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(54) Title: MODULATED IMMUNODOMINANCE THERAPY

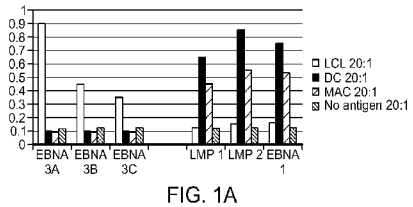

FIG. 1A

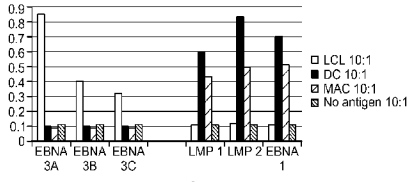

FIG. 1B

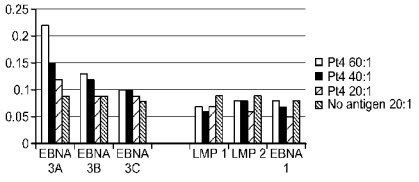

FIG. 1C

(57) Abstract: The invention involves generating a T cell response to subdominant antigens and using the cells to therapeutically change the cellular homeostasis and nature of the immune response. In a preferred embodiment, the cells are generated outside of the patient avoiding the influence of the patient's immunologic milieu. By stimulating and growing the T cells from a patient in a tissue culture to one or more subdominant antigens and the transplanting them into the patient, if enough cells are expanded and transplanted, the transplanted cells overwhelm the endogenous dominant T cells in the response to either break or induce immune tolerance or otherwise modify the immune response to the cells or organism expressing that antigen. When the memory cells are established they are then reflective of this new immunodominance hierarchy so that the desired therapeutic effect is long lasting. In effect, the transplantation exogenously generated T cells reactive to the subdominant antigens is recapitulating priming and rebalancing the patient's immune response to target previously subdominant antigens in the cells or organism to produce a therapeutic benefit.

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MODULATED IMMUNODOMINANCE THERAPY

RELATED APPLICATIONS

[0001] This application is related to provisional application U.S. Serial No. 61/490,505, filed on May 26, 2011, the contents of which are hereby incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

[0002] The present invention relates to a novel therapeutic for cancer, chronic infections, autoimmune diseases, and transplantation based upon the modification of the immunodominance hierarch by therapeutic manipulation of cellular homeostasis.

[0003] The clinical management of cancer is specific for each site of origin and, for the most part, depends upon stage of the disease (i.e., how far the tumor has invaded locally or spread to other organs by metastasis). Surgery and/or localized radiotherapy are generally the treatment of choice for the primary tumor with chemotherapy, monoclonal antibody or cytokine therapy or whole body irradiation being treatment for metastatic disease. Recently, the first dendritic cell therapy, Provenge, was approved for Prostate cancer with a 4-month progression benefit. Diagnosis is still based upon histologic analysis of the biopsy. Molecular markers are sometimes standard if they will be helpful in the selection of a drug (e.g., Herceptin). However, creating a profile of the immune response has not been done on a routine clinical basis.

[0004] The environment in which a T cell sees an antigen during the primary immune response determines the nature of subsequent recall response. The initial recognition event and microenvironment around that primary cell can result in many outcomes. If the antigen is presented by a nonprofessional APC, only a subset of the epitopes may be released during processing. If a costimulatory signal such as CD28 or TNF is missing, the T cells may be anergized. Depending on the microenvironment, the T cell may differentiate into a regulatory T cell, a T helper cell secreting Th1 cytokine (driving more of a cellular immune response with proliferation of $CD8^+$ CTL effectors) or a T helper cell secreting Th2 cytokine (driving more of

a humoral immune response with proliferation and maturation of B cells and antibody production). In addition, the immune response will evolve such that T cells responding to certain epitopes of an antigen or certain antigens in the milieu will grow at the expense of T cells in the population which are reactive with other epitopes or antigens in the milieu. Because of exponential cell growth, as the primary immune response subsides, the ratio of these T cells is further accentuated and stored in the form of memory such that upon secondary stimulation, the immune response within that individual is focused on a small subset of the possible epitopes. While there are multiple mechanisms at work, as an operative model, the T cells that grow out to dominate the population are those responding to the epitopes or antigens which come to dominate the immune response. In the primary immune response, they are growing out at the expense of the T cells responding to subdominant epitopes and, due to memory, dominate subsequent immune responses.

[0005] Over a period of days after a person's immune system first sees an antigen, a dominant population of T cells responding to a limited number of dominant epitopes is generated and these T cells determine the nature of the response to that antigen thereafter. While there are multiple types of cells involved, the working model associated with this invention is that if the T cells responding to the dominant epitope on the dominant antigens grow out as responsive T cells (e.g. $CD4^+$:TH1, TH2, Treg, T follicular helper, TH17, TH22, TH9; $CD8^+$ CTL's), a cellular or humoral immune response results. However, if the T cells in the dominant population are suppressive T cells (e.g., Treg, TH17, anergized T cells), tolerance is induced. T cells responding to subdominant antigens are overwhelmed by the clonal population of T cells responding to the dominant antigens.

[0006] In the case of cancer, chronic or latent infections, the local antigen processing/presenting and costimulatory environment impacts the primary immune response to the dominant antigens such that the T cell response to the dominant antigens in the tumor or infectious agent is balanced towards tolerance or ineffective response rather than a potent effector response. Due to differences in antigen processing and costimulation (CD28 and cytokines), this could be accentuated in organs where dendritic cells (DCs) are not the prevalent antigen presenting cells (APCs) unlike the body surfaces where DC are the

predominant antigen presenting cell. It is also well known that tumors and infectious agents create an immunosuppressive environment which is not optimal for a strong primary immune response. Alternatively, if a dominant antigen results in cells which are reacting to self, tolerance is broken and autoimmunity ensues. Such tolerance could be broken by the presence of a chronic or latent virus leading to a response (even chronically to weak subdominant antigens). There are multiple associations of viruses with autoimmunity. The inflammation at the site leads to release of other antigens while the viral antigens provide help to the T cells responsive to the organ, causing autoimmunity. After the dominance hierarchy is established in the primary response and reinforced by memory, the immune system in the patient will effectively replicate the same response each time the antigen is present.

[0007] An ongoing immune response against a dominant epitope can diminish the response to a subdominant epitope (Wolpert EZ 1998, Kedl RM 2003). The dominance/ subdominance hierarchy can be somewhat fluid. For instance, deleting or silencing T cell responses against a dominant epitope can lead to the appearance of a previously undetectable response against subdominant epitopes (Van derMost RG et al. 1997, Andreansky SS et al. 2005). Similarly, removal of a dominant sequence in an epitope does not eliminate the response to the antigen but rather results in the host responding more strongly to a previously subdominant epitope (Allan JE and Doherty PC 1985, Mylin LM et al. 2000).

SUMMARY OF THE INVENTION

[0008] The present invention relates to a novel therapeutic for cancer, chronic infections, autoimmune diseases, and transplantation based upon the modification of the immunodominance hierarch by therapeutic manipulation of cellular homeostasis.

[0009] Disclosed is a novel approach to rebalance the immune response to antigens to provide significant therapeutic benefit in, among others, cancer, chronic and latent infection, autoimmunity and transplantation. By generating immune responses to subdominant epitopes and subdominant antigens in a controlled microenvironment, the invention fundamentally changes the nature of the immune response to the disease to one that provides therapeutic

benefit. It can change the balance of the immune response before or after a prior immune response has occurred to the antigen or even if there is one ongoing.

[0010] The present invention features a method comprising identifying a dominant antigen or epitope and a subdominant antigen or epitope in a patient sample, cultivating a T cell capable of recognizing the subdominant antigen or epitope, and treating a patient with an effective number of the T cells to alter the immunodominance hierarchy of the patient.

[0011] The present invention also features methods for altering the immunodominance hierarchy of a patient comprising identifying at least one subdominant antigen or epitope in a patient sample, cultivating a T cell capable of recognizing the subdominant antigen or epitope, and treating the patient with an effective number of those T cells to provide therapeutic benefit.

[0012] In some aspects, the invention further comprises cultivating a T cell in the absence of a dominant antigen or epitope. In other aspects, the invention further comprises cultivating a T cell in the absence or presence of agents that enrich suppressive T cells or responsive T cells. Such agents can include, but are not limited to, growth factors, hormones, or other immune cells.

[0013] In some aspects, the invention further comprises administering the effective number of T cells via intradermal administration.

[0014] In other aspects, the invention further comprises pre-treating the patient with a conditioning agent to reduce the number of endogenous T cells prior to treating the patient with the cultivated T cells. The conditioning agent can be, but is not limited to, a chemotherapeutic agent.

[0015] In some aspects, the T cell is provided *ex vivo* from a patient.

[0016] The subdominant antigen or subdominant epitope is, for example, an antigen or epitope to which a cellular or humoral immune response is not detectable or is only detectable at a low level. Alternatively, the subdominant antigen or subdominant epitope is an antigen or epitope that evokes a weaker tolerance or immune response than that of a dominant antigen or

dominant epitope. The subdominant antigen is, for example, a viral antigen, a fungal antigen, a bacterial antigen, a parasitic antigen, a prion antigen, a tumor antigen, or an antigen associated with autoimmunity, allergy, inflammation, organ transplant rejection, or graft versus host disease. The viral antigen is, for example, a chronic or latent viral antigen. The viral antigen is be from EBV, HPV, HSV, VZV, Hepatitis B, Hepatitis C, HIV, HTLV, CMV, RSV, or influenza. The tumor antigen is, for example, a tumor-associated antigen, a tumor specific antigen, or an antigen associated with cancer stem cells or metastasis.

[0017] The present invention also features methods for identifying a dominant antigen or epitope and/or a subdominant antigen or epitope in a patient sample, cultivating a T cell capable of recognizing the subdominant antigen or epitope, wherein the T cell is a suppressive T cell, and treating a patient with an effective number of said T cell to alter the immunodominance hierarchy of the patient, thereby inducing tolerance in the patient for treatment or prevention of an autoimmune disease, allergy, inflammation, organ transplantation rejection, or graft versus host disease.

[0018] The present invention also features methods for identifying a dominant antigen or epitope and/or a subdominant antigen or epitope in a patient sample, cultivating a T cell capable of recognizing the subdominant antigen or epitope, wherein the T cell is a responsive T cell, and treating a patient with an effective number of said T cell to alter the immunodominance hierarchy of the patient, thereby inducing a cytotoxic immune response in the patient for treatment or prevention of an infection or cancer. The infection is, for example, a bacterial, viral, parasitic, or prion infection.

[0019] In any of the methods of the present invention, treatment or prevention of a disease, infection, cancer, or medical condition includes alleviating or ameliorating at least one symptom of a disease, infection, cancer, or medical condition. Therapeutic benefit includes any alleviation, amerlioration, improvement, prevention, or treatment of at least one symptom of a disease, infection, cancer, or medical condition.

[0020] In some aspects, the patient sample is a blood sample.

[0021] The present invention also features a method further comprising re-profiling of the patient by assaying for a tolerance or humoral or cellular immune response in response to the subdominant antigen or epitope to determine if the therapy successfully rebalanced the immune response.

[0022] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are expressly incorporated by reference in their entirety. In cases of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples described herein are illustrative only and are not intended to be limiting.

[0023] Other features and advantages of the invention will be apparent from and encompassed by the following detailed description and claims.

BRIEF DESCRIPTION OF THE FIGURES

[0024] Figures 1A, 1B and 1C show the results of a ^{51}Cr release assay.

[0025] Figure 2 shows the results of a ^{51}Cr release assay.

[0026] Figures 3A and 3B show the results of a ^{51}Cr release assay. Figure 3C shows the percentage of viable APCs.

[0027] Figure 4 shows IFNγ producing cells measured by Elispot.

[0028] Figures 5, 6A, 6B, 7 and 8 show the results of a ^{51}Cr release assay.

[0029] Figure 9 shows a mouse model of chronic hepatitis B.

[0030] Figure 10 shows treatment of the mouse model.

[0031] Figure 11 shows T cell responses to HBs and HBc.

[0032] Figure 12 and 13 show responses by method of administration.

[0033] Figure 14 shows a hierarchy of antigens.

[0034] Figure 15 shows responses by ICS.

[0035] Figure 16 shows a hierarchy of antigens.

[0036] Figure 17 shows immune response following an acute flair, then clearance of hepatitis.

[0037] Figure 18 shows a hierarchy of antigens following the clearance.

[0038] Figure 19 shows that the T cells completely resolved the patient's Hepatocellular carcinoma (before treatment – left; 8 weeks post-therapy – right).

[0039] Figure 20 shows antigens present in a patient's tumor.

[0040] Figure 21 shows cells responding to the NY-ESO-1 antigen.

[0041] Figure 22 shows antigens present after therapy in accordance with the present invention.

[0042] Figure 23 shows the rebalancing of immunodominance hierarchy.

[0043] Figure 24 is a CT scan from before and after T cell therapy in accordance with the present invention.

[0044] Figure 25 shows post treatment survival, progression free.

[0045] Figure 26 shows a comparison of therapy by the present invention and Rituxan + CHOP.

[0046] Figure 27 shows a characterization of the T cells administered to the animals.

[0047] Figure 28 shows clinical disease scores for trial mice.

[0048] Figure 29 shows the incidence of arthritis in trial mice.

[0049] Figures 30A, 30B and 30C show the histopathology of a normal rat, a rat immunized with human proteoglycan and a rat treated with T cells.

[0050] Figure 31 is a schematic of a bioreactor for use with the therapy of the present invention.

DETAILED DESCRIPTION

A. Definitions

[0051] The term "antibody" is used in the broadest sense and specifically covers human, non-human (e.g., murine) and humanized monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multi-specific antibodies (e.g., bispecific antibodies), and antibody fragments so long as they exhibit the desired biological activity.

[0052] The term "antigen," refers to a compound, composition, or substance that can stimulate the production of antibodies or a T cell response in an animal, including compositions that are injected or absorbed into an animal. An antigen reacts with the products of specific humoral or cellular immunity, including those induced by heterologous immunogens. The term "antigen" includes all related antigenic epitopes.

[0053] "Antigen presenting cells" or "APCs" are cells of the immune system used for presenting antigen to T cells. APCs include dendritic cells, monocytes, macrophages, marginal zone Kupffer cells, microglia, Langerhans cells, T cells, and B cells (see, e.g., Rodriguez-Pinto and Moreno (2005) Eur. J. Immunol. 35:1097-1105).

[0054] "Autoimmunity," "autoimmune disease," "autoimmune condition" or "autoimmune disorder" refers to a set of sustained organ-specific or systemic clinical symptoms and signs associated with altered immune homeostasis that is manifested by qualitative and/or quantitative defects of expressed autoimmune repertoires. Autoimmune disease pathology is manifested as a result of either structural or functional damage induced by the autoimmune response. Autoimmune diseases are characterized by humoral (e.g., antibody-mediated), cellular (e.g., cytotoxic T lymphocyte-mediated), or a combination of both types of immune responses to epitopes on self-antigens. The immune system of the affected individual activates inflammatory cascades aimed at cells and tissues presenting those specific self-antigens. The destruction of the antigen, tissue, cell type or organ attacked gives rise to the symptoms of the disease.

