0038] Peptide Elution and mass spectrometric sequencing. Elution of peptides from MHC followed by mass spectrometry sequencing has been used successfully to identify antigenic epitopes (Hunt DF, Science. 1992 Mar 6;255 (5049):1261-3.). Although a powerful methodology for identification, peptide elution is expensive, slow, and requires a large sample size, making it impractical for use in a clinical setting. This method provides a means of identifying antigenic epitopes but cannot be used to characterize the specific response (i.e., Type-1, Type-2, T-Regulatory) elicited by those epitopes.

[0039] Computer modeling of peptide MHC interactions. Computer modeling of peptide-major histocompatibility complex (MHC) interactions, based largely on X-ray crystal structures data of MHC peptide complexes, is often used to predict potential epitopes within proteins. Though this methodology has benefited greatly from the introduction of new prediction algorithms, it is, however, dependent on the size of the structure database and may therefore be less efficient at predicting MHC complexes not represented in that data set (Kuhara S. Pac Symp Biocomput. 1999;:182-9.). This method provides a means of identifying antigenic epitopes but cannot be used to characterize the specific response elicited by those epitopes.

[0040] Serological identification of antigens by recombinant expression cloning (SEREX). SEREX technology involves identification of epitopes recognized by serum antibodies through combinatorial expression library screening. Although SEREX technology may give an indication as to the specificity of the humoral immune response, no such indication is given as to either the magnitude or nature of the cellular immune response (Tureci O, Mol Med Today. 1997 Aug;3(8): 342-9.). This technology is both time and labor intensive, and therefore impractical in a clinical setting.

[0041] Real-time PCR for multiple cytokine analysis. Real-time PCR can be used to measure in a quantitative manner the expression of RNA encoding multiple cytokines (Giulietti A., Methods. 2001 Dec;25(4):386-401). This technology is, however, both time and labor intensive, requires large numbers of cells to isolate sufficient quantities of RNA for analysis, and is destructive to cells. Finally, the expression of RNA does not indicate the secretion of a biologically active cytokine.

[0042] As indicated above, technologies capable of predicting and characterizing antigenic epitopes have been reported, and in some cases, various technologies have been combined in order to maximize the amount of useful information, such as florescent based proliferation assays and intracellular cytokine assays. These combined assay approaches often suffer from the limitations of its individual component assays, such as the limitations associated with intracellular cytokine staining described above.

[0043] There is an urgent and thus far unfulfilled need for high throughput multiparameter technology, amenable to automation, non-destructive and requiring a small sample size making it applicable to a clinical diagnostic setting, allowing assessment of immune responses in humans. This need is particularly important in the view of emerging and re-emerging pathogens as well as biothreat agents where rapid assays providing the picture of immune response and/or identifying the nature of pathogens are needed. None of the methodologies described above provide a clear characterization of the qualitative and quantitative immune response to an antigenic epitope. With the exception of computer modeling, these methods do not provide the high throughput, limiting their utility from a diagnostic or immune-monitoring standpoint.

[0044] Subject matter of the present invention is defined in the claims. The present invention provides for identification

of immunomodulatory peptides and their derivatives, thus permitting evaluation of the type of immune response induced in healthy and diseased individuals with diagnostic, prognostic and therapeutic implications. This methodology also gives a measure of both the quality and magnitude of the response, while simultaneously identifying the epitope to which that response is generated.

[0045] In one aspect, the present invention is a method to assess antigen-specific immune responses, irrespective of the type of response induced, thus permitting the evaluation of the type of immune response induced in both healthy and diseased individuals. This methodology gives both a qualitative and quantitative measure of the immune response, while simultaneously identifying the epitope to which that response is generated.

[0046] The methods of the present invention are a combination of three technologies: cell culture of immune cells from either humans or animals, presentation of antigenic material that is non-MHC limited, and analysis for the presence of cytokines and/or chemokines which provides for the ability to observe all varieties of immune responses. In one embodiment, the three technologies involve cell culture of immune cells from either humans or animals, overlapping peptide libraries that are non-MHC limited, and multiplex analysis for the presence of cytokines and/or chemokines. Preferably, the present invention is a multiphasic method to assess antigen-specific immune responses, wherein the first phase is a high-throughput screening (Phase I), and the second phase provides for identification of specific antigenic epitopes (Phase II).

[0047] Methods of the present invention use cell culture analysis with multiple cell types present that are capable of diverse antigen processing, presentation and recognition for both peptide and non-peptide epitopes (e.g., glycolipid). Suitable cells for the methods of the present invention include but are not limited to T cells, B cells, dendritic cells, monocytes, neutrophils, mast cells and red blood cells. Isolated peripheral blood mononuclear cells (PBMCs) including T cells, B cells, NK cells, and NK-T cells provide an easily obtainable source of multiple cell types for use in the present invention. Moreover, any standardized procedure known in the art for culturing human or animal cells which also supports the production of cytokines is suitable for use in the present invention.

[0048] In the methods of the present invention, any antigenic material can be used to elicit an immune response. Sources for antigenic material suitable for the present invention include antigens, peptides, proteins, microbes, cells, tissues, tumors or any combination thereof. Suitable antigenic materials include but are not limited to compositions including peptides, glycopeptides, phosphopeptides, lipids, glycolipids, and phospholipids.

[0049] In one embodiment, overlapping peptide libraries are used in the present invention to identify antigenic epitopes on a protein of interest and to stimulate immune responses. This technique provides for the identification of all possible epitopes Class I and Class II for any given antigen and is suitable for both CD4 and CD8 T-cell responses. And identification of the epitopes is not limited by the HLA-haplotype.

[0050] The amino acid sequence of the protein of interest is divided into small overlapping peptides, for example, a series of 8- to 15-mer overlapping peptides with, e.g., 3 to 4 amino acid offsets. According to the present invention, an overlapping peptide library can either be prepared or purchased from commercially available sources. For example, from the flu matrix protein containing 296 amino acids, an overlapping peptide library of 60 overlapping 15-mer peptides (with 4 amino acid offset) can be prepared.

[0051] From the overlapping peptide library, a cell culture container is prepared with one or more peptides adhered to the culture container. In one multiphasic method, multi-well Phase-I culture plates are used for screening purposes, wherein each well contains a cluster of several overlapping peptides. The number of overlapping peptides per cluster and the number of clusters required for screening depends upon the size of the protein itself. Preferably, each cluster contains about 5-10 overlapping peptides; for a smaller protein, about 3-7 overlapping peptides. In one multiphase method, the overlapping peptide library for a protein of interest is distributed by clusters into coded, preconditioned 96-well Phase-I culture plates, with 5-10 peptides per cluster per well. For Phase-II, the overlapping peptides in a reactive cluster are distributed as a single peptide per well. The Phase-I culture plates can be prepared in advance to maximize throughput and minimize assay-to-assay variability. Plates prepared in advance and stored at -80oC have a shelf life of a minimum one year. And to the extent that specific Phase-I clusters are found to be repeatedly antigenic, Phase-II culture plates can also be prepared in advance.

[0052] To begin the immune response assay of the present invention, immune cells of interest are added to the culture container described above and incubated for a time and under conditions that support the production of cytokines in response to the antigenic peptide(s) adhered to the culture container. The immune cells that can be assessed using this methodology include but are not limited to PBMCs, lymphocytes, T cells, CD4+ T cells, CD8+ T cells, NK cells, NKT cells, TCR □□ T cells, B cells, monocytes, dendritic cells and granulocytes. In one method, peripheral blood mononuclear cells (PBMCs) are used. In the multiphase method, for example, PBMCs are seeded at 2 x 105 per well into one or more coded, preconditioned 96-well Phase-I culture plates described above that contain an overlapping peptide library of the antigen of interest with 5-10 peptides per cluster per well. The immune cells are then incubated under conditions that support the production of cytokines, with the culture medium, temperature and incubation conditions selected for the specific cell type. For example, PBMCs are preferably incubated for 18-48 hours at 37˚ C in complete medium as described in Example 1. Following incubation, cells are pelleted and the supernatants are isolated from each well and

analyzed for the presence of a wide variety of cytokines and chemokines. With respect to the present invention as described herein, the term "cytokines" is used to represent both cytokines and chemokines.

[0053] According to the present invention, any method known in the art that is capable of simultaneously analyzing the supernatants for the presence of several cytokines, preferably 20 or more cytokines, in the same sample can be used in the present invention. Such methods include mass spectroscopy, antibody-based array, and multiplex analysis bead technology. Cytokine multiplex analysis is used because it: i) allows for simultaneous measuring of multiple immune parameters, ii) requires small volume samples, iii) provides for a sensitive assay that does not destroy the sample; iv) supports coordinated expression of specific cytokines characteristic of specialized immune responses, and v) provides for a direct measure of immune effectors. A suitable commercially available cytokine multiplex analyzer is the Luminex 100 Cytokine Multiplex work station (Luminex Corp., Austin, Texas), which is capable of simultaneously measuring up to 100 analytes, requires a small volume sample, and rapidly detects the presence of cytokines within a dynamic range of 1-32,000 picograms per milliliter. Cytokine multiplex array technology (Luminex) permits the simultaneous quantitation of multiple cytokines and chemokines in a small volume (Earley MC, Cytometry. 2002 Oct 15;50(5):239-42). The assay is high throughput in nature and can be applied to the analysis of a wide variety of biological samples. By analyzing the coordinated cytokine expression in response to a stimulus or pathogenic insult, state specific biosignatures can be described corresponding to specific immune responses.

[0054] In one method, 50 microliters of the supernatants are transferred in 96 well format to a Luminex 100 Cytokine Multiplex work station (Luminex Corp., Austin, Texas) and analyzed according to manufacturer's instructions. Supernatants are rapidly screened in duplicate for the presence of a wide variety of cytokines using a master mix of pre-prepared and validated multiplex beads and reagents.

[0055] In the multiphase method, antigen clusters which prove to be positive for reactivity by Phase-I cytokine analysis are broken down into their constituent peptide components for antigenic peptide identification on an epitope focusing Phase-II culture plates as described above. In one method, Phase-II culture plates consist of coded, preconditioned well-strips containing the individual component peptides for each cluster in duplicate. Immune cells of interest are added to the well-strips and incubated for a time and under conditions that support the production of cytokines in response to the antigenic peptide(s) adhered to the well-strip. For example, thawed PBMCs are added at 2 x 10^5 cells per well in the well-strips and cultured for 18-48 hours at 37˚ C. Following incubation the cells are pelleted, supernatants isolated and analyzed for cytokine production. In one method, 50 microliters of the supernatants are transferred to the Luminex workstation for analysis. Multiplex screening in Phase-II for a given peptide strip consists of the cytokine or cytokines produced in response to its parent cluster during Phase-I analysis. Peptides identified in a Phase II screen represent antigenic epitopes recognized by the individual. The characteristic pattern of cytokines and chemokines produced in response to the identified peptide are an indication of the type of specialized response elicited by that epitope.

[0056] The methods of the present invention are a high throughput procedure for the rapid identification and characterization of peptide specific T-cell epitopes. As shown herein, development and validation of the assay has lead to the identification of a select group of cytokines and chemokines, which allow identification of Type-1, Type-2 and T-Regulatory cell phenotypes.

[0057] This methods of the present invention have several advantages over existing art including: a) high sensitivity: at picogram per milliliter detection for all cytokines tested; b) high throughput: assay can be completed within 48hrs; c) analysis of multiple analytes in a small volume of biological fluid, for example, assessment of at least 30 cytokines and chemokines in a 50 microliter volume; d) non-destructive: permitting the addition of cell based assays for multiparameter analysis; and e) identification of antigenic epitopes in a manner independent of HLA haplotype. The methods of the present invention use cytokine multiplexing to monitor multiple immune parameters and is therefore capable of characterizing the quality of the immune response including but not limited to antigenic, allergenic, and tolerogenic responses. By monitoring the coordinated response of multiple cytokines, identification and characterization of antigenic epitopes is also more sensitive than current state of the art technologies. None of the current art technologies either alone or in combination are able to provide the multiparameter assessment of cytokines and the identification and characterization of antigenic epitopes that the methods of the present invention can provide. Furthermore, the methods of the present invention encompass the advantages of a high-throughput technology. It should also be noted that as opposed to current state of the art technologies, the methods of the present invention are not destructive to the cells being analyzed; it can, therefore, be combined with any number of standard or custom immunoassays, including but not limited to proliferation, intracellular cytokine and surface receptor staining, CTL activity, and ELISPOT. Finally, the methods of the present invention require much smaller volumes of biological fluids and/or culture supernatant and much lower numbers of cells for analysis than any other currently available technology. Thus, the methods of the present invention are a high throughput, low volume, non destructive technology, amenable to automation and, therefore, practical for clinical diagnostic, prognostic and therapeutic use.

[0058] In one aspect, the present invention is a method that permits identification of immunomodulatory peptides and their derivatives, with diagnostic, prognostic and therapeutic applications. (a) Diagnostic: this technology can be used to assess the preexisting immune state of patients. By identifying and characterizing epitopes in a wide variety of antigens,

a clear picture of the complete T-cell repertoire for that individual can be determined. This technology is high throughput, amenable to automation, non-destructive and requires a small sample size, making it applicable to a clinical diagnostic setting. It can be used, therefore, as a tool in the diagnosis of a number of auto-reactive diseases and hyperimmune responses including but not limited to allergy, diabetes, arthritis, lupus and multiple sclerosis. (b) Prognostic: the epitope-specific immune status of an individual prior to treatment as assessed by this technology can serve as a prognostic indicator of treatment outcome. Therefore, the course of therapeutic intervention can be tailored to enhance or eliminate this immune state. This technology can be used for prognostic assessment of anti-tumor immunotherapy, organ transplant, allergy and autoimmune diseases treatments. (c) Therapeutic: methods of the present invention can be used during the course of treatment as an immunomonitoring tool. A clear picture of the specificity, magnitude and quality of the epitope-specific immune response during therapy can serve to assess the efficacy of ongoing treatment. Based on data from this approach, treatments can be adapted to enhance, modulate or inhibit specific immune responses. This technology can, therefore, be used to guide ongoing therapeutic treatment in the areas of vaccine development, anti-tumor immunotherapy, organ transplant, allergy and autoimmune diseases.

[0059] In another aspect, the present invention can be useful in the design of new drugs including but not limited to vaccines, bioactive peptides and targeting reagents. (a) Vaccines: the methods of the present invention can greatly facilitate the development of effective vaccines. Antigenic epitopes identified using this technology can themselves serve as effective vaccines to enhance the immune response against infectious disease or cancer, or to tolerize the immune response against proteins for the treatment of autoimmune disease, allergy, or organ transplant. (b) Bioactive peptides: bioactive peptides generated by proteolysis of larger proteins have been described with profound neuromodulatory and immunomodulatory functions. With this technology, peptides with these properties can be rapidly identified. (c) Targeting reagents: bioactive peptides identified using this technology which interact with specific receptor on cells can be used as a targeting reagent to deliver drugs to specific cellular populations.

[0060] The methods of the present invention are suitable for use in several medical applications including but not limited to chronic infections, allergy, neoplastic diseases, autoimmune diseases, Alzheimer's disease, acute infectious diseases, organ transplant, and atherosclerosis. It is to be understood that these applications are meant to be representative, and that other applications such as known in the art are contemplated as part of the present invention.

[0061] Chronic infection. A staggering number and variety of human diseases are caused by chronic pathogenic infection. Although the pathogenesis of these diseases may differ, they can all be characterized by an inability to mount and effective response, either Type-1 or Type-2, to the pathogen. Once a response is mounted, self epitope mimicry in pathogen associated proteins may also induce autoimmune responses which persist long after the infection is cleared, such as Lyme arthritis and Herpes associated demyelinating disease. The appearance of autoimmune reactions after pathogen immune recognition makes the selection of specific epitopes critical to a safe, effective therapy. Studies have implicated an inappropriate activation of regulatory response in some chronic infections. Methods of the present invention are suitable for diagnostic, prognostic and therapeutic applications regarding chronic infections. (a) Diagnostic: non-invasive diagnostic tests for some chronically infectious agents, such as PCR-based tests for HBV, demonstrate extreme variability in the clinical setting. The methods of the present invention can be applied to the diagnosis of chronic infection through the rapid detection and characterization of specific responses to pathogen-associated peptides. Diagnosis of pathogen-associated autoimmune complications can also be facilitated through the use of this technology. (b) Prognostic: the methods of the present invention can be used to detect and characterize reactivity, either Type-1, Type-2, or T-Regulatory, to specific pathogen-associated epitopes, which, in turn, can serve as a prognostic indicator of disease outcome, thereby guiding antibiotic therapy. The methods of the present invention are suitable for detecting and characterizing reactivity to specific pathogen-associated epitopes which can also serve as a prognostic indicator as to the onset of infection-associated autoimmune disease. (c) Therapeutic: the identification and characterization of antigenic epitopes to pathogen-associated proteins by means of the present invention can facilitate the development of safe, effective prophylactic and therapeutic vaccines. These would include the implementation of vaccines which avoid self epitope mimicry. Vaccines would also include the identification of pan-variant epitopes, targeting invariant epitopes within the pathogen proteome, thus providing protection against multiple strains of the same pathogen as well as common mutant variants of each pathogen as in the case of HIV and malaria. Identification and elimination of T-Regulatory epitopes and responding cells through application of this technology can play a role in initiating a protective anti-pathogen response.

[0062] Allergy. An allergy is a hyperimmune response to substances that are generally not harmful. It has been reported that an inappropriate shift to a Type-2 response plays a critical role in triggering an allergic immune response. Many allergens have been identified including inhalation allergens such as house dust mite, pollen, and mold; food allergens such as egg, wheat, soy, and nuts; or some drugs such as penicillin. A severe, often life-threatening reaction, called anaphylaxis, can result. Activation of a T-Regulatory response in the context of allergy, would, therefore, be advantageous. (a) Diagnostic: a skin test and blood test such as an allergen-specific IgE level is commonly used to identify allergens. However, a skin test can be dangerous to some patients who may experience anaphylaxis. Methods of the present invention can be applied as a safe diagnostic tool to identify patient-specific allergenic epitopes. (b) Prognostic:

methods of the present invention allow identification of allergen epitopes for an individual allergy patient. This is useful to monitor immune response during treatment or for following-up. Tolerogenic T-Regulatory stimulatory epitopes can also be identified after treatment and can serve as a prognostic indicator of successful therapy. (c) Therapeutic: identification of an antigen epitope can lead to a new peptide-based vaccine which can be used to switch immune response to T-Regulatory or to selectively eliminate or suppress inappropriate Type-2 cells.

[0063] Neoplastic diseases. Neoplastic disease is defined as uncontrolled growth of abnormal cells that have mutated from normal tissues. These include cancer, solid tumors and malignancies of lympho/hematopoietic system, all of which are generally referred to as tumors. It has been reported that tumor environment induces immune tolerance against tumor antigens through several different mechanisms. While the development of Type-1 T cells against tumor cells is related to regression or suppression of tumor growth, the shifted immune response to tolerance inhibits the development of cancer-specific Type-1 T cells. However, in some cancers, tumor-antigen specific Type-1 T responses induce autoimmune disease by antigenic mimicry. For example, it has been reported that cytotoxic T cells specific to CDR2, a breast and ovarian tumor antigen, induce paraneoplastic cerebellar degeneration (PCD) in some patients.

[0064] The appearance of autoimmune reactions in the course of anti-tumor immunity makes the selection of specific epitopes critical to a safe, effective therapy. (a) Diagnostic: diagnosis of tumor-associated autoimmune complications can be facilitated through the use of the methods of the present invention. (b) Prognostic: detecting and characterization of reactivity, either Type-1, Type-2, or T-Regulatory, to specific tumor-associated epitopes using this technology can serve as a prognostic indicator of disease outcome. Methods of the present invention allow evaluation of any kind of vaccine treatment by comparing the quality and magnitude of tumor-specific immune responses between pre-vaccine and post-vaccine. Detection and characterization of reactivity to specific tumor-associated epitopes using this technology can serve as a prognostic indicator as to the onset of tumor-associated autoimmune disease. (c) Therapeutic: the identification and characterization of antigenic epitopes to tumor-associated proteins by means of the present invention can facilitate the development of safe effective therapeutic vaccines. This would include the implementation of vaccines, which avoid self epitope mimicry.

[0065] Autoimmune diseases. Autoimmune diseases can be defined as disorders caused by an immune response against the body's own tissues. An inappropriate shift away from T-Regulatory function in the context of self antigen presentation is thought to be a critical factor in the onset and progression of autoimmune disease. The specific nature of the resulting disease is dependent on the dominant response. For example, in the case of Type-1 diabetes, Type-1 cells recognizing pancreatic islets have been isolated in patients. (Arif S, J Clin Invest. 2004 113:451). Furthermore, in multiple sclerosis patients, there is evidence indicating that myelin basic protein (MBP)-reactive Type-1 polarized T-cells undergo in vivo activation and clonal expansion, which has been reported to play a major role in the pathogenesis. A restoration of T-Regulatory function would be critical to reconstitution of normal immune homeostasis. (a) Diagnostic: methods of the present invention allow identification of dominant self epitopes responsible for expansion of a given type of autoreactive immune cells. (b) Prognostic: methods of the present invention allow identification of self antigen epitopes for an individual autoimmune patient. This is useful to monitor immune response during treatment or for following-up. Tolerogenic T-Regulatory stimulatory epitopes can also be identified after treatment and can serve as a prognostic indicator of successful therapy. (c) Therapeutic: identification of an antigen epitope can lead to a new peptide-based vaccine which can switch immune response to T-Regulatory or to selectively eliminate or suppress inappropriate Type-1 or Type-2 cells.

[0066] Alzheimer's disease. Alzheimer's disease (AD) is a slowly progressive form of dementia, which is a progressive, acquired impairment of intellectual functions. AD is characterized by the progressive deposition of the 42-residue amyloid beta protein (Abeta) in brain regions. Reports indicate that a significantly higher proportion of healthy elderly subjects and patients with AD had strong Abeta-reactive T-cells responses of both Type-1 and Type-2 character when compared to age-matched adults. (a) Diagnostic: in many cases, differential diagnosis between Alzheimer's disease and senile dementia can only be conclusively determined post-mortem. Methods of the present invention can be used to identify A beta reactive T cell responses, which can, therefore, serve as a diagnostic indicator of AD. (b) Prognostic: identification of specific immune responses by means of the present invention can allow prediction of the clinical course of disease.

[0067] Acute infection. The induction of Type-1 response against pathogens is critical for recovery from infection of intracellular microbes such as bacteria and viruses. In contrast, extracellular pathogens such as helminthes induce the development of Type-2 cells, whose cytokines direct IgE and eosinophils-mediated destruction of pathogens. Some viruses, such as Ebola virus or Dengue virus, cause severe illness with high mortality rate, for both of which licensed vaccine is not established. In a mouse model, vaccines with virus-like particles (VLPs) derived from Ebola virus glycoprotein (GP) and matrix protein (VP40) has been proved to activate both T cells and B cells, and protects from viral challenge. (a) Diagnostic: methods of the present invention are suitable for the identification of novel epitopes that diagnose the presence of emerging and re-emerging pathogens and biothreat agents. (b) Therapeutic: methods of the present invention can identify T cell epitopes for viral antigens by screening recovered patients from these diseases. Thus, new peptide-based vaccines can be made against acute fatal viral infections.

[0068] Organ Transplant. Rejection of an organ transplant is a hyperimmune response generally driven by a dominant

Type-1 response against the engrafted organ or, as in the case of graft versus host disease (GVHD), against the recipient. Regulatory function may play a critical role in developing tolerance and long term graft acceptance. Activation of T-Regulatory, or selective elimination of dominant Type-1 responses in the context of organ transplant, would therefore be advantageous. (a) Diagnostic: methods of the present invention are suitable for determining a pattern of immune reactivity which can be predictive of the development of acute GVHD and most particularly chronic GVHD. (b) Prognostic: prior to transplant or emergence after, the existence dominant Type-1 epitopes in either donor or recipient samples can be a prognostic indicator of graft rejection and the present invention is suitable for determining the presence of dominant Type-1 epitopes. Tolerogenic T-Regulatory stimulatory epitopes can also be identified by means of the present invention after treatment and can serve as a prognostic indicator of graft tolerance. (c) Therapeutic: identification of antigen epitopes by means of the present invention can lead to a new peptide-based vaccine which can switch immune response to T-Regulatory, or to selectively eliminate or suppress inappropriate Type-1 cells.

[0069] Atherosclerosis. Atherosclerosis is a common disorder of the arteries. Fat, cholesterol and other substances accumulate in the walls of arteries and form "atheromas" or plaques. It is currently appreciated that chronic inflammatory cell-mediated immune responses are involved in the pathogenesis of atherosclerosis. T-cell activation in atherosclerotic plaques is reported to be initiated by plaque-derived antigens, such as oxidized LDL (oxLDL). Others reported detection of Chlamydia pneumoniae-reactive T cells in human atherosclerotic plaques of carotid artery. (a) Diagnostic: methods of the present invention are suitable for identification of early patterns of immune reactivity. (b) Prognostic: methods of the present invention allow for the quantitative measurement of epitopes of antigens related to pathogenesis of atherosclerosis such as oxLDL or cross-reactive Chlamydia antigen, which are recognized by T cells. (c) Therapeutic: methods of the present invention are suitable for determining specific epitopes that can be used to develop new peptide-based vaccines to prevent progression of atherosclerosis.

[0070] It is to be understood that the examples given below are representative of the invention and are intended to be illustrative of the invention, but are not to be construed to limit the scope of the invention in any way. Modifications may be made in the process features of the invention without departing from the scope of the invention. It will be readily apparent to those skilled in the art that alternative methods may also be used without departing from the scope of the invention. In particular, the method for measuring cytokine production presented in the examples is merely representative and any method known in the art for measuring cytokine concentrations can be used in the present invention.

[0071] Example 1: Antigen specific immune responses detected as early as 48 hours by simple incubation of normal donor PBMCs with a viral peptide. To determine if an antigen specific immune response can be detected by the methods of the present invention using PBMCs and single peptide, PBMCs freshly prepared from an HLA-A0201+ normal volunteer were incubated with HLA-A0201 restricted peptides. The donor was known to have Flu-MP-specific CD8+ T cells but not Mage 3-specific CD8+ T cells by other methods (data not shown).

[0072] Peripheral blood mononuclear cells (PBMC) were isolated from freshly drawn blood from a HLA-A0201+ healthy volunteer by Ficoll-Paque density gradient centrifugation. PBMC were resuspended at a concentration of 1 x 10^6 cells/ml in RPMI medium supplemented with 10% heat-inactivated human AB serum (Gemini Bio-Products), L-glutamine (2 mM), penicillin (200 UI/ml), streptomycin (200 μg/ml), sodium pyruvate (1 mM), 1% non-essential amino acid, 2-β-mercaptoethanol (50 μM, Sigma), and HEPES pH 7.4 (25 mM, Gibco) (complete medium: CM). 2 x 10^5 cells/well were seeded in triplicates in round-bottom 96-well plates, and incubated with 1 μl of either of HLA-A0201 restricted peptides (stock concentration 1 mg/ml, culture concentration 10 μg/ml); Flu-MP58-66 (GILGFVFTL), or MAGE-3271-279 (FLWGPRALV, MultiPeptide Systems, San Diego, CA), or peptide diluent (5% DMSO in H_2O). Culture supernatants were harvested 48 hours later, and cytokines and chemokines were measured with Luminex.

[0073] PBMCs stimulated with Flu-MP peptide induced a large array of cytokines including IFN-γ within 48 hours (data not shown). The results demonstrated that fresh PBMC from a normal donor respond to Flu-MP peptide and produce a large array of cytokines and that antigen specific immune responses can be detected as early as 48 hours by simple incubation of PBMCs with a peptide.

[0074] Example 2: Antigen specific immune responses detected as early as 48 hours for a broad repertoire of peptide reactive cells in PBMC including tumor antigens. Viral antigens are often able to induce stronger recall responses than tumor antigens. To determine if tumor-antigen specific immune responses can be detected by the methods of the present invention, the cryopreserved PBMCs in liquid nitrogen from 2 melanoma patients who received at least 8 injections of DC vaccine (autologous CD34+ hematopoietic progenitor cell-derived DCs loaded with 4 HLA-A2 peptides of melanoma associated antigens) were thawed with cold PBS. After a second washing with PBS, PBMCs were incubated in CM for 15 min at 37° C, and cell debris was removed with a nylon cell filter. Cells were washed again with CM, and resuspended in CM at 1 x 10^6/ml. Then, 2 x 10^5 cells/well were seeded in triplicates in round-bottom 96-well plates, and incubated with 1 μl of each of HLA-A0201 restricted peptides: (stock concentration 1 mg/ml, culture concentration 10 μg/ml); Flu-MP 58-66, CMV PP65 (NLVPMVATV, Biosynthesis, Lewisville, TX), MAGE-3 271-279, MART-1 27-35 (AAGIGILTV), gp100g209-2M (IMDQVPFSV), Tyrosinase 368-376 (YMDGTMSQV, NCI), or peptide diluent (5% DMSO in H2O).Culture supernatants were harvested 48 hours later, and produced cytokines were measured with Luminex technology. Additionally, ELISPOT assay was performed for the detection of antigen-specific IFN-γ-producing T cells according to the

manufacturer's protocol (Mabtech). Briefly, PBMCs (2 x 10^5 cells/well) were added to plates precoated with 10 μg/ml of a primary anti-IFN-γ monoclonal antibody (Mabtech, Stockholm, Sweden) in the presence or absence of 10 μg/ml peptides.

[0075] Patient 1, who was known to have CD8+ T cells for Flu-MP and CMV by direct IFN-γ ELISPOT (data not shown), demonstrated induction of IL-1 a, IFN- y, TNF- a, and IP-10 in response to Flu-MP or CMV peptide, but not with 4 melanoma peptides (data not shown). This shows melanoma peptides do not modulate immune response nonspecifically. Patient 2, who was demonstrated to have MART-1 specific CD8+ T cells as well as Flu-MP or CMV (data not shown), displayed slight increase of IL-1α and marked increase of IP-10 in response to MART-1 peptide, but not with other melanoma peptides (data not shown). Results indicate that fresh PBMC from the normal donor respond to Flu-MP peptide and produce IL-1 a and IP-10. The cytokine/chemokine production profiles are different between two different normal donors, suggesting that different types of CD8+ T cells recognizing the same Flu-MP peptide can be identified with EPIMAX assay. Given the fact that IP-10 can be upregulated in response to IFNs, DC-T interactions may induce IFN- y production from T cells, which leads to IP-10 production from DCs. Thus, IP-10 could be a useful marker for Type-1 T cell response.

[0076] Example 3: The induction of IP-10 in response to Mart-1 peptide in a melanoma patient vaccinated with peptide-loaded CD34-DCs is dependent on IFN-γ. To examine whether IP-10 production is dependent on IFN-γ, PBMCs obtained from a melanoma patient, who was vaccinated with autologous CD34-DCs loaded with HLA-A2 melanoma peptides, were stimulated with either MART-1 peptide#6 (10 μM) or Flu-MP HLA-A2 peptide (10 μg/ml) in the presence of blocking anti-IFN-γR1 mAb. IP-10 production was completely abrogated by blocking of IFN-γR1 (data not shown). Thus, IP-10 production was dependent on IFN-γ production, indicating IP-10 can be a surrogate marker for IFN-γ production.

[0077] Thawed cryopreserved PBMCs in liquid nitrogen from 2 melanoma patients who received at least 8 injections of DC vaccine (autologous $CD34^+$ hematopoietic progenitor cell-derived DCs loaded with four (4) HLA-A2 peptides of melanoma associated antigens) were seeded in triplicates in round-bottom 96-well plates, and incubated with 10 μg/ml of each of HLA-A0201 restricted peptides: Flu-MP_{58-66}, CMV PP65 (NLVPMVATV), MAGE-$3_{271-279}$(FLWGPRALV), MART-1_{27-35} (AAGIGILTV), $gp100_{g209-2}$ M (IMDQVPFSV), $Tyrosinase_{368-376}$ (YMDGTMSQV), or peptide diluent (5% DMSO in H_2O); and culture supernatants were harvested 48 h later and evaluated quantitatively for the presence of cytokines and chemokines using Luminex technology. ELISPOT assay was performed for the detection of antigen-specific IFN-γ-producing T cells, wherein PBMCs (2 x 10^5 cells/well) were added to plates precoated with 10 μg/ml of a primary anti-IFN-γ monoclonal antibody in the presence or absence of 10 μg/ml peptides.

[0078] The results demonstrated a broad repertoire of peptide reactive cells in PBMC were identified by the method of the present invention.

[0079] Example 4: Methods of present invention allow identification of specific peptide sequence recognized by T cells. To determine if antigen epitope(s) recognized by T cells and an immune response induced by that epitope can be identified with the methods of the present invention, 5 clusters of peptides consisting of 4-7 peptides from the 15-mer MART-1 peptide library, were incubated with PBMCs obtained from an HLA-A0201+ melanoma patient who received CD34-DC vaccine. 15-mer overlapping peptides with four (4) amino acid offsets were designed to cover whole amino acid sequence of MART-1 (Mimotopes, San Diego, CA). Each peptide was reconstituted with 50% Acetonitrile at 2 mM, and kept -80° C until use. PBMCs were obtained from a melanoma patient who received DC vaccines. 4-6 clustered peptides (1 μl per peptide), or single peptide (1 μl) were seeded in triplicates in 96-well plates, and dried at room temperature. Then, 2x10^5 PBMCs in 200 μl CM were added to each well and incubated for 48 hours at 37° C in a humidified 5% CO_2 incubator. The cytokine or chemokine levels in culture supernatant were measured with Luminex.

[0080] It was found that a significantly higher amount of IP-10 was detected in the cluster #1 culture. Next, a second culture of PBMCs with a single peptide from MART-1 library was made to identify the responsible peptide within cluster #1. Peptide #6 induced marked IP-10, indicating that this peptide is the epitope for the melanoma patient's T cells. Interestingly, this peptide contains HLA-A0201 dominant epitope sequence (MART-127-35; AAGIGILTV). PBMCs were obtained from a melanoma patient who received DC vaccines. This patient is HLA-A^*0201^{pos}, and was shown to have MART-1 specific $CD8^+$ T cells recognizing HLA-A*0201-restricted dominant epitope (MART-1_{26-35}) with tetramer binding assay (data not shown). When PBMCs were cultured with this HLA-A*0201 dominant MART-1 peptide, two cytokines, IL-1 α and IP-10, were specifically induced, but no upregulation of IFN-γ was noted (data not shown). Given that IP-10 is secreted by many cell types in response to type I or type II interferons, we surmised that IP-10 was produced in response to IFN-γ secreted from MART-1 specific T cells. Accordingly, IP-10 production was completely abrogated by blocking the function of IFN-γ (Figure 4C). The results indicate that the induction of IP-10 in response to MART-1 peptide in the melanoma patient vaccinated with peptide-loaded CD34-DCs is dependent on IFN-γ.

[0081] To check if this patient's PBMCs contain CD8+ T cells recognizing this dominant epitope, PBMCs were cultured with autologous monocyte-derived DCs loaded with MART-127-35 or other HLA-0201 peptides (e.g., Mage-3, Tyrosinase, and Flu-MP), and examined for the induction of antigen-specific CD8+ T cell population with specific tetramers. As shown in Figure 4B-4E, MART-1 specific CD8+ T cells were easily expanded within 7 days, indicating that this patient has memory or effector CD8+ T cells for MART-1 and that this CD8+ T cell population was recognized according to

methods of the present invention with the MART-1 library.

[0082] Example 5: Methods of present invention allow identification of various types of immune responses. Methods of the present invention allow simultaneous identification of epitopes for immune response as well as the type of response induced. According to general methods given in Example 4, PBMCs from melanoma patients either pre-vaccine or post-vaccine were incubated with 5-6 peptide clusters from MART-1 or NY-ESO1 peptide libraries for 48 hours (MART-1 with 5 clusters; NY-ESO1 with 8 clusters). Typical results were shown to represent signatures of cytokine profiles corresponding to different immune responses (data not shown). The peptide clusters displayed the pattern of each immune response were shown in gray bars. In a Type-1 response, IL-10 production is suppressed and IP-10 is induced. On the contrary, in a T-Regulatory response, more IL-10 is induced and IP-10 production is abrogated because of the inhibition of Type-1 responses. IL-13 and eotaxin are detected in Type-2 responses without affecting the level of IP-10. Thus, the present invention identifies various types of immune responses induced by any kind of antigens in as fast as 48 hours. $2x10^5$ fresh PBMCs from an HLA-A0201+ donor were stimulated with HLA-A0201 restricted Flu-MP58-66, Mage3271-279, or diluent in triplicates for the indicated periods. Cytokines in the culture supernatant were measured with multiplex bead-based cytokine assay. Mean $\pm$ SD are shown from triplicate data. This result shows that the peptide-specific production of IL-1 a and IP-10 reached plateau at 48 h of peptide stimulation.

[0083] Example 6: Methods of present invention allow identification of other types of immune responses induced by non-T cells. It is known that 15-mers peptides are recognized by other immune cells, such as B cells, NK cells, or NK-T cells, and that some peptides bind to an unidentified receptor, leading to modulation of immune responses. Another example of the present invention was used to identify an immune response induced by non-T cells with a peptide that exhibited a regulatory signature in an MHC-non restricted. PBMCs from a melanoma patient were incubated with peptide clusters from survivin library. As shown in Figure 6A-6H, peptide #15 from the survivin cluster #3 induced a strong regulatory pattern of cytokine production. Namely, IL-10 and IL-1β were strongly induced, and IL-1α and IP-10, which are markers for Type-1 responses, were severely abrogated.

[0084] To determine if this response is restricted to a certain type of MHC-molecule, PBMCs derived fresh blood taken from 5 normal healthy volunteers were incubated with survivin peptide #15 for 48 hours, and cytokine concentrations were measured according to the methods of the present invention. Peptide #15 inhibited IP-10 production in all five normal volunteers (data not shown), indicating that this peptide is not restricted to a certain HLA subtype. Survivin Peptide #15 was shown to have a strong capacity to modify immune response, because PBMC from a melanoma patient stimulated with live flu virus in the presence of Peptide#15 exhibited strong abrogation of IP-10 induction, apparently due to IL-10 production (data not shown). Furthermore, Peptide #15 partially inhibited TSST-1 induced T cell proliferation (data not shown). Depletion studies showed that CD56+ cells were responsible for IL-10 production and IP-10 blockade (data not shown), indicating that survivin Peptide #15 acts on the NK, NK-T cell or gamma/delta T cell population. Peptide#15 was also shown to maintain the survival of CD3-CD56+ NK cells (data not shown). Thus, the methods of the present invention are useful for identification of any kind of immune modulation induced by certain types of peptides.