[0055] The term "cancer" refers to a disease or disorder that is characterized by unregulated cell growth. Examples of cancer include, but are not limited to, carcinoma, lymphoma, blastoma and sarcoma. Examples of specific cancers include, but are not limited to, lung cancer, colon cancer, breast cancer, testicular cancer, stomach cancer, pancreatic cancer, ovarian cancer, liver cancer, bladder cancer, colorectal cancer, and prostate cancer. Additional cancers known to those of skill in the art are also contemplated.

[0056] "Dominant antigen" or "dominant epitope" refers to an antigen or epitope that evokes a strong tolerance or immune response, which may be characterized by the presence of T cells specific for that antigen or epitope in an amount greater than about 70% of the total number of responding T cells.

[0057] The term "epitope" refers to a set of amino acid residues that is involved in recognition by a particular immunoglobulin, or in the context of T cells, those residues necessary for recognition by T cell receptor proteins and/or Major Histocompatibility Complex (MHC) receptors. In an immune system setting, in vitro or in vivo, an epitope is the collective features of a molecule, such as primary, secondary and tertiary peptide structure, and charge, that together form a site recognized by an immunoglobulin, T cell receptor or HLA molecule.

[0058] "Hepatitis" refers to a medical condition defined by the inflammation of the liver.

[0059] "Human Leukocyte Antigen" or "HLA" is a human class I or class II Major Histocompatibility Complex (MHC) protein (see, e.g., Stites, et al., IMMUNOLOGY, 8TH ED., Lange Publishing, Los Altos, Calif. (1994).

[0060] An "immune response" refers to a response of a cell of the immune system, such as a B cell, T cell, or monocyte, to a stimulus. In one embodiment, the response is specific for a particular antigen (an "antigen-specific response"). In one embodiment, an immune response is a T cell response, such as a CD4+ response or a CD8+ response. In another embodiment, the response is a B cell response, and results in the production of specific antibodies.

[0061] Immunodominance is the observation that in spite of a large number of possible epitopes (antigen fragments) in an antigen, the immune system focuses its response on a limited number of epitopes and can be ordered as a reproducible hierarchy (Sercarz et al. 1993). Immunodominance holds true for immune responses to artificial antigens, human viruses including influenza and vaccinia, and intracellular bacteria (Chen WS 1994, Belze GT et al. 2000, Chen W 2000, Tscharke DC 2005). The final outcome of immunodominance is determined by a number of steps, including MHC binding affinity, efficiency of cellular processing to generate appropriate MHC binding peptides, availability of TCRs to recognize complexes between the MHC bin0ding peptides, and MHC followed by cellular immunoregulatory mechanisms (Yewdell JW 2006, Sette A et al. 2009).

[0062] "Lymphocytes" refers to a type of white book cell that is involved in the immune defenses of the body. There are two main types of lymphocytes: B cells and T cells.

[0063] "Major Histocompatibility Complex" or "MHC" is a generic designation meant to encompass the histocompatibility antigen systems described in different species, including the human leukocyte antigens ("HLA").

[0064] "Subdominant antigen" or "subdominant epitope" refers to an antigen or epitope that evokes a weaker tolerance or immune response than that of a dominant antigen or dominant epitope.

[0065] The term "treatment" refers to a clinical intervention made in response to a disease, disorder or physiological condition manifested by a patient or to be prevented in a patient. The aim of treatment includes the alleviation and/or prevention of symptoms, as well as slowing, stopping or reversing the progression of a disease, disorder, or condition. 'Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already affected by a disease or disorder or undesired physiological condition as well as those in which the disease or disorder or undesired physiological condition is to be prevented.

[0066] "Tumor" refers to all neoplastic cell growth and proliferation, whether malignant or benign, and all pre-cancerous and cancerous cells and tissues.

[0067] "__-mer" refers to a linear sequence of __ amino acids that occur in a target antigen.

B. Assays that recognize/distinguish the subdominant epitope from the dominant one

[0068] The patient's tumor or infection is first assayed for the presence of a panel of tumor associated, viral or other antigens. This is generally done by immunohistochemistry on the tumor biopsy or FACS in the case of hematologic malignancies. The patient's tumor or infection is assayed for the presence of a panel of tumor associated, viral or other antigens. This is generally done by immunohistochemistry on the tumor biopsy or FACS in the case of hematologic malignancies. The patient's blood is drawn and tested for both humoral and cellular immune response to the antigens which are present. The antigens to which the immune response is not detectable or detectable at a low level are proactively selected to grow T cells *in vitro*. After these T cells are grown, they can be tested for response to the antigens re-infused, and the patient's blood can be assayed for the response to the antigens. In this way, the patient's immune response can be effectively rebalanced to provide therapeutic benefit. In a preferred embodiment, the assays of humoral immunity can include but is not limited to an ELISA assay. In a preferred embodiment, the assay of cellular immunity can include but is not limited to Intracellular cytokine staining for cytokines (ICS), including but not limited to Interferon γ (IFNγ)and Tumor necrosis Factor α (TNFα). The subsets of T cells responding (e.g., CD8, CD4, Treg) can also be assayed in this assay. Alternatively, the assay of cellular immunity can include but is not limited to ELISPOT assay for IFNγ or TNFα. In an alternative embodiment, the Elispot or ICS can assay for IL-4, IL-12 (TH2 and TH1), IL-10 (Treg) or IL-21 (T follicular helper cell subset). In still another embodiment, the cellular immune profiling assay can be Intracellular Staining (ICS) for these or other cytokines. The antigens tested can be full antigens, antigens with epitopes deleted or dominant or subdominant epitopes. In the case of epitopes, bioinformatics software can be used to predict epitopes which would bind to the patients MHC and these epitopes are then assayed. In one embodiment, this software is the

Net MHCpan or the consensus epitope immunoinformatics software described in the assay section. In the case of epitopes, tetramer binding can be used as an alternative assay to quantify the CTL providing one knows the HLA type. Tetramers including the peptides are combined with the cells and cells stained and FACS used to determine the % of cells recognizing each tetramer. This is useful if the patient is of a known HLA type such as HLA A2. However, the preferred method is the ELISA for humoral response and ICS or Elispot for the cellular response. In a preferred embodiment, the CTL's are generated from peripheral blood. Alternatively, the CTL's are generated from the tumor infiltrating lymphocytes (TIL) or from the DTH surrounding the injection site.

[0069] DTH infiltrating lymphocytes can be prepared by taking a 4 mm punch biopsy from the skin minced in RPMI medium 1640 with 10% FCS (CSL). Single cell suspensions were stimulated with 1 μg/ml phytohemagglutinin (Sigma) and cocultured with irradiated autologous PBMC'S with 10 IU/ml IL-2 (Cetus) and 10 ng/ ml IL-7 (Peprotech, Rocky Hill, NJ). Medium was replenished each 2-3 days.

[0070] For humoral immune response profiling, serum from the patient is serially diluted 1:4 from 1/100 to 1/100000 and used in a standard ELISA with purified recombinant tumor antigens (generally made in E. coli). From 2 to 10000+ antigens can be assayed. One microgram of each purified protein is absorbed to microwell plates (Nunc) overnight at 4 degrees C. Plates are washed with PBS and blocked with 2% FCS/PBS. Patient serum is diluted in 2% FCS/PBS and added for 2 hours. Plates are washed and goat anti-human IgG-AP (Southern Biotechnology Assoc) is added. Plates are washed, incubated with Attophose substrate (JBL Bioscientific) for 25 min, and immediately read (CytoFluor 2350, Millipore). Readout is UV Absorbance.

[0071] There are two methods for cellular response profiling. The first method involves the Enzyme-linked immunosorbent spot ("ELISPOT") assay for IFNγ. Ninety-six well polyvinylidene diflouride backed plates (Millipore, Bedford, MA) are coated with 5-15 μg/ml of anti-IFNγ monoclonal antibody 1-DIK (MABTECH, Stockholm, Sweden) at 4 degrees C overnight. The wells are washed and blocked with 5% human AB serum (Valeant Pharm).

5×10^6 PBMCs (or 5x 10^5 CTL's when the assay is performed post *in vitro* expansion) are added per well with peptide mixes 2 μM each from each of the antigens. Incubate overnight (18 hours) at 37 degrees C 5% CO_2. Cells are discarded and the wells are washed with PBS containing 0.05% Tween 20. 1 μg/ml biotinylated anti-IFNγ monoclonal antibody 7-B6-1 (MABTECH) is incubated for 2- 4 hours at room temperature followed by streptavidin conjugated alkaline phosphatase (MABTECH or Sigma Aldrich) for 2 more hours. This is followed by a 30 minute reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium from the alk-phos substrate kit (Bio-Rad Richmond, CA). The spots are to be counted using a dissection microscope (SZ CTV Olympus microscope). Spots can also be counted on an AIDELISPOT reader (Autoimmun Diagnostika, Strassberg, Germany). Each spot is a cell reported as spot forming cells (SFC)/10^5 PBMC's. 10 μg/ml PHA can be used as a positive control; cells alone without peptide can serve as the negative control.

[0072] The second method involves intracellular cytokine staining for IFNγ and TNFα. 5x 10^6 PBMC's (or 5x 10^5 CTL's when the assay is performed post *in vitro* expansion) are plated in 100 μl PBS 1% FCS 96 well plates together with the peptides(10^{-5} to 10^{-9} M final concentration) for each of the epitopes or antigens being studied. After 6 hours of incubation in the presence of IL-2 (150 U/Ml), 50 μM β mercaptoethanol and brefeldin A (1 μg/ ml) or Golgi Plug (BD Biosciences, San Diego, CA) (both of the latter components to increase accumulation of IFNγ or TNFα in responding cells), cells are pelleted, washed in 200 ml PBS 1% FCS and then labeled with stain for surface antigens (CD4 fluorescein isothiocyanate and CD8 allophycocyanin 0.25 μg/ml (Pharmingen, Becton Dickinson) for 30 minutes at 4 degrees C (for 30min on ice). After a wash, cells are permeabilized with Cytofix/Cytoperm for 20 min on ice and then stained with a phycoerythrin conjugated anti-IFNγ (0.4 μg/ml) or anti-TNFα (0.8 μg/ml) antibody (Pharmingen, Becton Dickinson). The cells are then washed, fixed and resuspended in PBS 1% FCS and tested on a FACScan flow cytometer and analyzed using Cell Quest software. Alternatively, FACS Canto (Becton Dickinson) can be used. Other cytokines, including but not limited to IL-12 and IL-4, can be assayed to measure TH1 or TH2 subsets. Cytokine panel to give a broader assay of T cells could measure IL-12, IFN g, IL-4, IL-10 and IL-17. T follicular helper cells can be measured as $CD4^+$, $CXCR5^+$, $ICOS^+$ cells. B cells can be

measured as $CD19^+$ and $B220^+$ cells. IL-21 in the T cells should be associated with B cell activation and affinity maturation of antibodies so this could be used to study this as well. As an alternative for profiling IFNγ, IL-4 (BD Biosciences) IL-12, IL-10, IL-17 (R&D Systems), and IL-21 antibody (R&D systems), ELISPOT can also be used. ICS actually profiles the % of CD8 or CD4 T cells responding to different antigens or epitopes. Other cell subsets can be analyzed as well including Treg. A cytochrome labeled CD25 monoclonal antibody can be used as a surface marker of most Tregs. Alternatively, cytochrome labeled Human FoxP3 monoclonal antibody clone 259D/C7 from BD Biosciences is used to stain the cells post permeabilization to measure % Treg cells and their status. IL-10 can also be assayed.

[0073] NetMHCpan is a bioinformatics method for quantitative predictions of peptide binding to HLA-A and -B (Nielsen M 2007). A consensus epitope prediction approach has also been developed (Mouaftsi M 2006). These methods can be used to sort all of the potential MHC I epitopes for an antigen and rank the top 1% of peptides and thus predict epitopes. These predicted epitopes would then be synthesized as 9-10 mer peptides and tested (e.g., to patient PBMCs or in a transgenic mouse for the HLA type of interest).

[0074] Tetramers with specific MHC (e.g., HLA A A2) are synthesized together with 8 mer peptide epitopes (in the case of class I MHC and 15 mer peptide epitopes in the case of MHC Class II. The cultured T cells are stained with the tetramer diluted 1/200 at room temperature for 20min; anti CD8 antibody was then added and stained for a further 30minutes. Cells were then washed and 100,000 acquired on FACS Calibur (BD Biosciences) and analyzed with Flowjo software (Tree Star).

[0075] Following therapy, the patient is re-profiled by assaying for a tolerance or humoral or cellular immune response in response to the subdominant antigen or epitope to determine if the therapy successfully rebalanced the immune response.

C. Rebalancing the immune response

[0076] In one embodiment of the present invention, the immune response can be rebalanced by growing T cells from a patient *ex vivo* to a subdominant antigen or subdominant

epitopes on an antigen followed by infusion or administration of these T cells into the patient. T cells to subdominant antigens or epitopes are grown in tissue culture, *ex vivo* (away from the patient's immunoregulatory milieu). After growing enough cells to overwhelm the previously dominant cells, the cells are re-infused into the patient to skew the cellular balance and therapeutically switch the dominance hierarchy. In a further preferred embodiment, this number of T cells introduced de novo as therapy is greater than 5% of the T cells responding to the antigen, infectious agent, tumor or organ. The ratio can be further skewed to favor the infused cells by pretreatment of the patient with conditioning agents which reduce the number of endogenous T cells (i.e., chemotherapy).

[0077] The present invention involves methods to optimize the growth of T cells to subdominant antigens in tissue culture. In one embodiment, the cells are grown in the absence of dominant antigens. This is accomplished by selection of a professional antigen presenting cell which has not been exposed to dominant antigen and the modification of the antigens to eliminate dominant epitopes or other components which limit the ability of an antigen to be processed.

[0078] As the therapeutic method requires T cells to be enriched for and ideally fully responsive to subdominant antigens/ epitopes, efficient methods for the growth of such T cells are important. The growth of other T cells to dominant epitopes and antigens increases the time in culture required to generate enough specific cells to skew this ratio. Moreover, the cells growing out to dominant epitopes is working against the achievement of the proper ratio upon reinfusion. Therefore, T cell culture methods which specifically limit the introduction of dominant antigens or epitopes have been developed. For example, while the method is broadly applicable to all tumors, it has distinct advantages for EBV malignancies because it does not use EBV transformed B cells (which express the EBV dominant antigens/epitopes). It is also more reliable when administered to cells in which a significant % of the CTL's are responding to subdominant antigens/epitopes.

[0079] Monocyte-derived dendritic cells are generated *in vitro* from peripheral blood mononuclear cells (PBMCs) from a patient. In a preferred embodiment, plating of PBMCs for 2

hours in a tissue culture flask permits adherence of monocytes. In an alternative embodiment, $CD14^+$ magnetic beads can be used to isolate dendritic cells from PBMC's (Miltenyi Biotec, Auburn, CA). At this point the nonadherent cells are removed and frozen at -80 to later serve as a source of T cells. Treatment of the adherent monocytes with interleukin 4 (IL-4) and granulocyte-macrophage colony stimulating factor (GM-CSF) leads to differentiation to immature dendritic cells (iDCs) in about a week. Subsequent treatment with tumor necrosis factor (TNF) or macrophage conditioned media for 2 days further differentiates the iDCs into mature dendritic cells. These cells are then pulsed with a peptide or a plasmid containing a subdominant antigen for 2 hours and then the PBMC's are thawed and added to the pulsed dendritic cells. After a few hours, the cells are pooled and resuspended in media containing IL-2 or IL-15 (with IL-15 being preferred) to generate *in vitro* expansion of the T cells which have recognized the antigen. For certain protocols, IL-7 and IL-15 are added to increase T cell survival. For other protocols, culture conditions are adjusted to optimize growth of certain subsets of T cells. For example, IL-12 can be added to polarize to TH1 cells. Alternatively, IL-4 can be added to polarize to TH2. In certain protocols, IL-6 can be added to prevent the growth of Treg. In still another variation which is useful in autoimmune or organ transplantation applications, low level $IL\text{-}2^+$ rapamycin can be added to accentuate the growth of Treg. More detailed protocols are outlined in the various examples and *in vivo* comparison of the cells produced with protocols to polarize the cells to certain T cell subsets is described in Example 3, Figure 3. If the T cells are grown in a tissue culture flask, media must be replaced at day 14 and day 21. However, in a preferred embodiment, a bioreactor can be used to mitigate this need, e.g., a gas permeable bioreactor such as Grex (Wilson Wolf) or Hyperstack (Corning). Generally, enough cells to be administered to a patient can be generated within 2 to 6 weeks as opposed to 12-24 weeks with traditional methods.

[0080] In another embodiment of the invention, the inventors have developed T cells for adoptive transfer against antigens which have been deleted for their dominant epitopes and demonstrate that more T cells are generated to subdominant epitopes. Such proteins or corresponding DNA vaccines can be used to generate T cells with a broad immune response against subdominant epitopes. This approach should be broadly applicable across a wide range

of diseases to achieve a balance of the immune response towards subdominant epitopes including but not limited to EBV, cancer, HIV or hepatitis. In another embodiment, the antigen, or a plasmid/ recombinant vaccine encoding it, is used to vaccinate the patient to induce a de novo broad immune response to the subdominant antigen. In another embodiment, the subdominant reactive T cells can be administered followed by a vaccination with the subdominant antigen to boost the response. In still another embodiment, the approach could be used therapeutically or prophylactically wherein a patient's immune profile can be determined to measure risk of coming down with diseases and the patient can then be primed to appropriate subdominant antigens using any of the approaches disclosed herein.

[0081] In an alternative embodiment, the grown T cells induce tolerance to prevent or treat an autoimmune disease, allergy, inflammation, organ transplantation rejection, or graft versus host disease. Depending upon the type of T cells desired, culture conditions can be modified to preferentially grow, or enrich for, the relevant subset including but not limited to CD8, CD4, TH1, TH2, or Treg. For example, T cells can be grown in presence or absence of certain growth factors, cytokines, drugs, small molecules, or other immune cells. In a preferred embodiment, subdominant antigen reactive T cells are generated from Peripheral Blood Mononuclear Cells (PBMC's) in tissue culture in the presence of a stimulated professional Antigen Presenting Cell (e.g., monocyte derived dendritic cell, macrophage or EBV immortalized B cell).

[0082] In another embodiment, various techniques are used to modify antigen processing to favor subdominant epitopes. In one embodiment of the invention, this is accomplished by modification of the antigens to eliminate dominant epitopes, regions which inhibit antigen processing, or to limit the number of dominant or subdominant epitopes presented at one time to an antigen presenting cell. These modifications increase the response and diversity of subdominant epitopes recognized (Example 1, Figure 5). In an alternative embodiment, the modified LMP1, LMP 2 and EBNA-1 sequences can be delivered to the APC using a viral vector such as adenovirus or vaccinia virus. In the case of other antigens (LMP1 and EBNA-1), eliminating regions of the protein which lead to poor antigen processing greatly enhances the immune response to subdominant epitopes on those antigens (Example 1, Figure

6). The modified LMP1, LMP2 and EBNA-1 sequences can be delivered to the APC using peptides, proteins, plasmids or viral vectors such as adenovirus or vaccinia.

[0083] In an alternative embodiment, a proteosome antagonist may be added to the APC's and antigen during CTL production to increase the number of subdominant epitopes recognized and enhance the response to the subdominant antigen (Example 2, Figure 7). There are many available proteosome antagonists having different mechanisms (e.g., bortezomib, clioquinol, lactacystin, epoxomycin, MG-132, MLN9708, carfilzomib (PR-171)).

[0084] In an alternative embodiment, an antigen may be administered in complexes with antibodies having various isotypes regarding responses to subdominant determinants (Example 3, Figure 13). The antigen is injected with an antibody binding a determinant flanking the intended T cell epitope to target professional antigen presenting cells and direct antigen processing to the flanking epitopes.

[0085] In another embodiment of the present invention, plasmids containing subdominant epitopes or antigens re used to generate T cells which are administered or are directly administered into the patient either directly by various routes of administration, in combination with IFNγ, IL-21 or other cytokines or on pulsed dendritic cells to induce a response to subdominant antigens. IFN-gamma or other cytokines may be induced before T cell stimulation to increase T cell responsiveness to subdominant epitopes and modify the immunodominance hierarchy.

[0086] In another embodiment, the route of administration is modified to alter the immunodominance hierarchy. The route of administration of a vaccinia response determines the degree of dominance of the dominant determinant. It has been found that when administered intraperitoneally, the dominant determinant accounted for only a quarter of the response as opposed to half, as was the case with intradermal administration (Tscharke DC et al. 2006, Tscharke DC et al 2005). As shown in Example 3, Figure 11, administration of antigen by the IM route develops a stronger response and broader response to the subdominant epitope than does IP or IV routes. Thus, modifying the route of administration is another *in vivo* mechanism which the inventors claim to modify the immunodominance hierarchy. In a

preferred embodiment, the T cells cultivated are delivered by intradermal administration. By targeting different APCs (e.g., macrophages, dendritic cells), the route of administration changes the dominance hierarchy (Example 3, Figure 12).

[0087] In a preferred embodiment, the antigens are viral antigens particularly latent viral antigens or chronic viral antigens with subdominant epitopes on them. For example, the viral antigens are from a virus selected from the group comprising: EBV, HSV, VZV, Hepatitis B and C, HIV, and HTLV. The viral antigens are, for example, EBV LMP1, LMP2, EBNA-1, HPV E6, or HPV E7. For example, the viral antigens are associated with EBV, HSV, VZV, Hepatitis B and C, HIV, and HTLV, CMV, RSV, or influenza. In another embodiment, the antigens are antigens on other chronic and latent infectious agents, for example, agents associated with, bacteria, fungi, parasites, or prions. In still another embodiment, the antigens are tumor antigens including but not limited to: tumor associated antigens, tumor specific antigens, antigens associated with cancer stem cells or metastasis. In other embodiments, the antigens are associated with autoimmunity, allergy, inflammation or organ transplantation rejection or graft vs. host disease.

[0088] In one embodiment, the immunodominance hierarchy of a patient is altered by identifying a dominant antigen or epitope and a subdominant antigen or epitope in a patient sample, cultivating a T cell capable of recognizing the subdominant antigen or epitope, and treating a patient with an effective number of the T cells.

[0089] In one embodiment, the immunodominance hierarchy of a patient is altered by identifying at least one subdominant antigen or epitope in a patient sample, cultivating a T cell capable of recognizing the subdominant antigen or epitope, and treating the patient with an effective number of those T cells to provide therapeutic benefit.

[0090] In another embodiment, the T cell is a responsive T cell, and treating a patient with an effective number of said T cell to alter the immunodominance hierarchy of the patient, thereby inducing a cytotoxic immune response in the patient for treatment or prevention of an infection or cancer. The infection is, for example, a bacterial, viral, parasitic, or prion infection.

[0091] In any of the methods of the present invention, treatment or prevention of a disease, infection, cancer, or medical condition includes alleviating or ameliorating at least one symptom of the disease, infection, cancer, or medical condition.

D. Therapeutic methods

1. Cancer

[0092] Work Flow of Clinical Use of Immune Profiling of Dominant and Subdominant antigens

Step 1: Tumor Biopsy (Immunohistochemistry) or Blood (IHC,FACS or Elisa) Antigen 1, 2, 3, 4, 5, 6, 7, 8, 9, 10

Result: Antigens 1, 2 &6 on tumor, others in panel not

Step 2: Immune Response Profiling

Humoral Profile	Cellular Profile
ELISA on Serum and	Elispot or ICS (for IFNg at least, also IL-10, IL-4 IL-12, IL-21 to assay T cell subsets) on PBMCs stimulated with each antigen

Result: Antigen 1 strong response (dominant); Antigen 2&6 No/ Modest Response (subdominant)

Step 3: Grow T cells (CD8 and CD4) to Subdominant antigens *in vitro*

Result: T cells responsive to antigens 2&6

Step 4 (optional once therapy is well established): Confirm >5% T cells grown respond to subdominant antigens using Cellular Immune Profiling (Elispot or ICS)

Result: 25% of the T cells respond to Antigens 2 & 6

Providing at least 5% of T cells respond to subdominant antigens, proceed to step 5

Step 5: IV Infuse cells into patient with or without prior conditioning (e.g., cyclophosphamide)

Step 6 (may become optional once therapy is well established): Isolate PBMC's from Blood 2-3 weeks post infusion and Profile Immune Response

Result: Cellular Profile Antigen 1 No/Moderate response; Antigens 2 & Strong response (dominant) to at least 1 of the antigens

Step 7: Assess Clinical responses

RECIST (CR, PR); Survival or Progression Free Survival

Result: Improved response rate and survival

[0093] In Example 4, this systematic method is used to treat melanoma. In Example 5, the method is applied to treat lymphoma with the multiplasmid LMP2. In fact the T cell therapy changes the natural course of lymphoma from one that is relapsing remitting to one that is a durable remission. This antigen as well as deleted LMP1 and EBNA-1 can also be used to treat other tumors that include EBV antigens including Nasopharyngeal carcinoma, Burkitts Lymphoma, CLL, Hodgkins, and some gastric cancer among others. In Example 3, a similar method is used to treat Hepatocellular carcinoma. These examples are incorporated into the invention by reference and demonstrate that rebalancing of the immune response towards subdominant antigens is broadly applicable to all forms of cancer.

2. General use of a large number of subdominant antigens on a tumor to produce a pan tumor type therapy

[0094] In another embodiment, the inventors propose that if enough subdominant antigens can be identified for a particular type of tumor, that that type of tumor could be treated with T cells to multiple subdominant antigens without the need to test the patient. Similarly,

targeting multiple antigens on the same tumor would decrease resistance, e.g., combination chemotherapy. For example, the ability to develop a single T cell line targeting the subdominant antigens EBV LMP2 which is on 40% of lymphomas and surviving (which is on 50% of lymphomas) would allow one to target approximately 80% of lymphomas with a T cell line specific to these 2 antigens. Clinical testing of the Pan-lymphoma product is demonstrated in Example 5. By year 3, progression free survival of patients treated with the Pan lymphoma product is comparable to the product where antigens were tested before therapy. This could be because, like combination chemotherapy, the response of CTL's to multiple antigens on the same tumor could decrease the likelihood of escape. The ability to treat all lymphoma (not having to only treat that subset which is positive for a single antigen) with a single T cell product is a novel product concept. In other embodiments of the invention, the inventors also claim similar Pan-cancer products for virtually any cancer.

3. Chronic Infections

Hepatitis and Hepatocellular carcinoma

[0095] In patients with chronic Hepatitis, HBs antibodies are not generated but HBc antibodies are (Ganem D et al 2004). In patients with acute hepatitis, antibodies to both antigens are generated. >90% of neonates and 30% of children ages 1-5 develop the chronic form while adults acutely clear the virus >90% of the time. 95% of Hepatocellular carcinoma is associated with chronic infection with Hepatitis B virus and. HBsAg often is on the cell surface of HCC. Given these observations, the inventors chose to study whether HBs was subdominant in hepatitis and hepatocellular carcinoma patients and if the growth of T cells could generate CTLs to HBs antigen which could rebalance the immune response to subdominant antigens and be therapeutic.

[0096] Based upon this finding, we administered the CTL's grown to HBs Ag to treat chronic hepatitis in an animal model of hepatitis and ultimately in HBV associated HCC patients. In the animal model, HBVtgRAG cells were administered and the tested. In the animal which received control cells, chronic Hepatitis developed. Furthermore, when the animal

developed hepatitis and received CTL's to subdominant antigens, the animal developed acute hepatitis but cleared the hepatitis virus (Example 3, Figures 9-11).

[0097] Given the encouraging animal data, patients with hepatitis were treated with the T cell rebalancing therapy.

[0098] Work Flow of Clinical Use of Immune Profiling of Dominant and Subdominant antigens

Step 1: Immune Response Profiling Hepatitis Surface Antigen and Hepatitis Core Antigen

Humoral Profile	Cellular Profile
ELISA on Serum and	Elispot or ICS (for IFNγ at least, also IL-10, IL-4 IL-12, IL-21 to assay T cell subsets) on PBMCs stimulated with antigen

Result: Hbc strong response (dominant); Hbs No/ Modest Response (subdominant)

Step 2: Grow T cells (CD8 and CD4) to Subdominant antigen *in vitro*

Result: T cells responsive to HBs antigen

Step 3 (optional once therapy is well established): Confirm >5% T cells grown respond to subdominant antigen using Cellular Immune Profiling (Elispot or ICS)

Result: 25% of the T cells respond to HBs Antigen

Providing at least 5% of T cells respond to subdominant antigens, proceed to step 4

Step 4: IV Infuse cells into patient with or without prior conditioning (e.g., cyclophosphamide)

Step 5 (may become optional once therapy is well established): Isolate PBMC's from Blood 2-3 weeks post infusion and Profile Immune Response

Result: Cellular Profile HBc No/Moderate response; HBs Strong response (dominant)

Step 6: Assess Clinical responses

Result: Clearance of Infection

[0099] Immune profiling was conducted on 5 patients with HBV and HCC. As expected, ELISA demonstrated high titers of antibody to hepatitis B core antigen (HBc Ag) but not to hepatitis B surface antigen (HBs Ag) (Example 3, Figure 17). However, when PBMC's were induced into dendritic cells, pulsed with hepatitis B core antigen (HBc Ag) and hepatitis B surface antigen (HBs Ag) and used to grow CTL's from PBMC's from the same patient, a surprising result was observed. By frequency of IFN producing T cells on Elispot a hierarchy was observed: no-antigen (10 sfc)<HBsAg(15 sfc)<HBcAg(45 sfc) (Example 3, Figure 14). This indicates that Hepatitis B surface antigen was indeed subdominant relative to Hepatitis core. Furthermore, it appeared that one epitope (FLL) of HBs was dominant by Elispot (Example 3, Figure 14) and by ICS (Example 3, Figure 15). The peptide containing this epitope was excluded from the pepmix that was used to grow cells from that patient.