[0085] Example 7: Methods of present invention allow for immunomonitoring of melanoma patient during vaccination. Cells were seeded at 2 $x10^5$ per well in coded, preconditioned 96 well Phase-I culture plates. Phase-I culture plates contain 5 peptide clusters each, of the tumor antigens GP100, MAGE3, NY-ESO, and MART-1, in quadruplicate with 12 overlapping peptides, each 15 amino acids in length, per cluster, as well as KLH and killed Colo cell controls. Plates may be prepared in advance to maximize throughput and minimize assay to assay variability. Cells are incubated for 5 days at 37˚ C. In parallel, cells designated for restimulation are cultured for 7 days in the presence of autologous mature DCs loaded with the full spectrum of antigenic peptide clusters at a ratio of 30:1 PBMC to DC. On the seventh day, these cells are extensively washed and transferred to a Phase-I culture plate for 24 hours. Following incubation, these restimulated cells will are pelleted, and 150 microliters of each supernatant is transferred in 96-well format to a fully automated Luminex100 Cytokine Multiplex work station equipped with a TECAN Genesis RPS robotic sample processor (TECAN).

[0086] A workstation capable of operator independent sample processing may be used with barcode identification and random microplate analysis. Supernatants are rapidly screened in duplicate for the presence of IFN-γ, TNF-α, IL-13, IL-10 and Granzyme-B, using a master mix of pre-prepared and validated multiplex beads and reagents. Remaining supernatant samples are frozen for later analysis. It is anticipated that DCs from the co-culture may contribute to background cytokine production during the 24-hour incubation in the Phase-I plate. Supernatants from mature DCs serve, therefore, as a control in the cytokine multiplex analysis. Cells from the primary stimulation assay are spun down and resuspended in media containing H3 thymidine and incubated 37˚ C. After 5 days, antigen specific proliferation is assessed in quadruplicate by a thymidine incorporation assay. Supernatants from the primary stimulation assay are frozen at -80˚ C for analysis at a later date. Those peptide clusters, which are positive for cytokine production after restimulation, are screened for cytokine production during primary stimulation.

[0087] Tumor antigen clusters which prove to be positive for reactivity by Phase-I cytokine multiplex array, are broken down into their constituent components for antigenic peptide identification on Phase-II culture plates. Phase-II culture plates includes well-strips containing the 12 component peptides for each cluster in duplicate. Phase-II peptide well-strips are prepared in advance and set into bar code labeled frames. Thawed PBMCs are cocultured for 7 days at 37˚

C with autologous mature DCs loaded with the specific peptide cluster identified in Phase-I, washed and transferred to a Phase-II culture plate for 24 hours at 2 x 10^5 per well. Following incubation, cells are pelleted, and 150 microliters of supernatants are transferred to the Luminex workstation for analysis. Multiplex screening in Phase-II for a given peptide strip consists of the cytokine or cytokines produced in response to its parent cluster during Phase-I analysis. Mature DCs alone serve as a background control for Phase-II analysis. Once the antigenic peptide or peptides of a given cluster are identified, they are used to isolate the antigen specific effector populations.

[0088] Phase-III monitoring consists of identification and characterization of peptide specific effector cells. In this procedure, 2x10^6 PBMCs are thawed and incubated for 7 days at 37˚ C with autologous mature DCs loaded with each antigenic peptide isolated in Phase-II. Cells are then washed and subjected to a 24 hour restimulation. Following restimulation, cells are brefeldin-A treated, fixed, permeabilized and stained with a panel of antibodies to characterize both surface marker and intracellular cytokine expression. Identification of antigen specific effector populations is accomplished by multicolor flowcytometric analysis using a FACSaria flow cytometer/sorter (Becton-Dickinson), capable of 9 color analysis and sorting. The CTL function of stimulated T cells is determined in vitro by a Cr51 release assay using peptide loaded, EBV transformed, autologous B-LCLs as targets. Following identification, antigen specific effector cell populations are subcloned by coculturing for 7 days with autologous mature antigenic peptide loaded DCs in the presence of IL-2 and IL-6, followed by weekly restimulations with peptide loaded mature DCs in the presence of IL-2 and IL-7. Once clonal effector cell populations are established, an extensive characterization of gene expression patterns and cytotoxic function is undertaken.

[0089] Example 8: Methods of present invention allow selection of vaccine epitopes in cancer. Detecting and characterization of reactivity, either Type-1, Type-2, or T-Regulatory, to specific tumor-associated epitopes using this technology can serve as a tool for the identification and characterization of antigenic epitopes to tumor-associated proteins that elicit a desired type of immune response when used for vaccination.

[0090] To select vaccine epitopes, a patient's PBMCs are exposed to a series of peptide libraries representing antigenic epitopes from tumor associated proteins. After 48 hours culture, supernatants are harvested, and secreted cytokines are analyzed according to the teaching of the present invention. Clusters of peptides that induce a Type-1 T cell response, for example, production of IFN-γ but no production of IL-10 (T-Regulatory response) or Type 2-cytokines are identified. In the next step, peptides corresponding to a given patient HLA type can be further selected and used for vaccination of this particular patient. Thus, agents for vaccination tailored to produce a desired type of immune response in a patient can be determined with the present invention.

[0091] Example 9: Methods of present invention allow selection of vaccine epitopes in infectious diseases. It is known that many of currently available vaccines against microbial agents do not confer protective immunity, for example Dengue virus vaccine. Lack of the protective immunity can be due to the induction of an inappropriate immune response, for example, some of the current vaccine epitopes may induce Type-2, or Regulatory T cells rather than Type-1 T cells, thus skewing and/or inhibiting the immune response towards a non-protective one. In this example, the technology of the present invention can serve as a tool for the identification and characterization of antigenic epitopes of microbe associated antigens that elicit a desired type of immune response when used for vaccination.

[0092] To select vaccine epitopes, a patient's PBMCs are exposed to a series of peptide libraries representing antigenic epitopes from a microbe, for example Dengue virus. After 48 hours culture, supernatants are harvested, and secreted cytokines are analyzed according to the teaching of the present invention. Clusters of peptides that induce a Type-1 T cell response, for example, production of IFN-γ but no production of IL-10 (T-Regulatory response) or Type-2 cytokines are identified. In the next step, peptides are further selected and used for vaccination. Thus, the methods of the present invention allows for the selection of epitopes that permit the induction of protective antimicrobial immunity upon vaccination.

[0093] Figure 1 is a graph that depicts the immune response of a normal donor evaluated according the present invention, wherein peripheral blood mononuclear cells (PBMC) were isolated from freshly drawn blood from an HLA-A0201$^+$ healthy volunteer, seeded at 2 x10^5 cells/well in triplicates in round-bottom 96-well plates, and incubated with 1 μl of either of HLA-A0201 restricted peptides Flu-MP_{58-66} (GILGFVFTL) or MAGE-$3_{271-279}$ (FLWGPRALV), or peptide diluent (5% DMSO in H_2O) and culture supernatants were harvested 48 h later and evaluated quantitatively for the presence of cytokines and chemokines using Luminex technology. Results indicate that fresh PBMC from the normal donor respond to Flu-MP peptide and produce a large array of cytokines.

[0094] Figure 2 is a graph that depicts another example of immune response to HLA-A0201 restricted peptides Flu-MP_{58-66} (GILGFVFTL) in a normal donor according the present invention. PBMCs were isolated from freshly drawn blood from another HLA-A0201$^+$ healthy volunteer, seeded at 5 x 10^5 cells/well in triplicates in round-bottom 96-well plates, and incubated with 10 μg/ml of either of HLA-A0201 restricted peptides Flu-MP_{58-66} (GILGFVFTL) or MART-1_{27-35} (AAGIGILTV), or peptide diluent (5% DMSO in H_2O); and culture supernatants were harvested 48 h later and evaluated quantitatively for the presence of cytokines and chemokines using Luminex technology. Results indicate that fresh PBMC from the normal donor respond to Flu-MP peptide and produce IL-1α and IP-10. The cytokine/chemokine production profiles are different between two different normal donors, suggesting that different types of CD8+ T cells

recognizing the same Flu-MP peptide can be identified with EPIMAX assay.

[0095] Figures 3A-3D are graphs that depict the immune response of 2 melanoma patients as evaluated according the present invention. In Figures 3A and 3C, for Patient 1 and 2, respectively, thawed cryopreserved PBMCs in liquid nitrogen from 2 melanoma patients who received at least 8 injections of DC vaccine (autologous $CD34^+$ hematopoietic progenitor cell-derived DCs loaded with four HLA-A2 peptides of melanoma associated antigens) were seeded in triplicates in round-bottom 96-well plates, and incubated with 10 μg/ml of each of HLA-A0201 restricted peptides: Flu-MP_{58-66}, CMV PP65 (NLVPMVATV), MAGE-$3_{271-279}$(FLWGPRALV), MART-1_{27-35} (AAGIGILTV), $gp100_{g209-2}$ M (IMDQVPFSV), $Tyrosinase_{368-376}$ (YMDGTMSQV), or peptide diluent (5% DMSO in H_2O); and culture supernatants were harvested 48 h later and evaluated quantitatively for the presence of cytokines and chemokines using Luminex technology. In Figures 3B and 3D for Patient 1 and 2, respectively, ELISPOT assay was performed for the detection of antigen-specific IFN-γ-producing T cells, wherein PBMCs (2 x 10^5 cells/well) were added to plates precoated with 10 μg/ml of a primary anti-IFN-γ monoclonal antibody in the presence or absence of 10 μg/ml peptides. The results indicate that a broad repertoire of peptide reactive cells in PBMC was identified by the method of the present invention.

[0096] Figures 4A-4C are graphs that depict the immune response of a melanoma patient as evaluated according the present invention. For Figure 4A, PBMCs were obtained from an HLA-A*0201 ^{pos} melanoma patient who received DC vaccines, and stimulated with autologous DCs loaded with HLA-A*0201-restricted dominant MART-1 peptide (MART-1_{26-35}). The patient was shown to have MART-1 specific CD8+ T cells recognizing HLA-A*0201-restricted dominant epitope (MART-1_{26-35}) with tetramer binding assay (Figure 4A). When PBMCs were cultured directly with this HLA-A*0201 dominant MART-1 peptide, two cytokines, IL-1α and IP-10, were specifically induced, but no upregulation of IFN-γ was noted (Figure 4B). Given that IP-10 is secreted by many cell types in response to type I or type II interferons, we surmised that IP-10 was produced in response to IFN-γ secreted from MART-1 specific T cells. Accordingly, IP-10 production was completely abrogated by blocking the function of IFN-γ using IFNγR blocking mAb (Figure 4C). The results indicate that the induction of IP-10 in response to MART-1 peptide in the melanoma patient vaccinated with peptide-loaded CD34-DCs is dependent on IFN-γ.

[0097] Figure 5 is a graph that depicts the kinetics of the immune response of a normal donor evaluated according the present invention. 2 x 10^5 fresh PBMCs from an HLA-A0201$^+$ donor were stimulated with HLA-A0201 restricted peptides Flu-MP_{58-66}, or $Mage3_{271-279}$, or diluent in triplicates for the indicated periods. Cytokines in the culture supernatant were measured with multiplex bead-based cytokine assay. Mean±SD are shown from triplicate data. These result shows that the peptide-specific production of IL-1a and IP-10 in EPIMAX assay reached plateau at 48 h of peptide stimulation.

[0098] Figure 6 depicts the scheme of epitope focusing according to the present invention. First, 15-mer overlapping peptide libraries encoding antigenic proteins are split into clusters of peptides (5-10 peptides/cluster) in duplicates. Five x 10^5 PBMCs are first stimulated with each cluster of peptides (Cluster Analysis). Multiple cytokines at culture supernatant are measured with a multiplex bead-based cytokine assay, and a "hit" cluster was determined. In the second culture, Epitope Focusing, PBMCs were cultured with individual peptides from the "hit" cluster for 48 h, and subsequently the antigenic peptide was determined with multiple cytokine analysis.

[0099] Figure 7 is a graph that depicts the immune response of a melanoma patient as evaluated according the present invention, which allows identification of epitope recognized by specific T cells. PBMCs were obtained from a melanoma patient who received DC vaccines. This patient is HLA-A*0201^{pos}, and was shown to have MART-1 specific CD8+ T cells recognizing HLA-A*0201-restricted dominant epitope (MART-1_{26-35}) with tetramer binding assay (Figure 4A). 15-mer overlapping peptides with 4 amino acid offsets covering the whole amino acid sequence of MART-1 as either 4-6 clustered peptides (10 μM per peptide) or a single peptide (10 μM) were seeded in triplicates in 96-well plates; and 2 x 10^5 cells PBMCs in 200 μl CM were added to each well and incubated for 48 hours at 37 degrees C in a humidified 5% CO2 incubator; and the cytokine or chemokine levels in the culture supernatant were measured with Luminex. As shown in Figure 7, MART-1 #6 peptide induces strong production of IP-10 in PBMCs obtained from a vaccinated melanoma patient. MART-1 peptide #6 contains 10-mer HLA-A2 dominant epitope, as depicted in Figure 4A. Thus, the methods of the present invention allow identification of peptide-specific T cell responses.

[0100] Figure 8 is a graph that depicts the immune response of a melanoma patient as evaluated according the present invention. PBMCs were obtained from a melanoma patient who received DC vaccines. This patient is HLA-A*0201^{pos}, and was shown to have MART-1 specific $CD8^+$ T cells recognizing HLA-A*0201-restricted dominant epitope (MART-1_{26-35}) with tetramer binding assay (Figure 4A). PBMCs were stimulated for 48 h with A2-MART-1 peptide or 15-mer MART-1 peptide#6 in the presence of blocking IFN γR mAb (20 μg/ml) or control mAb. A2-HIVpol peptide and 15-mer MART-1 peptide#15 were used as control peptides. The levels of IL-1a and IP-10 in the culture supernatant are shown. This result indicates that both-IL-1a and IP-10 production are dependent on IFN-γ production from peptide-specific T cells, demonstrating that IL-1a and IP-10 are surrogate markers for Type 1 T cells responses, i.e. Th1 and Tc1 cells.

[0101] Figure 9 is a graph that depicts the immune response of melanoma patients as evaluated according the present invention. PBMCs obtained from melanoma patients were stimulated for 48 h with either clusters of overlapping peptides or individual peptides encoding melanoma antigens. The levels of cytokines/chemokines in the culture supernatants at

48 hours of cultures were measured with Luminex. This result indicates that the concept of epitope focusing can be applied to any antigens and any cytokines in the method of the present invention.

[0102] Figure 10 depicts the immune response of a melanoma patient as evaluated according the present invention. PBMCs obtained from a melanoma patient who received DC vaccination series were stimulated with either clusters of overlapping peptides or individual peptides encoding MART-1, a melanoma differentiation antigen. The levels of IL-1a and IP-10 in the culture supernatants at 48 h of culture are shown. With the cluster analysis (Top), cluster #2 and #3 induced upregulation of IL-1a and IP-10. With the epitope focusing (Bottom), peptide#6 from cluster #2 and peptides#13 from cluster #3 were identified as epitopes for specific T cells. This result indicates that the method of the present invention allows identifying epitopes for specific T cells capable of making IFN-γ.

[0103] Figure 11 depicts the immune response of a melanoma patient as evaluated according the present invention. PBMCs were obtained from the same patient with Figure 10 with a leukopheresis, made aliquots in cryotubes, and kept in liquid nitrogen until use. Thawed PBMCs were stimulated with MART-1 15-mer peptide#6, peptide#13, or diluent. This experiment was repeated three times separately. The levels of IL-1a a and IP-10 in the culture supernatants at 48 h of culture are shown. Three out of 3 experiments revealed upregulation of IL-1a and IP-10 upon stimulation with peptide#6 or #13, demonstrating a high reproducibility of this EPIMAX assay.

[0104] Figures 12A is a graph that depicts the immune response of a melanoma patient as evaluated according the present invention. PBMCs obtained before DC vaccination and post 8 DC vaccinations from a melanoma patient (same as Figure 10) were stimulated with single 15-mer MART-1 peptides or diluent. The levels of IL-1a and IP-10 in the culture supernatants at 48 h of culture are shown. While Post-PBMC showed upregulation of IL-1a andIP-10 in response to identified two (2) 15-mer peptides, peptide#6 and #13, Pre-PBMC failed upregulation of IL-1a and IP-10 in response to peptide#13. IL-1a and IP-10 production from Pre-PBMC in response to peptide#6 was less than that from Post-PBMC. These results suggest that DC vaccination induced peptide#13-specific T cell responses and enhanced peptide#6-specific T cell responses. To examine this hypothesis, we used a different well-established methodology, intracellular cytokine detection assay. The same PBMCs were stimulated for 8 h with MART-1 15-mer single peptides in the presence of anti-CD28/CD49d mAb and monensin. After permeabilization of the cell membrane, the cells were stained with anti-IFN-γ mAb and the IFN-γ+ populations were analyzed with a flowcytometer (Figure 12B). While about 0.15% of CD8+ T cells produced IFN-g in response to either peptide#6 or #13 in Post-PBMC, no IFN-g production was detected in Pre-PBMC. These results demonstrate that EPIMAX can be used to monitor antigen-specific T cell responses in cancer patients, and suggest that the magnitude of each specific T cell response is predictable by looking at the magnitude of cytokine/chemokines modulation in EPIMAX.

[0105] Figure 13 depicts the immune response of a melanoma patient as evaluated according the present invention. CFSE (5,6-carboxyfluorescein diacetate succinimidyl ester) is a tracer dye that permits to identify the number of cell division with a flowcytometer. As EPIMAX is a non-destructive assay, we combined with EPIMAX and CFSE technology to determine the proliferative capacity of antigen-specific T cells. It was confirmed that CFSE staining does not alter the cytokine production in EPIMAX. PBMCs obtained from the same melanoma patient (same as Figure 10) were first stained with 1 uM of CFSE, and stimulated with either clusters of overlapping peptides or individual peptides encoding MART-1. On day 8 of culture, the cells were stained with CD8-PE, CD3-PerCP, and CD4-APC, and analyzed T cell proliferation in response to peptide stimulation. The figure shows the analysis of CD8+ T cell proliferation. MART-1 cluster #2 and #3, and peptide#6 and #13 induced dilution of CFSE intensity in some CD8+ T cell populations, demonstrating that peptide stimulation induced proliferation of antigen-specific CD8+ T cells. This result indicates that combination with other methodology permits to obtain more phenotypical and/or functional characterization of antigen-specific T cells.

[0106] Figure 14 depicts the immune response of a melanoma patient as evaluated according the present invention. PBMCs obtained from the same melanoma patient (same as Figure 10) were first stained with 1 μM of CFSE, and stimulated with individual peptides encoding MART-1 in triplicates. On day 8 of culture, the cells were stained with CD8-PE, CD3-PerCP, and CD4-APC, and analyzed T cell proliferation in response to peptide stimulation. The figure shows the % of CFSE-diluted CD8+ T cell populations within the CD8+ T cell populations. MART-1 peptide#6 and #13 induced dilution of CFSE intensity in some CD8+ T cell populations, demonstrating that peptide stimulation induced proliferation of antigen-specific CD8+ T cells.

[0107] Figure 15 depicts the immune response of a melanoma patient as evaluated according the present invention. $CD8^+$ T cells recognize 8-10mer peptide in the context of MHC-class I molecules expressed on antigen-presenting cells. To determine the precise epitope for identified $CD8^+$ T cells, overlapping 10mer peptides were generated with 1 amino acid lagging within 15mer MART-1 peptide#13, and used to stimulate the same patient's PBMCs. The levels of IL-1a and IP-10 in the culture supernatant at 48 h, and CFSE dilution assay on day 8 of culture are shown. These results demonstrate that MART-1_{54-62} is the epitope for the CD8+ T cells, which allows production of IFN-γ and proliferation of specific CD8+ T cells. Thus EPIMAX permits to identify precise epitopes for CD8+ T cells.

[0108] Figures 16A-D depicts the immune response of a melanoma patient as evaluated according the present invention. PBMCs obtained from a $HLA\text{-}A2^{neg}$ melanoma patient were stimulated with either clusters of overlapping peptides or individual peptides encoding NY-ESO1, a cancer-testis antigen broadly expressed in most of melanoma

cells. NY-ESO1 peptide#24 within cluster#5 induced upregulation of IP-10 at 48 h (Figure 16A). These results indicate that NY-ESO1$_{93-107}$ is recognized as an epitope by specific T cells. To examine which T cell subsets recognize this epitope, the patient's PBMCs were stimulated with NY-ESO1 peptide#24 in the presence of anti-MHC class I blocking mAb (W6/32) or isotype control mAb. As shown in Figure 16B, the IP-10 production in response to peptide#24 was completely abrogated by W6/32, indicating that the production of IP-10 is dependent on MHC-class I molecule, thus that CD8$^+$ T cells recognize peptide#24.

[0109] To identify precise epitope for CD8+ T cells, 9mer overlapping peptide within peptide#24 were generated, and used to stimulate the patient's PBMCs. As shown in Figure 16C, NY-ESO1$_{94-102}$ induced strong upregulation of IP-10, indicating that this is the epitope for CD8+ T cells. To identify HLA-restriction element, the PBMCs were stimulated with autologous monocyte-derived DCs pulsed with NY-ESO1 peptide#24 in the presence of IL-2 (10 IU/ml). At day 9 of culture, the cells were re-stimulated with LCLs lacking MHC-class I molecules transfected with expression plasmids encoding each HLA, pulsed or unpulsed with NY-ESO1$_{94-102}$ peptide. IFN-γ secretion during 8 h stimulation was measured with ELISA. As shown in Figure 16D, HLA-B*3501 transfectant pulsed with NY-ESO1$_{94-102}$ peptide induced IFN-γ production from T cells, indicating this novel peptide is presented by HLA-B*3501 molecule, These results indicate that EPIMAX allows to identify novel epitopes for CD8+ T cells in HLA-A2^{neg} patients, and combination with other methodologies allows us to identify their HLA restriction elements.

[0110] Figure 17 depicts the immune response of a melanoma patient as evaluated according the present invention. The PBMCs obtained from a melanoma patient (same as Figure 10) were stimulated with 10mer overlapping peptides within MART-1 15-mer peptides #6 and #13 for 48 h. Figure 17 shows the levels of IP-10 in the culture supernatant at 48 hours is shown. MART-1$_{26-35}$ (known epitope) and MART-1$_{53-62}$ (novel epitope) are the epitopes.

[0111] Figure 18 depicts the immune response of melanoma patients as evaluated according the present invention. The PBMCs obtained from two HLA-A2^{neg} melanoma patients were stimulated with 10mer overlapping peptides within MART-1 15-mer peptides #6 for 48 hours. The levels of IP-10 in the culture supernatant at 48 hours are shown. MART-1$_{26-35}$ (known epitope) is the epitopes for the both patients. This epitope is known as restricted to HLA-A2. As these patients are HLA-A2^{neg}, and the HLA-class I subtypes are completely different between these two patients, this epitope is presented by at least three different MHC class I molecules. This is another example of identification of epitopes for CD8+ T cells with EPIMAX.

[0112] Figure 19 depicts the immune response of a melanoma patient as evaluated according the present invention. The PBMCs obtained from a melanoma patient (same as Figure 10) were stimulated in triplicates with individual MART-1 15-mer peptides from #6 to #15 for 48 h. The levels of IL-2 in the culture supernatant at 48 hours are shown. MART-1 peptides#10 and #11 induced upregulation of IL-2. This result demonstrates another example of epitope identification with EPIMAX.

[0113] Figure 20 depicts IL-2 production from CD4$^+$ T cells upon stimulation with the identified 15-mer peptide as shown in Figure 19. The same patient's PBMCs were stimulated for 8 hours with MART-1 15-mer peptide#10 in the presence of antiCD28/CD49d mAb and monensin. The production of IL-2 were analyzed by staining with specific mAbs. The figure demonstrates the CD4+ T cell populations producing IL-2 in response to MART-1 peptide#10. These results indicate that EPIMAX can also identify epitopes for CD4$^+$ T cells.

[0114] Figures 21A-B depicts the immune response of a melanoma patient as evaluated according the present invention. PBMCs obtained from the same melanoma patient (same as Figure 19) were first stained with 1 μM of CFSE, and stimulated with individual peptides encoding MART-1 in triplicates. On day 8 of culture, the cells were stained with CD8-PE, CD3-PerCP, and CD4-APC, and analyzed T cell proliferation in response to peptide stimulation. The figure 21A shows the % of CFSE-diluted CD4+ T cell populations within the CD4$^+$T cell populations. MART-1 peptide#10 and #11 induced more CFSE-diluted CD4$^+$ T cell populations than no peptide, demonstrating that these peptides induced proliferation of antigen-specific CD4$^+$ T cells. Figure 21B depicts the CFSE-diluted CD4+ T cell populations in response to each MART-1 15-mer peptide within the CD4$^+$ T cell population. MART-1 peptide#10 and #11 induced significantly more proliferation of CD4$^+$ T cells than the other 15-mer peptides. These results strongly indicates that EPIMAX allows identification of epitopes for CD4$^+$ T cells, and permits to determine the proliferative capacity of specific CD4$^+$ T cells by combining with the CFSE technology.

[0115] Figures 22 A-C depicts the immune response of a melanoma patient as evaluated according the present invention. PBMCs obtained from a HLA-A2^{neg} melanoma patient who received DC vaccinations were first stained with 1 μM of CFSE, and stimulated with clusters of peptides encoding NY-ESO1. On day 8 of culture, the cells were stained with CD8-PE, CD3-PerCP, and CD4-APC, and analyzed T cell proliferation in response to peptide stimulation was analyzed based on CFSE dilution with a flowcytometer. Figure 22A shows the percent (%) of CFSE-diluted CD4$^+$ T cell populations within the CD4+ T cell populations. NY-ESO1 cluster#8 induced more CFSE-diluted CD4$^+$ T cell populations than the other clusters, demonstrating that these peptides induced proliferation of antigen-specific CD4+ T cells. Figure 22B depicts the IL-2 levels in the culture supernatant at 48 hours of culture. IL-2 was produced in the culture with NY-ESO1 cluster#8. To identify the epitope for CD4+ T cells, CFSE-stained PBMCs were stimulated with individual peptides within NY-ESO1 cluster#8. The proliferating CD4+ T cells were analyzed based on CFSE dilution with a flowcytometer

on day 8. As shown in Figure 22C, NY-ESO1$_{149-167}$ and NY-ESO1$_{165-180}$ induced more proliferation of CD4$^+$ T cells, demonstrating that these are the epitopes. This result indicates another example of identification of CD4$^+$ T cell epitopes in melanoma antigens.

[0116] Figure 23 depicts the immune response of a melanoma patient as evaluated according the present invention. The PBMCs obtained from a melanoma patient (same as Figure 10) were stimulated in triplicates with individual MART-1 15-mer peptides from #6 to #15 for 48 h. The levels of IL-5 in the culture supernatant at 48 hours are shown. MART-1 peptides#10 and #11 induced upregulation of IL-5. This result demonstrates another example of epitope identification with EPIMAX.

[0117] Figure 24 depicts IL-5 production from CD4$^+$ T cells upon stimulation with the identified 15-mer peptide as shown in Figure 23. The same patient's PBMCs were stimulated for 8 hours with MART-1 15-mer peptides#11 in the presence of antiCD28/CD49d mAb and monensin. The production of IL-5 were analyzed by staining with specific mAbs. The figure demonstrates the CD4$^+$ T cell populations producing IL-5 in response to MART-1 peptide#11. This result indicates EPIMAX can identify epitopes as well as type of cytokines produced by specific CD4$^+$ T cells.

[0118] Figure 25 depicts the immune response of melanoma patients as evaluated according the present invention. CFSE-stained PBMCs obtained from two melanoma patients were stimulated with individual 15-mer peptides either from TRP-1 cluster#25 or gp 100 cluster# 15. The cytokines in the culture supernatants were measured at 48 h, and the proliferation of T cells were analyzed on day 8 of culture based on CFSE dilution assay. The results indicate that TRP-1 peptide#124 and gp100 peptide#152 are epitopes for CD4$^+$ T cells, both of which are novel epitopes for CD4$^+$ T cells. Peptide stimulation induced various effector cytokine production, such as IL-2, IL-5, IL-13, and IP-10 (IFN-γ), and proliferation of specific CD4$^+$ T cells. These results indicate another example of identification of CD4$^+$ T cell epitopes in melanoma antigens, and suggest that EPIMAX can be used to identify different type of T cell responses.

[0119] Figure 26 depicts the immune response of three melanoma patients as evaluated according the present invention. EPIMAX permits to identify different T cell subsets, such as Typel (Th1, and Tc1), Type 2 (Th2 and Tc2) cells. PBMCs obtained from patients with metastatic melanoma were stimulated with newly-identified melanoma peptides (15-mers) for 48 h, and cytokines in the culture supernatants were measured with a multiplex bead-based assay. Colored columns represent the levels of cytokines with indicated peptides (bottom), and open columns represent those with diluent. The representative cytokine production for three different T cell subsets, Type 1, 2, and Type-IL-10 are shown. Type 1 response is identified by upregulation of IL-1a and IP-10, while Type 2 response is identified by upregulation of type 2 cytokines, such as IL-4, IL-5, and IL-13 without upregulation of IP-10. EPIMAX was used to identify a novel T cell response characterized by upregulation of IL-10 (Type IL-10).

[0120] Figure 27 depicts a summary of new epitopes for CD4$^+$ and CD8$^+$ T cells within 4 melanoma antigens, i.e., gp100, MART-1, NY-ESO1, and TRP-1, which are identified with EPIMAX methodology. These new epitopes were identified by screening 13 melanoma patients PBMCs with EPIMAX.

Table 1 depicts the summary of epitopes for CD8$^+$ T cells identified with EPIMAX. The identified 15-mer peptides and their amino acid sequence number are shown. Some 15-mer peptides were focused either with 9-mer or 10-mer overlapping peptides, and shown in the table.

Patient	Pre or Post	peptide library	identified epitopes	epitope sequence	Precise epitope	Type of response
094-001	Pre	MART-1	p6	MART-1$_{21-35}$	MART-1$_{25-35}$	1
	Post	MART-1	p6	MART-1$_{21-35}$	MART-1$_{25-35}$	1
	Pre	NY-ESO1	p24	NY-ESO1$_{93-107}$	NY-ESO1$_{94-102}$	1
	Post	NY-ESO1	p24	NY-ESO1$_{03-107}$	NY-ESO1$_{94-102}$	1
	Pre	gp100	p60	gP100$_{237-251}$		1
094-004	Pre	MART-1	p6	MART-1$_{21-35}$	MART-1$_{25-35}$	1
094-007	Pre	NY-ESO1	p39	NY-ESO1$_{153-167}$		1
094-009	Post	MART-1	p6	MART-1$_{21-35}$	MART-1$_{26-35}$	1
	Post	MART-1	p13	MART-1$_{40-63}$	MART-1$_{53-62,54-62}$	1
	Post	gp100	p52	9P$^{100}_{205-219}$	gp100$_{209-217}$	1

(continued)

Patient	Pre or Post	peptide library	identified epitopes	epitope sequence	Precise epitope	Type of response
094-010	Post	NY-ESO1	p21	NY-ESO1$_{61-85}$		10
	Post	gyp100	p140	gp100$_{557-571}$		1
094-012	Post	NY-ESO1	p31	NY-ESO1$_{153-167}$		1
094-015	Post	NY-ESO1	p38	NY-ESO1$_{149-183}$		0
	Post	NY-ESO1	p40	NY-ESO1$_{157-171}$	NY-ESO1$_{160-100}$	0
	Post	NY-ESO1	p41	NY-ESO1$_{161-175}$	NY-ESO1$_{101-170}$	1/10
	Post	gap100	p73	gp100$_{280-303}$		10

Table 2 depicts the summary of epitopes for Th0, Th1, and Th2 cells identified with EPIMAX. The identified 15-mer peptides and their amino acid sequence number are shown.

Patient	Pre or Post	peptide library	identified epitopes	epitope sequence	Type of response	proliferation
094-004	Post	NY-ES01	p38/p39	NY-ESO1$_{149-167}$	0	Yes
		NY-ESO1	p42/p43	NY-ESO1$_{165-183}$	0	Yes
		TRP-1	p118	TRP-1$_{468-483}$	1	Yes
		TRP-1	p124	TRP-1$_{493-507}$	1	Yes
094-009	Post	MART-1	p10/p11	MART-1$_{37-55}$	2	Yes
		gp100	p152	gp100$_{605-610}$	0	Yes
		TRP-1	p48	TRP-1$_{169-203}$	0	Yes
094-012	Post	gp100	p32	gp100$_{125-139}$	2	Yes
		gp100	p40	gp100$_{157-171}$	2	Yes
		gp100	p71	gp100$_{281-285}$	2	Yes
		gp100	p78	gp100$_{300-323}$	2	Yes
		gp100	p152	gp100$_{605-610}$	2	Yes
094-015	Post	gp100	p1/p2	gp100$_{1-16}$	0	Yes
		gp100	p152	gp100$_{605-519}$	0	Yes
		MART-1	p11	MART-1$_{41-55}$	0	Yes (CD4/CD8)

Table 3 depicts the summary of epitopes for Type-IL-10 CD4+ T cells identified with EPIMAX. The identified 15-mer peptides and their amino acid sequence number are shown.

Patient	Pre or Post	peptide library	identified epitopes	epitope sequence	Type of response	proliferation
094-004	Pre	MART-1	p10	MART-1$_{37-51}$	10	No
	Post	MART-1	p10	MART-1$_{37-51}$	10	No
	Pre	TRP-1	p113	TRP-1$_{440-483}$	10	No
094-005	Pre	gp100	p93	gp100$_{300-384}$	10	No

(continued)

Patient	Pre or Post	peptide library	identified epitopes	epitope sequence	Type of response	proliferation
094-006	Pre	MART-1	p8	MART-1_{37-51}	10	No
094-007	Pre	NY-ESO1	p43	NY-$ESO1_{10-183}$	10	No
094-009	Pre	NY-ESO1	p39/p40	NY-$ESO1_{100-183}$	10	Marginal
	Pre	TRP-1	p50	TRP-$1_{107-211}$	10	Marginal
094-010	Post	NY-ESO1	p23	NY-$ESO1_{80-103}$	10	Yes
094-*013	Post	NY-ESO1	p23	NY-ESO_{80-103}	10	

[0121] Figure 28 are graphs that depict the immune response of three melanoma patients as evaluated according the present invention. EPIMAX analysis of PBMCs obtained from patients with metastatic melanoma (13 patients) with MART-1, gp100, NY-ESO1, and TRP-1 overlapping peptides yielded 16 peptides inducing Type 1 responses, 15 peptides inducing Type 0/2 responses, and 9 peptides inducing Type-IL-10. The cytokines were measured at day 2 of cultures. IL-α and IP-10 levels in each T cell subsets are shown respectively. Each dot represents cytokine levels in each separate well from these patients' PBMC culture stimulated with identified single peptides. This result clearly indicates that IL-1α and IP-10 completely correlate in the Type 1 response.

[0122] Figures 29A-D are graphs that depict the immune response of a melanoma patients as evaluated according the present invention. PBMCs obtained from a melanoma patient were stimulated with clusters of peptides or individual peptides encoding NY-ESO1. IL-10 levels in culture supernatant at 48 hours of culture are shown (Figure 29A). NY-ESO1 peptide#39 and #40 are the epitopes. To explore the origin of IL-10 production, the PBMCs were stimulated with peptide#39 in the presence of anti CD28/CD49d mAbs and monensin for 8 hours, and stained with IL-10 specific mAb. As shown in Figure 29B, about 0.11 % of CD4+ T cells produced IL-10 in response to NY-ESO1 peptide#39. This result indicates that EPIMAX permits IL-10 producing melanoma-antigen specific $CD4^+$ T cells in melanoma patients. Next, to evaluate whether this IL-10 producing T cells had regulatory properties, PBMCs stimulated with peptide #39 or irrelevant 15-mer peptide were added in transwells to a "third party" MLR to test suppression of T cell expansion by the release of soluble mediators. The MLR used CFSE-labeled $CD4^+$T cells and allogeneic mature DCs, both from healthy volunteers, in order to avoid the effect of peptide-specific T cell response. The patient's PBMC stimulated with NY-ESO1 peptide #39, but not with control peptide, significantly suppressed proliferation in the MLR (Figures 29C, 29D). These results indicate EPIMAX approach can identify tumor-antigen specific regulatory T cells.

[0123] Figure 30 are graphs that depict the immune response of a melanoma patient as evaluated according the present invention. PBMCs obtained from the same melanoma patient as in Figures 29A-D at two different time points, i.e., Pre DC vaccination and Post 8 DC vaccination were stimulated with individual peptides encoding NY-ESO1. The levels of cytokine/chemokines in culture supernatant at 48 h of culture are shown. This result demonstrates that the signature of Type-IL-10 disappeared at Post vaccine, suggesting the function of IL-10 producing $CD4^+$ T cells was lost at Post vaccine. These results demonstrate another example of modulation of T cell responses by DC vaccine with EPIMAX assay.

[0124] Figure 31 is a graph that depicts the immune response of melanoma patients as evaluated according the present invention. We have identified 9 different epitopes in melanoma antigens inducing Type-EL-10 responses in 7 melanoma patients. PBMCs obtained from patients with melanoma were incubated with each patient-specific identified 15-mer peptide for 48h. The figure shows the inhibition of spontaneous IP-10 production by incubation with identified Type-IL-10 peptides. This is another demonstration of suppressive function of IL-10 producing $CD4^+$ T cells identified with EPIMAX.

[0125] Figure 32 is a graph that depicts the immune response of melanoma patients as evaluated according the present invention. IL-1β and IP-10 levels in each PBMC culture with identified IL-10 inducing 15-mer peptides are shown. Each dot represents cytokine levels in each separate well in PBMC culture with the identified unique single 15-mer peptides. This result shows that IL-1β and IL-10 correlate in identified Type-IL-10 responses, thus both cytokines can be a good marker to identify Type-IL-10 cells in EPIMAX.