[00100] In patients with HCC, 3/5 patients who received the T cells to subdominant HBs (Example 3, Figure 16) cleared the HBs antigen and demonstrated CR's to the HCC following a transient elevation of Alanine transaminase (ALT) in Liver Function Tests (Example 3, Figure 17 & 19). When PBMC's from those patients were tested 2 weeks post administration, the dominant response was to HBs rather than to HBc and to the previously subdominant epitopes of HBs (Example 3, Figure 18) indicating that the patients had rebalanced their immunodominance hierarchy. As a result of the therapy and the switch in immunodominance hierarchy was providing the therapeutic benefit (Example 3, Figures 17 & 19).

[00101] Thus, T cell adoptive immunotherapy to subdominant antigens can be used to eradicate chronic viruses and to treat cancers on which they are present. Further, immune profiling to direct the production of T cells to subdominant antigens and epitope opens a novel therapeutic avenue in multiple diseases.

[00102] Virtually any viral, bacterial, fungal, prion, parasitic or other infectious disease can be treated including but are not limited to Hepatitis C virus and HIV virus. Other chronic infectious disease can also be treated using rebalancing. For example, Mycobacterial tuberculosis is a chronic infection in many patients but only reactivates when the immune system is repressed. In its dormant state, it is contained by a granuloma usually in the lung. Recent studies of the proteome of the mycobacterial phagosome indicate that MTB represses antigen presentation in dendritic cells to a greater degree than macrophages (Li et al. 2011). To this end the inventors proposed that rebalancing the immune system to respond to subdominant epitopes is a broad therapeutic approach for virtually any cancer or infectious disease.

4. Autoimmunity

[00103] Epidemiological studies demonstrate that the risk of patients developing multiple sclerosis correlates with EBV antibody titers. By following hundreds of thousands of individuals before they were infected with EBV and following up with them for several years post initial infection, Ascherio et al. were able to study the 305 patients who developed MS. Their risk increased sharply following EBV infection (Ascherio A et al. 2010). Memory CTL's responsive to EBNA-1 400-641 were elevated in MS patients relative to other EBV antigens when compared to healthy individuals who were also EBV carriers (Lunemann JD et al. 2006). EBNA-1 specific Th1 cells appear capable of sustaining autoimmunity by cross recognition of auto-antigens or bystander mechanisms. Further, transgenic mouse studies suggest that B cells expressing LMP2a bypass normal tolerance checkpoints and enhances development of autoimmune disease (Swanson-Mungerson M 2007).As such, while we have studied T cells reactive to EBV subdominant epitopes as a treatment for EBV and EBV related cancers, T cells reactive to EBV subdominant epitopes may also be useful in reinstating balance in autoimmunity. By growing and introducing the T cells responsive to EBV latent antigens (EBNA-1, LMP1 and LMP2), the inventors propose that the immune response can be rebalanced, reinducing tolerance in the autoimmune diseases with an EBV association. The same would hold true for other viruses associated with other autoimmune disease. For example, Picornaviruses such as Coxsackievirus B3 cause myocarditis/ dilated cardiomyopathy, type 1 diabetes, encephalitis, myositis, orchitis, hepatitis.

[00104] In still other embodiments, T cells can be generated against antigens associated with organs in which organ specific autoimmunity is developed. Because the phenomenon of immunodominance involves epitopes from all of the antigens which are being processed at any one time, there is not a completely required need to know the exact autoantigen which is driving the autoimmune response at that time. For example, generating and introducing T cells to reactive subdominant epitopes of collagen into the inflamed joint of rheumatoid arthritis patients, the ongoing immune response will be rebalanced and tolerance will be reinstated. It is contrarian and unexpected to think that introducing active T cells into an inflammatory site would be beneficial, but according to our operative model, the rebalancing of the immune response in that site will reinstate appropriate control over the aberrant immune response. The treatment of autoimmune diseases is another embodiment of the invention.

5. Treg *in vitro* expansion

[00105] One of the principles of immune regulation is the balance of Tregs to other subsets of T cells. Another embodiment of the invention is polarizing the *ex vivo* T cell growth towards Treg cells. In this case, instead of targeting subdominant epitopes, Treg to dominant epitopes are generated as another way to rebalance the immune response. In one embodiment, the Treg T cells so generated are used as the therapeutic product alone. In another embodiment, the Treg subset is used in combination with T cells from other subsets grown to subdominant epitopes or subdominant antigens While Tregs have been expanded in a non antigen specific way using FACS sorting followed by expansion with anti-CD23 and anti-CD28 coated beads (Putnam et al. 2009) , to date no one has expanded Tregs in an antigen specific way for adoptive immunotherapy. Described herein are methods which are useful in the establishment of Tregs specific for different antigens especially those which are dominant in the immune response observed in autoimmune disease, graft vs. host disease or transplant rejection.

[00106] Tregs were isolated from PBMC's of autoimmune or Transplant patients by FACS sorting (BD FACS Aria II high speed cell sorter) using an aseptic technique in a GMP clean room using CD4-PerCP(SK3), CD127-PE (hIL-7R-M21), CD25 APC (2A3),

CD45RA-PE.Cy7 (L48) and CD45RO-PE.Cy5 (UCHLI). $CD4^+CD127^{lo/-}CD25^+$ and $CD4^+CD127^{lo/-}$ T cells were sorted and collected in 3ml X-Vivo 15 media (Lonza, Walkersville, MD) containing 10% human heat-inactivated pooled AB serum (Valley Biomedical, Winchester, VA). (Alternatively, Tregs can be separated using magnetic beads coated with the same antibodies (Miltenyi Biotec, Auburn, CA). These cell were plated at 2.5×10^5 Tregs per well in a 24 well plate (Costar, Cambridge, MA), each well containing dendritic cells prepared from the PBMC's (as described for T cell stimulation above) which had been pre-pulsed with dominant antigen at a Treg:APC ratio of 1:5. Following 18 hours incubation, cells received rapamycin (100ng/ml; Wyeth, Madison, NJ), from day1 to 7 in culture. At day 2, culture volume was doubled and 300 units/ml IL-2 was added (Chiron, Emeryville, CA). Cells were resuspended and fresh media and IL-2 were added days 2, 5, 7, 9 and 12. On day 9 cells were restimulated with peptide pulsed dendritic cells. Alternatively, anti-CD23/anti-CD28 coated microbeads (Invitrogen, Carlsbad, CA) can be used for this second stimulation. Further, the gas permeable bioreactor (such as Grex) can be used to perform this cell growth with fewer manipulations in a closed system and improve the kinetics of growth. In another variation IL-10 is added at the time of stimulation with the coated beads to drive further differentiation of the Treg to Treg1 which secrete high levels of IL-10 and regulate TH1 and Th2 responses.

[00107] Work Flow of Clinical Use of Immune Profiling of Dominant and Subdominant Antigens in Autoimmunity

Step 1: Immune Response Profiling

Humoral Profile	Cellular Profile
ELISA on Serum and	Elispot or ICS (for IFNγ at least, also IL-10, IL-4 IL-12, IL-21 to assay T cell subsets) on PBMCs stimulated with each antigen

Result: Antigen 1 strong response (dominant); Antigen 2 No/ Modest Response (subdominant)

Step 2: Grow T cells (CD8 and CD4) to Subdominant antigens *in vitro*; Grow Treg to dominant antigens *in vitro*

Result: T cells responsive to subdominant antigens; Treg responsive to dominant antigens

Step 3 (optional once therapy is well established): Confirm >5% T cells grown respond to subdominant antigens and Treg grow to dominant antigens using Cellular Immune Profiling (Elispot or ICS)

Result: 25% of the T cells respond to Antigens

Providing at least 5% of T cells respond to subdominant antigens and/or at least 5% of Treg cells respond to dominant antigen, proceed to step 4

Step 4: IV Infuse cells into patient with or without prior conditioning (e.g., cyclophosphamide)

Step 5 (may become optional once therapy is well established): Isolate PBMC's from Blood 2-3 weeks post infusion and Profile Immune Response

Result: Cellular Profile Antigen 1 No/Moderate effector response; Antigens 2 & Strong Treg response (dominant)

Step 6: Assess Clinical responses

In autoimmunity (MS- flare ups decrease, Rheumatoid Arthritis- joint swelling reduced, Asthma- number of attacks decreases, Early Type I diabetes, pancreas is maintained), In transplant- organ rejection rate and Graft vs. host disease decrease

Result: Improved clinical outcome

6. Administration of Tregs to dominant antigens in autoimmunity or transplant

[00108] In still another embodiment, Treg against dominant antigens could be combined with T cell of the TH1, TH2 or CTL subsets which are themselves responsive to

subdominant antigens. Such a combination would more fully switch the balance of the response towards subdominant epitopes. The inventors have demonstrated synergy between the two types of T cells in arthritis (Example 6).

7. Transplant

[00109] In an alternative embodiment, Treg are grown using the above Treg culture conditions from PBMC' s of healthy donors using dendritic cells(or irradiated PBMC's) from other healthy donors. In this way alloreactive Treg lines to each of the MHC are established and banked. As we discussed above, 80% of the MHC could be covered with Treg lines generated against 20 to 50 MHC haplotypes. Each of these lines could be frozen in single dose aliquots at –80 degrees C. When an organ or BMT is performed, 5×10^7 cells /m^2 Treg reactive to the mismatched MHC are also transplanted into the patient. In this way, the allogeneic rejection or graft vs. host disease is mitigated.

8. Automated Immune profiling assays and Closed system cell culture Device

[00110] Gas permeable membrane devices are a preferred embodiment of the culture techniques. Because the membrane is gas permeable, the scale of the culture is determined by the surface area of the membrane and the volume of media required to grow the cells. Examples of these gas permeable devices include Hyperstack (Corning) or Grex (Wilson Wolf). One of the useful features of this type of bioreactor is that the cell culture process is linearly scaleable. As part of the standardization of our approach, we have designed versions of bioreactors which slide into standard CO_2 incubators for use in production suites. In a separate embodiment, we have designed bioreactors which fit into standard stacks for warm rooms in an automated production facility The bioreactors intended for automation are made in two sizes: one for growth of autologous cells for an individual patient and a second larger version for the commercial production of allogeneic T cell lines. In an improved method, the cell culture devices are modified for automation into a rectangular shape so as to slide into the slots on a standard CO_2 incubator. This is a significant advantage as the gas permeable membrane is on the bottom of the flask and hence is blocked if it is sitting on the shelf. By having the flask be the shelf, there is better airflow to the membrane. A key attribute is the flanges on the side of

the flask which allow them to hold the weight of the bioreactor. In one embodiment, the bioreactor sets into a stainless steel frame which forms slides into the shelf supports. In another embodiment, they are molded as part of the plastic. In one of the embodiments, they have the footprint of an entire shelf. In another embodiment, they comprise ½, ¼ or 1/3 0r 1/5 of a shelf. In a preferred embodiment, the incubator is made by New Brunswick, Forma, ThermoElectron, Nuaire, ESCO. In a preferred embodiment, the incubator can be air or water jacketed or other design. In still another embodiment, they fit within a shelf which is a metal frame or on a flat in a warm room. In another preferred embodiment, the flat is moved and processed robotically. In still another embodiment, the bioreactor fits various commercial processing equipment including but not limited to a rocker which brings the cells back into suspension prior to harvesting.

[00111] Each bioreactor is a closed system with access ports to introduce media, components and cells and from which to harvest the cells for freezing and quality control. In a preferred embodiment, these access ports are tubing which ports fluids and cells in and out of the bioreactor the cap for which has integrated solid tubes which reach the bottom of the bioreactor. In an alternative embodiment, the access port is a sterile sheet of rubber through which a needle or other probe can be inserted into the bioreactor to inject or remove fluid cells or other reagents either manually or using an automated robot. The devices are bar coded in order to track them so that each patient and each cell line will have its own dedicated bioreactor. The bioreactor is disposable after it has been used. The bioreactors are also sized to fit standard robotic automation equipment in automated cell culture including but not limited to stacks, transporters and rocking agitators. Figure 31 is an example of such a bioreactor.

[00112] Like the bioreactors, commercial multiplexed immune profiling assays are also designed to enable processing of measuring titers of antibodies and T cell responses to panels of antigens. In order to do this, the ELISPOT assay is used as standard 96 well plate format can be applied. In a preferred embodiment, the AIDELISPOT reader (Autoimmun Diagnostika, Strassberg, Germany) is used to count the spots. Alternatively, the 96 well plate is used to input cells into the FACS for the ICS assay. In any event, each patient has their own

dedicated 96 well plate which is bar coded and all plates are disposable. These commercial assays and manufacturing processes are embodiments of the invention.

E. Examples

1. Example 1: EBV latent infection, lymphomas and Nasopharyngial carcinoma

[00113] 90% of the world's population has been exposed to EBV (the causative virus in mononucleosis) as measured by antibodies in the blood. EBV becomes latent in B cells and shuts off the majority of its proteins but express very low levels of latent antigens LMP1, LMP2 and sometimes EBNA-1. These proteins are weakly immunogenic but are required to maintain the virus even in its latent state. Because they derive predominantly from B cells, 40% of lymphomas test positive for EBV latent antigens. Thus, these antigens can serve as targets for the generation of CTL responses in adoptive cell therapy. In addition, Nasopharyngial carcinoma also expresses EBV latent antigens as do other tumors (e.g.-10% of gastric cancer). While CTL's have been used to treat EBV lymphomas, the current production methods are time consuming (3-6 months) and cumbersome using B cells transformed with EBV as repeat stimulation. Further, CTL's made in this way to LMP2 produced T cell batches only half of which had a detectable response to LMP2 post production. The inventors believed that the reasons for this were due to the presence of dominant epitopes from EBV proteins in the EBV transformed LCL cells which generated CTL's which outgrew the cells against LMP2 50% of the time. In patients with bulky tumors, 52% of the patients treated with CTL's produced by the traditional process had a complete response. While the prior art considered all CTL's equivalent regardless of the presence or lack of response to LMP2, the inventors felt that this may be one reason for the variable clinical response. Furthermore, the operative model of the invention predicted that this was indeed the case. Hence increasing the efficiency of CTL generation to LMP2 may be clinically important.

[00114] While other methods of CTL production are also embodied in the invention, the following methods were used to create the following experimental data: 40 ml to 100 ml of peripheral blood was collected from the patients in Vacutainer tubes. Peripheral Blood Mononuclear cells (PBMC's) were isolated by centrifugation on Lymphoprep

(Nycomed, Oslo, Norway), resuspended in RPMI 1640 (Gibco, Grand Island, NY) including 2mM L glutamine, 100 IU/ml penicillin, 100 μg streptomycin/ ml, with 10% Fetal calf serum (FCS) (5×10^6 cells/ml) and seeded onto 6 well plates (Costar Corp, Cambridge, MA) at 10^7 cells/well. After 2 hours at 37 degrees C, nonadherent cells were removed and resuspended in FCS with 10% polyethylene glycol (PEG) placed in test tubes, frozen on dry ice and stored in a -80 freezer. The adherent cells still in the 6 well plate was cultured in RPMI + 10% FCS supplemented with 50 ng of GM-CSF and 1000 U of IL-4 per ml. Half of the media was replaced with fresh media including the same growth factors described above on day 2 and day 4. On day 6, the media was completely replaced with the media described as well as the addition of 25% volume of macrophage conditioned medium to stimulate maturation. Macrophage conditioned media was produced by PBMCs adherent to immunoglobulin coated plates (prepared by immunoglobulin in PBS, plating and incubating at 4 degrees C overnight) for 24 hours at 37 degrees C in RPMI 10% FCS, harvesting the supernatant, filtration through a 0.2 mm pore size membrane (Acrodisc, Gelman Sciences) and storage at -20 degrees C for up to 8 weeks before use. Nonadherent cells were harvested 2 days later and used as a source of dendritic cells. Immunofluorescence staining with monoclonal antibodies for surface markers including CD54, CD80, CD83 and CD86 was performed to assure dendritic cell quality (>50% of cells +).

[00115] DC stimulators were preexposed for 2 hours at 37 degrees C to proteins at a concentration of μg/ml (50 for peptides) in serum free RPMI 1640 supplemented with human β2 microglobulin at 3 μg/ml. They were then washed and seeded at 10^5 cells/ 2 ml well in RPMI 10% FCS supplemented with IL-7 5 ng/ml. 2×10^6 PBMC's were added to each well for a responder to stimulator ratio of 20:1. The cultures were restimulated (and split into additional wells, if necessary) on days 14 and 21 with autologous peptide loaded dendritic cells in RPMI 10% FCS supplemented with IL-2 at 20 U/ml.

[00116] Release testing of CTL's to be used for treating patients included viability of >70%, negative culture for bacteria and fungi after 7 days, endotoxin testing less than 5 EU/ ml, negative results for Mycoplasma, less than 20% killing of recipient

lymphoblasts at a 20:1 ratio in ^{51}Cr release assays, less than 2% $CD19^+$ B cells, less than 2% $CD14^+$ monocytes and HLA identity.

[00117] Polyclonal T cell populations were harvested and used as effectors in a 5 hr chromium release assay. For the chromium release assays, monolayer cultures of fibroblasts established from skin biopsies of CTL donors and exposed to recombinant vaccinia virus (2x 10^6 cells per 9 cm petri dish) Cells harvested 18 hours post transfection and labeled for 1 hour with $^{51}CrO_4$, washed three times, and used as targets in a 5 hour chromium release assay. Supernatants from the assay were harvested into 1% formaldehyde before counting on a γ counter.

a. Experiment 1: The relative frequency of T cells responding to subdominant epitopes is enhanced when Dendritic cells or activated Macrophages are used as antigen presenting cells instead of LCL

[00118] T cell lines were prepared from 10 patients using 3 different antigen presenting cells as stimulators: EBV transformed lymphoblastoid cell lines (LCL) presentation and expansion; Dendritic cell (DC) presentation with cytokine expansion; IFNγ Macrophage (MAC) presentation with cytokine expansion. Each of the 3 were stimulated during antigen presentation with a mixture of 3 plasmids expressing subdominant epitopes of EBNA-1 (EBNA-1 with deletion of aa 90 to 325), LMP1 (LMP1 with deletion of aa 1-43 and aa 260-315) and LMP2 (LMP2A in 2 plasmids one expressing aa 1-399 and the second plasmid expressing aa 400-497). The T cell lines so generated using the protocol of the invention were then studied in ^{51}Cr release assay.

[0100] Figures 1A and 1B: ^{51}Cr release at two different effector:target (E/T) ratios, 20:1 and 10:1, respectively, (CTL line: HLA matched fibroblasts) HLA matched fibroblasts pre-pulsed with peptide mixes from the indicated antigens, CTL's expanded from representative patient using different indicated APC's to grow CTL line.

[0101] Conclusion: Dendritic cells (DC) and Macrophages (MAC) more selectively stimulate the growth of CTL's to subdominant antigens than EBV transformed B cells (LCL's).

[0102] Figure 1C: ^{51}Cr release at three different effector:target (E/T) ratios (CTL's: HLA matched fibroblasts)HLA matched fibroblasts pre-pulsed with peptide mixes from the indicated antigens CTL's from PBMC's of the same patient directly after blood harvested (before culture).

[0103] Conclusion: Patients have some response to EBV dominant antigens but at significantly higher E/T ratios than after T cell culture

[0104] Figure 2: ^{51}Cr release at 20:1 effector:target (E/T) ratios (CTL Lines: HLA matched fibroblasts)HLA matched fibroblasts pre-pulsed with peptides representing specific HLA A2 restricted subdominant epitopes from LMP2 with CTLs made from the same patient using three different methods as above:

[0105] Conclusion: Dendritic cells and Macrophages both lead to a response to a broader number of subdominant epitopes & higher levels of CTL activity than does LCL and the magnitude of the response to different epitopes is different between the Dendritic cells and Macrophages

[0106] Figures 3A and 3B: ^{51}Cr release at 20:1 effector:target (E/T) ratios (CTL Lines: HLA matched fibroblasts)HLA matched fibroblasts pre-pulsed with peptides from LMP2: LCL Stimulation in Figure 3A and DC/MAC stimulation in Figure 3B.

[0107] Conclusion: Dendritic cells and Macrophages lead to CTL Lines in 90% of the patients, in contrast to the LCL which only led to 50% of the CTL lines from the patients having a detectable LMP2 response. Therefore, the DC/MAC process is more robust and reproducible for generating T cells to subdominant antigens and epitopes.

[0108] Figure 3C: % of Viable $CD3^+$ Cells which are $CD4^+$, $CD8^+$ and $CD25^+$ in CTL Lines grown using Macrophage, Dendritic Cells or LCL cells as Antigen Presenting Cells. Lines were stained with antibodies for CD3, CD4, CD8 and CD25 and analyzed by Flow Cytometry.

[0109] Conclusion: While all methods establish $CD8^+$ cells and not Tregs, the use of Dendritic cells and macrophages as APC's appears to increase the % of $CD4^+$ cells relative to that produced with LCLs.

ELISpot Assays:

[0110] Elispot assays were performed to determine the number of T cells produced to particular antigens. Elispot γ IFN 96 well polyvinylidene diflouride backed plates (Millipore, Bedford, MA) were coated with 15 μg/ml of anti-IFNγ monoclonal antibody 1-DIK (MABTECH, Stockholm, Sweden). 5×10^6 PBMCs were added per well with peptide mixes 2 μM each from each of the proteins and incubated overnight at 37 degrees C 5% CO_2. Cells were discarded and 1 μg/ml biotinylated anti-IFNγ monoclonal antibody 7-B6-1 (MABTECH) was incubated 2- 4 hours at room temperature followed by streptavidin conjugated alkaline phosphatase (MABTECH) for 2 more hours. After a 30 minute reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium from the alk-phos substrate kit (Bio-Rad Richmond, CA), depending upon the number of samples, the spots were counted either using a dissection microscope or on an AIDELISPOT reader (Autoimmun Diagnostika, Strassberg, Germany). Each spot was a cell reported as spot forming cells (SFC)/ 10^5 PBMC's. In these assays, the positive control was cells + 10 μg/ml PHA and negative control was cells alone without peptide.

[0111] Figure 4: IFN producing cells (SFC's) / 105 T cells grown in vitro as measured by Elispot

[0112] Conclusion: Dendritic cells and Macrophages more selectively expand T cells to subdominant antigens than LCL

Optimizing antigens to establish responses to subdominant epitopes

[0113] LMP2 was chosen for optimization as it is a subdominant antigen relative to EBNA-3 and the majority of EBV subdominant epitopes recognized appeared to be on this protein as identified using consensus software and PBMC testing. In an effort to increase the

number of subdominant epitopes recognized, LMP2 was split onto two or more plasmids. The following plasmids were constructed in the p shuttle or pUC19 plasmid under control of the CMV promoter and artificial ATG and poly A:

Plasmid 1 LMP2B (aa 1-497)

Plasmid 2 LMP2A 1st exon (aa 1-119)

Plasmid 3 LMP2A 2nd exon (aa 120-497)

Plasmid 4 LMP2A (aa 120-399)

Plasmid 5 LMP2A (aa 400-497)

Plasmid 6 LMP2A (aa 120-440)

Plasmid 7 LMP2A (aa440-497)

Plasmid 8 LMP2A (aa 1-399)

Plasmid 9 LMP2A (aa400-497)

[0114] All plasmids were generated using standard procedures in SCS110 bacteria strains (Stratagene, La Jolla, CA) and purified with Endo free Plasmid Maxi kit (Qiagen, Hilden, Germany). Antigen presenting cells were transfected 24 hours after maturation using the Amaxa DC Nucleofection Kit (Amaxa, Koeln, Germany) with 2-20 μg plasmid DNA per 10^6 cells.

[0115] T cell immunosubdominant epitopes on LMP2 include but are not limited to:

LLW 329-337 LLWTLVVLL HLA A 2.01

CLG 426-434 CLGGLLTMV HLA A 2.01

IED 200-208 IEDPPFNSL HLA B 40.01

SSC 340-350 SSCSSCPLSKI HLA A11.01

TYG 419-427 TYGPVFMCL HLA A24.02

LLS 447-455 LLSAWILTA HLA A2

LTA 453 461 LTAGFLIFL HLA A2

FLY 356-364 FLYALALL HLA A2

[0116] Peptides for these epitopes were synthesized and used to test CTL responses in ^{51}Cr release assays on HLA-A2 expressing fibroblasts isolated from skin of PBMC donors.

[0117] Figure 5: ^{51}Cr release at 20:1 effector:target (E/T) ratios (CTL Lines: HLA matched fibroblasts)HLA matched fibroblasts pre-pulsed with peptides from LMP2: CTL Lines established with LMP2 split on 1, 2 , 3 or 4 plasmids (1P: 497 aa LMP2B; 2P: LMP2A 1st exon (119 aa) on 1 plasmid, LMP2A 2nd exon (378 aa); 3P LMP2A 1st exon (119 aa) on 1 plasmid, LMP2A (120-399 aa) on plasmid 2 and LMP2A (400-497 aa) on 3rd plasmid; 4P: LMP2A 1st exon (119 aa) on 1 plasmid, LMP2A (120-399 aa) on plasmid 2 LMP2A (400-440 aa) on 3rd plasmid LMP2A (440-497 aa) on 4th plasmid; 2P 2: Plasmid 1 (aa 120-440), Plasmid 2 (aa 440-497)

[0118] Conclusion: Splitting the LMP2 into at least two plasmids one containing aa 400 to 497, the other containing residues before 399 results in stronger responses to a greater number of subdominant epitopes

[0119] EBNA-1 amino acid 90 to 325 corresponding to Gly Ala repeat domain were deleted from the EBNA-1 sequence and inserted into the p shuttle plasmid under the control of the CMV promoter. This sequence was selected for deletion because it was demonstrated to inhibit peptide processing. The following HLA-A2 restricted peptide was used to assess response: VLK 574-582 HLA A2.

[0120] Furthermore, LMP1 sequence was prepared with aa 1-43 deleted (to prevent its aggregation/protection from proteosome processing) and 260-315 deleted (5 copies of 11

amino acid tandem repeats). These sequences were constructed in the p shuttle plasmid under control of the CMV promoter and compared with wild type LMP1. To assess breadth of response, the following HLA-A2 restricted epitopes of LMP1 were prepared and tested:

YLL 125-133 YLLEMLWRL HLA A2

YLQ 159-167 YLQQNWWTL HLA A2

TLL 166-174 TLLVDLLWLL HLA A2

LLV 167-175 LLVDLLWLL HLA A2

LLL 92-100 LLLIALWNL HLA A2

RLG 132-140 RLGATIWQL HLA A2

[0121] Figures 6A and 6B: ^{51}Cr release at 20:1 effector:target (E/T) ratios (CTL Lines: HLA matched fibroblasts)HLA matched fibroblasts pre-pulsed with peptides from EBNA-1 or LMP1: Comparison of CTL Lines established with EBNA-1 wild type vs. EBNA-1 deleted and LMP1 wild type vs. LMP1 deleted.

[0122] Conclusion: Deletion of certain regions of proteins which evade antigen processing results in stronger responses to a greater number of subdominant epitopes

b. Example 2

[0123] In the following experiments, T cells were grown in the same way as outlined in Example 1 above, with different variations designed to enhance antigen processing to favor T cells reactive with the production of subdominant epitopes.

[0124] Figure 7: (^{51}Cr release) Cr release at 20:1 effector:target (E/T) ratios (CTL Lines: HLA matched fibroblasts)HLA matched fibroblasts pre-pulsed with peptides representing Dominant antigen (EBNA-3A), Subdominant antigen (LMP2) and specific HLA A2 restricted subdominant epitopes from LMP2 with CTLs made from the same patient using

two different methods : one with 100nM to 300nM bortezomib added during antigen presentation, one without.

[0125] Conclusion: Addition of the Proteosome antagonist bortezomib during CTL antigen presentation modifies antigen processing to generate CTL's to a broader number of subdominant epitopes with a modest decrease in the response to dominant epitopes

[0126] Figure 8: (^{51}Cr release) Cr release at 20:1 effector:target (E/T) ratios (CTL Lines: HLA matched fibroblasts)HLA matched fibroblasts pre-pulsed with peptides representing Dominant antigen (EBNA-3A), Subdominant antigen (LMP2) and specific HLA A2 restricted subdominant epitopes from LMP2 with CTLs made from the same patient using two different methods : one with 10 ng/ml Interferon γ (IFNγ) added 12 hours before and during antigen presentation, one without.

[0127] Conclusion: Addition of IFNγ during CTL antigen presentation modifies antigen processing to generate CTL's to a broader number of subdominant epitopes with a modest decrease in the response to dominant epitopes. The effect is more dramatic in Macrophages than in dendritic cells.

c. Example 3: Hepatitis and Hepatocellular carcinoma

[0128] A mouse model of chronic hepatitis has been recently developed which allows the study of the primary and secondary immune response in hepatitis B (Publicover J et al. 2011). This HBVtgRAG mouse is a cross between HBV-replication transgenic mice (HBVRpl) in the C57BL/6 background for 15 generations which constitutively allows viral replication and release of virions and RAG-1 deficient mice making them unable to generate T and B cells. When 10^8 spleenocytes are transferred from C57BL/6 mice, the immune system is reconstituted and the primary immune response to hepatitis infection is modeled. If one administers the spleenocytes to young (3-4 week old) mice, the animals (similar to young children infected with Hepatitis B) develop chronic Hepatitis. Like the humans, they clear HBc but HBs remains at high levels in the serum (Figure 1). This model of chronic hepatitis was used in the following experiments.