[0126] Figure 33 are graphs that depict the immune response of normal healthy volunteers as evaluated according the present invention. The fresh PBMCs obtained from three normal healthy volunteers were split into 3 batches. PBMCs

from each batch were stimulated with clusters of 15-mer overlapping peptides encoding influenza virus matrix protein (Flu-MP) in duplicates (2 x 10^5 cells/well, 10 μM per peptide), thus created 6-plicates of samples. The levels of IP-10 at 48 h of culture are shown. The black bars and shades represent the mean±3SD value of IP-10 in the cultures with no peptides. The levels of IP-10 were determined above mean+3SD of this background are positive induction of IP-10 in response to peptide clusters. In normal door#1 , 9 out of 12 peptide clusters scored positive more than 4 out of 6-plicates of samples, indicating that this donor had very wide repertoire of T cells specific to Flu-MP. In contrast, normal donor#3, no clusters induced upregulation of IP-10. EPIMAX allows identification of the full breadth of T cell responses against a given antigenic protein. Furthermore, this studies shows that EPIMAX is highly reproducible assay.

[0127] Figure 34 are graphs that depict the immune response of normal healthy volunteers as evaluated according the present invention. The levels of IL-6 and IP-10 in the same studies shown in Figure 33 were shown. Strikingly, in normal donor#3, no hits were identified with IP-10 production, but many clusters of peptides scored positive with IL-6 production. This result demonstrates an example showing that measurement of different sets of cytokines can identify different subsets of T cells in EPIMAX.

[0128] Figure 35 are graphs that depict the immune response of normal healthy volunteers as evaluated according the present invention. The levels of IL-1a and IP-10 in the studies shown in Figure 33 were shown. Each dot represents cytokine levels in each separate well in PBMC culture with the peptide clusters. In all the donors, the levels of IL-1a and IP-10 correlate very well in EPIMAX, confirming that these two markers completely correlate in Type 1 responses.

[0129] Figure 36 are graphs that depict the immune response of normal healthy volunteers as evaluated according the present invention. The frozen PBMCs obtained from the donor#1 were thawed and stimulated with either clusters of 15-mer peptides or single peptides encoding Flu-MP. The levels of IL-1a and IP-10 are shown. Each dot represents cytokine levels in each separate well in PBMC culture with the peptide clusters or single peptides. Even with the frozen PBMCs, the levels of It-1a and IP-10 correlate very well ($P<0.0001$, $r=0.9121$), indicating that these two markers can be used to identify Type 1 responses in EPIMAX no matter how the PBMCs are obtained freshly or frozen.

[0130] Figure 37 are graphs that depict the immune response of normal healthy volunteers as evaluated according the present invention. The fresh or frozen PBMCs obtained from the donor#1 were stimulated with clusters of 15-mer peptides encoding Flu-MP. The levels of IL-1a and IP-10 are shown. As shown in Figure 37, both clusters#6 and#12 induced the upregulation of IL-1a and IP-10 in fresh and frozen PBMCs. This study demonstrates that frozen PBMCs can be used to monitor antigen-specific T cell responses with EPIMAX.

[0131] Figure 38A, 38B are graphs that depict the immune response of normal healthy volunteers as evaluated according the present invention. The frozen PBMCs obtained from donor#1 were thawed and stimulated with a cluster of peptides or individual peptides encoding Flu-MP in 6-plicates (2 x 10^5 cells/well, 10 μM per peptide). The levels of IP-10 at 48 hours of culture are shown (Figure 37A). In another study, the higher number of PBMCs (2 x 10^6 cells/well) were stimulated with the same concentration of 15-mer peptide in triplicates. As shown in Figure 37B, while peptide#58 and #59 turned on the IP-10 production in both studies, the contrast between samples and control culture is greater where more PBMCs/well were used in EPIMAX. Thus, higher number of PBMCs per well helps to reduce the variability and sensitivity in EPIMAX assay. Considering the low frequency of antigen specific T cells in PBMCs (usually 1 out of 10^5 PBMCs), it is plausible that T cell responses may not be detected with EPIMAX (or virtually any kind of assays) when few cells responded to the peptide stimulation. Thus, in regular EPIMAX assay, we are using 5 x 10^5 PBMCs/well.

[0132] Figure 39A and 39B are graphs that depict the immune response of normal healthy volunteers against the peptides identified in the EPIMAX assay. PBMCs were resuspended in CM at 1 x 10^6 cells/ml, and 1 ml of cell suspensions were put in culture tubes, and stimulated with indicated single peptides from Flu-MP library for 7 days. Cultured PBMCs were harvested and restimulated with DCs pulsed with indicated peptide for 5 h in the presence of monensin. Cytoplasmic cytokines were examined with specific mAbs after cell permeabilization. The percentages of cytokine producing cells in $CD3^+CD4^+$ T cell population were shown (Figure 39A). Stimulation with dominant peptides induced proliferation of specific $CD4^+$ T cells. The donor's PBMCs were first stained with 1 mM of CFSE. PBMCs were resuspended in CM at 1 x 10^6 cells/ml, and 1 ml of cell suspensions were put in culture tubes, and stimulated with indicated single peptides from Flu-MP library for 6 days. Stimulated cells were stained with anti-CD3 and CD4 mAb, and proliferating $CD4^+$ T cells were analyzed with a flowcytometer based on CFSE dilution. The percentages of CFSE- cells in $CD3^+CD4^+$ T cell population were shown (Figure 39B). These results demonstrate the proof that EPIMAX allowed identification of epitope for functional Flu-MP specific $CD4^+$ T cells.

[0133] Figure 40 are graphs that depict the immune response of a normal healthy volunteer as evaluated according the present invention. The PBMCs were obtained from donor #2 in Figure 33. The frozen PBMC were thawed and stimulated with either Flu-MP cluster#11 or single 15-mer peptides within cluster#11 (5 x 10^5 cells/well, 20 μM per peptide). The levels of cytokines at 48 h of culture are shown. These results indicate that peptide#52 and #53 are epitopes for Flu-MP specific T cells, showing another example of identification of novel epitopes for specific $CD4^+$ T cells.

[0134] Figure 41A, 41B are graphs that depict the immune response of normal healthy volunteers against the peptides identified in the EPIMAX assay. The PBMCs used in the study in Figure 40 were labeled with CFSE, and stimulated with single peptides within Flu-Mpcluster #11. The CFSE-diluted $CD4^+$ T cells were analyzed on day 8 of culture (Figure

41 A). In another study, the CFSE-diluted $CD4^+$ T cell populations were analyzed on day 6, 7, 8 and 10 of culture (Figure 41B). These studies demonstrate that the identified peptide (Flu-MP peptide#52 and #53) with EPIMAX induced proliferation of peptide-specific $CD4^+$ T cells, and the population of proliferating $CD4^+$ T cell reached at a peak on day 7 or 8 of peptide stimulation. This study shows another example of identification of novel epitopes for specific $CD4^+$ T cells, and justifies performing an analysis of proliferating $CD4^+$ T cell population on day 8 of culture.

[0135] Figure 42 are graphs that depict the immune response of a melanoma patient as evaluated according the present invention. PBMCs were obtained from a melanoma patient who underwent DC vaccinations. The patient received autologous DC vaccine loaded with killed allogeneic melanoma cell lines. It is possible that DC vaccine induced T cells in this patient specific to allogeneic antigens expressed on allogeneic melanoma cell lines used for DC vaccines. To examine this possibility, the patients PBMCs were stimulated with 15-mer overlapping peptides encoding HLA-B*4001, which is expressed on the melanoma cell line, but not on the patient cells. As shown in Figure 42, peptide#5 and peptide#7 within cluster#I induced IP-10 production in PBMC culture. As expected, these two epitopes can be antigenic epitope for the patient, as both the peptides contain amino acid mismatches. These results demonstrate that EPIMAX can identify allogeneic antigen-specific T cell responses. Thus this methodology can be useful to identify allogeneic-antigen specific T cells in a setting of organ transplantation.

[0136] Figure 43 is a graph that depicts the immune response of a type-1 diabetic patient as evaluated according to the present invention. PBMCs were obtained from a patient with Type 1 diabetes taking immunosuppressive drugs, such as Cellcept and Predonisolone. The fresh PBMCs (5 x 10^5 cells/well) were stimulated with single 15-me peptides encoding IA-2, one of the auto-antigens specifically expressed on pancreatic β-islet cells. The levels of IP-10 at 48 h of culture are shown. 15-me peptide#47, 60, 61, 62, 64, and 66 induced upregulation of IP-10 production in PBMC culture.

[0137] This study demonstrates that this patient has wide repertoire of IA-2 specific Type- cells. Furthermore, 5 out of 6 (peptide#47, 60, 61, 62, and 66, sequences are shown in Table 4) are novel epitopes for T cells. Thus, this study indicates that EPIMAX allows us to identify epitopes and type of T cell response in an autoimmune disease model even under medication of immunosuppressive drugs.

peptide#47	$IA-_{789-803}$
peptide#60	$IA-2_{841-855}$
peptide#61	$IA-2_{845-859}$
peptide#62	$IA-2_{849-863}$
peptide#64	$IA-2_{857-871}$
peptide#66	$IA-2_{865-879}$

[0138] Figure 44A and 44B are graphs that depict the immune response of a melanoma patient as evaluated according the present invention, which allows the identification- of various types of immune responses. PBMCs obtained from a pre-vaccine melanoma patient were incubated with 5 peptide clusters (7-8 peptides/cluster) of the survivin peptide library. Survivin cluster#3 induced down-regulation of IP-10 and upregulation of IL-10. PBMCs were then incubated with single peptide from cluster #3 to identify the responsible peptide, peptide#15. The upregulation of IL-10 was correlated with IL-1b Data are shown in Figure 44A and 44C for IP-10, Figure 44B and 44D for IL-10, Figure 44E and 44G for IL-1α, and Figure 44F and 44H for IL-1 β.

[0139] Figure 45 is a graph that depicts the immune response of normal healthy volunteers as evaluated according the present invention, which allows the identification of various types of immune responses by non-T cells. PBMCs were isolated from 5 healthy volunteers' fresh blood, incubated with survivin Peptide#15 for 48 hours and evaluated according to the methods of the present invention. IP-10 produced in the culture supernatant was measured, and the data in Figure 45 are given in relative values (%) to cultures where no peptide was added. Survivin Peptide #15 was shown to inhibit IP-10 production in all five volunteers in a non-MHC restricted fashion.

[0140] Figure 46A and 46B are graphs that depict the immune response in a melanoma patient as evaluated according to the present invention, which allows the identification of various types of immune responses by non-T cells. PBMCs from a melanoma patient were cultured for 18 hours in the presence or absence of survivin Peptide #15 and/or live flu virus (Charles River Laboratories, CT). IP-10 (Figure 46A) and IL-10 (Figure 46B) concentrations were measured with Luminex. Survivin Peptide #15 was shown to inhibit IP-10 production from PBMCs stimulated with live flu virus.

[0141] Figure 47 is a graph that depicts the immune response in a melanoma patient as evaluated according to the present invention, which allows the identification of various types of immune responses by non-T cells. PBMCs from a melanoma patient were stimulated for 5 days with TSST-1 at titrated concentrations in the presence of survivin Peptide#15, or Peptide#16 (11 amino acids are identical to Peptide#15). After 5 days, tritiated thymidine (1 μCi/well) was added. The plates were harvested 16 hours later, and incorporated radioactivity was measured by a Wallac scintillation counter. Survivin Peptide #15 was shown to inhibit T cell proliferation stimulated with TSST-1.

[0142] Figure 48A-48D are graphs that depict the immune response in a melanoma patient as evaluated according

to the present invention, which allows the identification of various types of immune response by non-T cells. $CD4^+$, $CD8^+$, $CD14^+$, $CD56^+$, BDCA-4^+, or BDCA-1 or 3^+ cells were depleted from a melanoma patient's PBMCs by using microbeads conjugated with each mAb (Miltenyi). As a negative control, PBMCs were passed through a column without any beads (no depletion). Depleted cells were resuspended in CM at 1 M/ml, and stimulated with live flu virus in the presence or absence of survivin Peptide #15. Supernatants were harvested at 48 hours, and IL-10 and IP-10 levels were analyzed with Luminex. The data show that $CD14^+$ or $CD56^+$ cells are necessary for Peptide #15 to induce E-10. secretion.

[0143] Figure 49A and 49B are graphs that depict the immune response in a melanoma patient as evaluated according to the present invention, which allows the identification of various types of immune responses by non-T cells. PBMCs from melanoma patients were stained with 5 μM CFSE, and cultured for 4 days with 10 μM of survivin Peptide#15 (Figure 49B) or control Peptide#6 (Figure 49A). The NK population was analyzed with FACS. Those $CD56^+$ cells did not decrease the intensity of CFSE, showing that they are not proliferating (not shown). Thus, Peptide#15 was shown to maintain the survival of CD3-$CD56^+$ NK cells,

[0144] It will be understood that particular embodiments described herein are shown by way of illustration and not as limitations of the invention. The principal features of this invention can be employed in various embodiments without departing from the scope of the invention. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of this invention and are covered by the claims.

Claims

1. A method for identifying one or more immunomodulatory peptides comprising the steps of:

   culturing isolated immune cells from a subject with a
   cluster of several overlapping peptides from an overlapping peptide library;
   analyzing simultaneously a supernatant of a culture medium for multiple parameters of immune reactivity comprising the specificity and cytokine response of cytokines and
   chemokines, which allow identification of Type-1, Type-2 and T-Regulatory cell phenotypes to the cluster of several overlapping peptides;
   culturing isolated immune cells from the subject with individual peptides from the cluster of overlapping peptides and analyzing simultaneously the supernatant of the culture medium for said cytokines and chemokines; and
   determining the type of immune response based on the coordinated cytokine expression in response to the individual peptides in the culture medium.

2. The method of claim 1, further comprising identifying peptide specific effector cells.

3. The method of claim 1, further comprising identifying multiple peptide fragment-specific effector cells.

4. A method for assessing the immune status of a subject comprising the steps of:

   identifying a series of immunomodulatory peptides according to claim 1, wherein the type of immune reactivity is indicative of the subject's immune status.

5. The method of claim 4, **characterized in that** the degree of immunosuppression of the subject is assessed.

6. The method of claim 4, **characterized in that** the degree of hyperactivation of the immune system of the subject is assessed.

7. The method of claim 4, **characterized in that** the immune status of a subject is assessed for multiple immunomodulatory peptides to provide a complete T-cell repertoire for the subject.

8. The method of claim 4, **characterized in that** the immunomodulatory peptide and the type of immune reactivity is indicative of the subject's risk of immune-related diseases.

9. The method of claim 8, **characterized in that** the immune related diseases are selected from the group consisting of infectious diseases, autoimmune diseases, autoinflammatory disease, cancer, chronic inflammation and allergy.

10. The method of claim 4, further comprising predicting the subject's response to therapy by determining epitope-specific immune status of the subject as an indicator of treatment outcome.

11. The method of claim 10, **characterized in that** the therapy is immunotherapy such as vaccination, passive immunotherapy and adoptive cell transfer.

12. A method for identifying and characterizing new therapeutic agents for immunotherapy comprising the steps of:

    identifying a series of immunomodulatory peptides according to claim 1, wherein the immune cells are cultured in the presence and absence of an immune modulator and one or more peptides from the overlapping peptide library, wherein the inhibition of the immune reactivity is indicative of an immunosuppressive therapeutic agent and wherein the enhancement of the immune reactivity is indicative of an adjuvant.

13. The method of claim 12, **characterized in that** the therapeutic agents are a combination of immunomodulatory peptides that drive the type of immune response to a Th1, a Th2, a Tc1, a Tc2, and combinations thereof.

14. The method of claim 12, **characterized in that** the new therapeutic agents are vaccine epitopes.

15. The method of claim 14, **characterized in that** sequences of the one or more peptides are determined after isolation.

16. The method of claim 14, **characterized in that** antigenic agent comprises a virus, a bacteria, a fungi, a protozoan, a parasite or a helminth.

17. The method of claim 14 or 16, **characterized in that** antigenic agent is a self-antigen.

18. The method of claims 14 to 17, **characterized in that** the cell culture is analyzed and the cytokine secretion profile of one or more T cells, B cells, dendritic cells, monocytes, neutrophils, mast cells and red blood cells is determined.

Patentansprüche

1. Verfahren zum Identifizieren von einem oder mehreren immunmodulatorischen Peptiden, welches die Schritte umfasst:

    Kultivieren von isolierten Immunzellen von einem Subjekt mit einem Cluster von mehreren überlappenden Peptiden von einer überlappenden Peptidbibliothek;
    gleichzeitiges Analysieren eines Flüssigkeitsüberstands eines Kulturmediums auf multiple Parameter von Immunreaktivität, umfassend die Spezifität und Zytokinantwort von Zytokinen und Chemokinen, welche Identifikation von Typ-1, Typ-2 und T-regulatorischen Zellphänotypen zum Cluster von mehreren überlappenden Peptiden erlauben;
    Kultivieren von isolierten Immunzellen vom Subjekt mit individuellen Peptiden vom Cluster von überlappenden Peptiden und gleichzeitiges Analysieren des Flüssigkeitsüberstands des Kulturmediums auf besagte Zytokine und Chemokine; und
    Bestimmen des Immunantworttyps, basierend auf der koordinierten Zytokinexpression als Antwort auf die individuellen Peptide in dem Kulturmedium.

2. Verfahren nach Anspruch 1, welches ferner Identifizieren von Peptid-spezifischen Effektorzellen umfasst.

3. Verfahren nach Anspruch 1, welches ferner Identifizieren von multiplen Peptidfragment-spezifischen Effektorzellen umfasst.

4. Verfahren zum Bewerten des Immunstatus eines Subjekts, welches die Schritte umfasst:

    Identifizieren einer Serie von immunmodulatorischen Peptiden gemäß Anspruch 1, wobei der Typ der Immunreaktivität für den Immunstatus des Subjekts indikativ ist.

5. Verfahren nach Anspruch 4, **dadurch gekennzeichnet, dass** der Grad der Immunsuppression des Subjekts bewertet wird.

6. Verfahren nach Anspruch 4, **dadurch gekennzeichnet, dass** der Grad der Hyperaktivierung des Immunsystems des Subjekts bewertet wird.

7. Verfahren nach Anspruch 4, **dadurch gekennzeichnet, dass** der Immunstatus eines Subjekts bezüglich multipler immunmodulatorischer Peptide bewertet wird, um ein vollständiges T-Zellen-Repertoire für das Subjekt vorzusehen.

8. Verfahren nach Anspruch 4, **dadurch gekennzeichnet, dass** das immunmodulatorische Peptid und der Typ der Immunreaktivität indikativ für das Risiko des Subjekts für immunbezogene Krankheiten ist.

9. Verfahren nach Anspruch 8, **dadurch gekennzeichnet, dass** die immunbezogenen Krankheiten ausgewählt sind aus der Gruppe bestehend aus Infektionskrankheiten, Autoimmunkrankheiten, autoinflammatorischer Krankheit, Krebs, chronischer Entzündung und Allergie.

10. Verfahren nach Anspruch 4, welches ferner Vorhersagen der Antwort des Subjekts auf Therapie durch Bestimmen von Epitopspezifischem Immunstatus des Subjekts als Indikator für Behandlungsergebnis umfasst.

11. Verfahren nach Anspruch 10, **dadurch gekennzeichnet, dass** die Therapie Immuntherapie wie Impfung, passive Immuntherapie und adoptiver Zelltransfer ist.

12. Verfahren zum Identifizieren und Kennzeichnen von neuen therapeutischen Agenzien für Immuntherapie, welches die Schritte umfasst:

    Identifizieren einer Serie von immunmodulatorischen Peptiden gemäß Anspruch 1, wobei die Immunzellen in Anwesenheit oder Abwesenheit von einem Immunmodulator und einem oder mehreren Peptiden von der überlappenden Peptidbibliothek kultiviert werden,

    wobei die Hemmung der Immunreaktivität indikativ für ein immunsuppressives therapeutisches Agens ist und wobei die Verstärkung der Immunreaktivität indikativ für ein Adjuvans ist.

13. Verfahren nach Anspruch 12, **dadurch gekennzeichnet, dass** die therapeutischen Agenzien eine Kombination aus immunmodulatorischen Peptiden sind, die den Typ der Immunantwort auf ein Th1, ein Th2, ein Tc1, ein Tc2 und Kombinationen davon lenken.

14. Verfahren nach Anspruch 12, **dadurch gekennzeichnet, dass** die neuen therapeutischen Agenzien Impfstoffepitope sind.

15. Verfahren nach Anspruch 14, **dadurch gekennzeichnet, dass** Sequenzen von dem einen oder mehreren Peptiden nach Isolierung bestimmt werden.

16. Verfahren nach Anspruch 14, **dadurch gekennzeichnet, dass** antigenes Agens ein Virus, eine Bakterie, einen Pilz, ein Protozoon, einen Parasit oder einen Helminthen umfasst.

17. Verfahren nach Anspruch 14 oder 16, **dadurch gekennzeichnet, dass** antigenes Agens ein Selbstantigen ist.

18. Verfahren nach Ansprüchen 14 bis 17, **dadurch gekennzeichnet, dass** die Zellkultur analysiert wird und das Zytokinsekretionsprofil von einer oder mehreren T-Zellen, B-Zellen, dendritischen Zellen, Monozyten, Neutrophilen, Mastzellen und roten Blutkörperchen bestimmt wird.

Revendications

1. Procédé d'identification d'un ou plusieurs peptides immunomodulateurs, comprenant les étapes consistant à :

    mettre en culture des cellules immunitaires isolées d'un sujet avec un groupe de plusieurs peptides chevauchants provenant d'une bibliothèque de peptides chevauchants ;
    analyser simultanément un surnageant d'un milieu de culture pour de multiples paramètres d'immunoréactivité comprenant la spécificité et la réponse en cytokine des cytokines et des chimiokines, qui permettent l'identification de phénotypes de cellules de type 1, de type 2 et régulatrices des lymphocytes T selon le groupe de

plusieurs peptides chevauchants ;
mettre en culture des cellules immunitaires isolées du sujet avec des peptides individuels provenant de l'agrégat de peptides chevauchants et analyser simultanément le surnageant du milieu de culture pour lesdites cytokines et chimiokines ; et
déterminer le type de réponse immunitaire sur la base de l'expression de cytokines coordonnée en réponse aux peptides individuels dans le milieu de culture.

2. Procédé selon la revendication 1, comprenant en outre l'étape consistant à identifier des cellules effectrices spécifiques de peptides.

3. Procédé selon la revendication 1, comprenant en outre l'étape consistant à identifier des cellules effectrices spécifiques de multiples fragments peptidiques.

4. Procédé d'évaluation de l'état immunitaire d'un sujet, comprenant les étapes consistant à :

identifier une série de peptides immunomodulateurs selon la revendication 1, dans lequel le type d'immunoréactivité constitue une indication de l'état immunitaire du sujet.

5. Procédé selon la revendication 4, **caractérisé en ce que** le degré d'immunosuppression du sujet est évalué.

6. Procédé selon la revendication 4, **caractérisé en ce que** le degré d'hyperactivation du système immunitaire du sujet est évalué.

7. Procédé selon la revendication 4, **caractérisé en ce que** l'état immunitaire d'un sujet est évalué pour de multiples peptides immunomodulateurs afin d'obtenir un répertoire immunologique de lymphocytes T complet pour le sujet.

8. Procédé selon la revendication 4, **caractérisé en ce que** le peptide immunomodulateur et le type d'immunoréactivité constituent une indication du risque de maladies liées à l'immunité du sujet.

9. Procédé selon la revendication 8, **caractérisé en ce que** les maladies liées à l'immunité sont choisies dans le groupe constitué par les maladies infectieuses, les maladies auto-immunes, les maladies auto-inflammatoires, les cancers, les inflammations chroniques et les allergies.

10. Procédé selon la revendication 4, comprenant en outre l'étape consistant à prédire la réponse du sujet à la thérapie en déterminant l'état immunitaire spécifique des épitopes du sujet en tant qu'indication du résultat du traitement.

11. Procédé selon la revendication 10, **caractérisé en ce que** la thérapie est une immunothérapie telle qu'une vaccination, une immunothérapie passive et un transfert de cellules d'immunisation adoptive.

12. Procédé d'identification et de caractérisation de nouveaux agents thérapeutiques destinés à l'immunothérapie, comprenant les étapes consistant à :

identifier une série de peptides immunomodulateurs selon la revendication 1, dans lequel les cellules immunitaires sont mises en culture en présence et en l'absence d'un immunomodulateur et d'un ou plusieurs peptides provenant de la bibliothèque de peptides chevauchants, dans lequel l'inhibition de l'immunoréactivité constitue une indication d'un agent thérapeutique immunosuppresseur et dans lequel la facilitation de l'immunoréactivité constitue une indication d'un adjuvant.

13. Procédé selon la revendication 12, **caractérisé en ce que** les agents thérapeutiques sont une combinaison de peptides immunomodulateurs qui conduisent au type de réponse immunitaire du type TH1, TH2, TC1, TC2, et des combinaisons de ceux-ci.

14. Procédé selon la revendication 12, **caractérisé en ce que** les nouveaux agents thérapeutiques sont des épitopes de vaccin.

15. Procédé selon la revendication 14, **caractérisé en ce que** les séquences des dits un ou plusieurs peptides sont déterminées après isolement.

16. Procédé selon la revendication 14, **caractérisé en ce que** l'agent antigénique comprend un virus, une bactérie, un champignon, un protozoaire, un parasite ou un helminthe.

17. Procédé selon les revendications 14 ou 16, **caractérisé en ce que** l'agent antigénique est un auto-antigène.

18. Procédé selon les revendications 14 à 17, **caractérisé en ce que** la culture cellulaire est analysée et le profil de sécrétion de cytokines d'un ou plusieurs lymphocytes T, lymphocytes B, d'une ou plusieurs cellules dendritiques, d'un ou plusieurs monocytes, neutrophiles, mastocytes et globules rouges est déterminé.

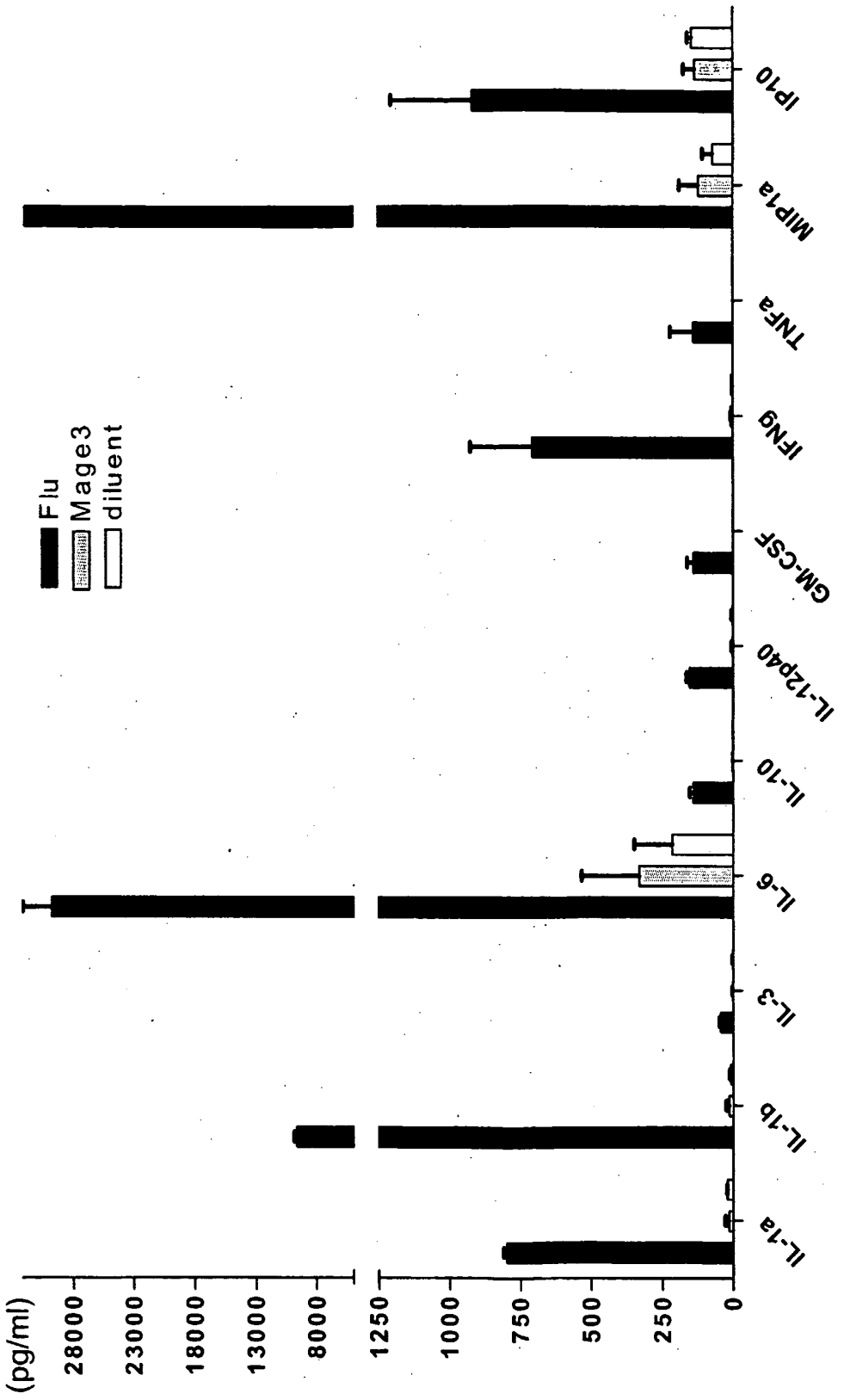

(pg/ml)
28000
23000
18000
13000
8000
1250
1000
750
500
250
0
Flu
Mage3
diluent
IL-1a
IL-1b
IL-3
IL-6
IL-10
IL-12p40
GM-CSF
IFNg
TNFa
MIP1a
IP10

Figure 1

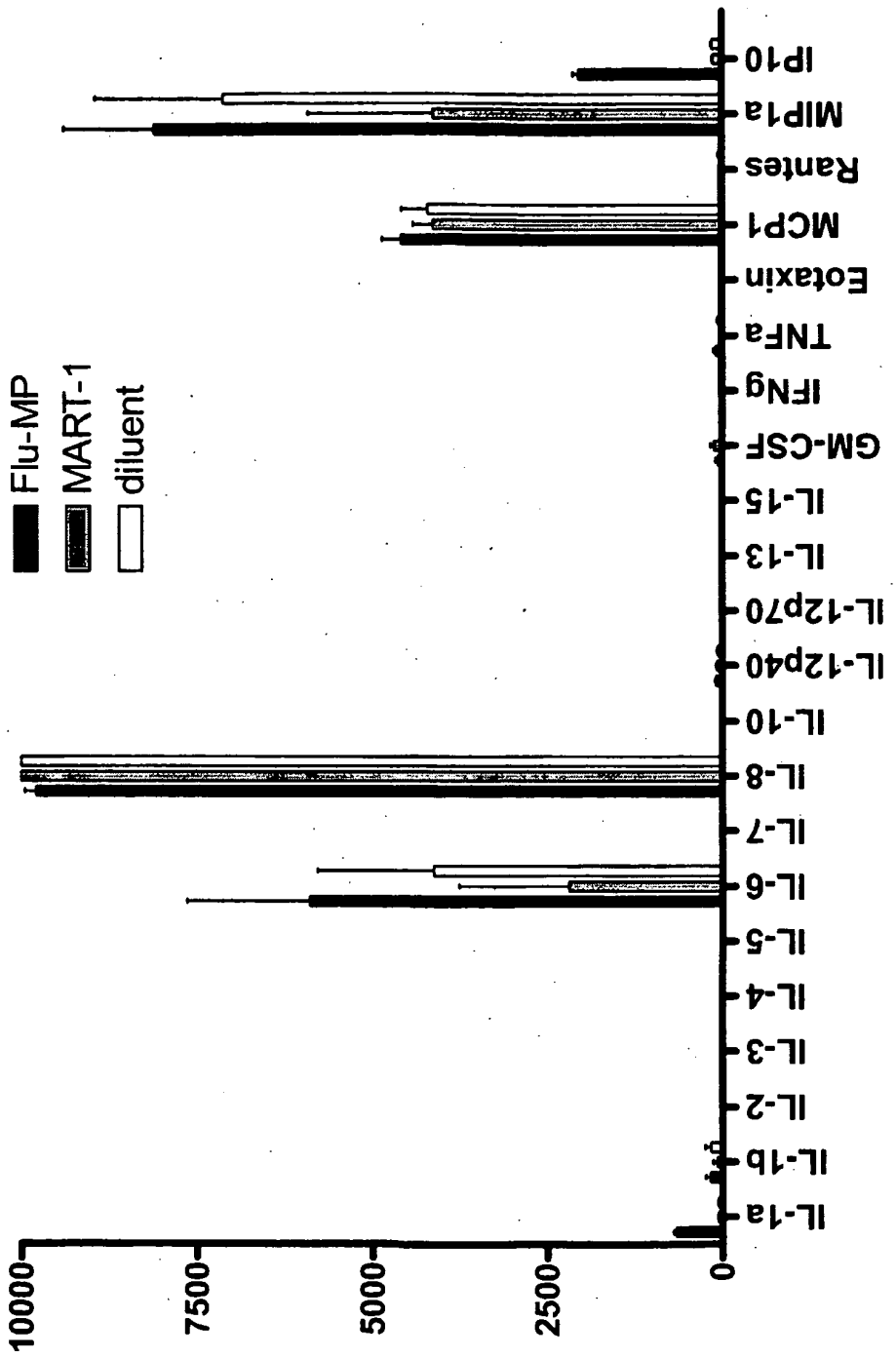
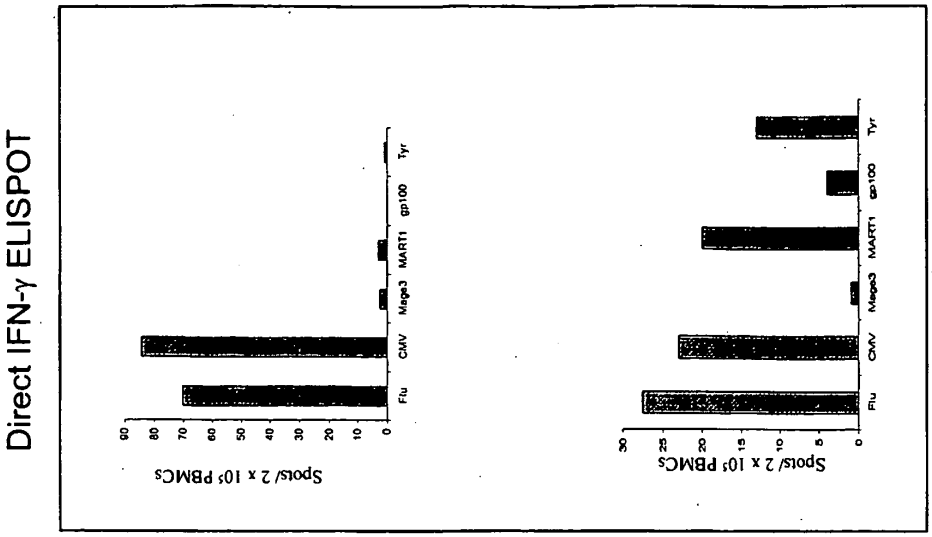

Flu-MP
MART-1
diluent
10000
7500
5000
2500
0
IL-1a
IL-1b
IL-2
IL-3
IL-4
IL-5
IL-6
IL-7
IL-8
IL-10
IL-12p40
IL-12p70
IL-13
IL-15
GM-CSF
IFNg
TNFa
Eotaxin
MCP1
Rantes
MIP1a
IP10

Figure 2


Direct IFN-γ ELISPOT
Spots/ 2 x 10⁵ PBMCs
Flu
CMV
Mage3
MART1
gp100
Tyr

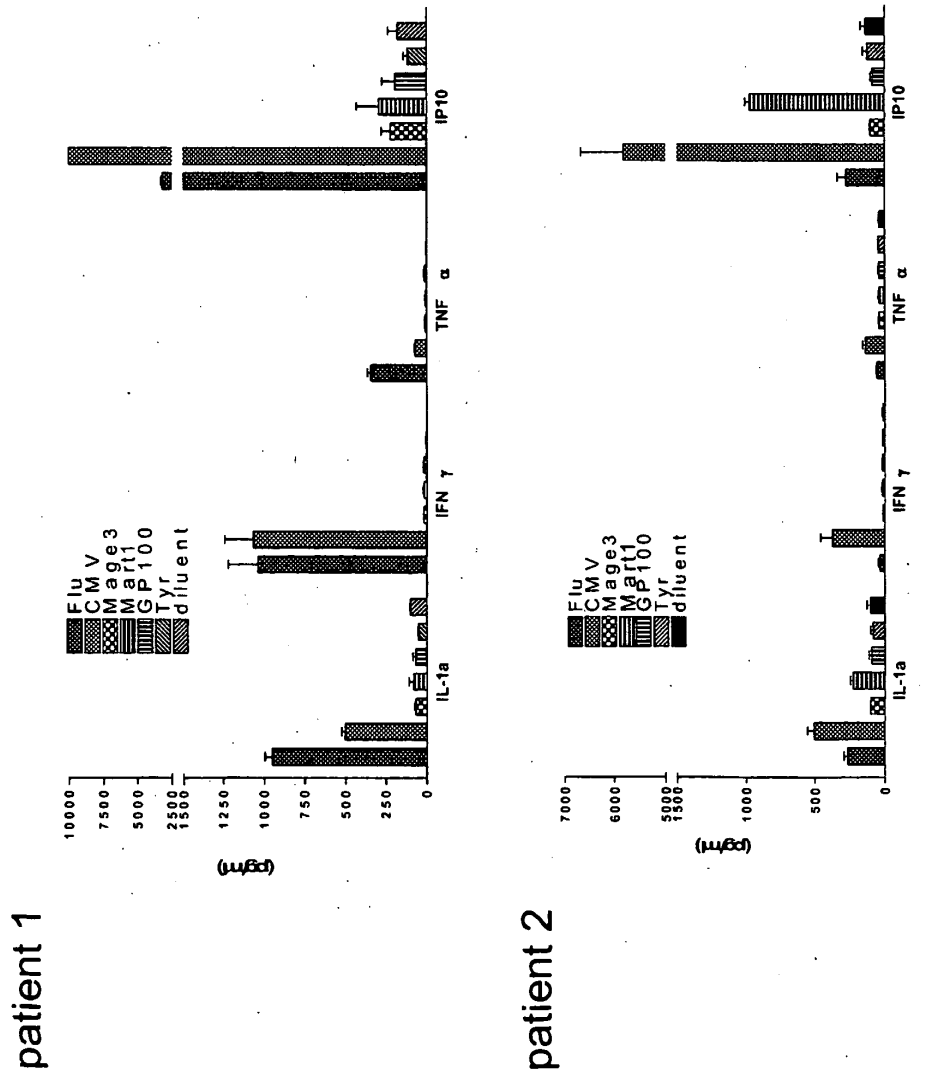

patient 1
patient 2
Flu
CMV
Mage3
Mart1
GP100
Tyr
diluent
IL-1a
IFN γ
TNF α
IP10
(pg/ml)