[0129] Alanine aminotranferase were measured using ALT-L3K kit (Diagnostic Chemicals Ltd) on a Cobas Miras Plus analyzer (Roche diagnostics). HBs Ag was measured using ETI-MAX 2 Plus (Diasorin; HBs Antibody was measured using ETI-AB-AUK-PLUS and ABAU standard set (Diasorin). HBcAB was measured using ETI-AB-COREK-PLUS (Diasorin). Assays were read on ELx800 (Biotek Instruments) wavelength 450nm and 630 nm.

[0130] Figure 9: HBVtgRAG mouse model of chronic hepatitis B.

[0131] T cells were grown to HBs *ex vivo* using our protocol and injected by tail vein injection 3-4 weeks post transfer of the original spleenocytes. A titration was performed from 1×10^5 to 1×10^8 cells with plateau achieved at 1×10^6 cells. As can be seen below, our T cell rebalancing therapy clears chronic hepatitis in the HBVtgRAG model.

[0132] Figure 10: Treatment of HBVtgRAG model with T cells reactive to HBs Ag lead to acute inflammation and clearance of the previously chronic infection.

[0133] When IL-4 or IL-21 were used as a supplement to the medium during antigen presentation, the amount of T cells required to achieve plateau effect was 5×10^5 and 7×10^4, respectively. This number of cells is more than a log lower than T cells expanded in the standard IL-15 protocol and indicates that polarization during culture towards T follicular helper cells and TH2 cells is advantageous. On the other hand, polarization with IL-12 increased the number of cells to 1×10^7 required for plateau and polarization to Treg with IL-2 and rapamycin eliminates the response to therapy.

[0134] Different Culture conditions result in polarization to different T cell subsets and different numbers of T cells to reach plateau in clearing Hepatitis in the HBVtgRAG mouse

Culture Conditions	**Number of T cells to Reach Plateau**
IL-15	1×10^6
IL-15+IL-4	5×10^5

IL-15+IL-12	1×10^7
IL-15+IL-21	7×10^4
IL-2+rapamycin	No response

[0135] Therefore, polarization of the T cells to different T cell subsets has important therapeutic effects.

[0136] To determine the effect of T cell therapy on the frequency of T cells to different epitopes, the % of IFNγ producing epitopes to different epitopes was determined by Elispot. At week 6 post transplant, spleenocytes were collected from HBVtgRAG mice who were untreated or treated with plateau levels of T cells raised to HBs Ag. The spleenocytes were pulsed with Hepatitis Core Antigen (HBc), Hepatitis Surface Antigen (HBs) or two Kb restricted peptides of HBs: ILS or WWL.

HBs Ag 190-197 VWLSVIWM K^b

HBs Ag 208-215 ILSPFLPL K^b

The following results were observed:

[0137] Figure 11: Treatment with HBs T cells rebalances the immune system to a new dominance hierarchy.

[0138] As can be seen, T cell lines produced using our protocol generated a significantly higher T cell response to HBs and a slightly decreased response to HBc. The HBs response was driven predominantly by a de novo response to a previously subdominant epitope (WWL). Thus, the dominance hierarchy within HBs was switched by T cell rebalancing therapy as the response which exists in untreated animals (albeit weak) is against the dominant epitope (ILS) switching the overall balance of the HBV response in favor of the previously subdominant HBs antigen relative to the previously dominant HBc antigen.

[0139] The HBVtgRAG mouse model was also used to investigate whether *in vivo* methods could also rebalance immunodominance hierarchies. In one approach, the inventors injected HBs Antigen in Freund's adjuvant by tail vein injection (IV) , intramuscularly (IM) or intraperitoneally (IP). Alternatively, 100μg plasmid DNA was administered per mouse (data not shown).

[0140] Figure 12: The IM route of administration results in a broader response to subdominant antigens as dose treatment with a protease inhibitor during vaccination.

[0141] As can be seen, a 4 X stronger response is generated by IM administration relative to the other two and this is associated with a response to the subdominant epitope. Furthermore, when this mouse was treated with the proteasome antagonist bortezomib, and the antigen was administered IM, the response to the subdominant epitope was further accentuated.

[0142] In a second, set of experiments the response alone and in complexes with two HBs reactive murine antibodies of different isotypes (IgG1 vs. IgG2a):

Monoclonal antibodies to HBs Ag

10-H05 Murine IgG1 HBsAg (Fitzgerald, Concord, MA)

10-H05A Murine IgG2a HBsAg (Fitzgerald, Concord, MA)

[0143] Figure 13: IgG2a monoclonal antibody but not IgG1 antibody specific for HBs Ag results in a different dominance hierarchy and significantly better antigen presentation by the IV route.

[0144] When the antigen antibody complex was administered intravenously and IM, the IgG2a complex generated more of a response to the subdominant epitope. Further, this protocol developed a near comparable level of immune response by IV administration to that achieved by IM administration indicating that IV administration of IgG2a immune complexes may be able to rebalance the immunodominance hierarchy *in vivo*.

[0145] The inventors hypothesize that the likely mechanism for this observation is that IgG2a binds the high affinity FcR which is preferentially expressed on dendritic cells and thus targets the HBs antigen to dendritic cells. Additionally, as HBs Ag *a* determinant first loop (aa 124-147) is the major epitope for recognition by neutralizing antibodies and the ILS epitope is further away, it could be that antigen processing for ILS is enhanced.

Clinical trial of T cell rebalancing therapy in HBV associated Hepatocellular carcinoma

[0146] 5 patients with HBV and HCC who were HLA-A *0201 were immune profiled for their Humoral and cellular immune response. Pretreatment clinical laboratory testing demonstrated high titers of antibody to hepatitis B core antigen (HBc Ag) but not to hepatitis B surface antigen (HBs Ag) (Figure 10).

[0147] PBMC's were induced into dendritic cells, pulsed with hepatitis B core antigen (HBc Ag) and hepatitis B surface antigen (HBs Ag) and used to grow CTL's from PBMC's which were tested by Elispot for IFNγ.

[0148] Figure 14: Number of Spot forming cells in 10^5 PBMCs from Patient 3.

[0149] By frequency of IFN γ producing T cells on Elispot a hierarchy was observed: no-antigen(10 sfc)<HBsAg(15 sfc)<HBcAg(45 sfc). This indicates that Hepatitis B surface antigen is subdominant relative to Hepatitis core antigen. Additionally, 3 peptides of HBs (FLL, GLS and ILS) were studied:

HBs Ag 20-28 FLLTRILTI HLA-A*201

HBs Ag 185-194 GLSPTVWLSV HLA-A*201

HBs Ag 208-216 ILSPFLPLL HLA-A*201

[0150] The FLL peptide appeared to be slightly dominant in what little response to HBs was detectable but, because of the weak response, the dominance hierarchy was confirmed by ICS assays:

Intracellular cytokine staining (ICS)

[0151] 5×10^5 CTL were resuspended and incubated for an hour in 100 µl of 1XPBS 1% FCS with peptide (10^{-5} to 10^{-9} M final concentration) Golgi Plug (BD Biosciences, San Diego, CA) was then added , cells were incubated at 37 degrees C 5% CO_2 for 5 hours, pelleted, washed in 200µl PBS 1%FCS and stained for surface antigens (CD4 fluorescein isothiocyanate and CD8 allophycocyanin(Pharmingen, Becton Dickinson) 30 minutes at 4 degrees C. Following resuspension in fixation and permeabilization solution, cells were stained with anti human IFNγ phycoerythrin (Pharmingen, BD) 1/20 dilution on ice for 30 minutes, washed once and resuspended in PBS 1% FCS and analyzed on FACS Canto (Becton Dickinson)

[0152] Figure 15: ICS Analysis of response to HBs epitopes.

[0153] Although the responses detectable by ICS weren't strong, the FLL epitope did again appear dominant relative to the other two epitopes.

[0154] 5×10^6 PBMC's were isolated from each patient and T cells were grown as per our protocol using HBs 15 mer peptides mixes as the stimulating antigen with the peptide with the exclusion of peptide 15-30 (to eliminate FLL). IL-4 + IL-15 were used during the expansion. 1×10^{10} T cells were obtained and frozen with an aliquot taken for Elispot testing.

[0155] Figure 16: HBs Cells are the majority of the T cells which are administered to the patient and these respond to previously subdominant antigens.

[0156] As can be seen, the CTL line generated was almost exclusively reactive to the previously subdominant HBs, a response which was primarily driven by the previously subdominant epitopes. CTL's were dosed by IV Infusion at 5×10^7 to 1×10^8 cells /m^2 and the patients were monitored.

[0157] Figure 17: The patient has an acute flair and then clears the hepatitis.

[0158] After developing acute hepatitis, the patient resolved and completely cleared the hepatitis. On day 14, PBMC's were again collected and a profile of the cellular immune response was re-determined.

[0159] Figure 18: The patient's immunodominance hierarchy has been rebalanced to the previously subdominant antigen (HBs) and the previously subdominant epitopes on that antigen.

[0160] The immunodominance hierarchy has been successfully rebalanced by the T cells grown by our process. In addition to clearing the chronic hepatitis, the patient also had a complete response to his Hepatocellular carcinoma.

[0161] Figure 19: The T cells completely resolved the patient's Hepatocellular carcinoma.

d. Example 4: Systematic method for treating cancer

[0162] The clinical diagnosis and therapy of cancer involves the biopsy and imaging of the tumor to determine its tissue of origin, differentiation and extent of local and systemic metastasis. While diagnostics to genetic defects in oncogenes or tumor suppressor genes and assays to determine sensitivity to chemotherapeutic or biologic agents is sometimes performed, generally patients are treated with a combination of surgery, chemotherapy and radiation depending upon their specific cancer and stage. Similarly, while the immune system in various patients has been studied using different techniques, this information has not been used in the clinical management of the patients. With the advent of their cellular therapy to rebalance the immune system, the inventors had to establish the use of immune profiling in the management of the cancer patient. The inventors have developed a standardized immune profiling methodology which is used to select antigens which are subdominant in that patient and can be used to grow T cells *in vitro* which can be reinfused into the patient to rebalance the immune response to a tumor. Following therapy, the patient is reprofiled to determine if the therapy successfully rebalanced the immune response. Such a therapeutic method is novel and can

result in clinical response and enhanced survival. The same approach can be used infectious diseases and autoimmunity and organ transplantation.

[0163] The first step is to identify which tumor associated antigens are present on the patient's tumor. This is generally done by immunohistochemistry on a biopsy from the patient's tumor. The panel antigens tested will depend on the type of tumor. For example, in melanoma antigens included NY-ESO-1, SSX-2, Melan A, gp100, MAGE A4, MAGE A1, Tyrosinase and would be supplemented as new tumor associated antigens were described. Not all tumors will express all of the antigens in the panel. For the antigens, which the patients tumor has, a baseline immune profile to the different antigens is determined using the systematic profiling for humoral and cellular immune response described above. Elisa is used to determine antibody titer to each antigen in the serum and the % of T cells responding to the antigen is determined by IFNγ ICS or Elispot. In this way, a base line profile is determined.

[0164] Figure 20: IFNγ ICS to a Panel of Tumor Antigens in Patient 1

[0165] In Patient 1, the patient's tumor had NYESO-1, SSX-2, Melan A, and MAGE A4. ICS demonstrated a strong response to MAGE A4 but modest/ no response to NYESO-1, SSX-2 and Melan A. The NYESO-, SSX-2 and Melan A antigens are thus chosen to grow and expand CTL in the *in vitro* culture using the following protocol:

[0166] 5×10^6 PBMC's are isolated from the blood. Monocyte-derived dendritic cells are generated *in vitro* from peripheral blood mononuclear cells (PBMCs) from a patient by plating of PBMCs for 2 hours in a tissue culture flask to permit adherence of monocytes. At this point the nonadherent cells are removed and frozen at -80 to later serve as a source of T cells. Treatment of the adherent monocytes with interleukin 4 (IL-4) and granulocyte-macrophage colony stimulating factor (GM-CSF) leads to differentiation to immature dendritic cells (iDCs) in about a week. Subsequent treatment with tumor necrosis factor (TNF) further differentiates the iDCs into mature dendritic cells. These cells are then separated into 3 separate flasks (or however many antigens to which one desires to grow T cells) in RPMI 1640 media supplemented with 45% Click's medium (Irvine Scientific, Santa Ana, CA), 2mM Glutamax 1

and 5% human serum. Each flask is pulsed with one of the 3 plasmids (or pepmix) containing the coding sequence for the antigen of interest (in this patient 1 containing NY-ESO-1,1 containing SSX-2 and the third containing Melan A). The cells are stored at 37 degrees C for 2 hours. In the meantime, PBMC's are thawed and added to the pulsed dendritic cells at a 1:20 to 1:100 PBMC to Dendritic cell ratio and incubated at 37 degrees C for 18 hours. The three flasks of cells are then pooled and resuspended in the same media, containing IL-15 (5ng/ml) to generate *in vitro* expansion of the T cells which have recognized the antigen. A Grex gas permeable bioreactor is used (Wilson Wolf Manufacturing Minneapolis, MN) obviating the need to change media and enabling exponential growth kinetics. Generally 3×10^8 to 1.5×10^{10} cells are obtained within 3 to 6 weeks enough cells to be administered to a patient.

T cells grow in Gas Permeable Bioreactor

[0167] T cells grow as a layer on the gas permeable membrane for excellent gas exchange and have a volume of media sufficient to grow to the required density.

[0168] At the end of *in vitro* culture, T cells are assayed in the ICS assay with the same antigens: NY-ESO-1, SSX-2, Melan A, gp100, MAGE A4, MAGE A1, Tyrosinase.

[0169] Figure 21: ICS Assay Patient 1

[0170] Based upon the assay, the % of the cells responding to each subdominant antigen/ epitope is determined in this case, NY-ESO-1, SSX-2 and Melan A. The total number of cells responding to each subdominant antigen or epitope can be calculated using this % and the number of CTLs in the culture. In the case of patient 1, 61.4% of the cells were responding to NY-ESO-1 (Figure 21).

[0171] Based upon this, a known dose of CTLs responsive to the subdominant antigens can be administered to the patient. CTL's were dosed at 5×10^6 to 2×10^8 cells /m^2.

[0172] 2 -3 weeks after infusion into the patient, PBMC's were again collected and a profile of the cellular immune response was re-determined by ICS:

[0173] Figure 22: Cellular Immune Profiling by ICS post therapy- Patient 1

[0174] Figure 23: Humoral Immune Profiling by ELISA- Patient 1 (Reciprocal titers of the humoral immune response are plotted below).