Figure 3

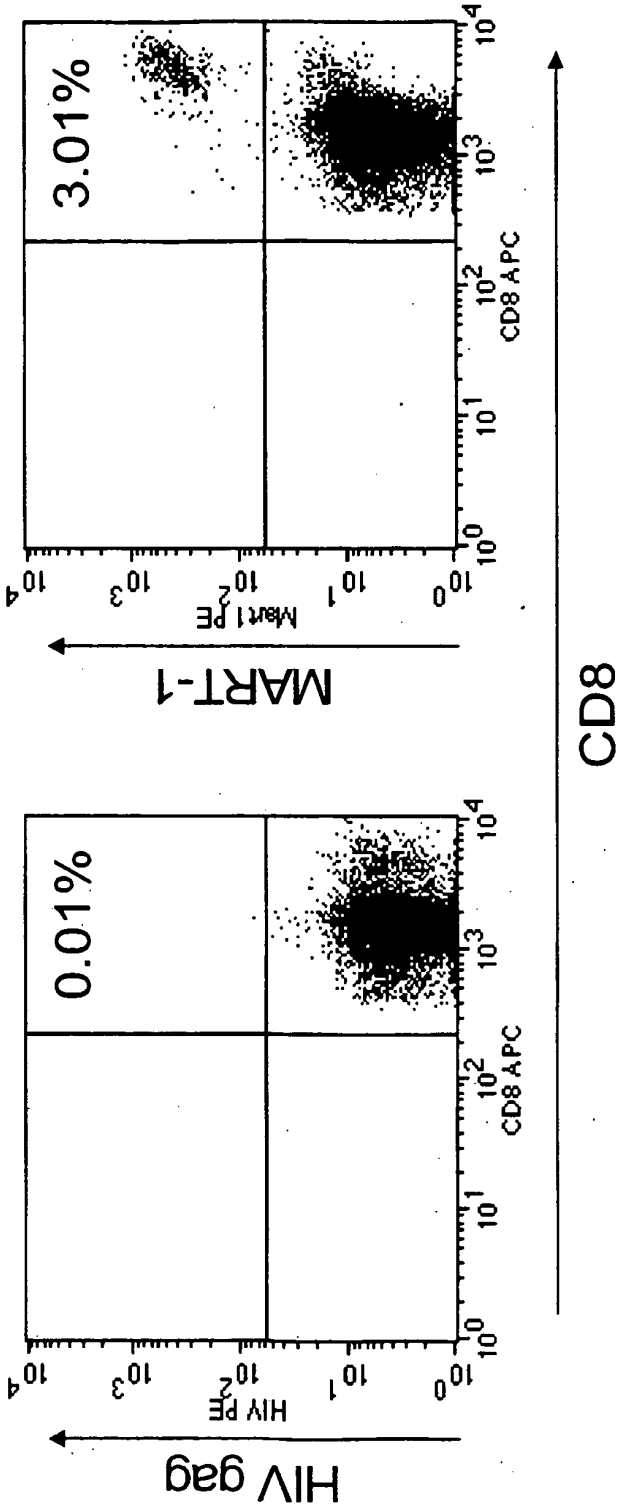


Figure 4A

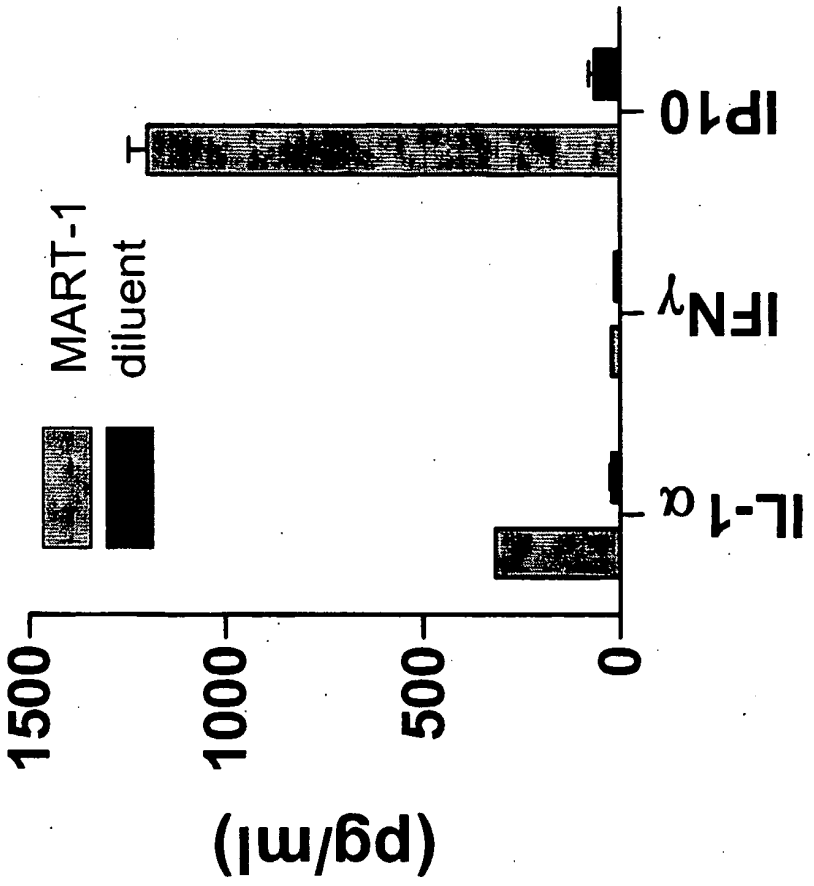


Figure 4B

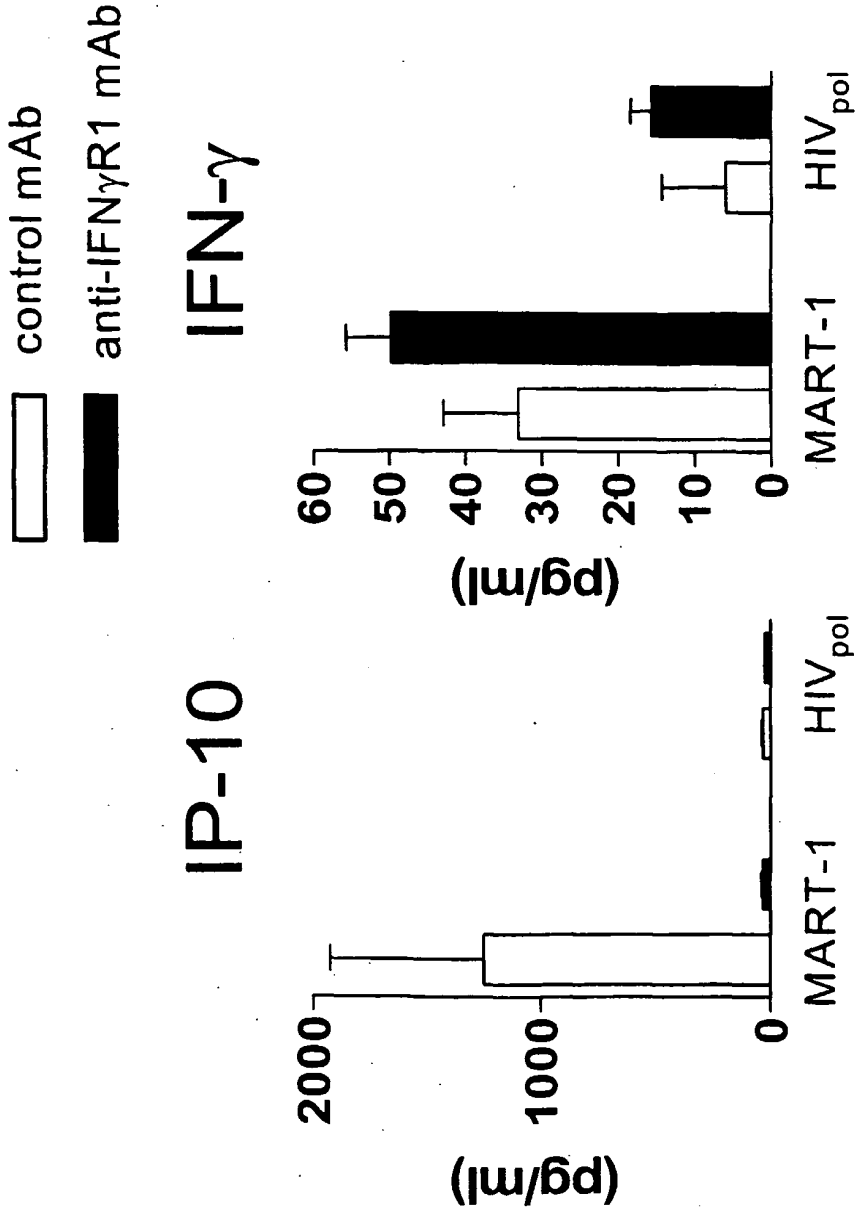

control mAb
anti-IFNγR1 mAb
IFN-γ
60
50
40
30
20
10
0
(pg/ml)
MART-1
HIV$_{pol}$
IP-10
2000
1000
0
(pg/ml)
MART-1
HIV$_{pol}$

Figure 4C

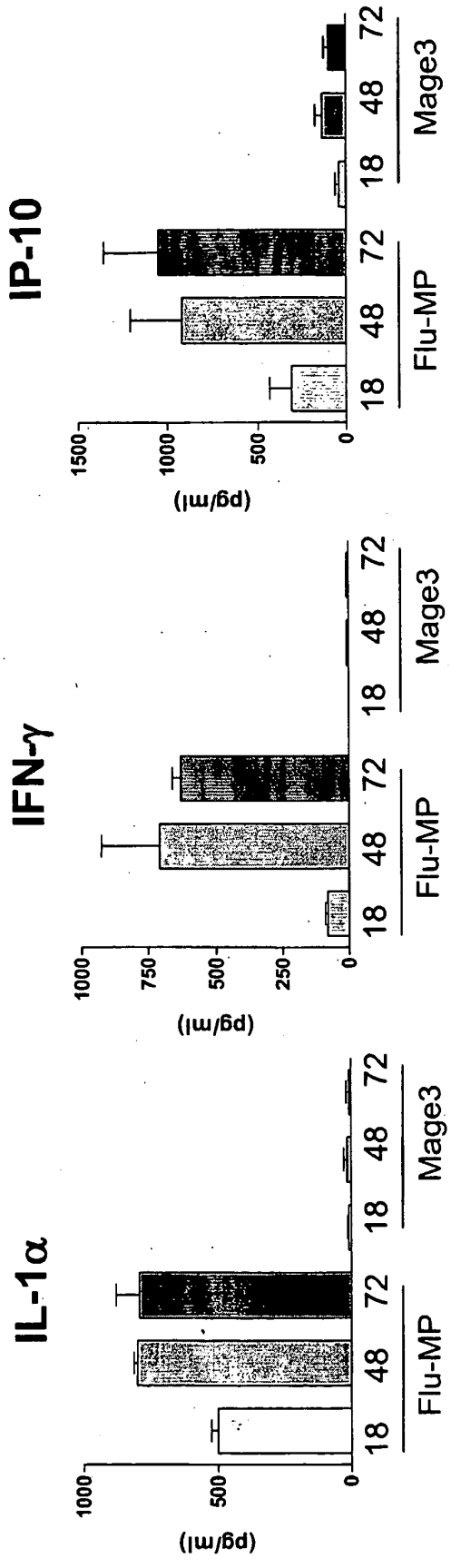

IL-1α
IFN-γ
IP-10
(pg/ml)
1000
500
0
750
250
1500
18
48
72
Flu-MP
Mage3

Figure 5

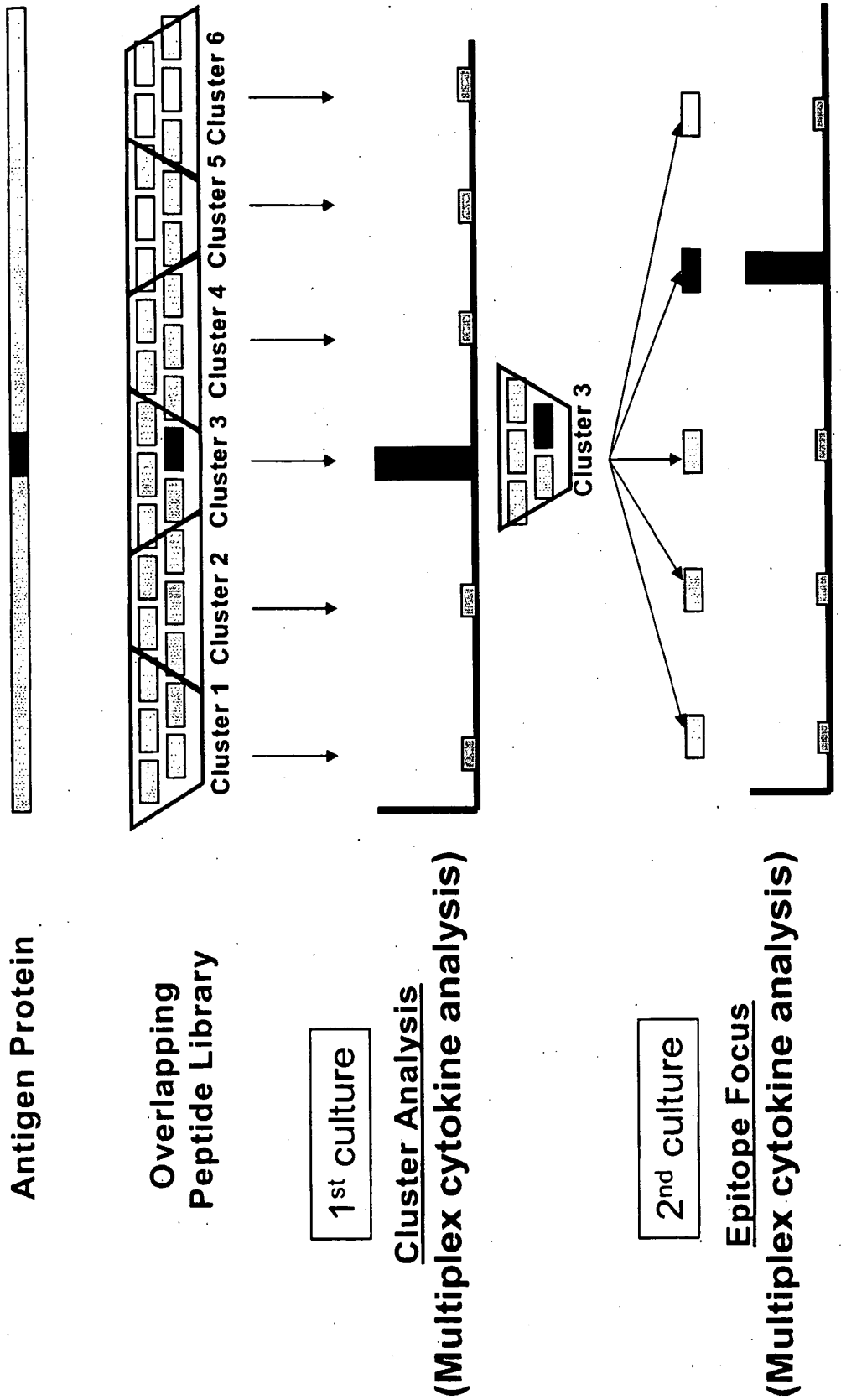
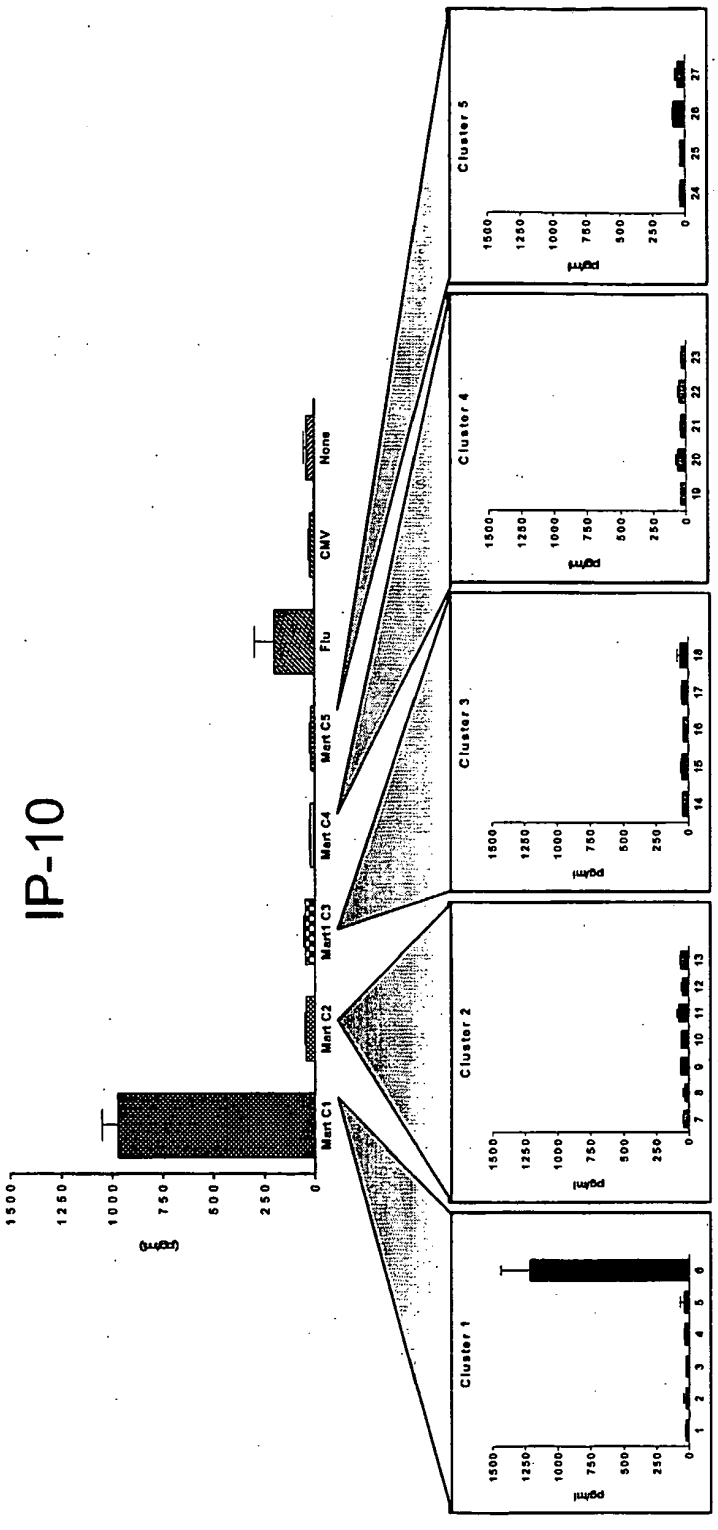

Antigen Protein
Overlapping
Peptide Library
Cluster 1 Cluster 2 Cluster 3 Cluster 4 Cluster 5 Cluster 6
1st culture
Cluster Analysis
(Multiplex cytokine analysis)
Cluster 3
2nd culture
Epitope Focus
(Multiplex cytokine analysis)

Figure 6



MART-1 peptide#6 contains MART1 A2 dominant epitope AAGIGILTV (27-35).

Figure 7

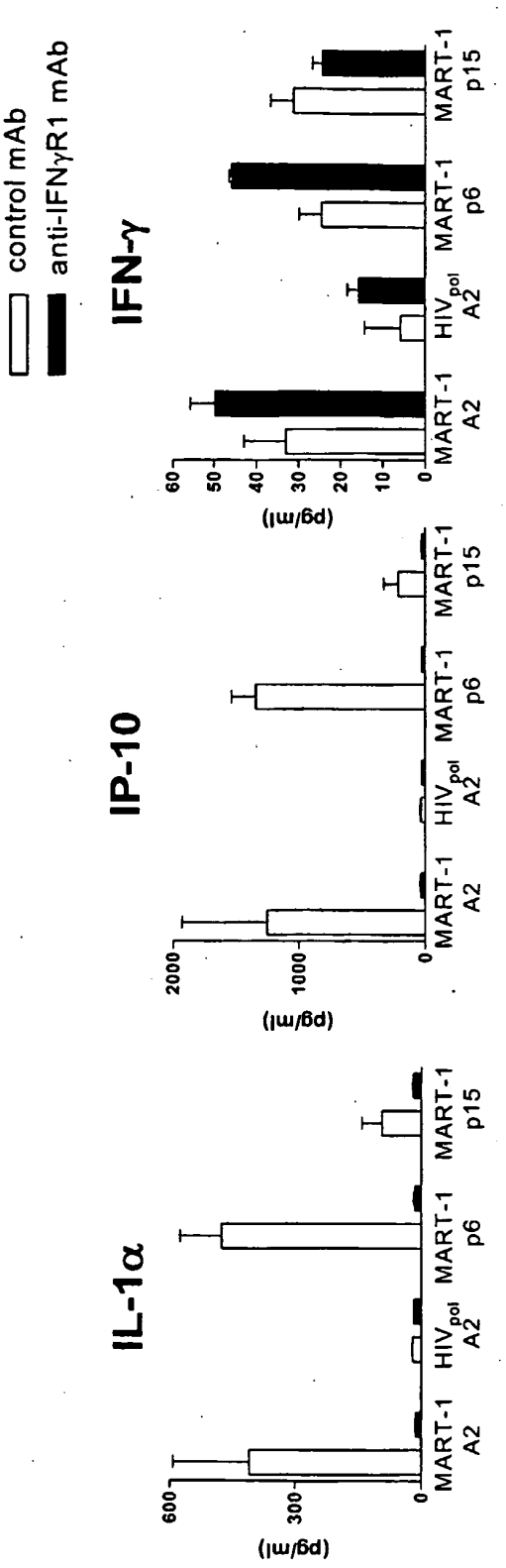

control mAb
anti-IFNγR1 mAb
IFN-γ
IP-10
IL-1α
(pg/ml)
MART-1 A2
HIVpol A2
MART-1 p6
MART-1 p15

Figure 8

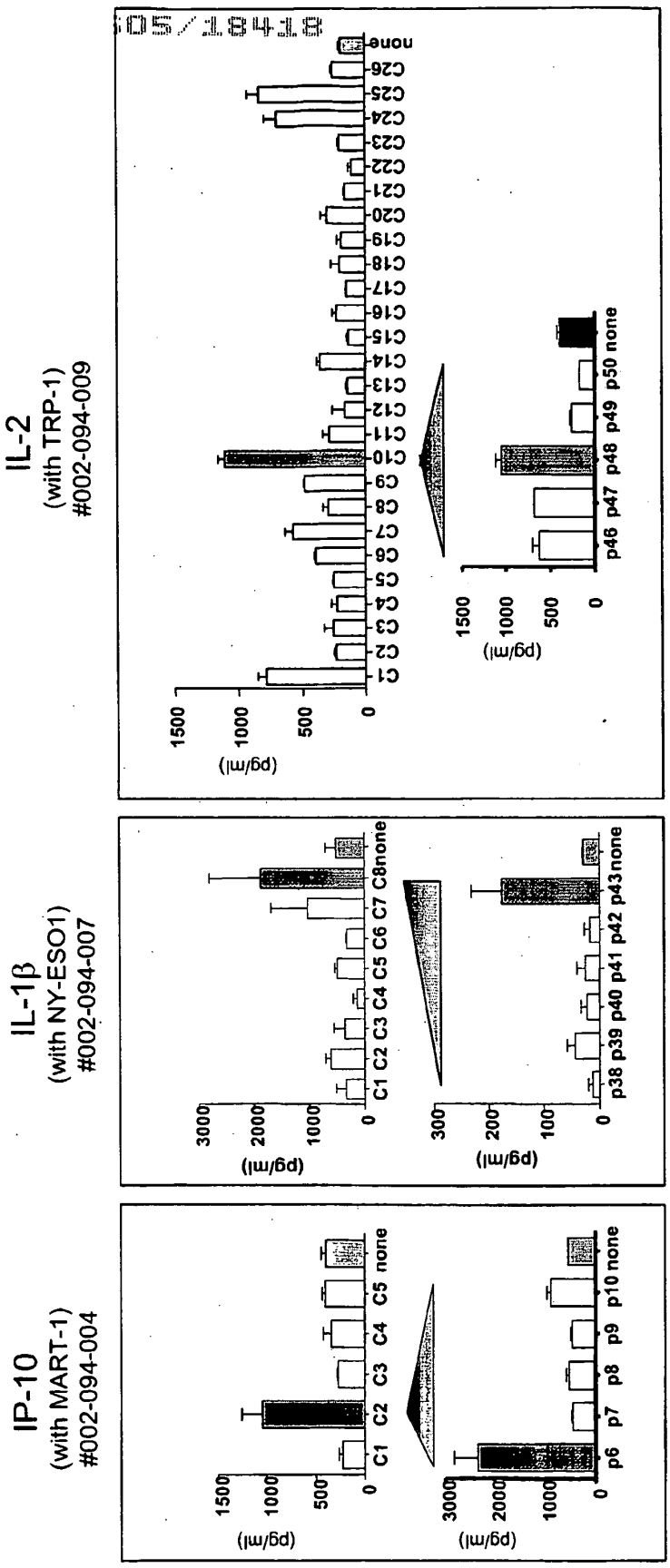

IP-10
(with MART-1)
#002-094-004
IL-1β
(with NY-ESO1)
#002-094-007
IL-2
(with TRP-1)
#002-094-009
(pg/ml)
C1 C2 C3 C4 C5 none
p6 p7 p8 p9 p10 none
C1 C2 C3 C4 C5 C6 C7 C8 none
p38 p39 p40 p41 p42 p43 none
C1 C2 C3 C4 C5 C6 C7 C8 C9 C10 C11 C12 C13 C14 C15 C16 C17 C18 C19 C20 C21 C22 C23 C24 C25 C26 none
p46 p47 p48 p49 p50 none

Figure 9

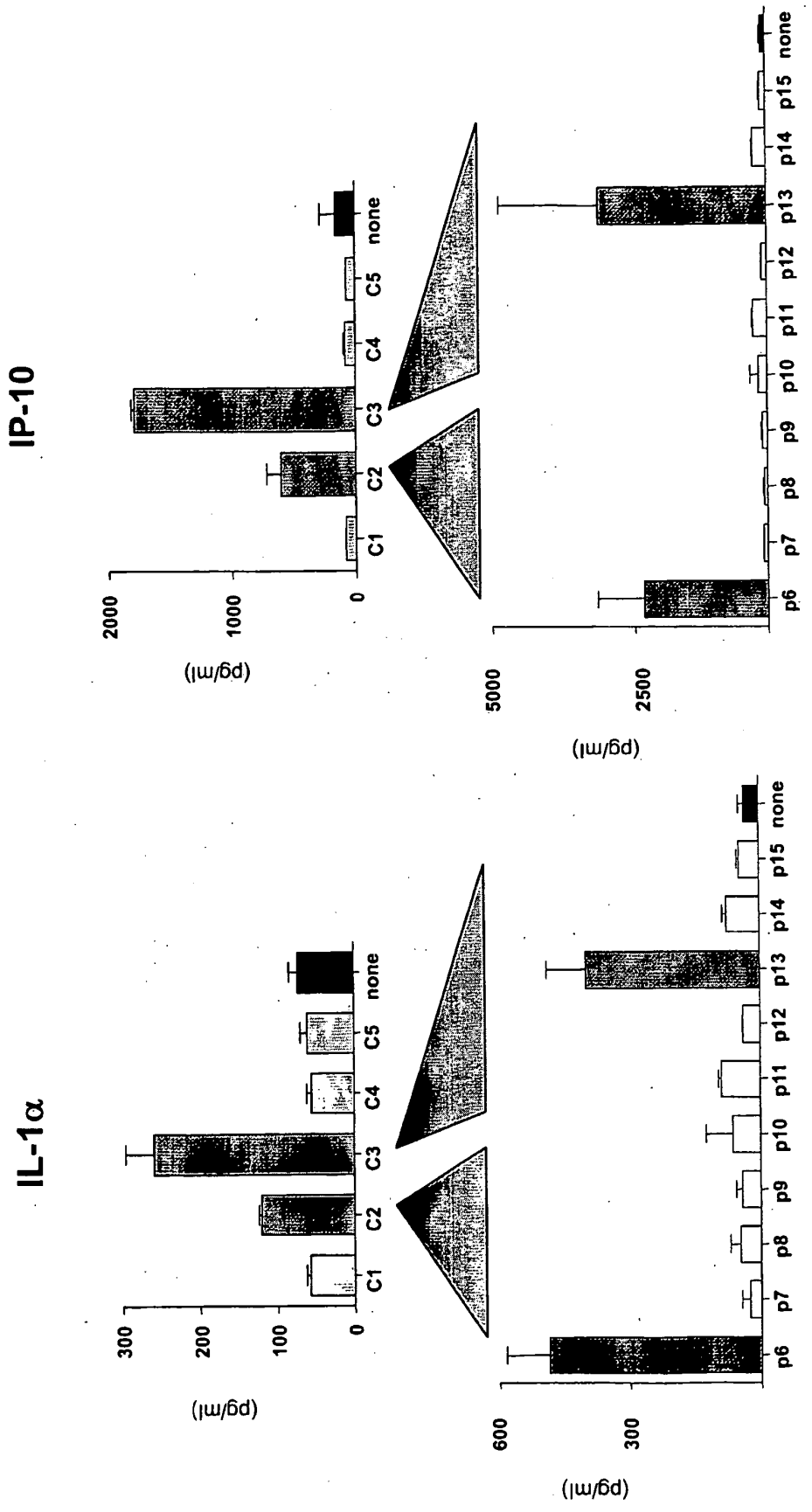

IP-10
2000
1000
0
(pg/ml)
C1
C2
C3
C4
C5
none
5000
2500
(pg/ml)
p6
p7
p8
p9
p10
p11
p12
p13
p14
p15
none
IL-1α
300
200
100
0
(pg/ml)
C1
C2
C3
C4
C5
none
600
300
(pg/ml)
p6
p7
p8
p9
p10
p11
p12
p13
p14
p15
none

Figure 10

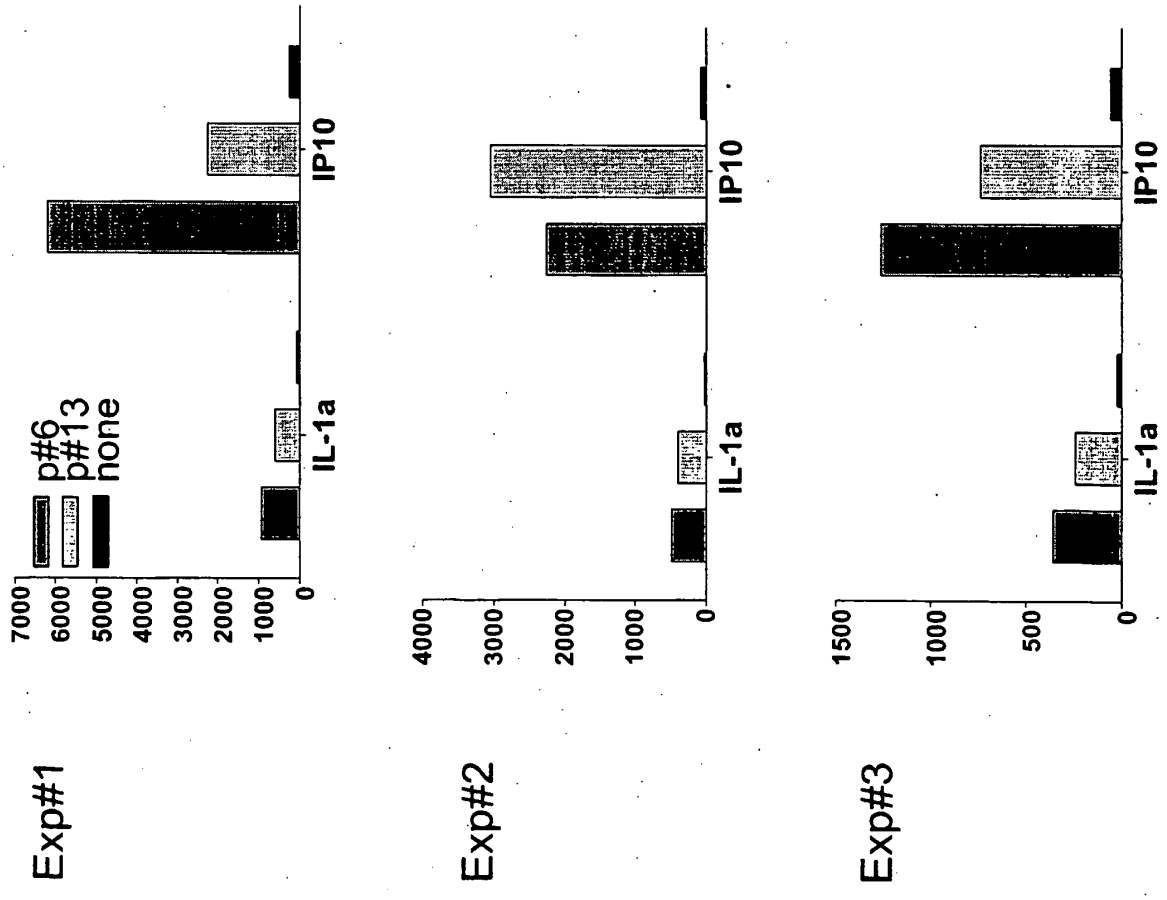

Exp#1
p#6
p#13
none
7000
6000
5000
4000
3000
2000
1000
0
IL-1a
IP10
Exp#2
4000
3000
2000
1000
0
IL-1a
IP10
Exp#3
1500
1000
500
0
IL-1a
IP10

Figure 11

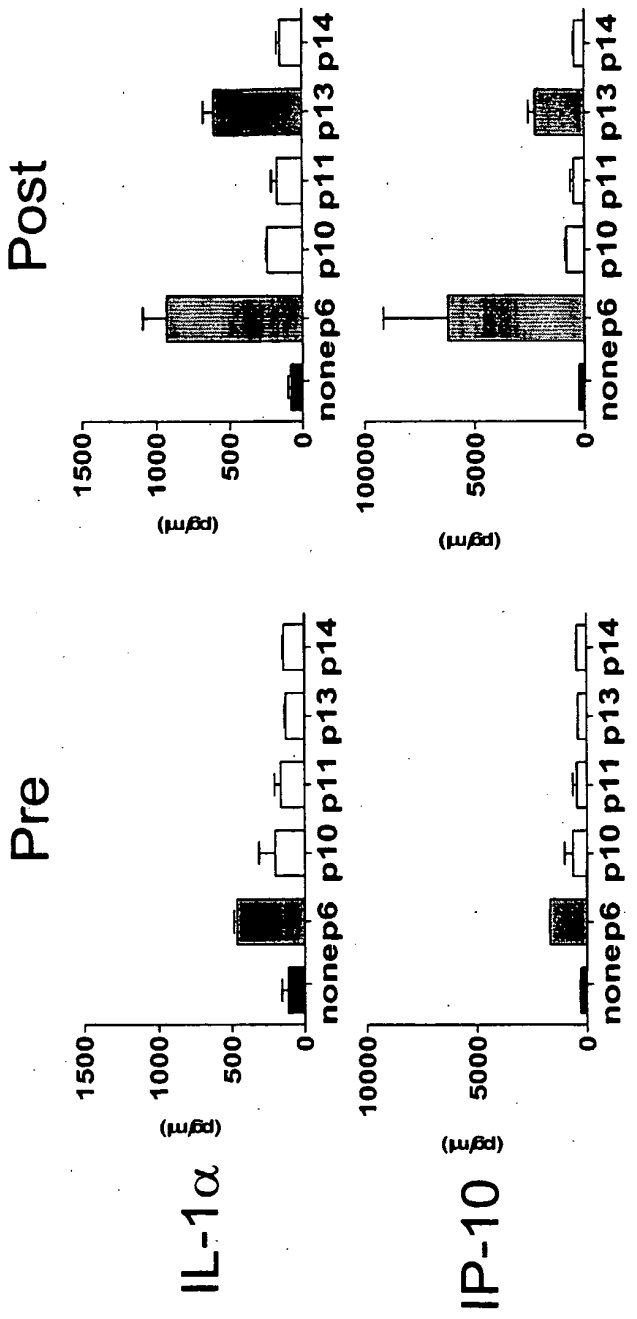

Post
Pre
IL-1α
IP-10
1500
1000
500
0
10000
5000
(pg/μl)
none
p6
p10
p11
p13
p14

Figure 12A

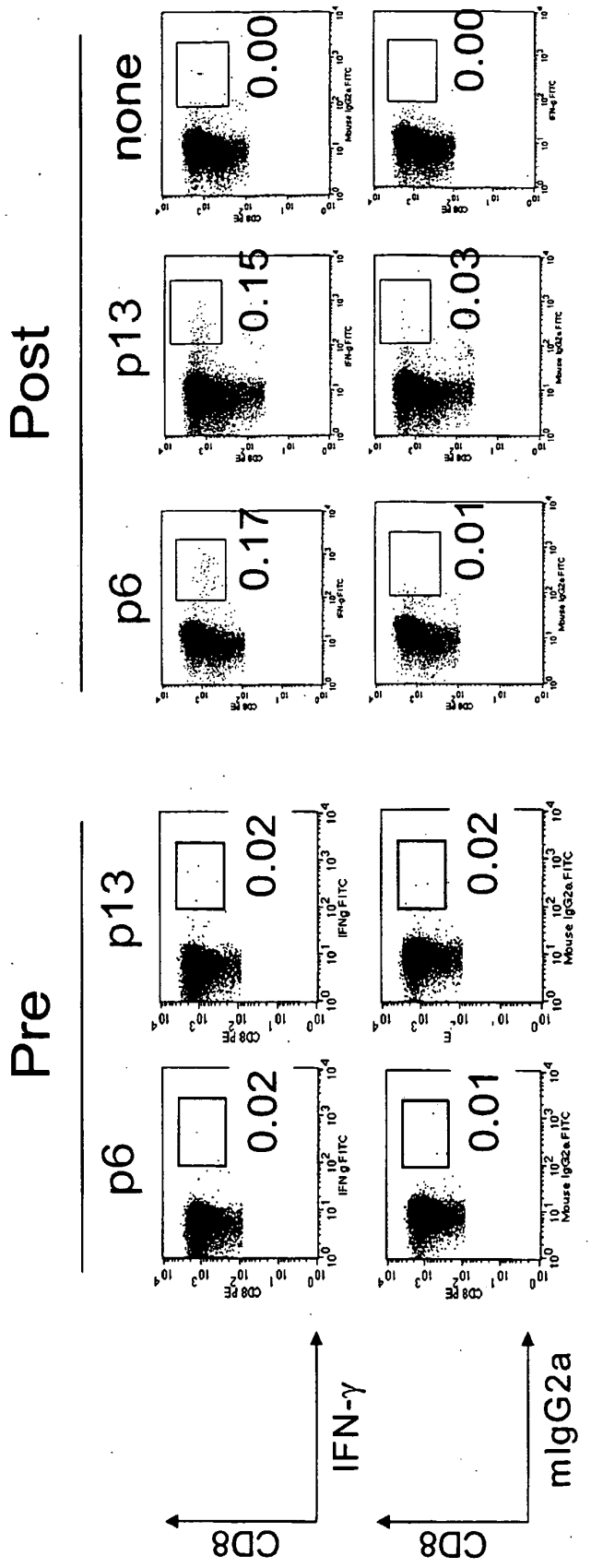

Pre
Post
p6
p13
p6
p13
none
0.02
0.02
0.17
0.15
0.00
0.01
0.02
0.01
0.03
0.00
CD8
IFN-γ
CD8
mIgG2a

Figure 12B

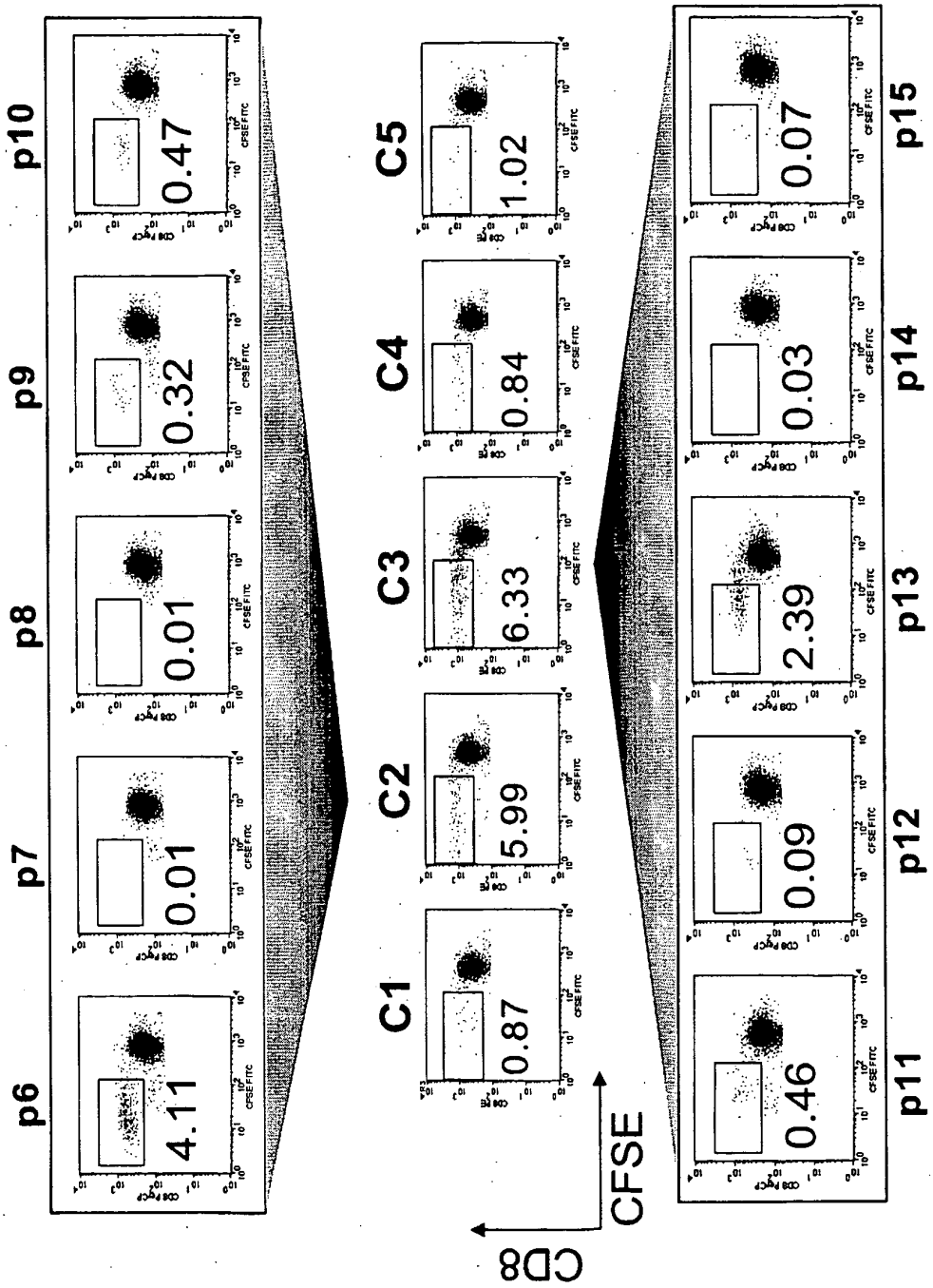

p6
p7
p8
p9
p10
C1
C2
C3
C4
C5
p11
p12
p13
p14
p15
4.11
0.01
0.01
0.32
0.47
0.87
5.99
6.33
0.84
1.02
0.46
0.09
2.39
0.03
0.07
CD8
CFSE

Figure 13

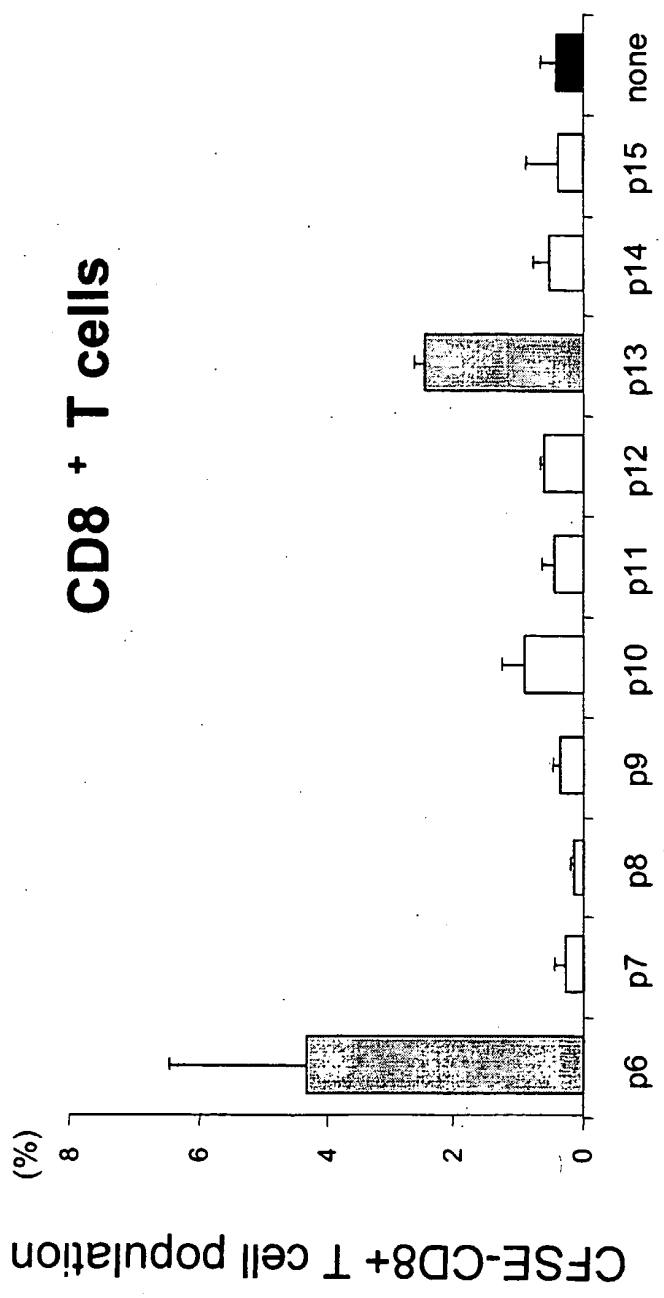

CD8 + T cells
CFSE-CD8+ T cell population
(%)
8
6
4
2
0
p6
p7
p8
p9
p10
p11
p12
p13
p14
p15
none

Figure 14

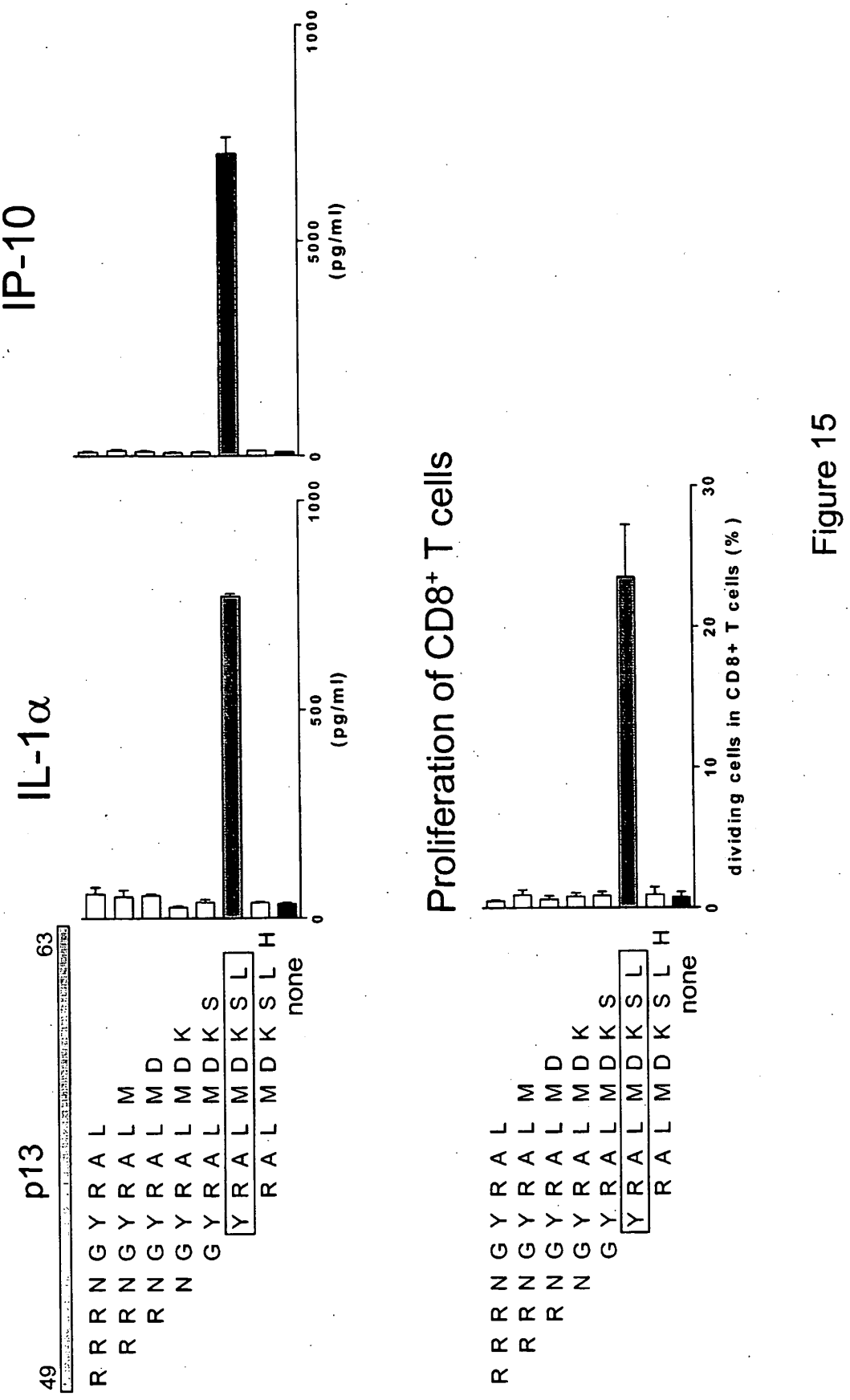

IP-10
IL-1α
p13
49
63
R R R N G Y R A L
R R N G Y R A L M
R N G Y R A L M D
N G Y R A L M D K
G Y R A L M D K S
Y R A L M D K S L
R A L M D K S L H
none
0
500
1000
(pg/ml)
0
5000
1000
(pg/ml)
Proliferation of CD8+ T cells
dividing cells in CD8+ T cells (%)
0
10
20
30

Figure 15

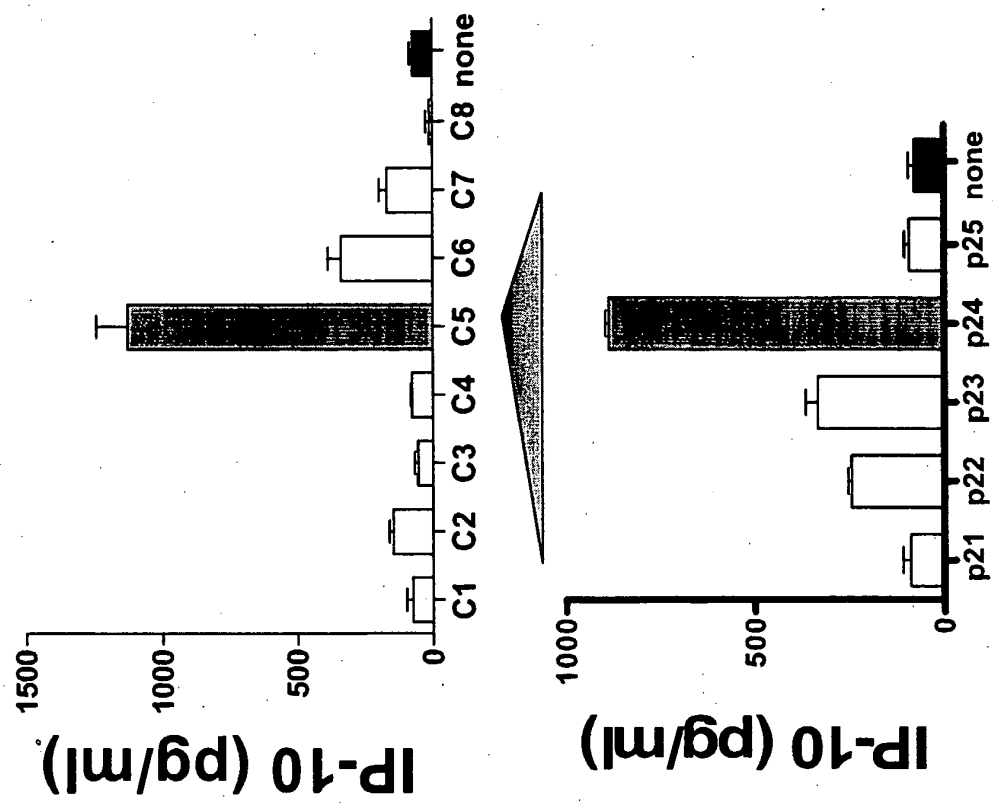

IP-10 (pg/ml)
1500
1000
500
0
C1
C2
C3
C4
C5
C6
C7
C8
none
IP-10 (pg/ml)
1000
500
0
p21
p22
p23
p24
p25
none

Figure 16A

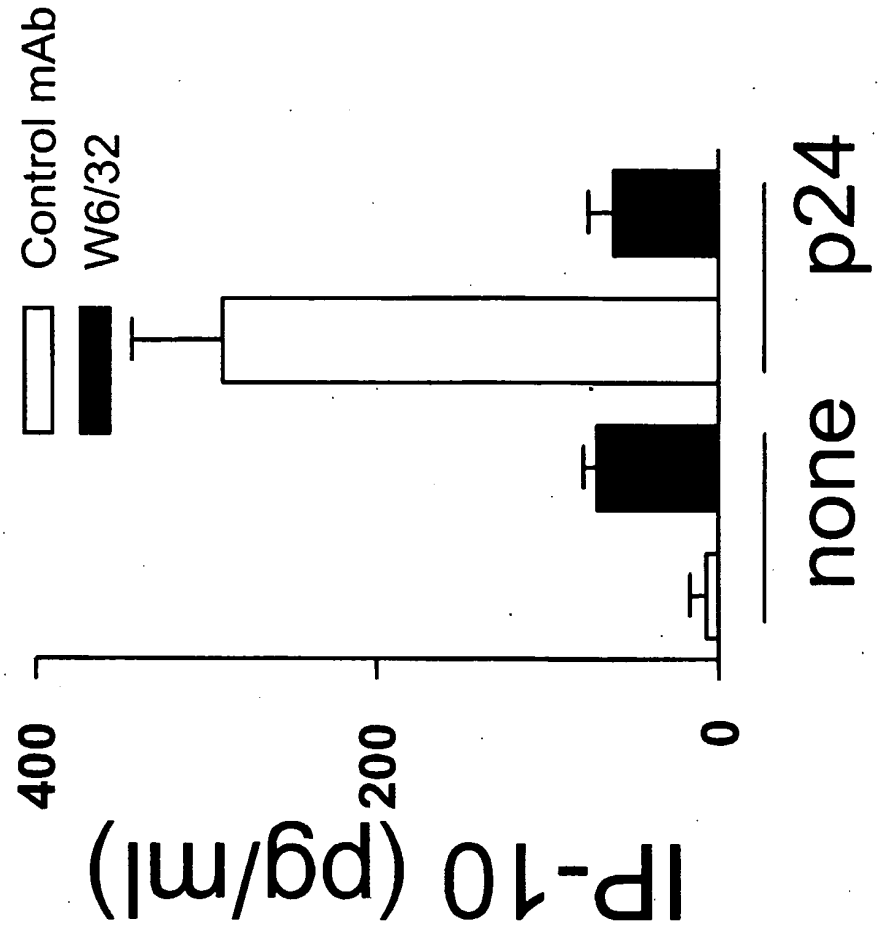


Figure 16B

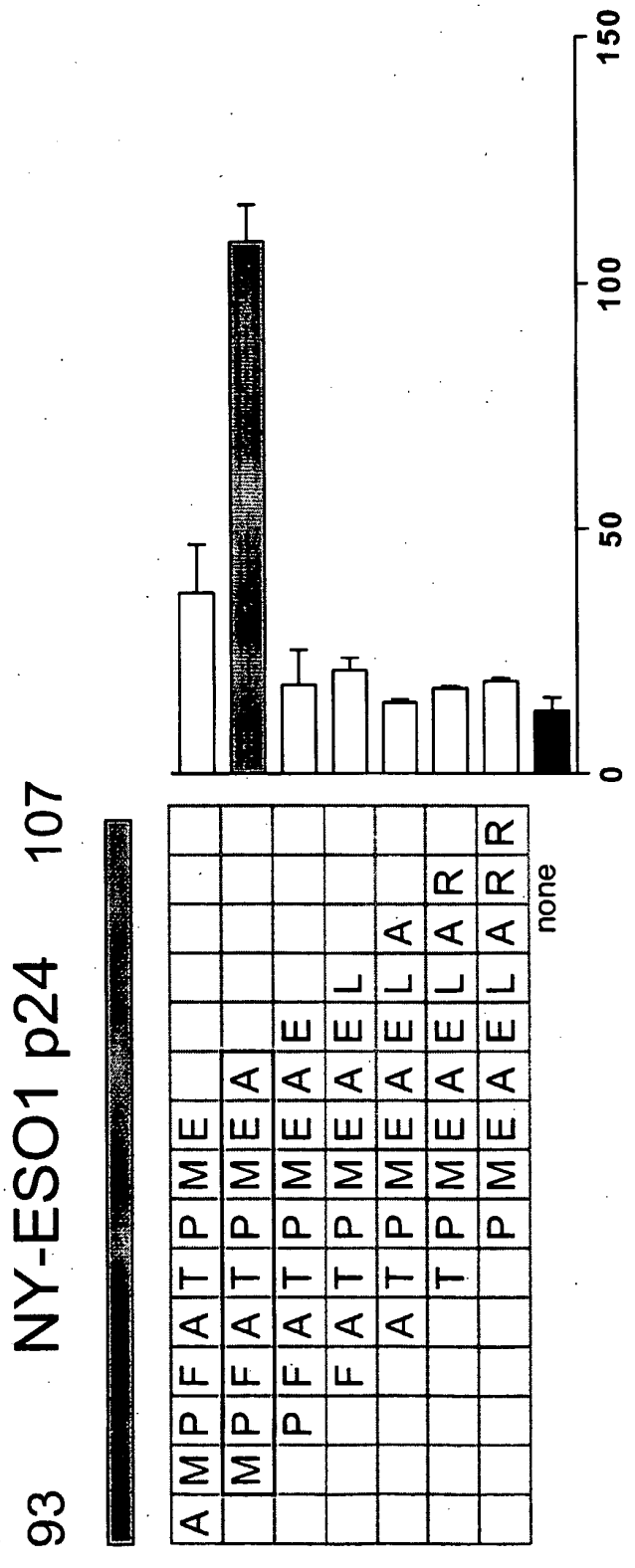

93
NY-ESO1 p24
107
A
M P F A T P M E
M P F A T P M E A
P F A T P M E A E
F A T P M E A E L
A T P M E A E L A
T P M E A E L A R
P M E A E L A R R
none
0
50
100
150
IP-10 (pg/ml)

Figure 16C

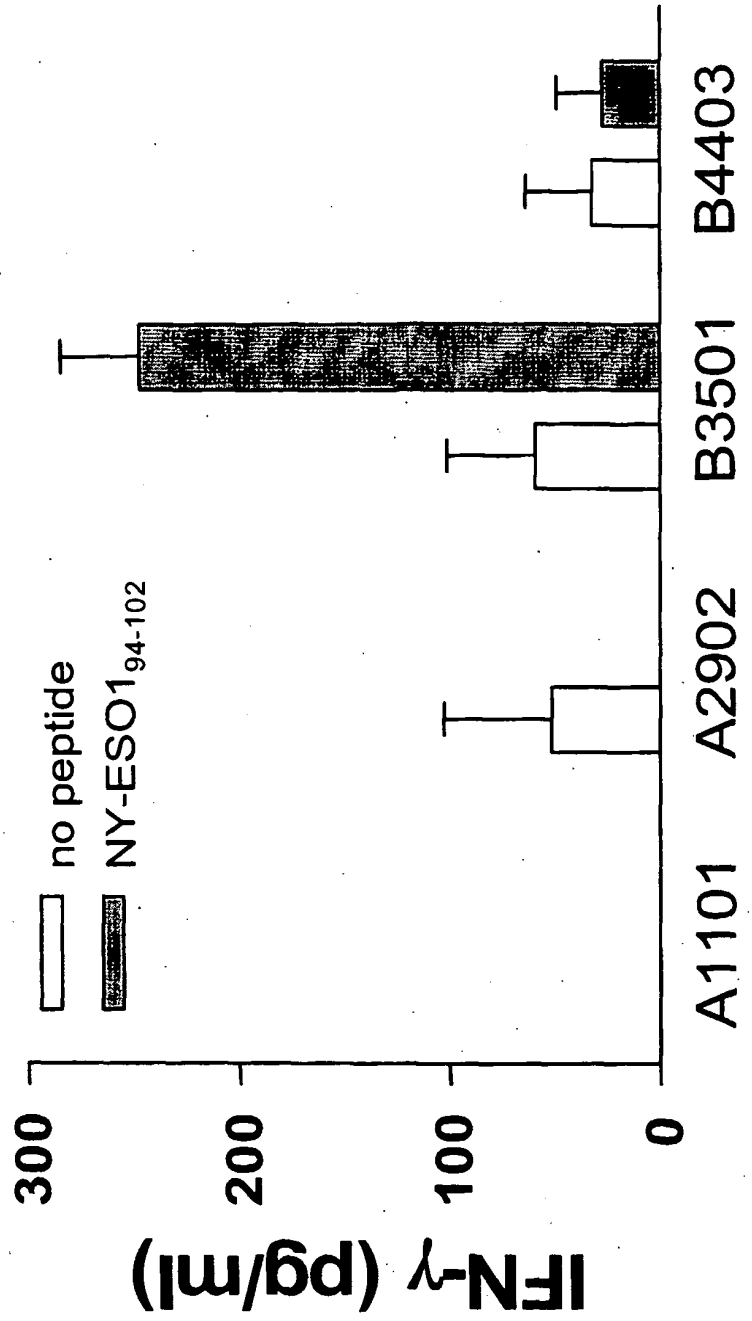

IFN-γ (pg/ml)
300
200
100
0
A1101
A2902
B3501
B4403
no peptide
NY-ESO1 94-102

Figure 16D

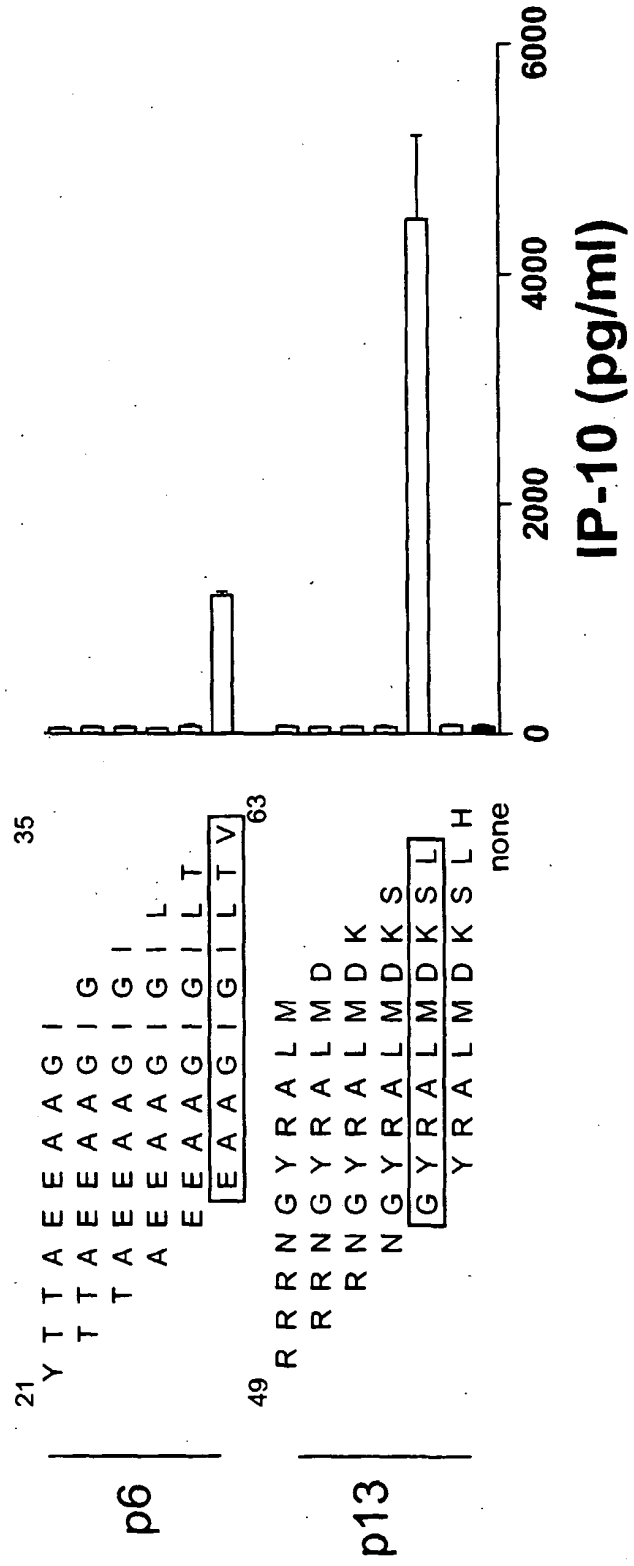


Figure 17

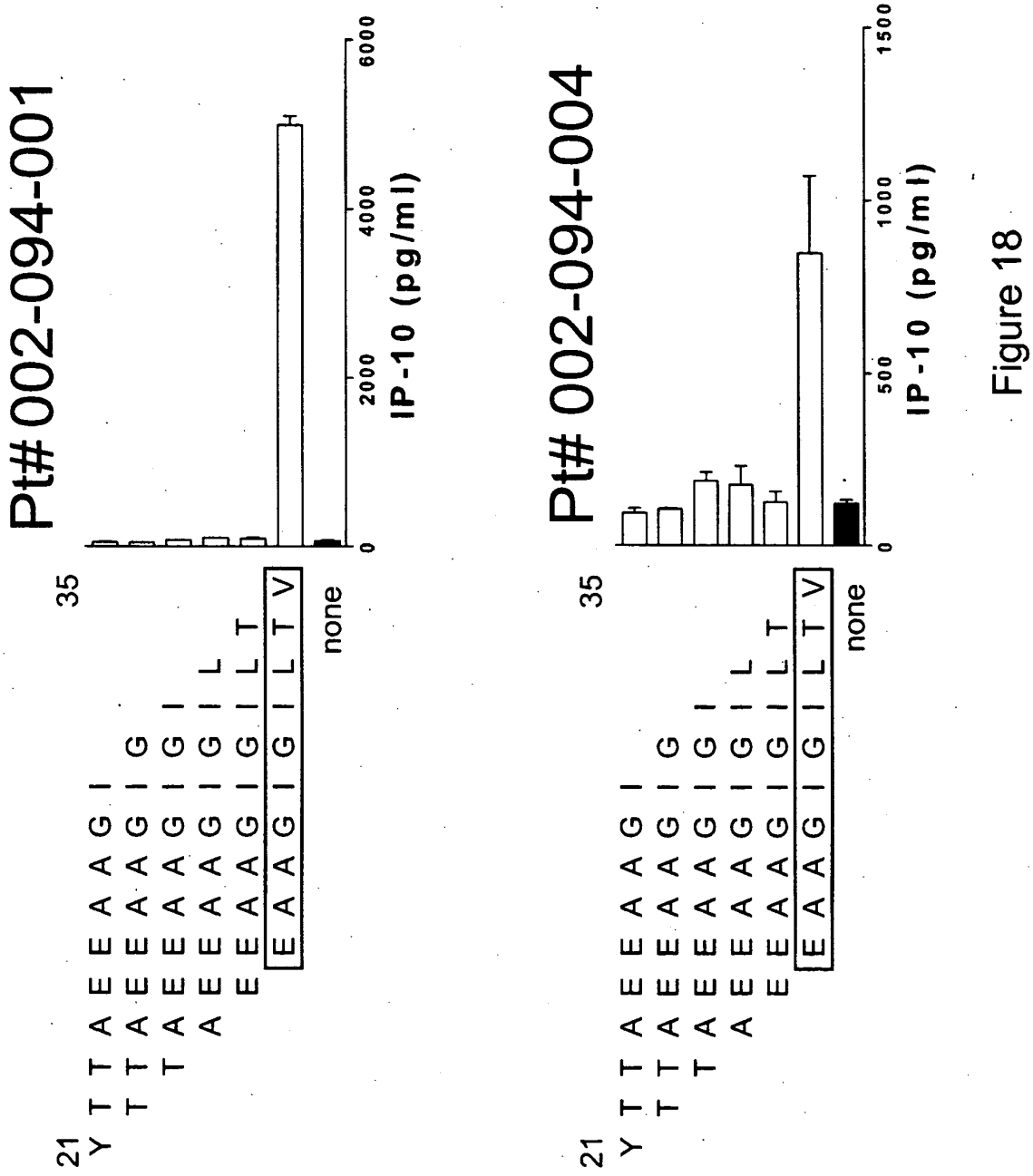

Pt# 002-094-001
IP-10 (pg/ml)
0
2000
4000
6000
21
35
Y T T A E E E A A G I
T T A E E E A A G I G I
T A E E E A A G I G I L
A E E E A A G I G I L T
E E E A A G I G I L T V
none
Pt# 002-094-004
IP-10 (pg/ml)
0
500
1000
1500
21
35
Y T T A E E E A A G I
T T A E E E A A G I G I
T A E E E A A G I G I L
A E E E A A G I G I L T
E E E A A G I G I L T V
none

Figure 18

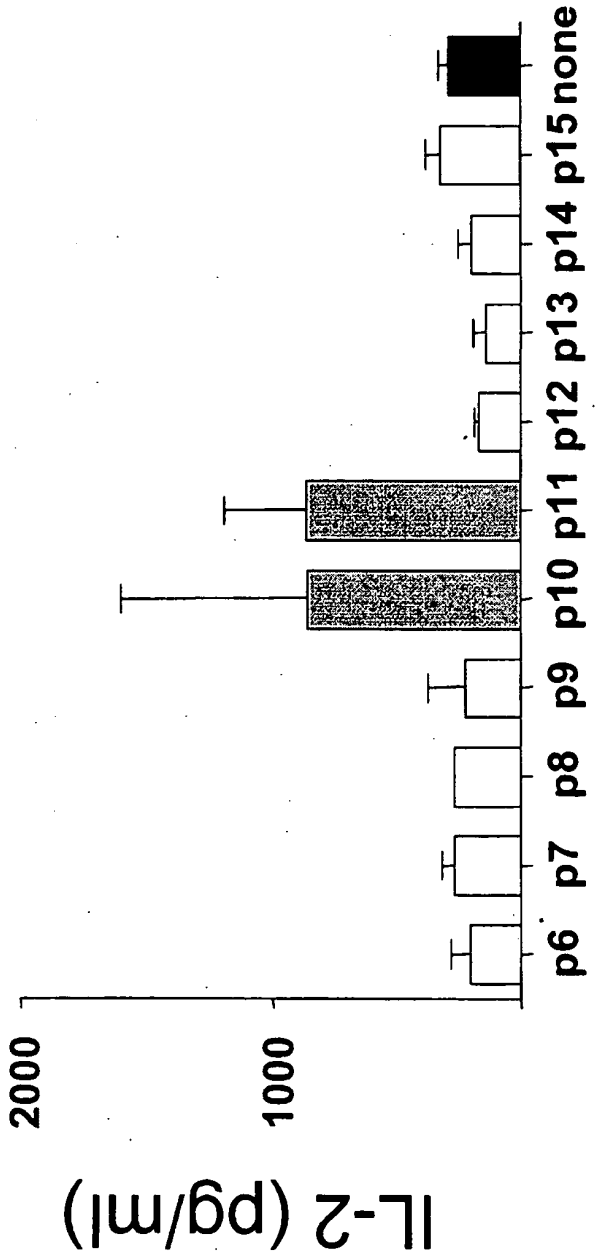


Figure 19

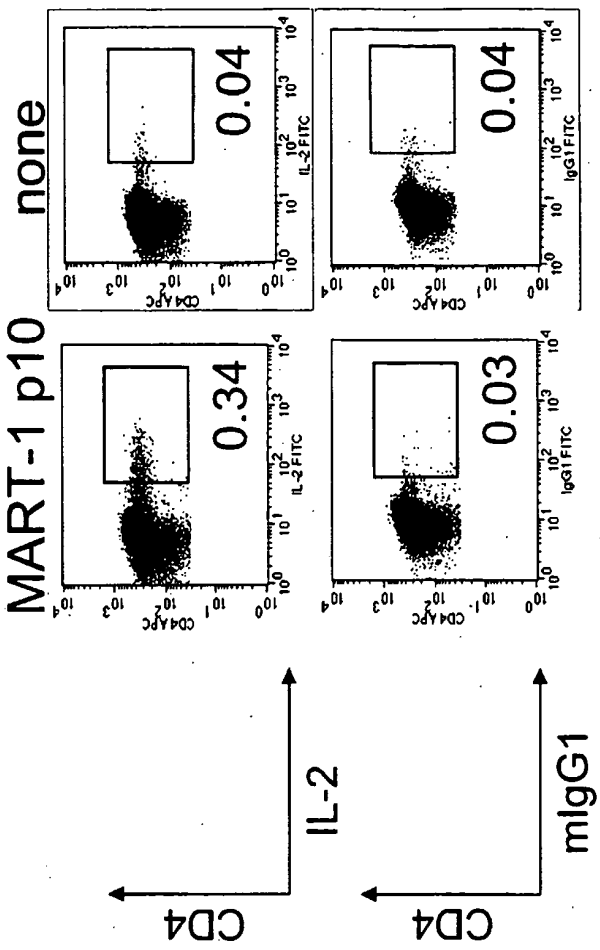

none
MART-1 p10
0.04
0.04
0.34
0.03
IL-2
mIgG1
CD4
CD4
CD4APC
IL-2FITC
IgG1FITC

Figure 20

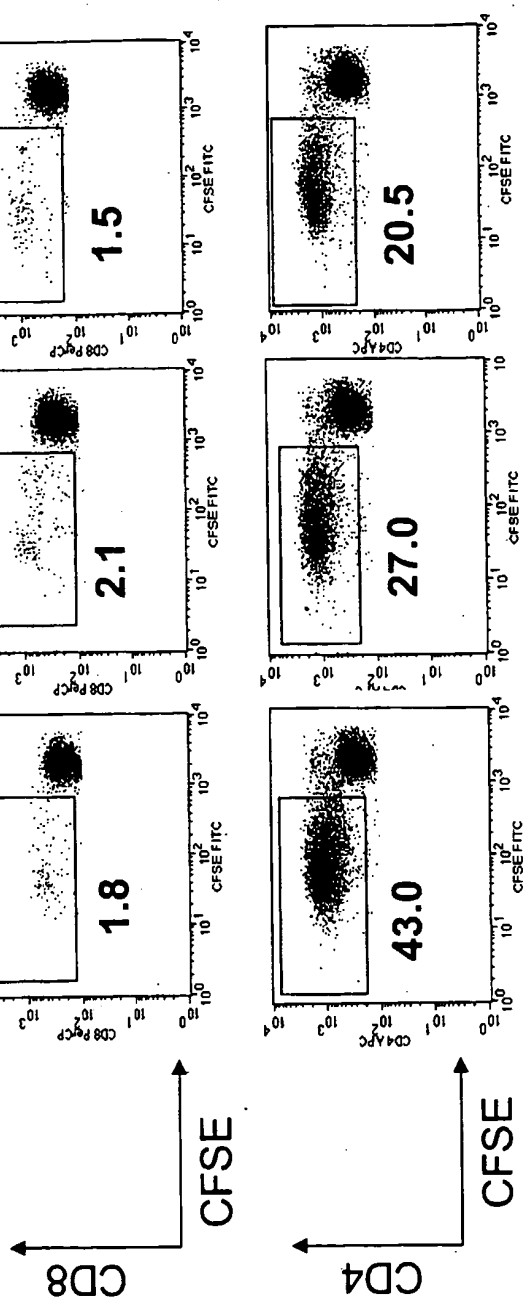

p15
p11
p10
1.5
20.5
2.1
27.0
1.8
43.0
CFSE FITC
CD8 PerCP
CD4 APC
CD8
CFSE
CD4
CFSE

Figure 21A

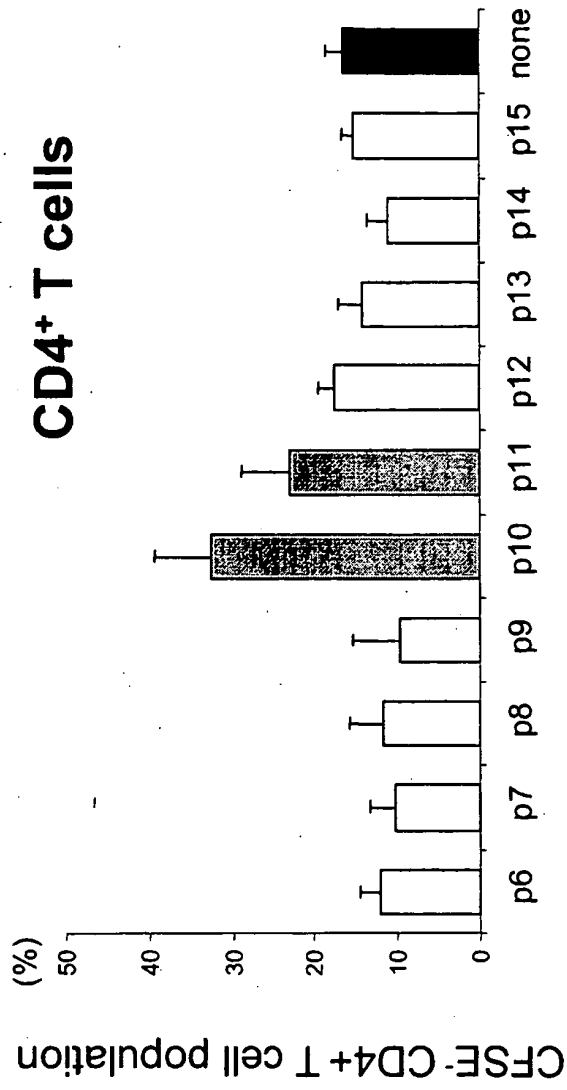

CD4+ T cells
CFSE- CD4+ T cell population
(%)
50
40
30
20
10
0
p6
p7
p8
p9
p10
p11
p12
p13
p14
p15
none