[0175] As can be seen from the Cellular and Humoral Profiles from the Immune Hierarchy Assays, the immunodominance hierarchy was rebalanced to favor the previously subdominant antigens.

Clinical Findings:

[0176] Clinically, the patient was a 52 year old male who was diagnosed with Stage IV metastatic melanoma. He had failed to respond to a combination regimen of Dacarbazine (DTIC) and Temodar (Temolzolomide) chemotherapy drugs in combination with IL-2.

[0177] Two years post T cell therapy, the patient is alive and has undergone a complete response. As can be seen in the Chest CT Scan the metastasis in the lung completely resolved within 6 months of Immune rebalancing Therapy and has remained stable.

[0178] Figure 24: CT Scan Pre and Post T Cell Therapy

e. Example 5: T cell therapy of Lymphoma

[0179] 150 adult patients with relapsed aggressive Non Hodgkins lymphoma were randomized into 3 arms: A: Rituxan + CHOP; B: Testing for EBV LMP1 & LMP2 antigens in tumor, followed by EBV LMP1 & LMP2 T cell rebalancing and C: Pan lymphoma: No assay for antigens; Therapy with T cells grown to respond to EBV LMP1, LMP2, surviving, MAGE A3. 40% of lymphoma biopsies test positive for EBV LMP2, 50% test positive for surviving and 15% test positive for MAGE A3. Standard of Care R-CHOP regimen was used and 5×10^7 to 2×10^8 T cells were dosed per m^2.

[0180] Figure 25: Progression Free Survival

[0181] As can be seen, T cells responsive to the tumor provide superior response rates to the current standard of care. While failures occur in year 1, post year 1 the CTL maintains patients in remission. This is evidence of a properly functioning immune system post rebalancing. Finally, while initially the EBV LMP T cells provide a better response, by year 3 the Progression Free Survival has approached that of the Pan lymphoma product. Furthermore, lymphoma today is a relapsing remitting disease with patients generally relapsing within 18 months to 2 years. CTL rebalancing therapy changes this course: once a patient's immune system is rebalanced, the patient develops a memory response which maintains a long term remission. Thus, unlike other therapies of lymphoma, T cell therapy provides a durable remission.

[0182] Figure 26: T Cell Therapy Changes the Natural History of Disease

f. Example 6: T cell therapy of Autoimmune Diseases

[0183] The collagen induced arthritis model (CIA) is a model of Rheumatoid Arthritis (RA) that can be induced by immunization with heterologous collagen II (CII) in DBA/ 1 mice.

[0184] DBA/1 male 6-8 week old mice were obtained from Jackson Laboratories (Bar Harbor, ME). 100 μg of bovine CII (Chondrex, Redmond, WA) emulsified in CFA containing 4 mg/ml M tuberculosis (Chondrex) were injected subcutaneously in the tail. By week 5 post injection, 80-100% of untreated mice showed fully developed disease.

[0185] T cells were grown to the following peptides using the protocols described for the *in vitro* growth of T cells.

[0186] Collagen Type II

263-270 immunodominant peptide used to stimulate the growth of Treg (IL-2 + rapamycin)

286-300 subdominant peptide used to stimulate growth of T cells (IL-15)

[0187] 5×106 cells were administered to each animal on Day 20 post induction alone or in a combination of equal parts. See Figure 27. CD25 is a marker for Treg cells. Control animals received PBS. See Figure 28.

[0188] Mice were scored for clinical disease three times per week using a score of 0-3 for each limb for a maximum total score of 12 possible: 0-1 Normal; 1 mild redness or swelling in single digits; 2 significant swelling of ankle or wrist with erythema; 3 severe swelling and erythema of multiple joints. The percent of animals with arthritic lesions in the group represent incidence of arthritis. Average clinical score in the group reflects severity of the disease. See Figure 28 and Figure 29.

Histopathology of Joint

[0189] Figure 30A shows a normal rat, Figure 30B shows a rat immunized with human proteoglycan and Figure 30C shows a rat treated with T cells.

[0190] As can be seen, the T cell therapy significantly decreased the incidence, severity and amount of inflammation in the joint of animals. There appeared to be a synergistic effect in rebalancing the immune response between T cells raised to subdominant epitopes and Treg grown to the dominant epitope.

What is claimed is:

1. A T cell capable of recognizing a subdominant antigen or epitope in a patient for use in a method of altering the immunodominance hierarchy of the patient.
2. A T cell for the use according to claim 1, wherein the T cell is obtained by:
    a. identifying at least one subdominant antigen or epitope in a sample obtained from the patient, and
    b. cultivating a T cell capable of recognizing said subdominant antigen or epitope.
3. A T cell for the use according to claim 1 or 2, wherein altering the immunodominance hierarchy treats/prevents/alleviates an infection, cancer, inflammation, organ transplantation rejection, or graft versus host disease in the patient.
4. Use of a T cell capable of recognizing a subdominant antigen or epitope in a patient in the manufacture of a medicament for altering the immunodominance hierarchy of the patient.
5. A use according to claim 4, wherein the T cell is obtained by:
    a. identifying at least one subdominant antigen or epitope in a sample obtained from the patient, and
    b. cultivating a T cell capable of recognizing said subdominant antigen or epitope.
6. A use according to claim 4 or 5, wherein altering the immunodominance hierarchy treats/prevents/alleviates an infection, cancer, inflammation, organ transplantation rejection, or graft versus host disease in the patient.
7. A T cell population for use in altering the immunodominance hierarchy of a patient obtainable by identifying at least one subdominant antigen or epitope in a sample obtained from the patient and cultivating a T cell population capable of recognizing said subdominant antigen or epitope.
8. A T cell population according to claim 7 for use in therapy.
9. A method comprising the steps of:
    a. identifying a dominant antigen or epitope and a subdominant antigen or epitope in a patient sample;
    b. cultivating a T cell capable of recognizing said subdominant antigen or epitope; and

c. treating a patient with an effective number of said T cell to alter the immunodominance hierarchy of the patient.

10. A method comprising the steps of:
    a. identifying at least one subdominant antigen or epitope in a patient sample;
    b. cultivating a T cell capable of recognizing said subdominant antigen or epitope; and
    c. treating a patient with an effective number of said T cell to alter the immunodominance hierarchy of the patient.
11. The method of claim 9 or 10, wherein said subdominant antigen or epitope are antigens or epitopes to which a cellular or humoral immune response is not detectable or is only detectable at a low level.
12. The method of claim 9 or 10, wherein said subdominant antigen is a viral antigen, other infectious agent antigen, tumor antigen, or an antigen associated with autoimmunity, allergy, inflammation, organ transplantation rejection, or graft versus host disease.
13. The method of claim 9 or 10, wherein step (b) further comprises cultivating a T cell in the absence of a dominant antigen or epitope.
14. The method of claim 9 or 10, wherein step (b) further comprises cultivating a T cell in the the presence or absence of agents to enrich either suppressive T cells or responsive T cells.
15. The method of claim 9 or 10, wherein step (c) further comprises administering the effective number of T-cell via intradermal administration.
16. The method of claim 9 or 10, further comprising a step prior to step (c), wherein the patient is pretreated with a conditioning agent to reduce the number of endogenous T cells.
17. The method of claim 9 or 10, wherein said T cell is a suppressive T cell, and wherein step (c) further comprises inducing tolerance in the patient to treat or prevent an autoimmune disease, allergy, inflammation, organ transplantation rejection, or graft versus host disease.
18. The method of claim 9 or 10, wherein said T cell is a responsive T cell, and wherein step (c) further comprises inducing a cytotoxic immune response to treat or prevent an infection or cancer.
19. The method of claim 9 or 10, further comprising profiling the tolerance or humoral or cellular immune response to the subdominant antigen or epitope to determine if the therapy successfully rebalanced the immune response of the patient.