Figure 21B

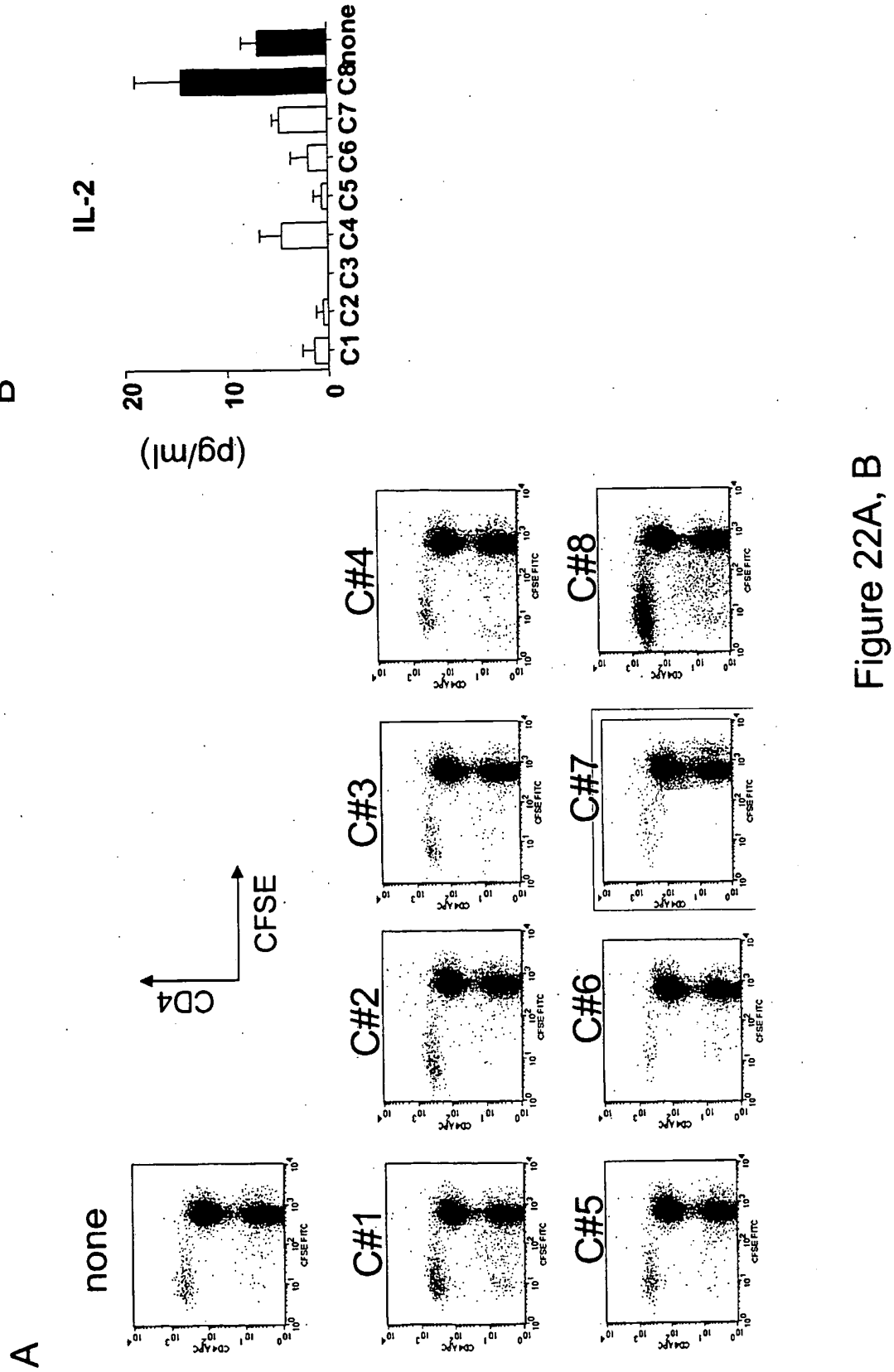

A
none
C#1
C#2
C#3
C#4
C#5
C#6
C#7
C#8
CD4
CFSE
B
IL-2
(pg/ml)
20
10
0
C1 C2 C3 C4 C5 C6 C7 C8none

Figure 22A, B

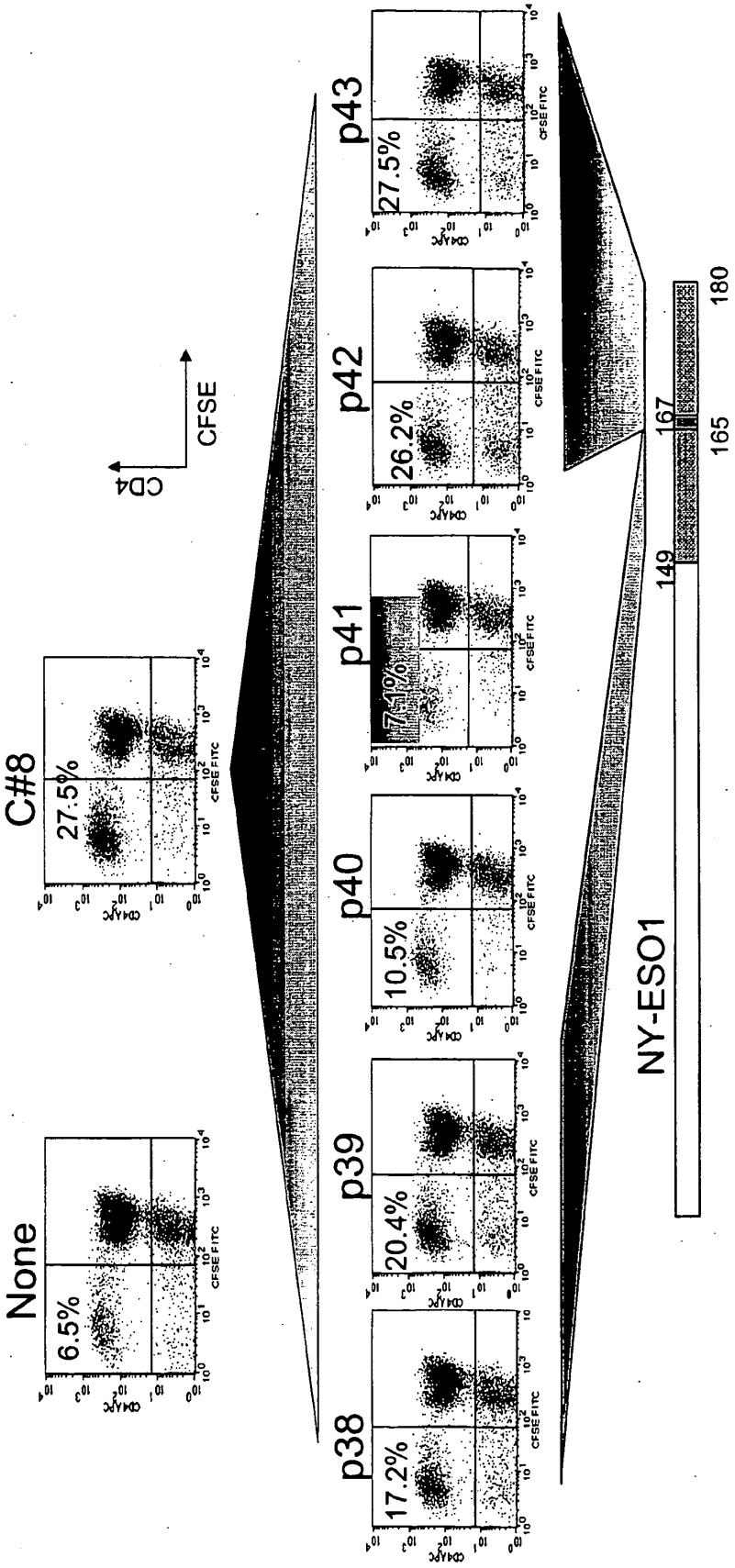

None
6.5%
C#8
27.5%
p38
17.2%
p39
20.4%
p40
10.5%
p41
7.1%
p42
26.2%
p43
27.5%
CD4
CFSE
NY-ESO1
149
165
167
180

Figure 22C

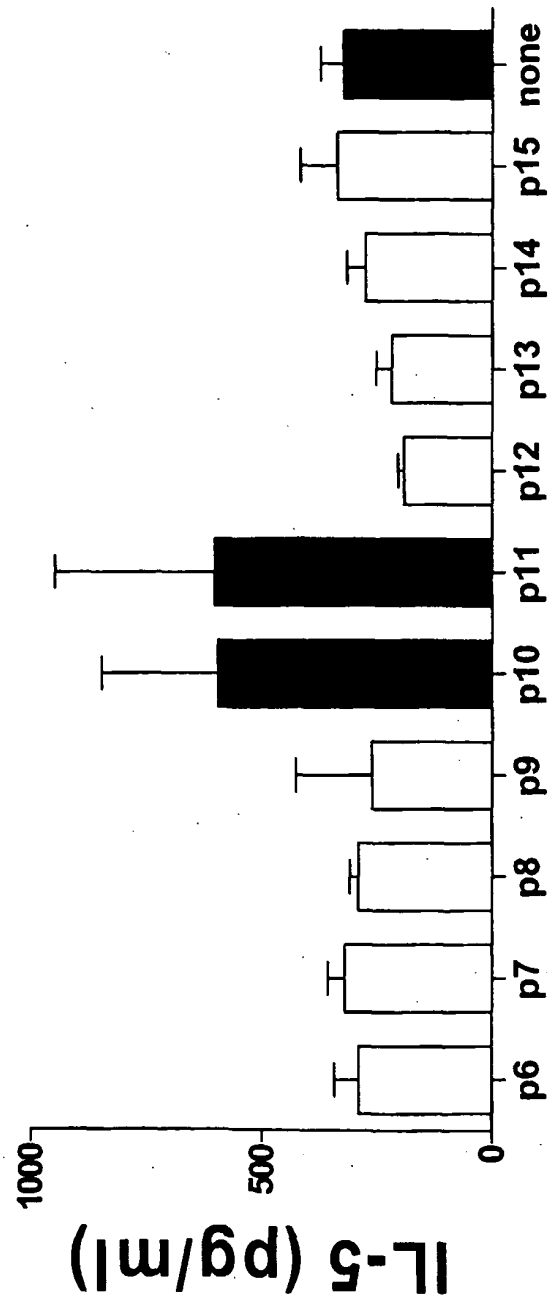


Figure 23

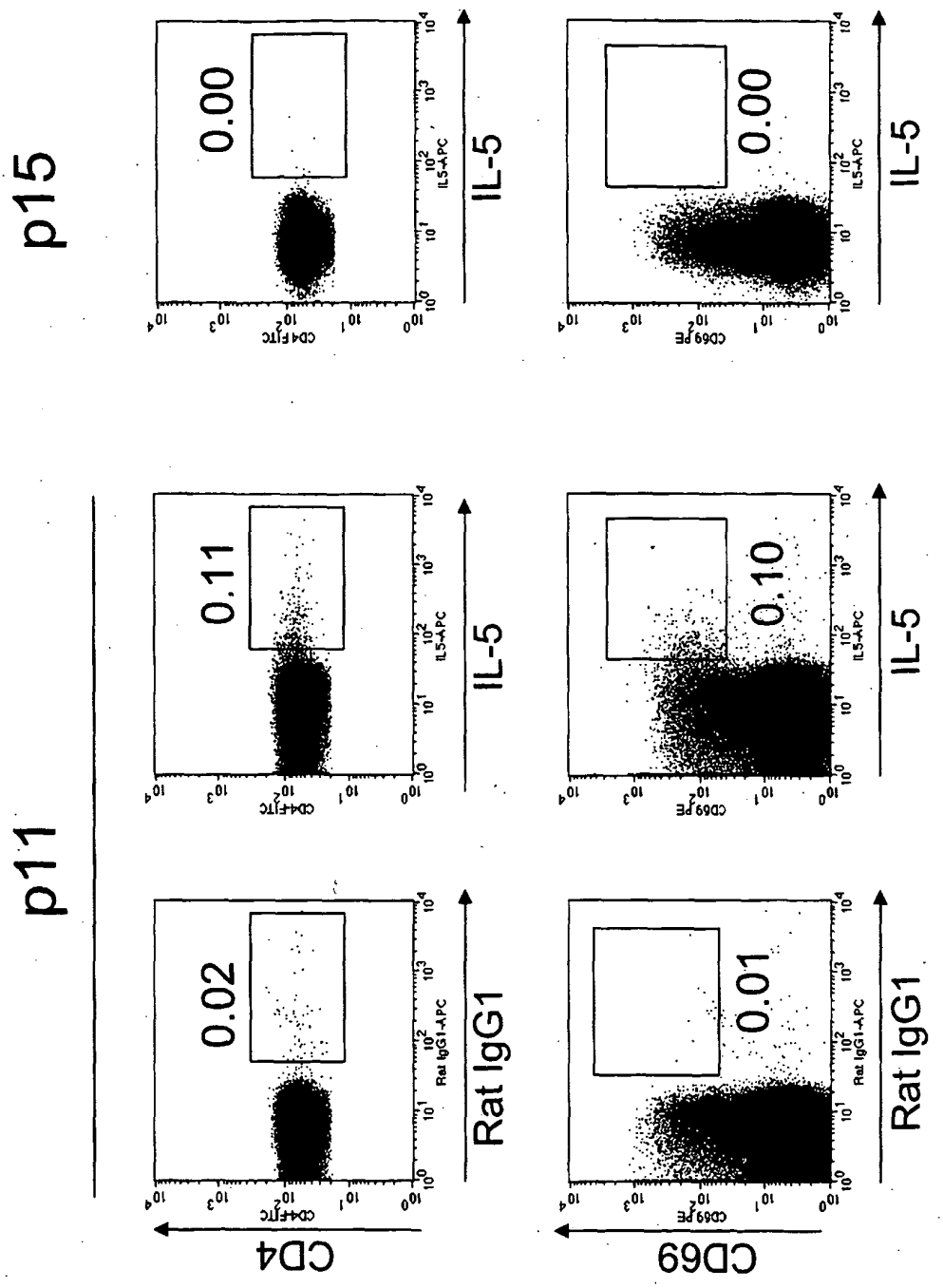

p15
p11
0.00
0.00
0.11
0.10
0.02
0.01
IL-5
IL-5
IL-5
IL-5
Rat IgG1
Rat IgG1
CD4
CD69
CD4-FITC
CD69-PE
IL5-APC
Rat IgG1-APC

Figure 24

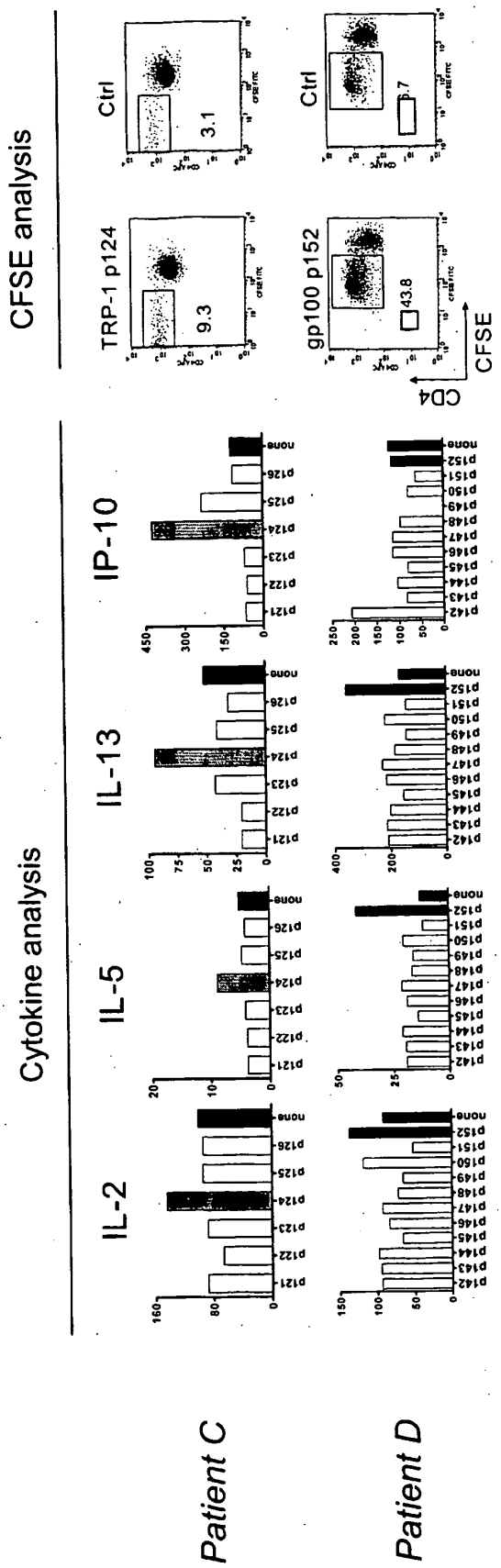

CFSE analysis
Ctrl
3.1
TRP-1 p124
9.3
Ctrl
3.7
gp100 p152
43.8
CD4
CFSE
Cytokine analysis
IL-2
IL-5
IL-13
IP-10
Patient C
Patient D

Fig. 25

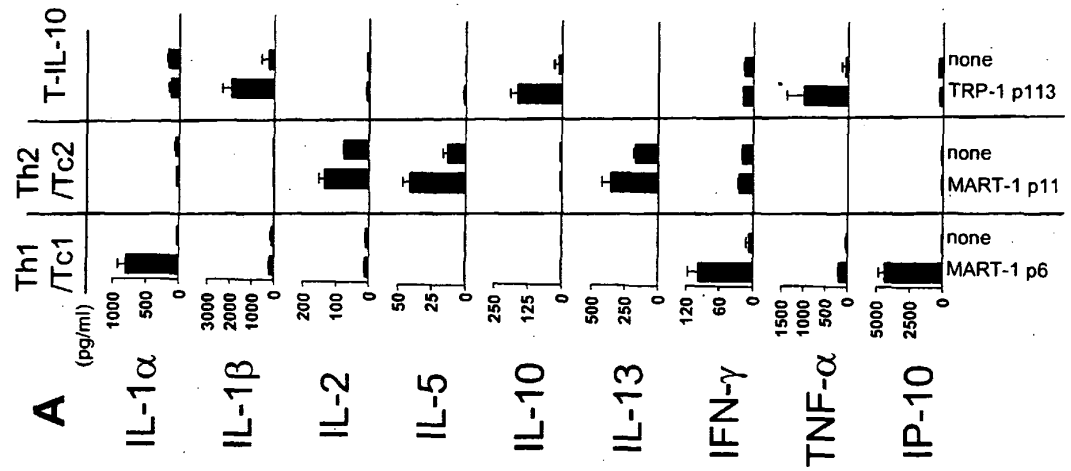

A
Th1 /Tc1
Th2 /Tc2
T-IL-10
(pg/ml)
IL-1α
IL-1β
IL-2
IL-5
IL-10
IL-13
IFN-γ
TNF-α
IP-10
none
TRP-1 p113
none
MART-1 p11
none
MART-1 p6

Figure 26

Novel epitopes identified with EPIMAX

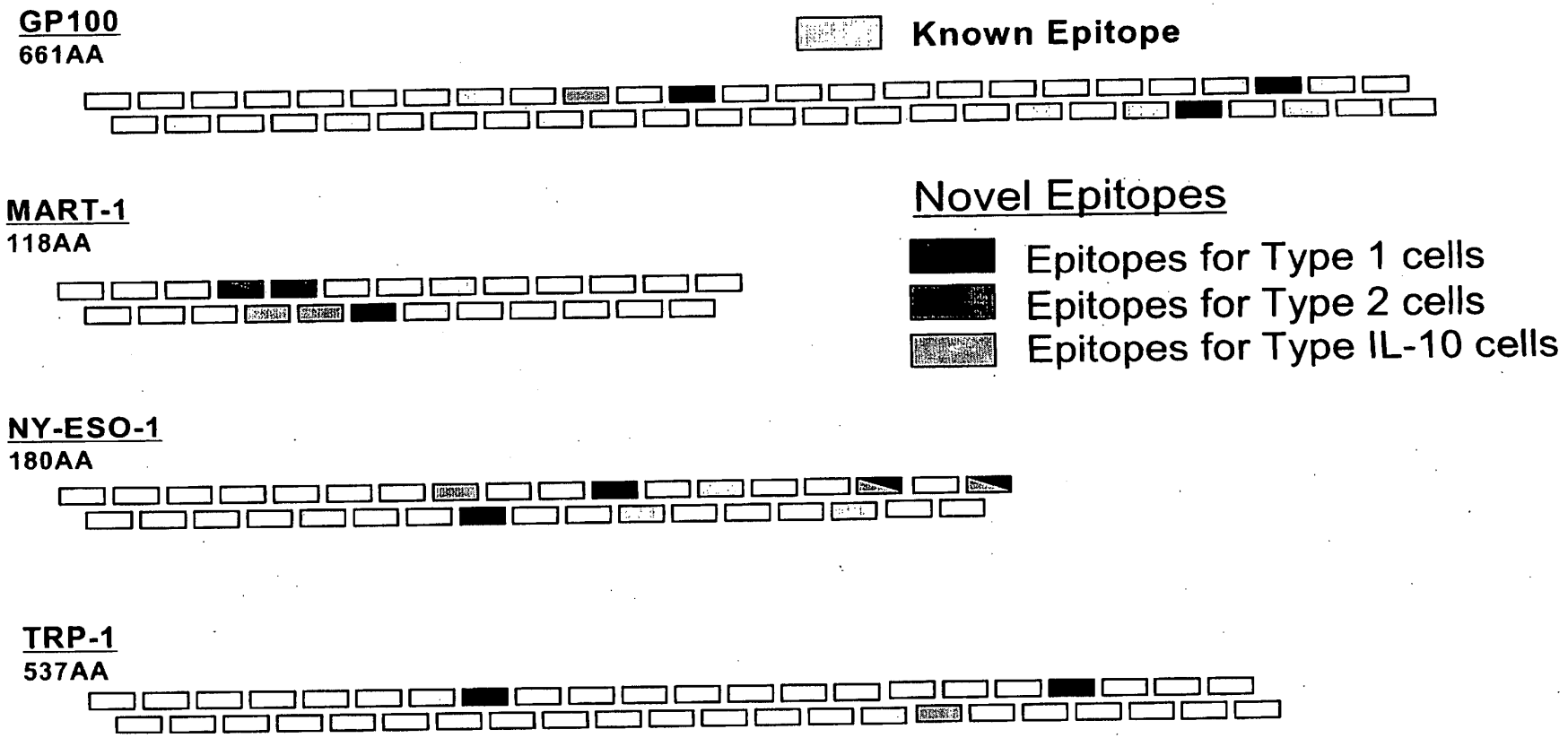


Figure 27

EP 1 774 332 B1

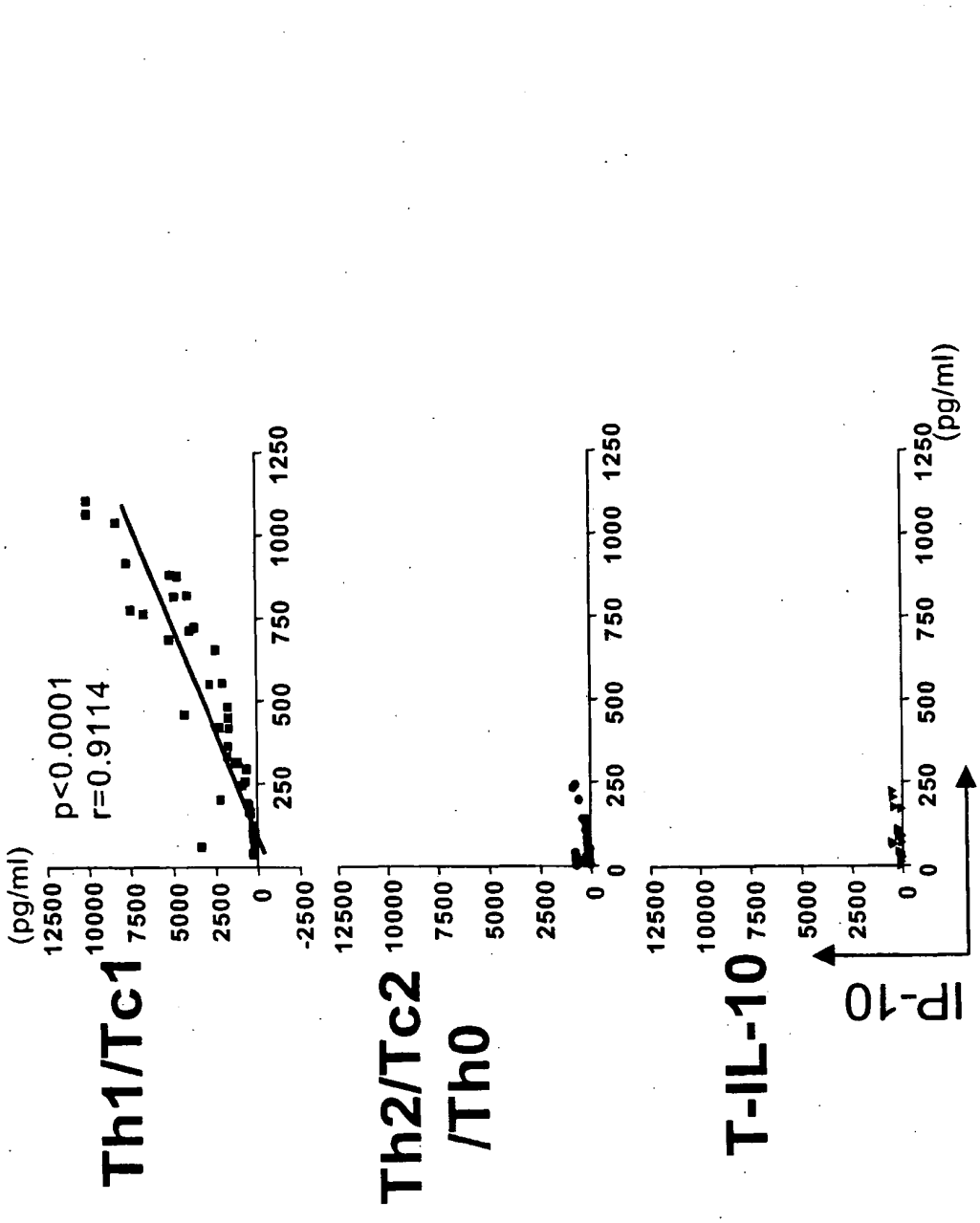

Th1/Tc1
p<0.0001
r=0.9114
(pg/ml)
12500
10000
7500
5000
2500
0
-2500
0
250
500
750
1000
1250
Th2/Tc2
/Th0
T-IL-10
IP-10
(pg/ml)
IL-1α

Figure 28

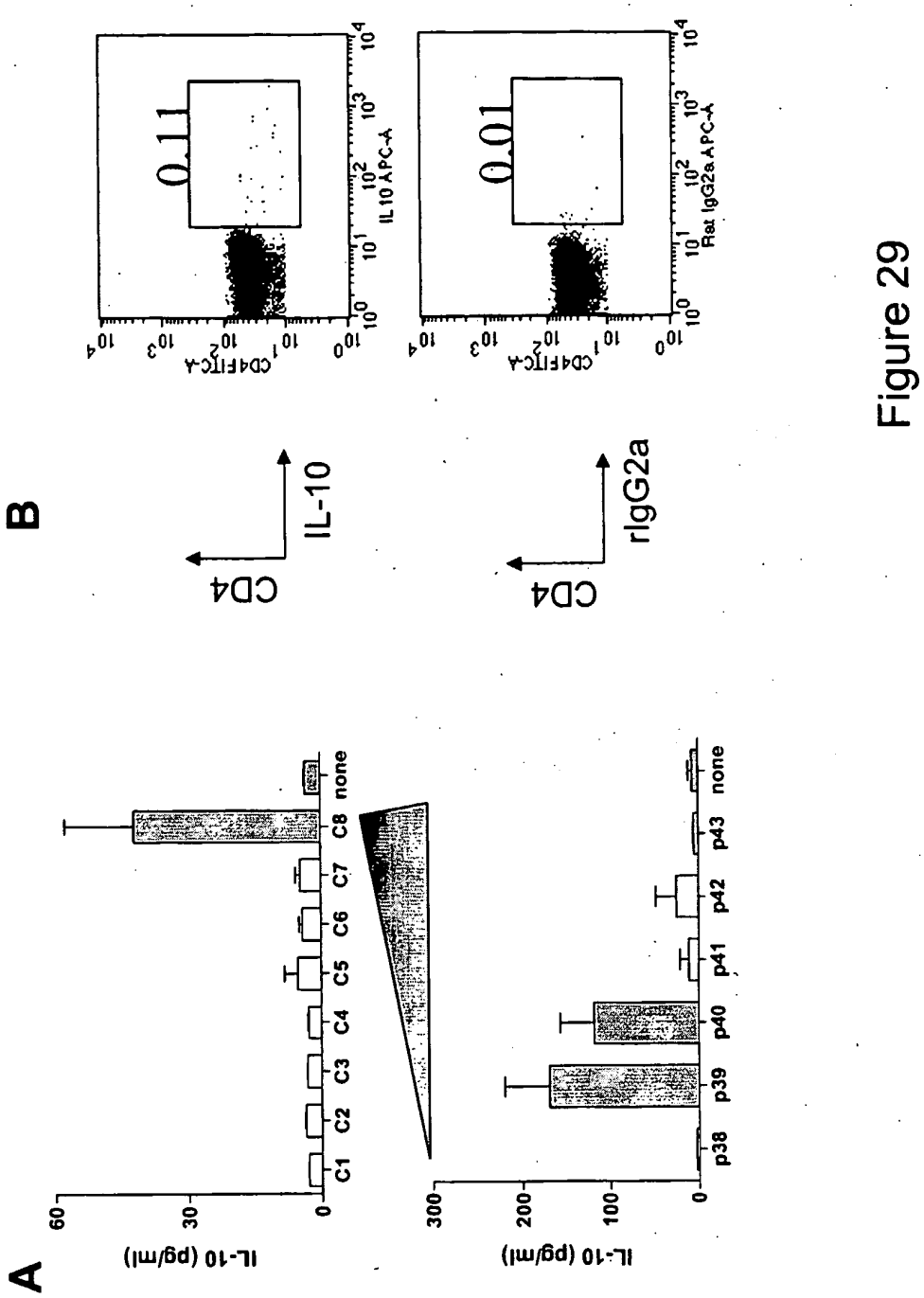
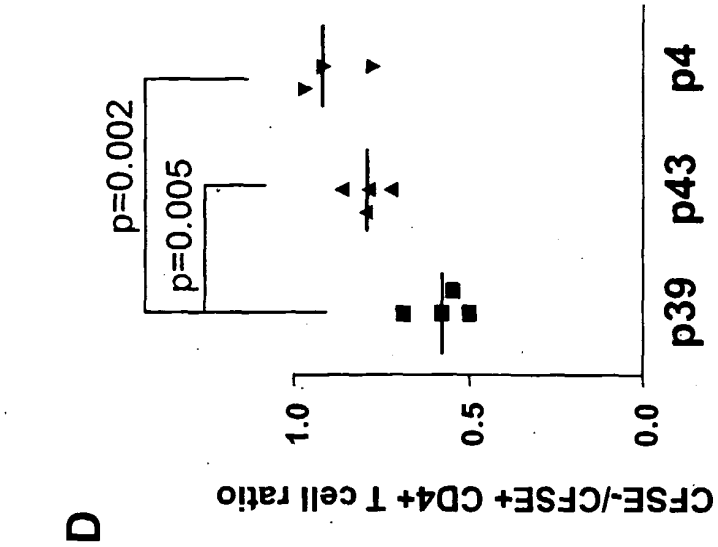

A
IL-10 (pg/ml)
60
30
0
C1
C2
C3
C4
C5
C6
C7
C8
none
IL-10 (pg/ml)
300
200
100
0
p38
p39
p40
p41
p42
p43
none
B
CD4
IL-10
CD4FITC-A
IL10 APC-A
0.11
CD4
rIgG2a
CD4FITC-A
Rat IgG2a APC-A
0.01

Figure 29


D
CFSE-/CFSE+ CD4+ T cell ratio
1.0
0.5
0.0
p=0.005
p=0.002
p39
p43
p4

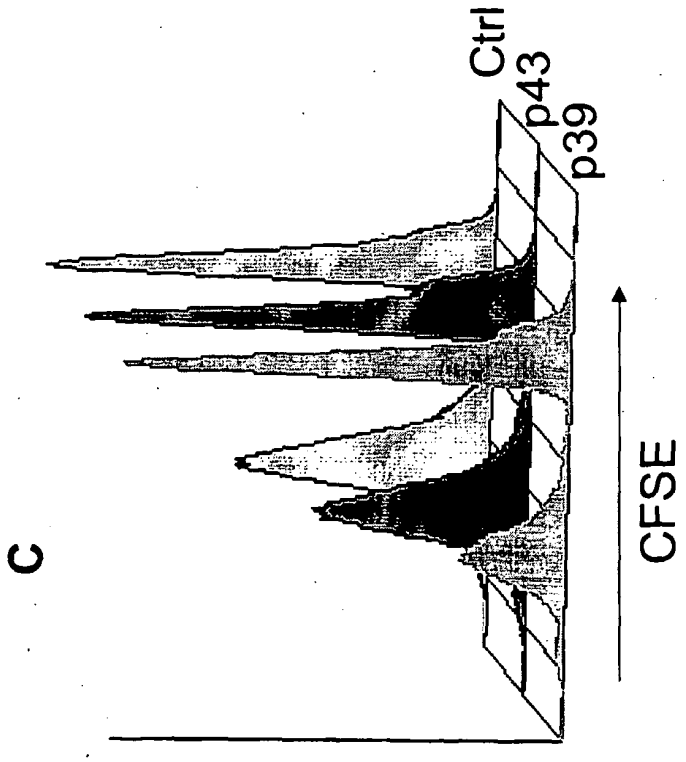

C
Ctrl
p43
p39
CFSE

Figure 29

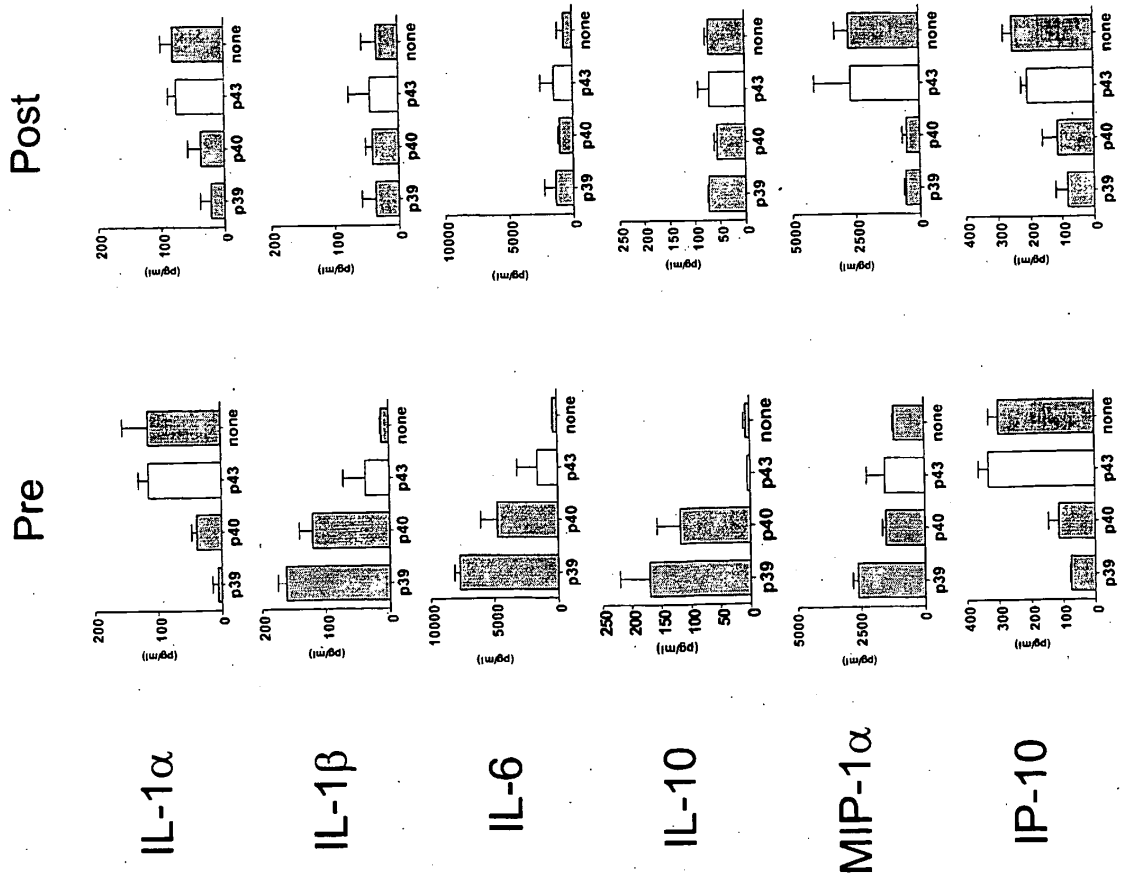


Figure 30

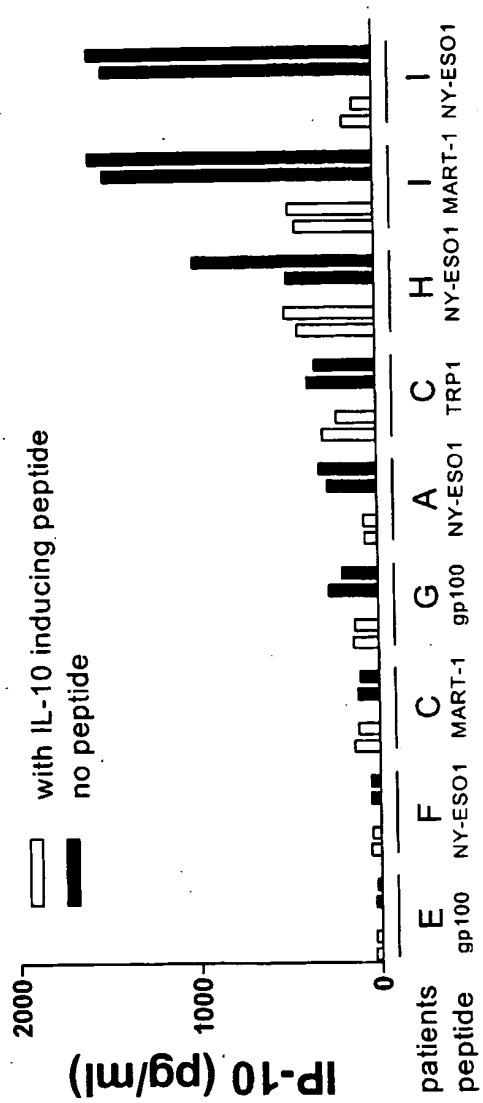

IP-10 (pg/ml)
2000
1000
0
with IL-10 inducing peptide
no peptide
patients
peptide
E
gp100
F
NY-ESO1
C
MART-1
G
gp100
A
NY-ESO1
C
TRP1
H
NY-ESO1
I
MART-1
I
NY-ESO1

Figure 31

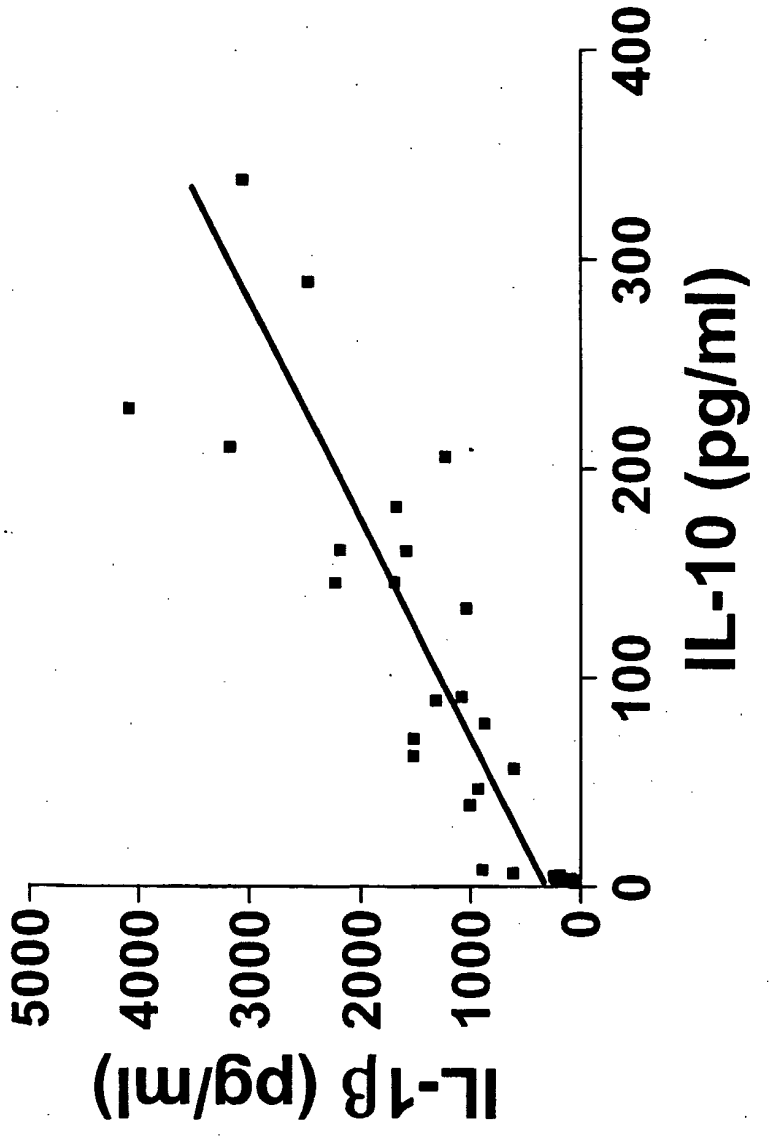

IL-1β (pg/ml)
5000
4000
3000
2000
1000
0
0
100
200
300
400
IL-10 (pg/ml)

Figure 32

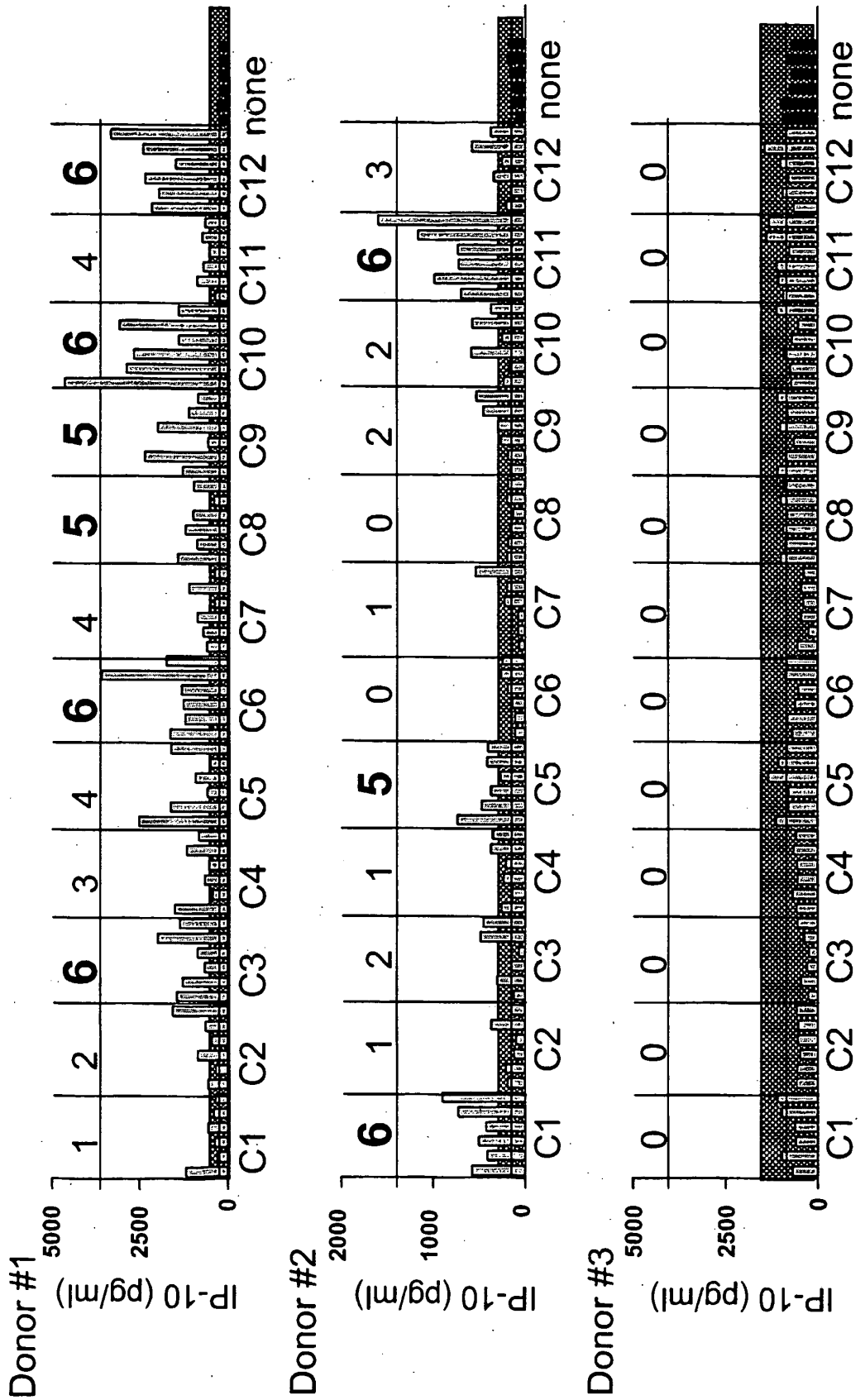

Donor #1
IP-10 (pg/ml)
5000
2500
0
1 2 6 3 4 6 4 5 5 6 4 6
C1 C2 C3 C4 C5 C6 C7 C8 C9 C10 C11 C12 none
Donor #2
IP-10 (pg/ml)
2000
1000
0
6 1 2 1 5 0 1 0 2 2 6 3
C1 C2 C3 C4 C5 C6 C7 C8 C9 C10 C11 C12 none
Donor #3
IP-10 (pg/ml)
5000
2500
0
0 0 0 0 0 0 0 0 0 0 0 0
C1 C2 C3 C4 C5 C6 C7 C8 C9 C10 C11 C12 none

Figure 33

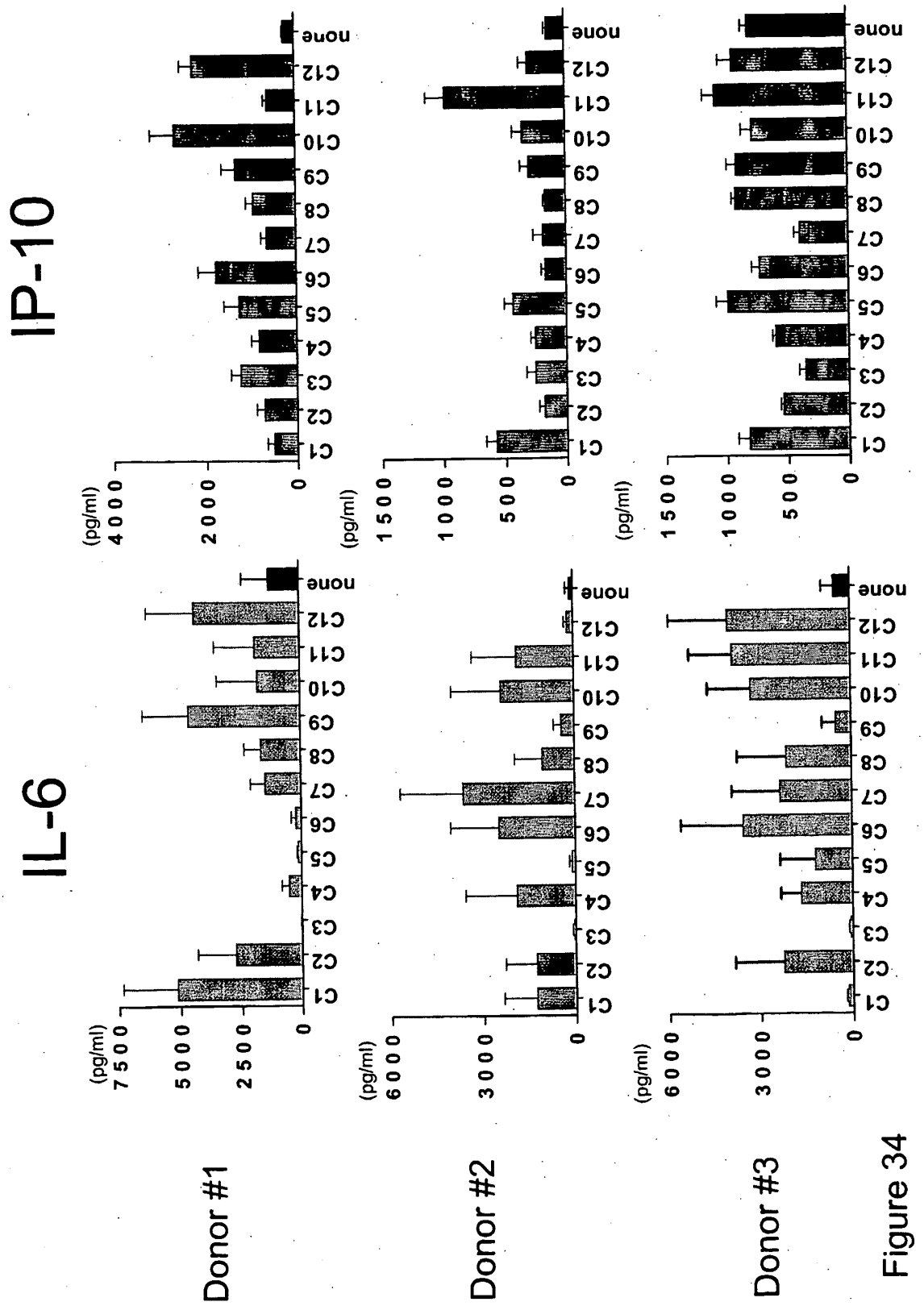

IP-10
IL-6
Donor #1
Donor #2
Donor #3
(pg/ml)
0
2000
4000
500
1000
1500
2500
5000
7500
3000
6000
C1
C2
C3
C4
C5
C6
C7
C8
C9
C10
C11
C12
none

Figure 34

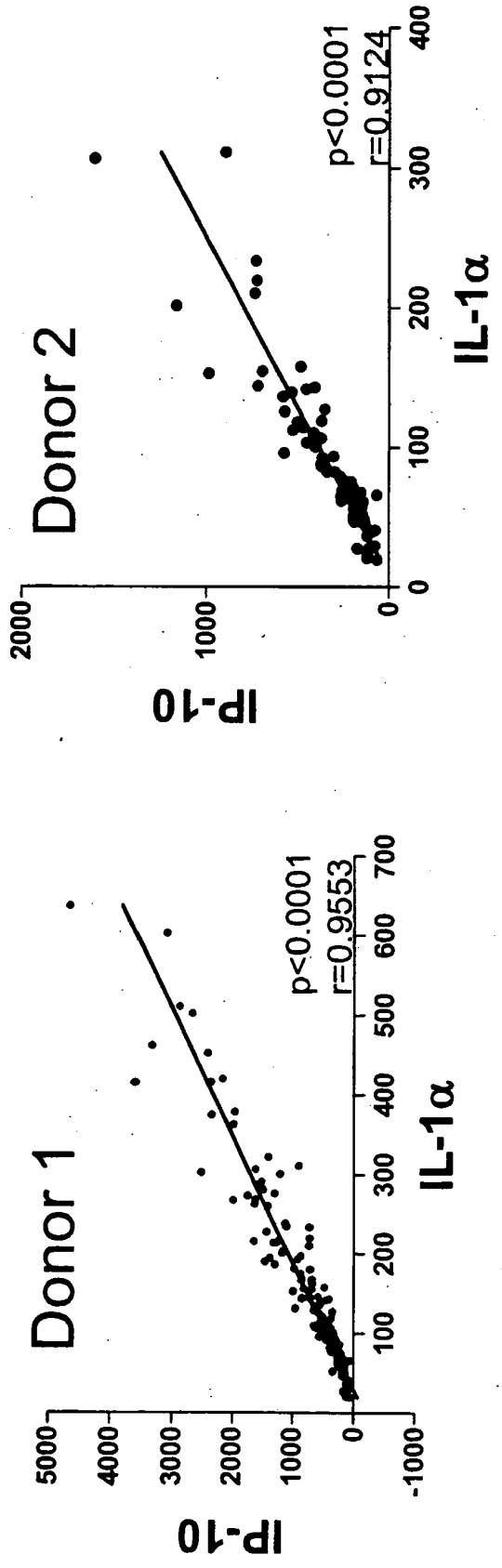

Donor 2
IP-10
IL-1α
p<0.0001
r=0.9124
2000
1000
0
0
100
200
300
400
Donor 1
IP-10
IL-1α
p<0.0001
r=0.9553
5000
4000
3000
2000
1000
0
-1000
100
200
300
400
500
600
700

Figure 35

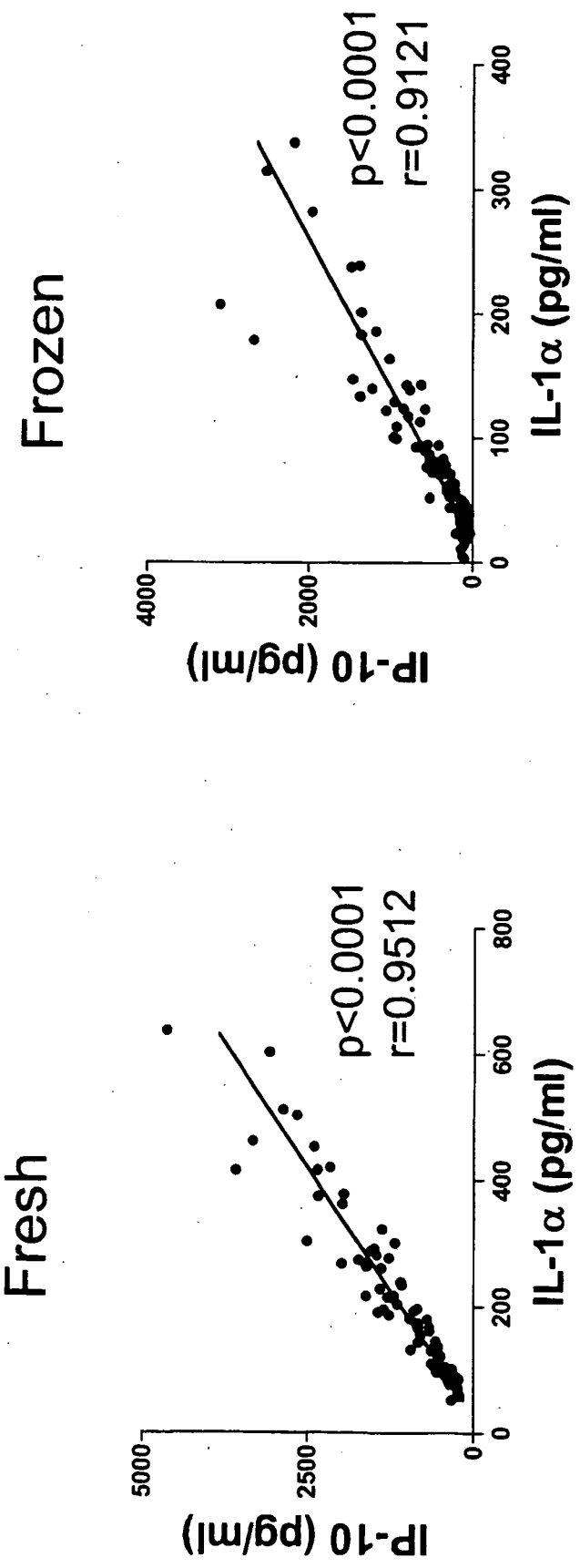

Frozen
p<0.0001
r=0.9121
IP-10 (pg/ml)
IL-1α (pg/ml)
0
2000
4000
0
100
200
300
400
Fresh
p<0.0001
r=0.9512
IP-10 (pg/ml)
IL-1α (pg/ml)
0
2500
5000
0
200
400
600
800

Figure 36

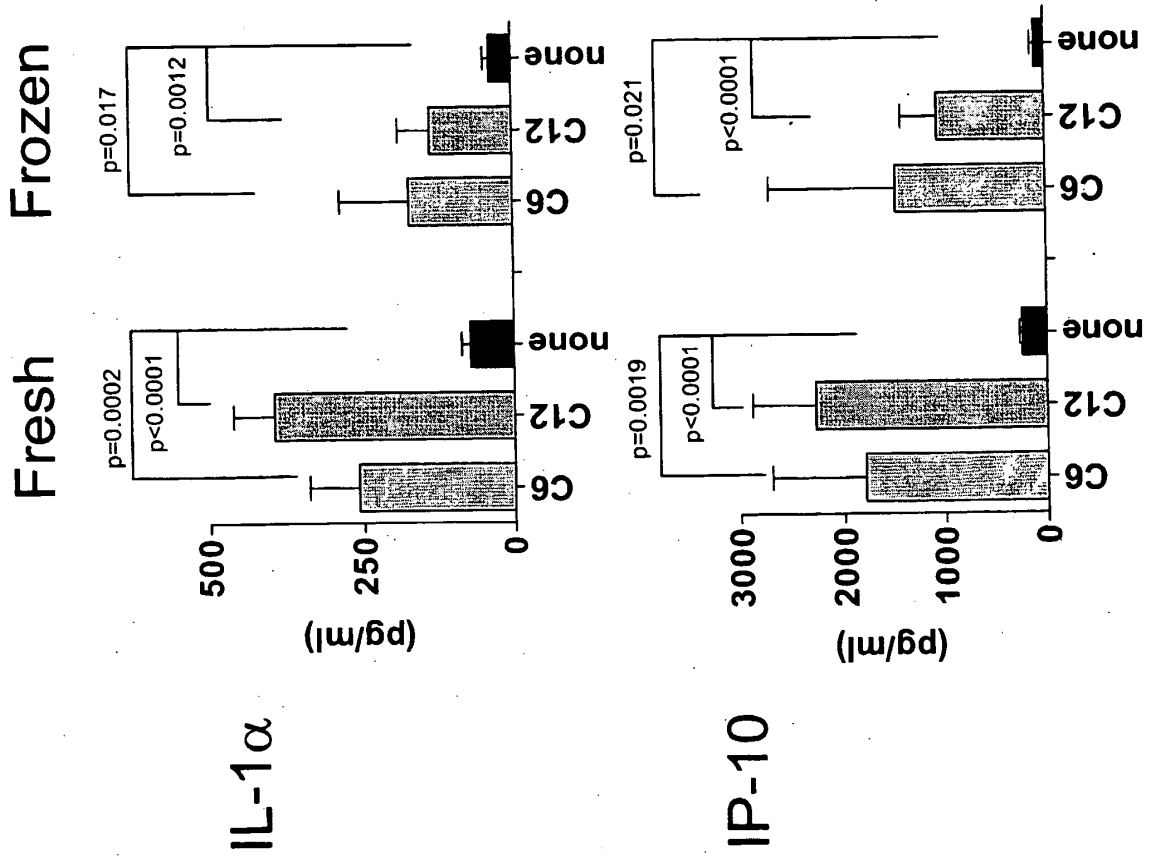

Fresh
Frozen
IL-1α
IP-10
(pg/ml)
500
250
0
3000
2000
1000
C6
C12
none
p=0.0002
p<0.0001
p=0.017
p=0.0012
p=0.0019
p<0.0001
p=0.021
p<0.0001

Figure 37

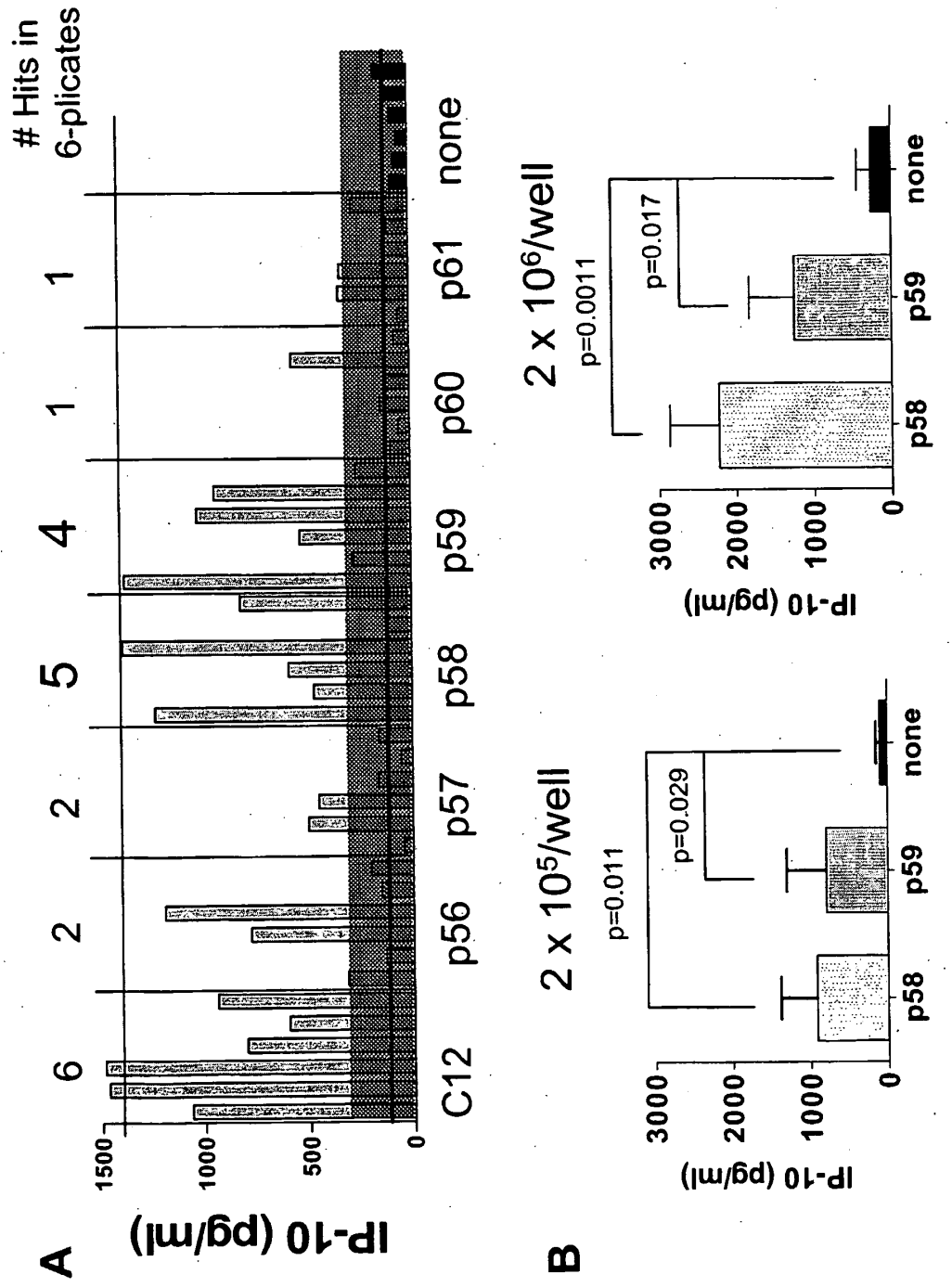

A
IP-10 (pg/ml)
1500
1000
500
0
Hits in 6-plicates
6
2
2
5
4
1
1
C12
p56
p57
p58
p59
p60
p61
none
B
2 x 10^5/well
p=0.011
p=0.029
IP-10 (pg/ml)
3000
2000
1000
0
p58
p59
none
2 x 10^6/well
p=0.0011
p=0.017
IP-10 (pg/ml)
3000
2000
1000
0
p58
p59
none

Figure 38

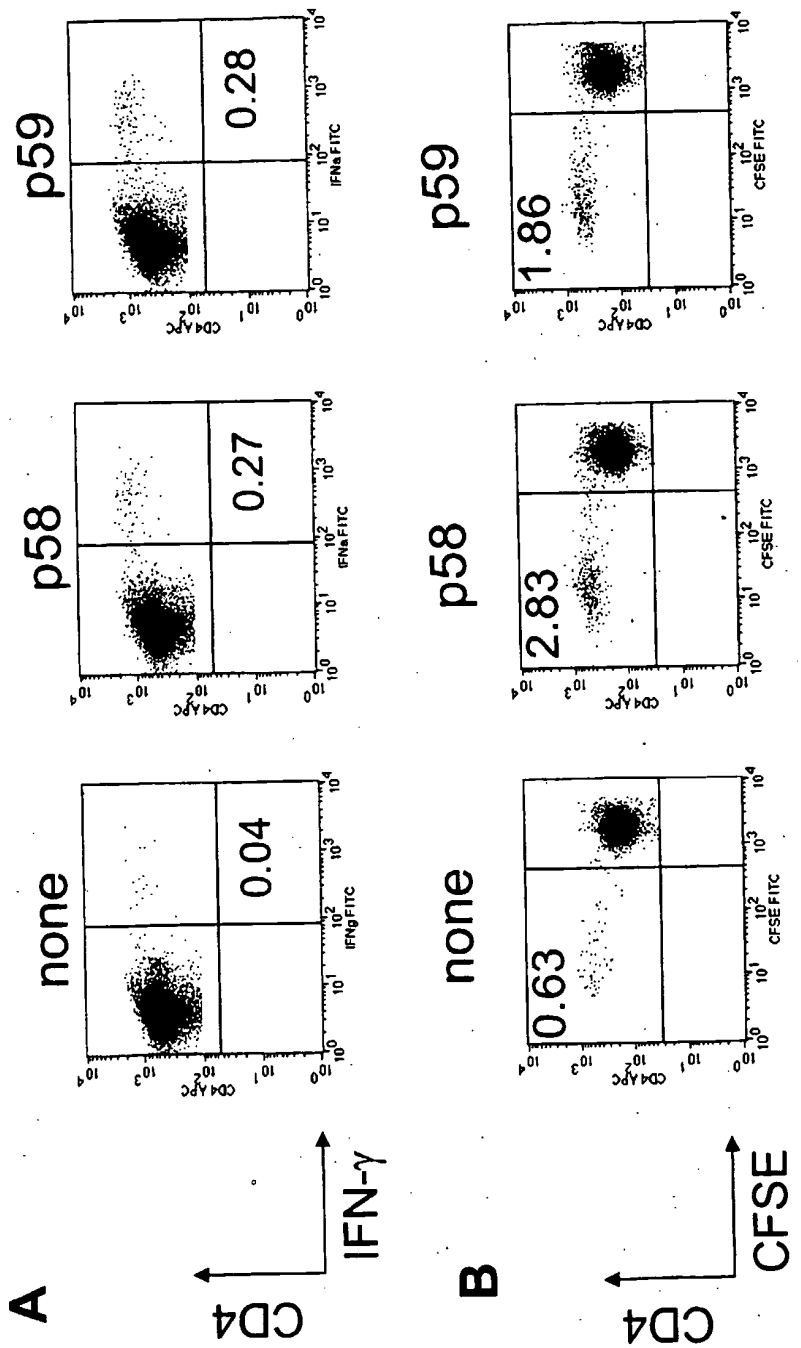

A
B
CD4
IFN-γ
CFSE
none
p58
p59
0.04
0.27
0.28
0.63
2.83
1.86
CD4APC
IFNgFITC
IFNaFITC
CFSEFITC

Figure 39

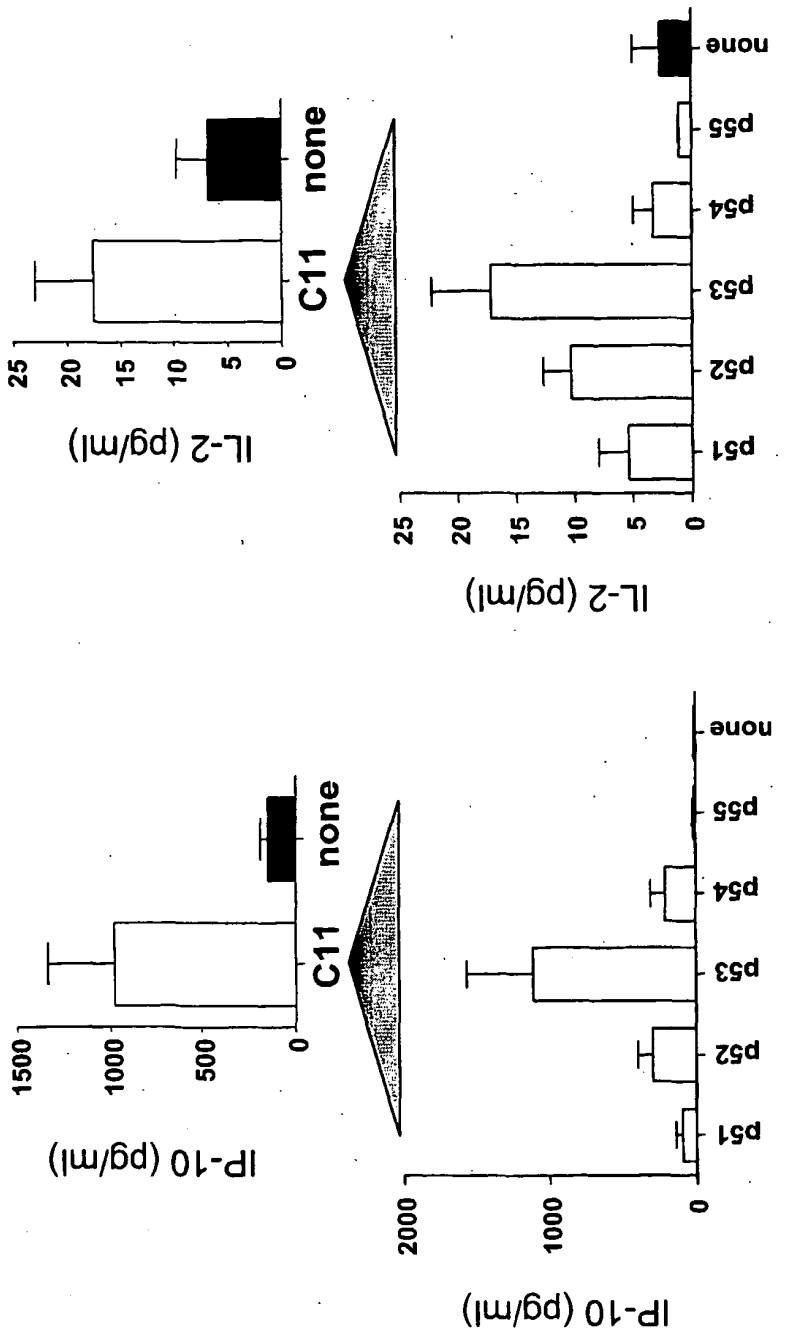

IP-10 (pg/ml)
2000
1000
0
p51
p52
p53
p54
p55
none
C11
none
IP-10 (pg/ml)
1500
1000
500
0
IL-2 (pg/ml)
25
20
15
10
5
0
p51
p52
p53
p54
p55
none
C11
none
IL-2 (pg/ml)
25
20
15
10
5
0

Figure 40

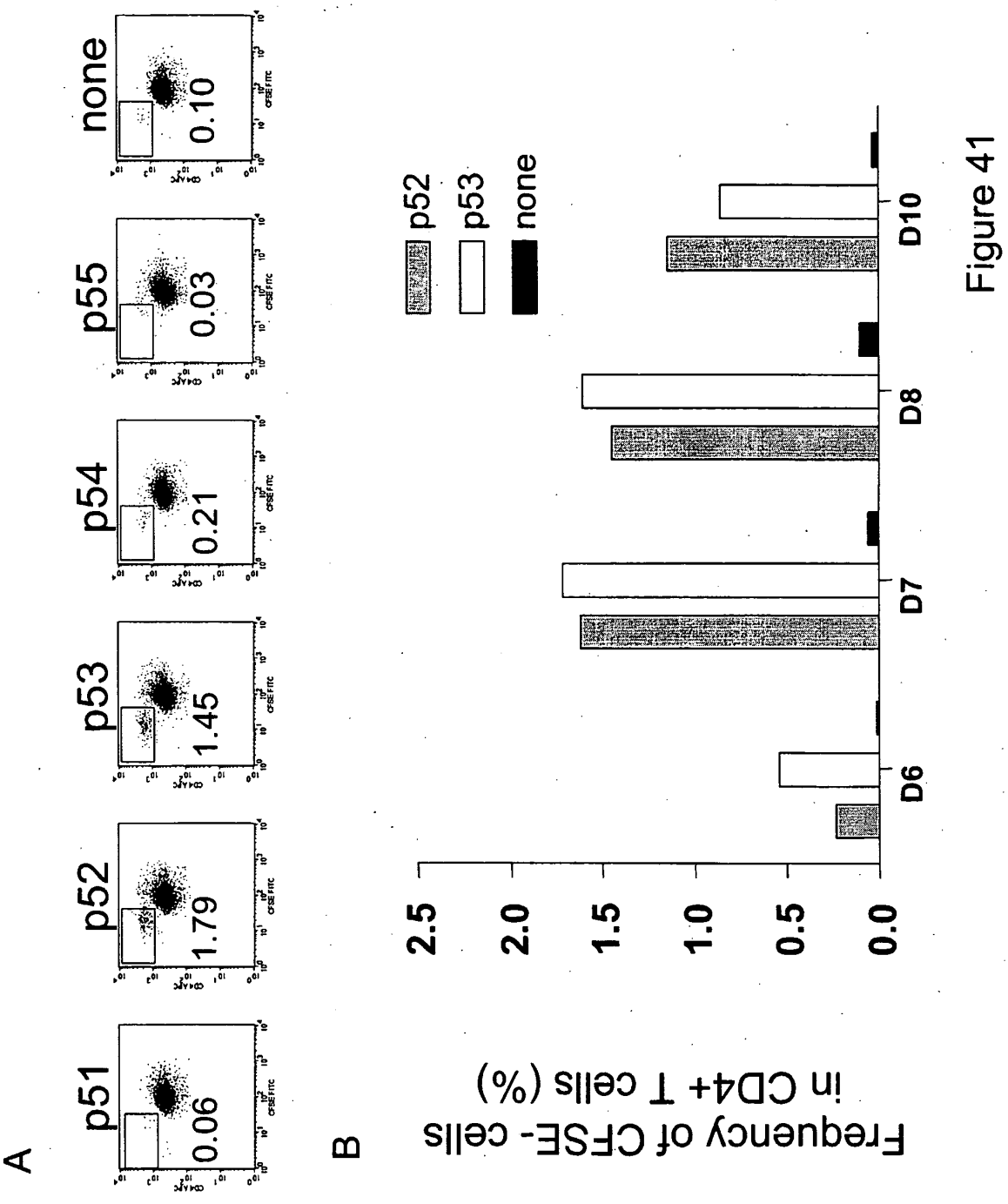

A
p51
0.06
p52
1.79
p53
1.45
p54
0.21
p55
0.03
none
0.10
B
Frequency of CFSE- cells
in CD4+ T cells (%)
2.5
2.0
1.5
1.0
0.5
0.0
D6
D7
D8
D10
p52
p53
none

Figure 41

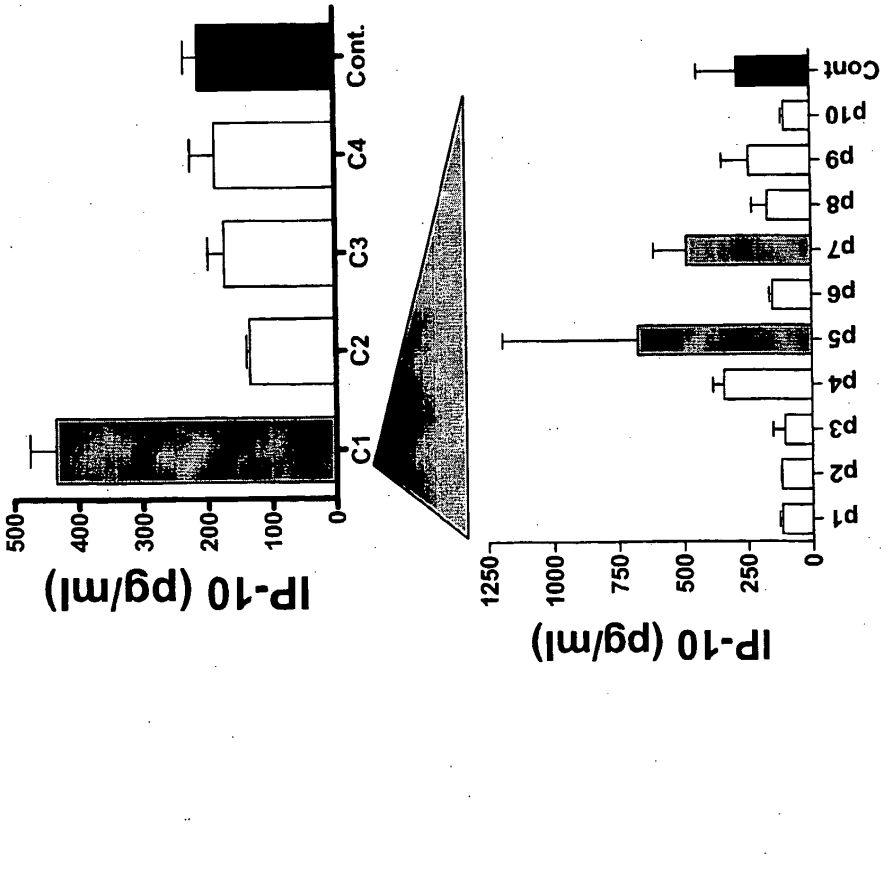

IP-10 (pg/ml)
500
400
300
200
100
0
C1
C2
C3
C4
Cont.
IP-10 (pg/ml)
1250
1000
750
500
250
0
p1
p2
p3
p4
p5
p6
p7
p8
p9
p10
Cont.
Cell line allele
Pt's allele
B*4002 1 GSHSMRYFHT 11 SVSRPGRGEP 21 RFITVGYVDD 31 TLFVRFDSDA 41 TSPRKEPRAP 51 WIEQEGPEYW
B*0801 GSHSMRYFDT AMSRPGRGEP RFISVGYVDD TQFVRFDSDA ASPREEPRAP WIEQEGPEYW
B*1402 GSHSMRYFYT AVSRPGRGEP RFISVGYVDD TQFVRFDSDA ASPREEPRAP WIEQEGPEYW
p5
p7

Figure 42

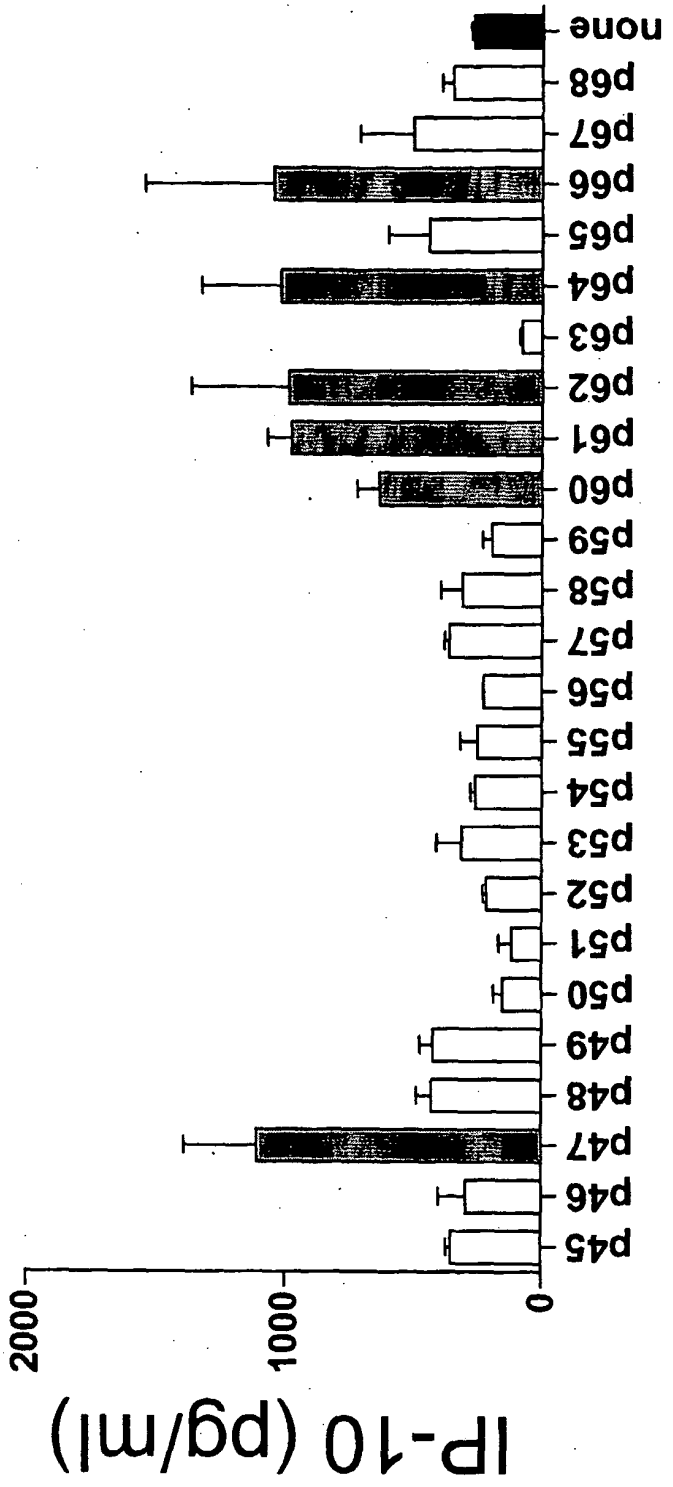

IP-10 (pg/ml)
2000
1000
0
none
p68
p67
p66
p65
p64
p63
p62
p61
p60
p59
p58
p57
p56
p55
p54
p53
p52
p51
p50
p49
p48
p47
p46
p45

Figure 43

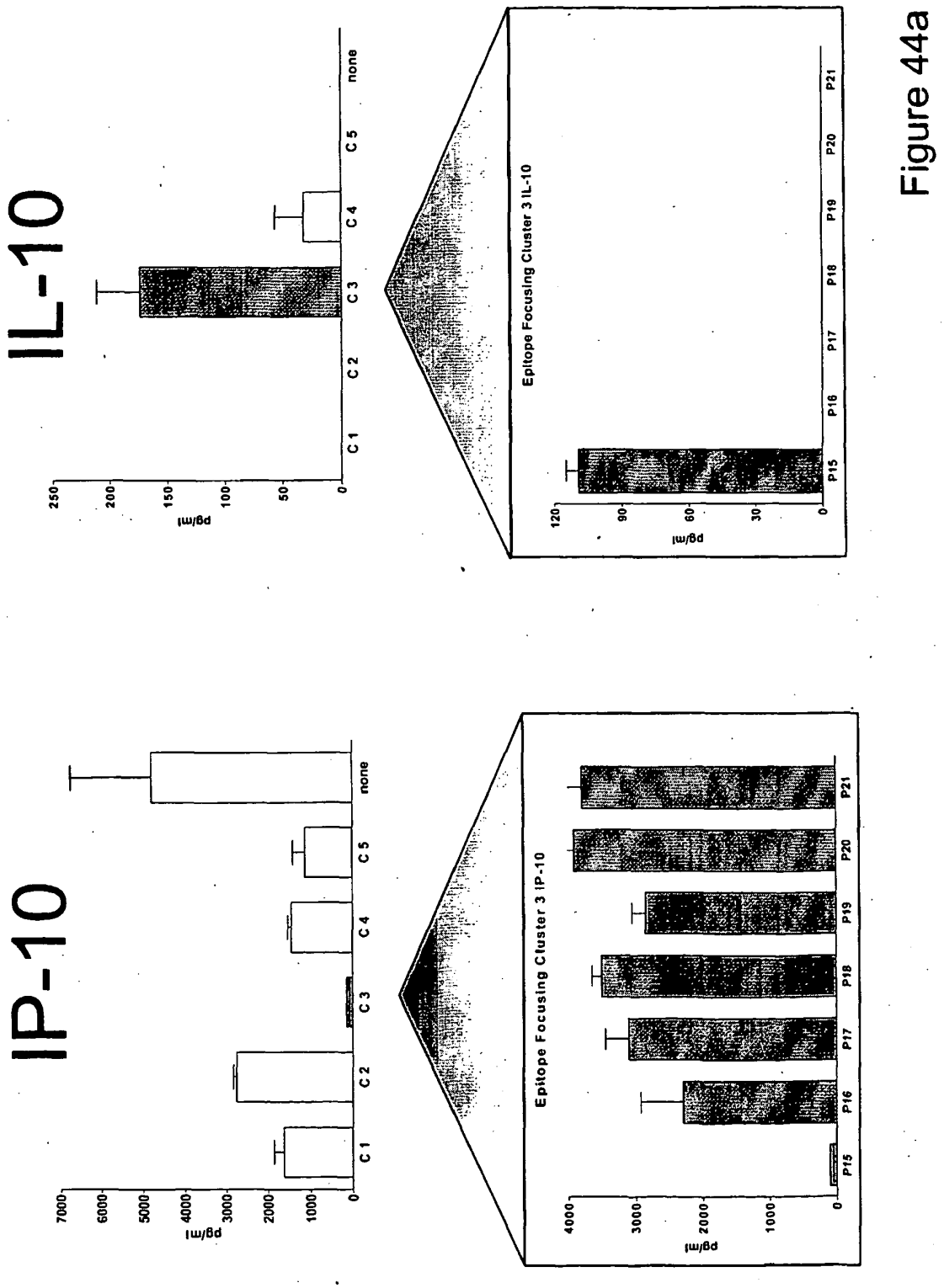

IL-10
pg/ml
250
200
150
100
50
0
C 1
C 2
C 3
C 4
C 5
none
Epitope Focusing Cluster 3 IL-10
pg/ml
120
90
60
30
0
P15
P16
P17
P18
P19
P20
P21
IP-10
pg/ml
7000
6000
5000
4000
3000
2000
1000
0
C 1
C 2
C 3
C 4
C 5
none
Epitope Focusing Cluster 3 IP-10
pg/ml
4000
3000
2000
1000
0
P15
P16
P17
P18
P19
P20
P21

Figure 44a

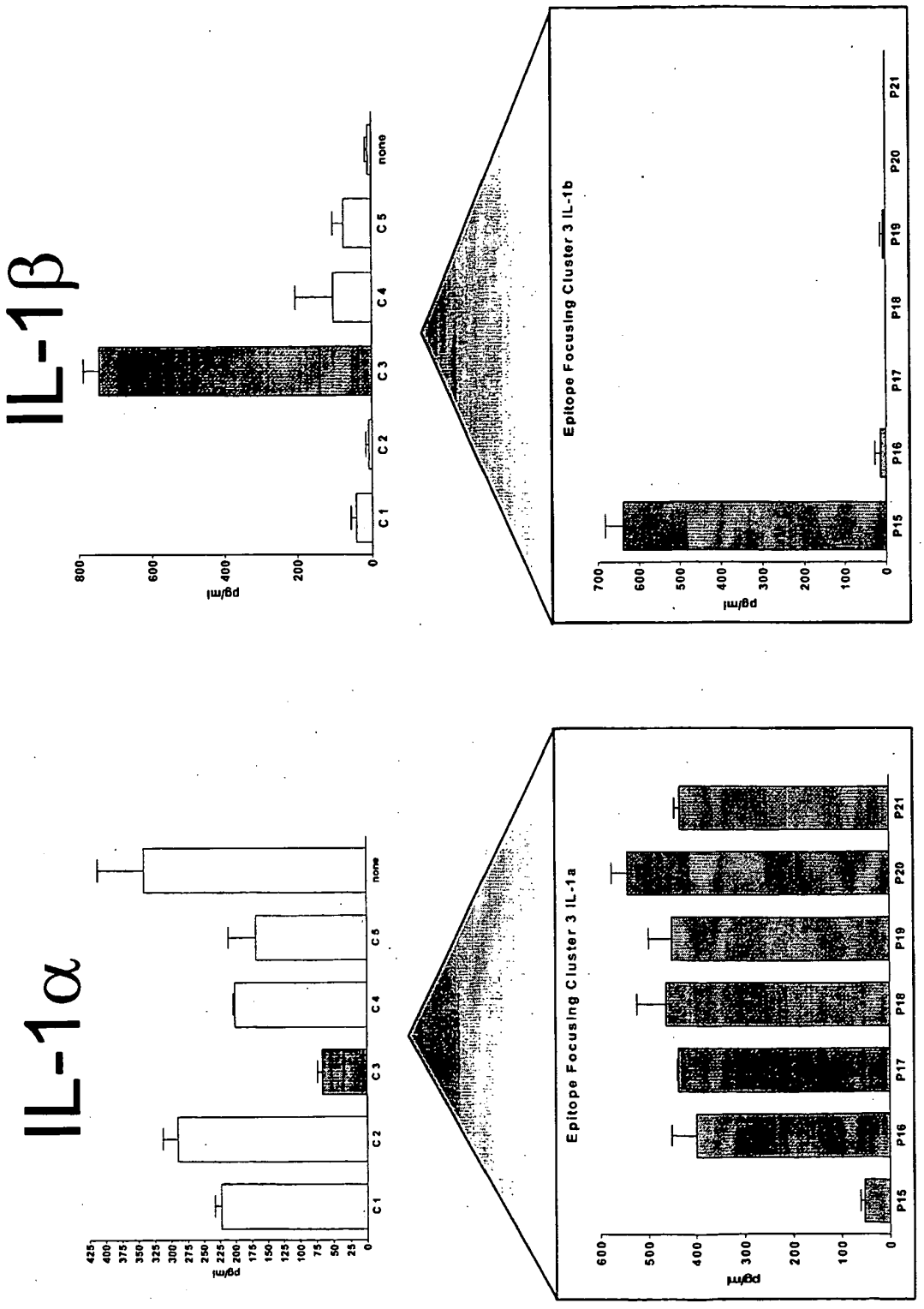

IL-1β
pg/ml
800
600
400
200
0
C1
C2
C3
C4
C5
none
Epitope Focusing Cluster 3 IL-1b
pg/ml
700
600
500
400
300
200
100
0
P15
P16
P17
P18
P19
P20
P21
IL-1α
pg/ml
425
400
375
350
325
300
275
250
225
200
175
150
125
100
75
50
25
0
C1
C2
C3
C4
C5
none
Epitope Focusing Cluster 3 IL-1a
pg/ml
600
500
400
300
200
100
0
P15
P16
P17
P18
P19
P20
P21

Figure 44b

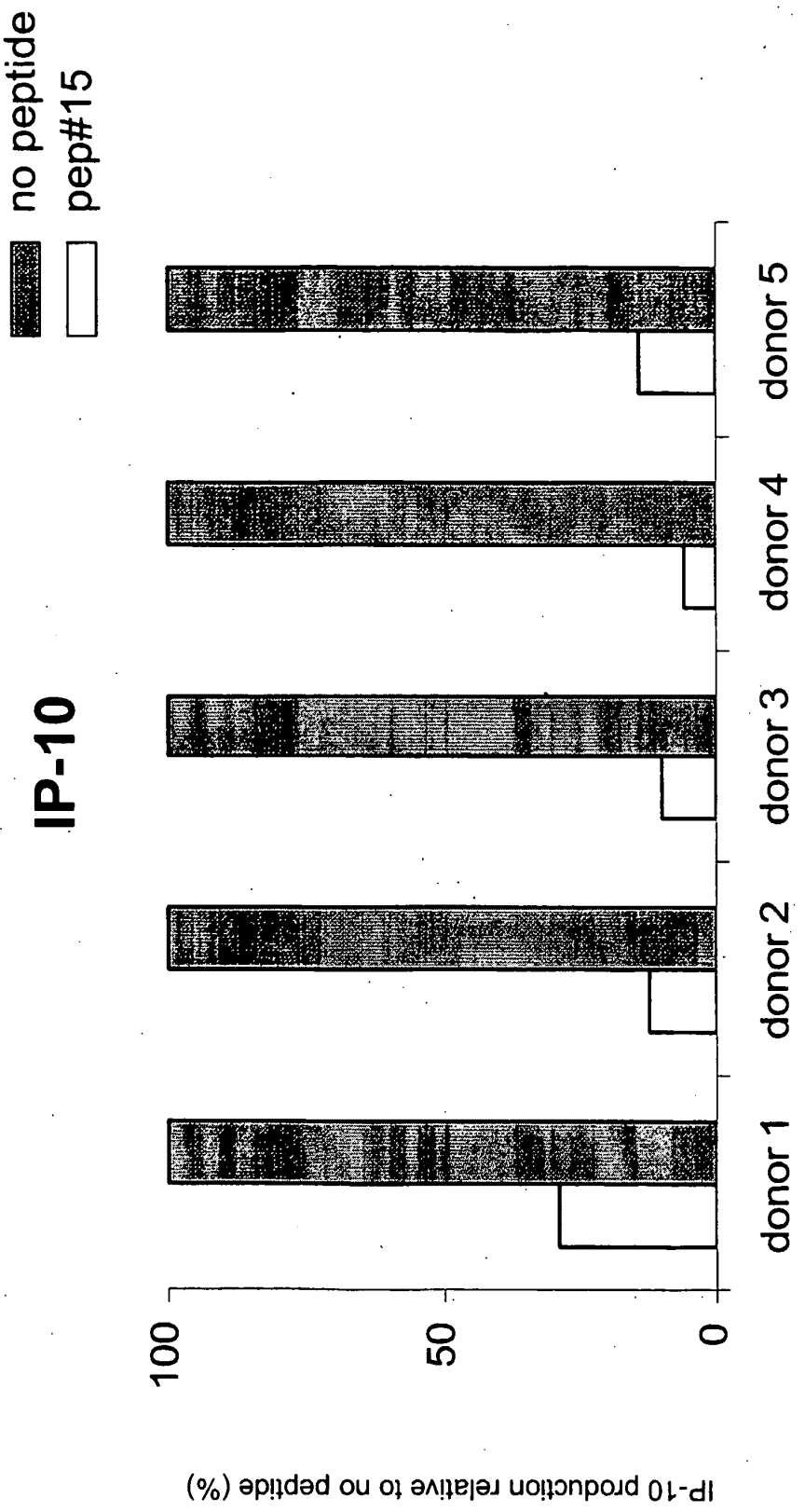
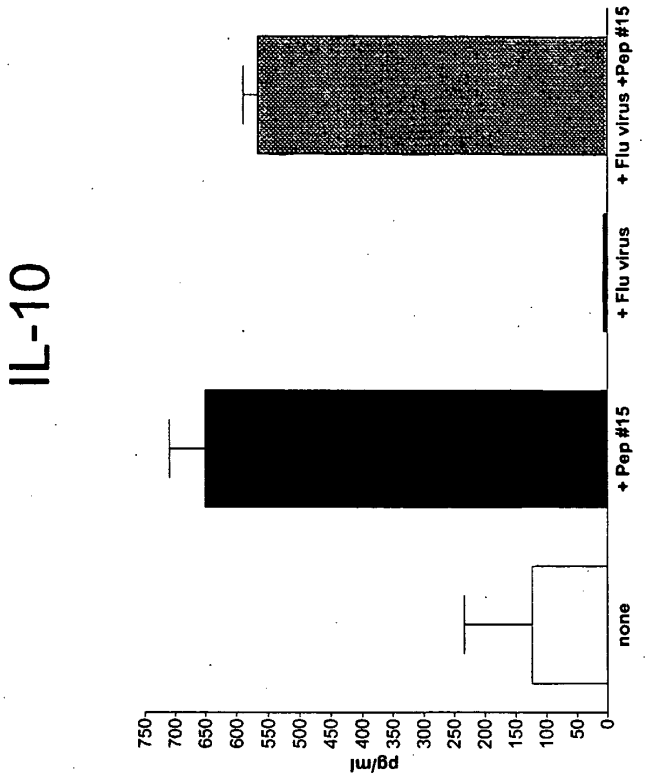

IP-10
no peptide
pep#15
donor 1
donor 2
donor 3
donor 4
donor 5
0
50
100
IP-10 production relative to no peptide (%)

Figure 45


IL-10
pg/ml
750
700
650
600
550
500
450
400
350
300
250
200
150
100
50
0
none
+ Pep #15
+ Flu virus
+ Flu virus +Pep #15

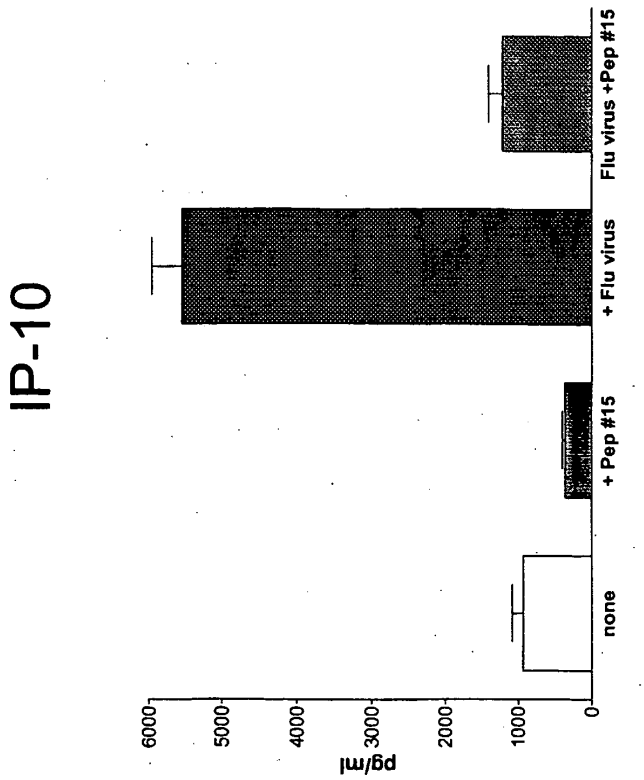

IP-10
pg/ml
6000
5000
4000
3000
2000
1000
0
none
+ Pep #15
+ Flu virus
Flu virus +Pep #15

Figure 46

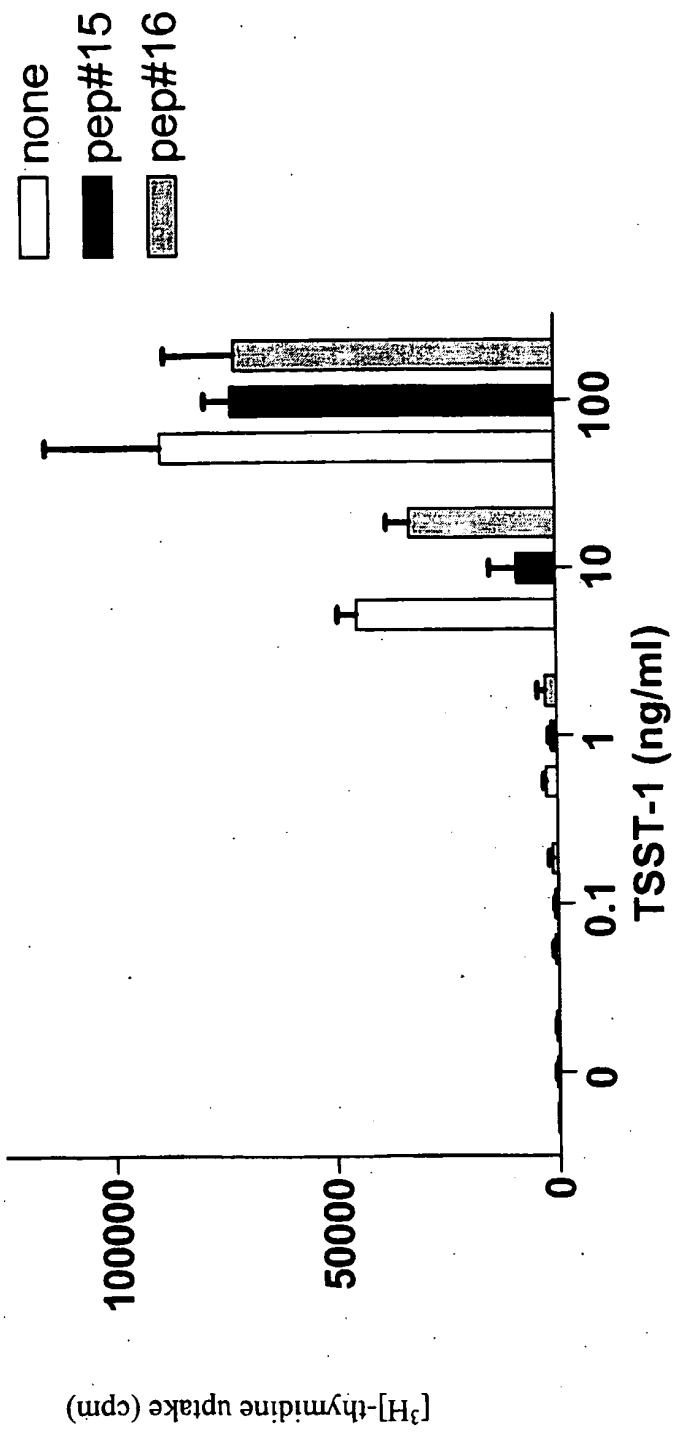

none
pep#15
pep#16
100000
50000
0
0
0.1
1
10
100
TSST-1 (ng/ml)
[3H]-thymidine uptake (cpm)

Figure 47

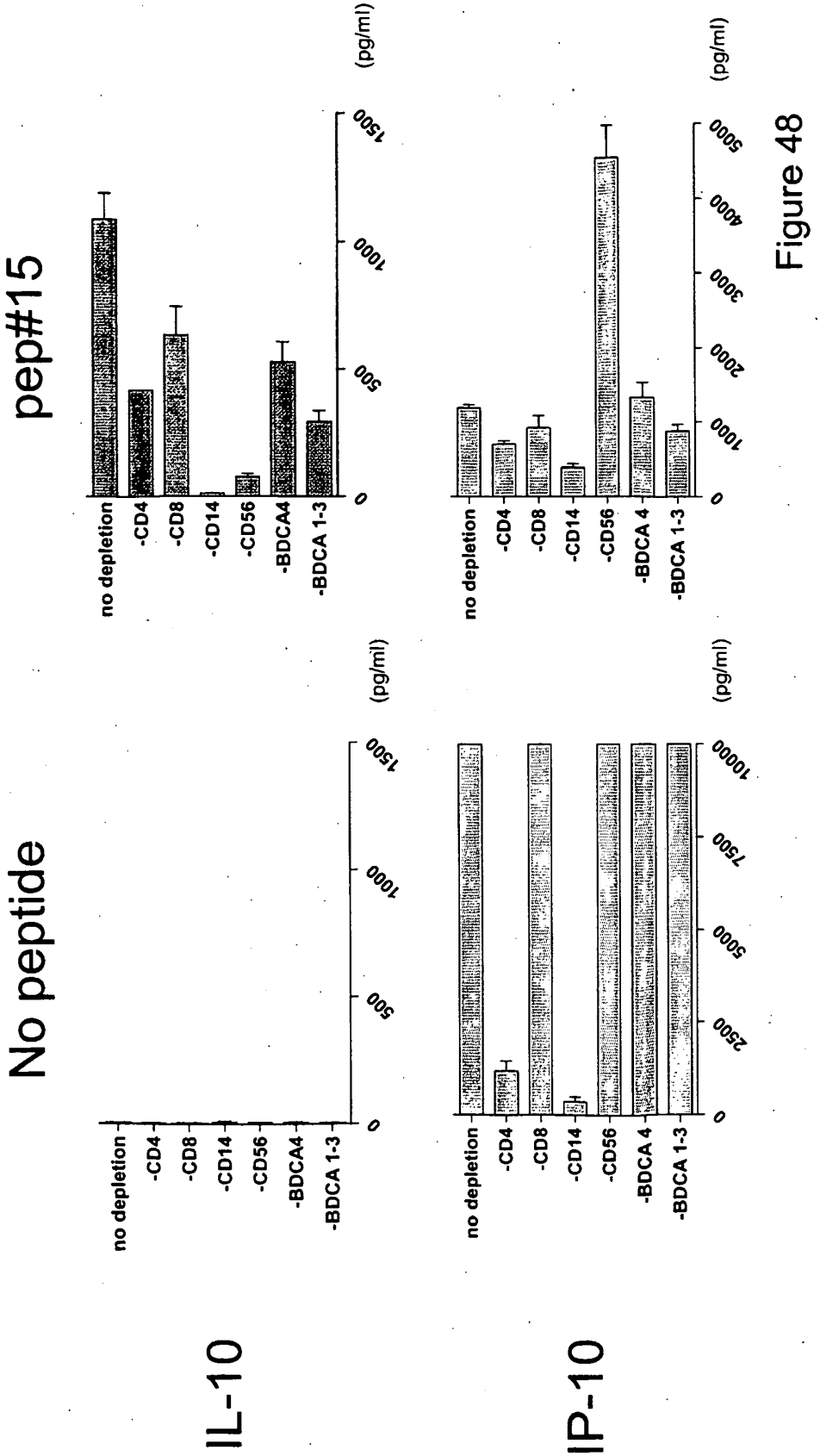

No peptide
pep#15
IL-10
IP-10
(pg/ml)
no depletion
-CD4
-CD8
-CD14
-CD56
-BDCA4
-BDCA 4
-BDCA 1-3
0
500
1000
1500
2500
3000
4000
5000
7500
10000

Figure 48

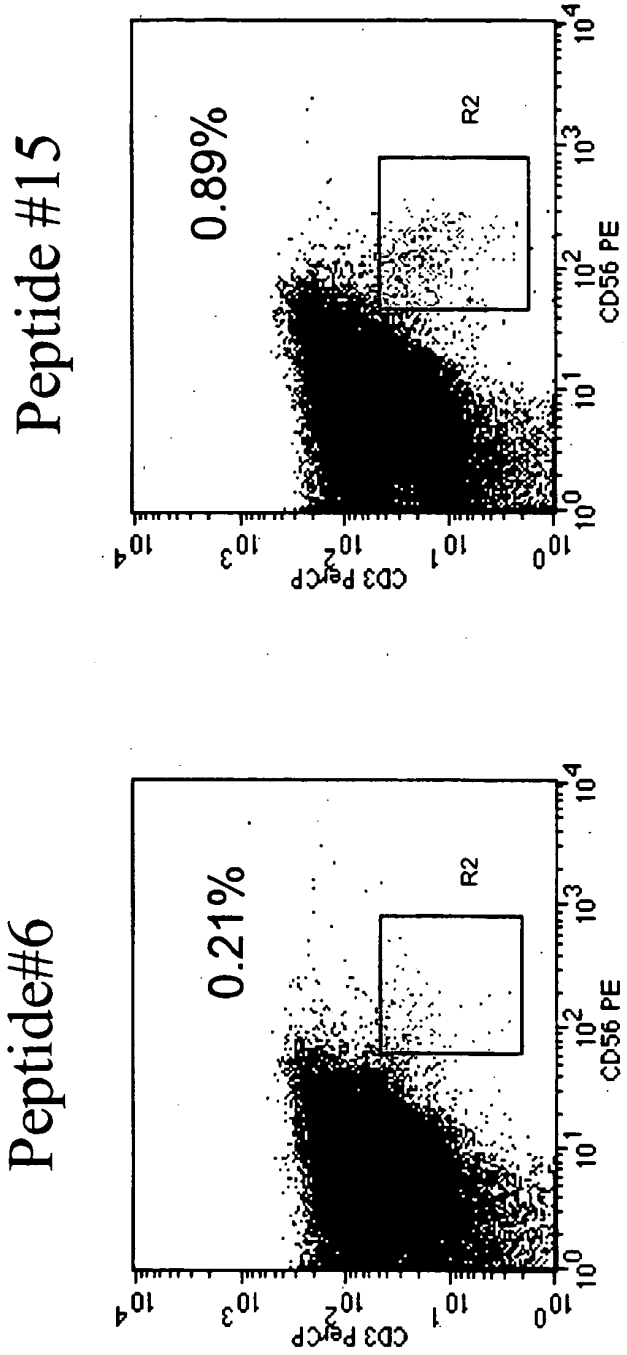

Peptide #15
0.89%
R2
CD56 PE
CD3 PerCP
Peptide#6
0.21%
R2
CD56 PE
CD3 PerCP

Figure 49

REFERENCES CITED IN THE DESCRIPTION

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专利名称(译)	免疫反应评估方法		
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申请号	EP2005804838	申请日	2005-05-24
[标]申请(专利权)人(译)	贝勒研究协会		
申请(专利权)人(译)	BAYLOR研究所		
当前申请(专利权)人(译)	BAYLOR研究所		
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发明人	BANCHEREAU, JACQUES CONNOLLY, JOHN, E. PALUCKA, ANNA, KAROLINA UENO, HIDEKI		
IPC分类号	G01N33/53 A61K38/00 A61K39/00 A61K49/00 C12N15/10 C40B30/02 C40B30/04 G01N33/00 G01N33/50 G01N33/531 G01N33/564 G01N33/566 G01N33/68		
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优先权	60/573912 2004-05-24 US		
其他公开文献	EP1774332B1 EP1774332A2		
外部链接	Espacenet		

摘要(译)

本发明包括通过同时分析培养物的免疫反应性的多个参数以鉴定至少一个表位来鉴定与受试者的HLS类型无关的T细胞表位的组合物和方法，以鉴定至少一个表位，其中鉴定了引起该亚组中感兴趣的免疫反应性的表位 作为子集的目标代理。

overlapping peptides, and shown in the table.

Patient	Pre or Post	peptide library	identified epitopes	epitope sequence	Precise epitope	Type of response
094-001	Pre	MART-1	p6	MART-1_{21-35}	MART-1_{25-35}	1
	Post	MART-1	p6	MART-1_{21-35}	MART-1_{25-35}	1
	Pre	NY-ESO1	p24	NY-ESO1_{93-107}	NY-ESO1_{94-102}	1
	Post	NY-ESO1	p24	NY-ESO1_{03-107}	NY-ESO1_{94-102}	1
	Pre	gp100	p60	gP$100_{237-251}$		1
094-004	Pre	MART-1	p6	MART-1_{21-35}	MART-1_{25-35}	1
094-007	Pre	NY-ESO1	p39	NY-ESO$1_{153-167}$		1
094-009	Post	MART-1	p6	MART-1_{21-35}	MART-1_{26-35}	1
	Post	MART-1	p13	MART-1_{40-63}	MART-$1_{53-62,54-62}$	1
	Post	gp100	p52	9P$^{100}_{205-219}$	gp$100_{209-217}$	1