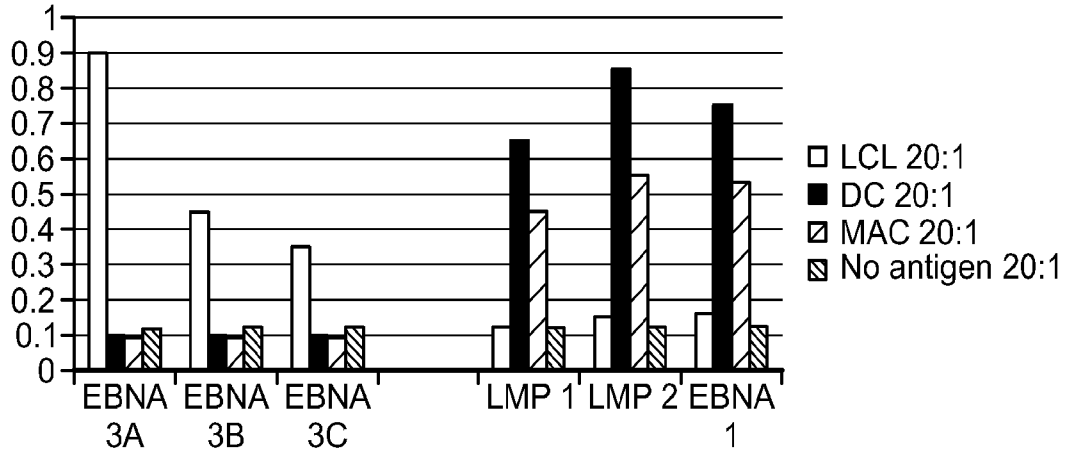


FIG. 1A

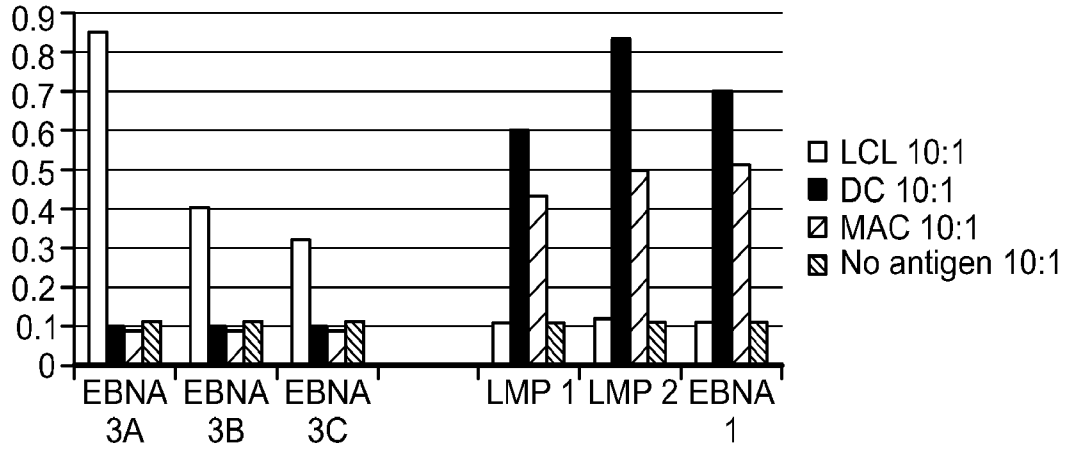


FIG. 1B

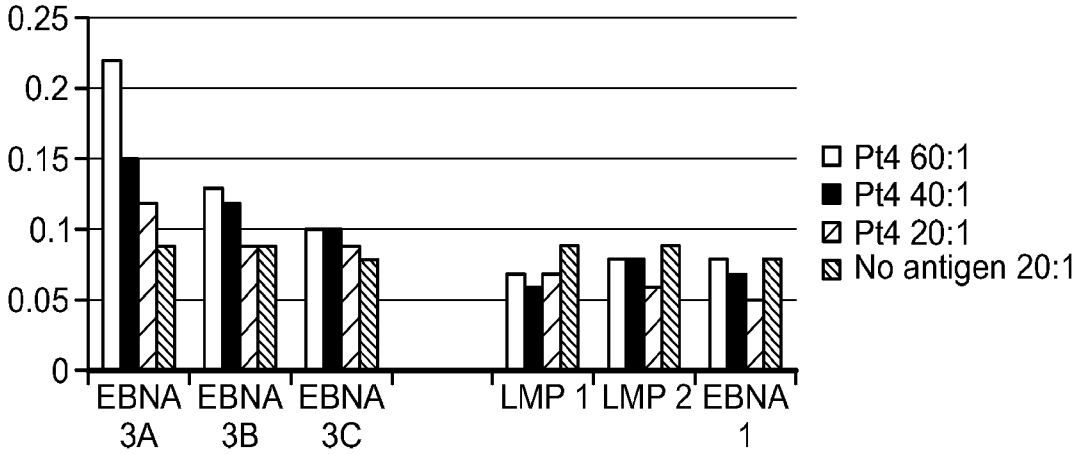


FIG. 1C

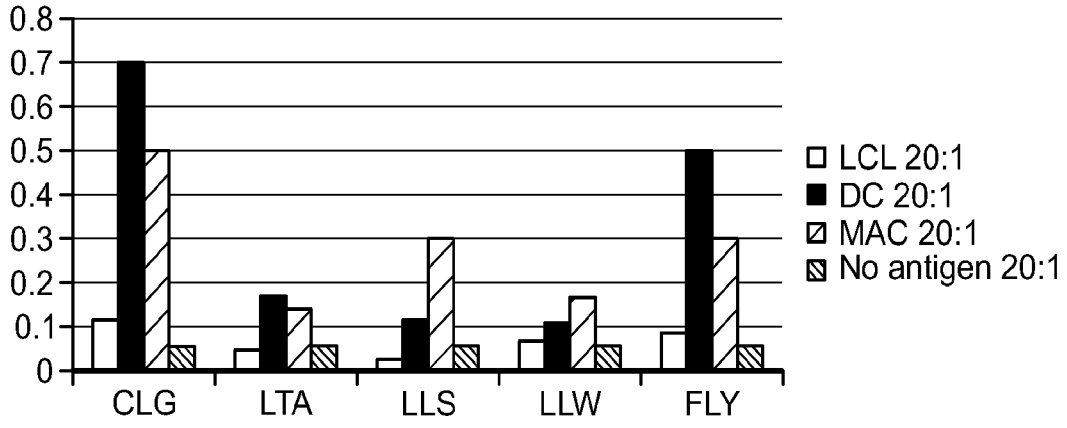


FIG. 2

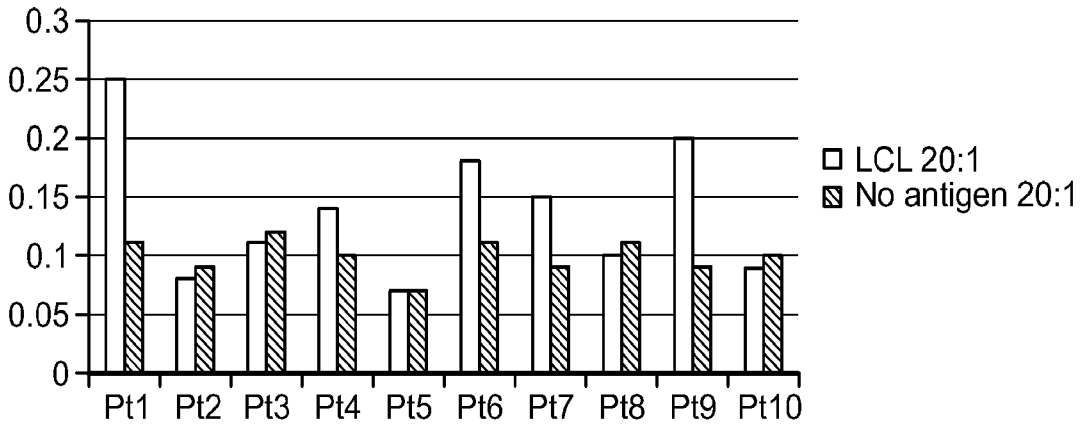


FIG. 3A

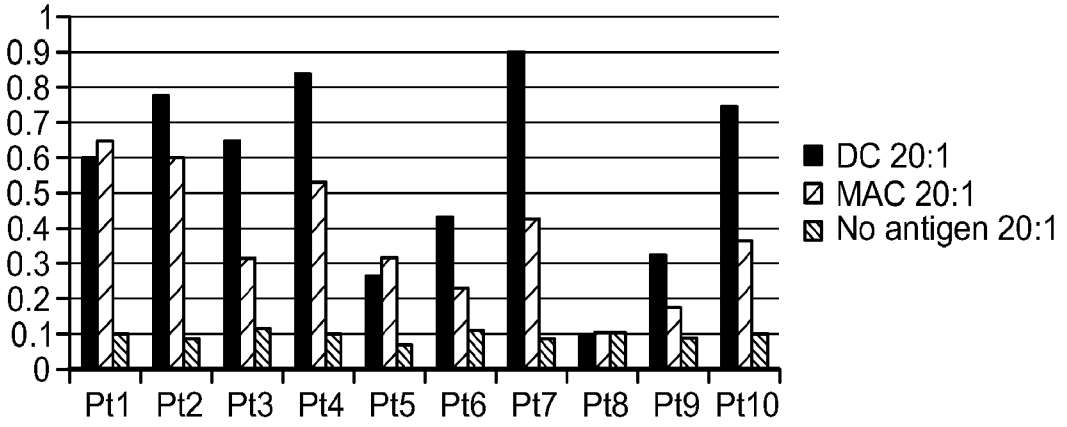


FIG. 3B

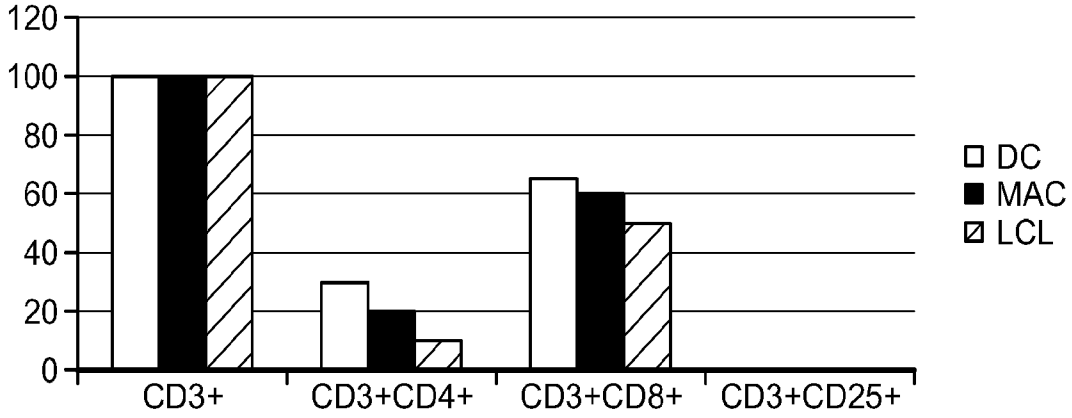


FIG. 3C

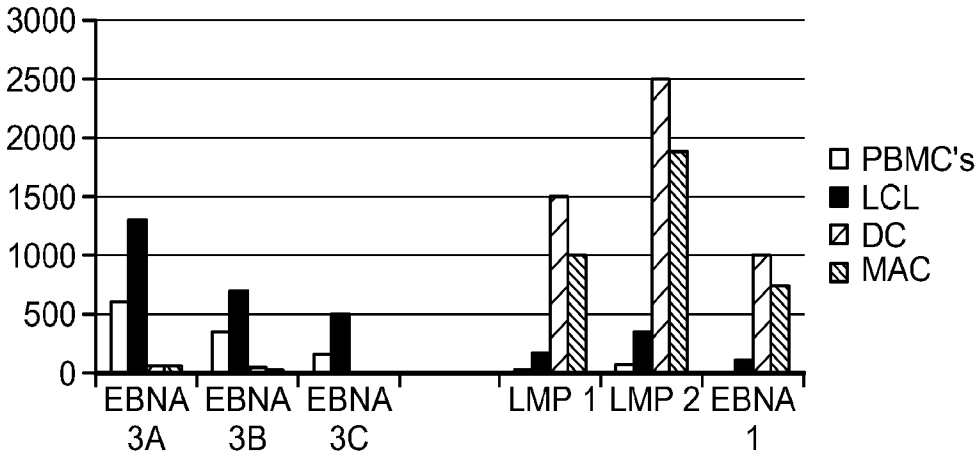


FIG. 4

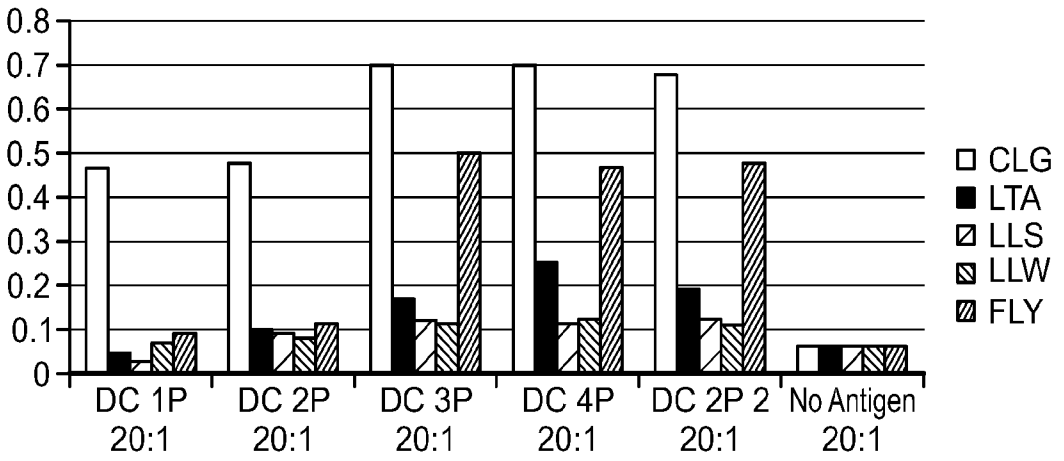


FIG. 5

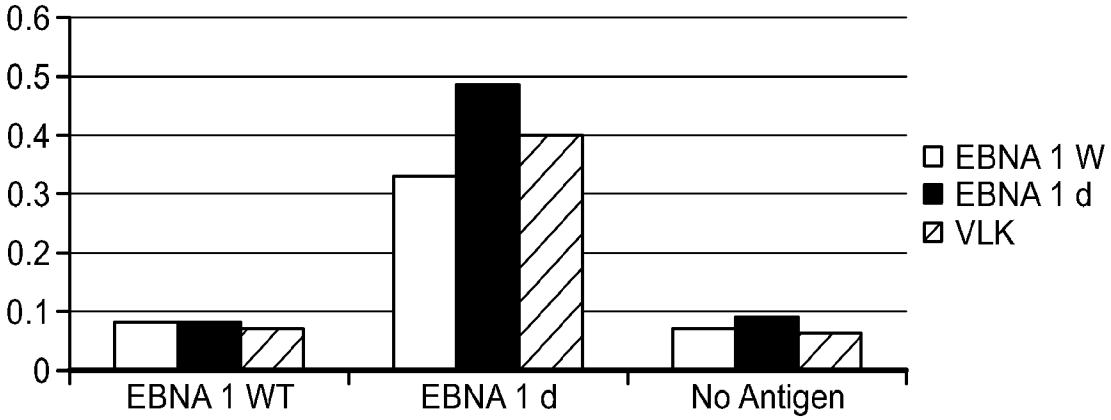


FIG. 6A

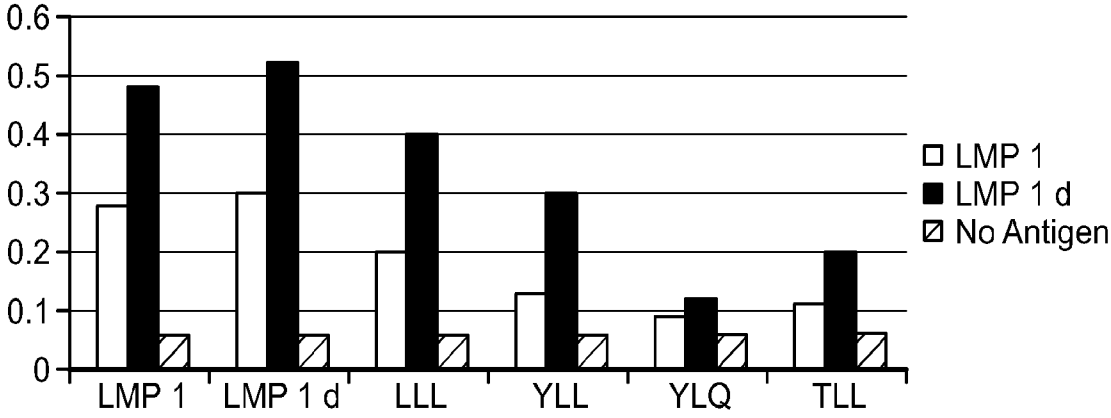


FIG. 6B

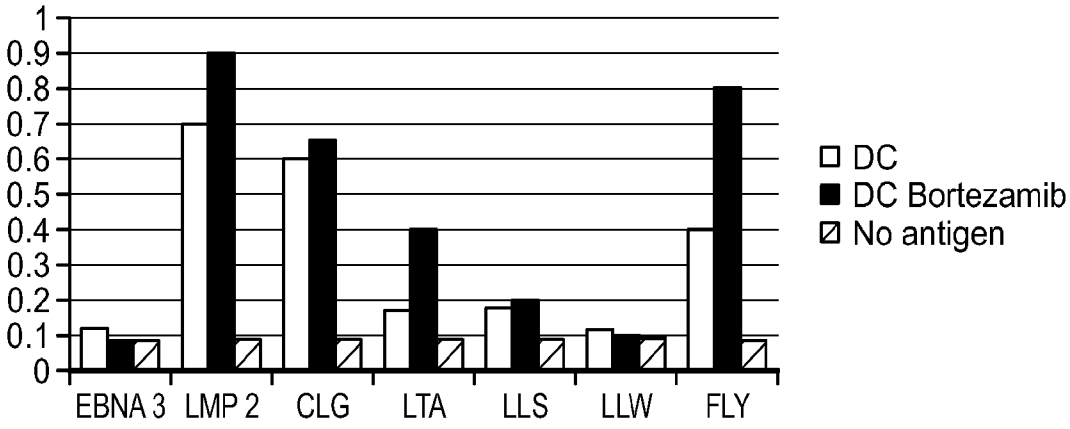


FIG. 7

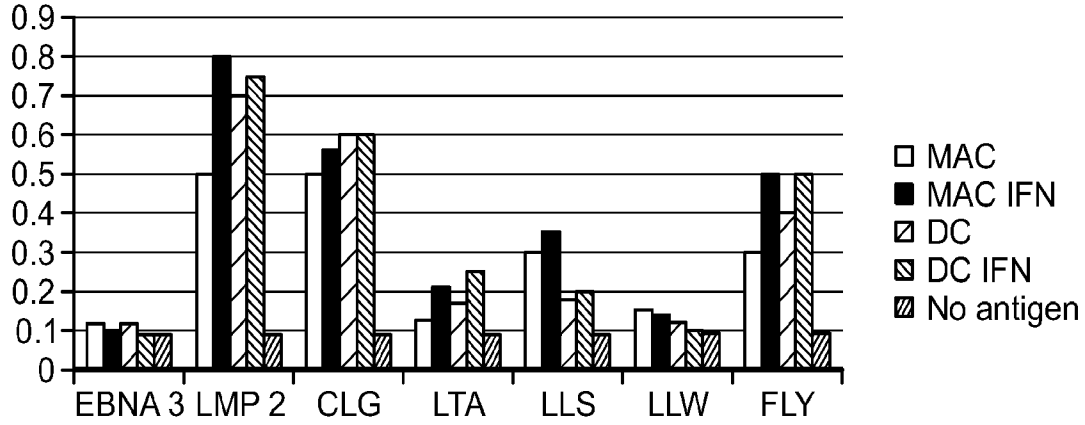


FIG. 8

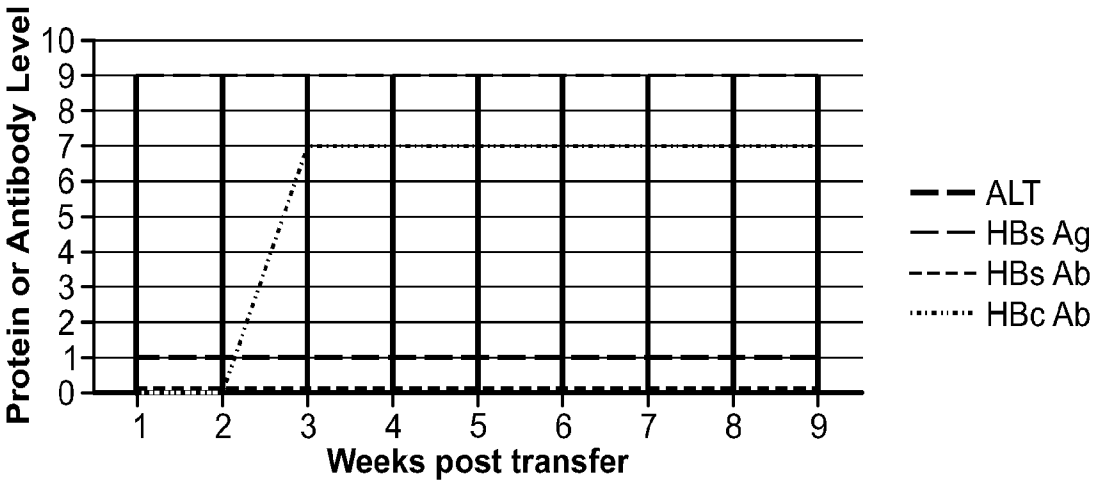


FIG. 9

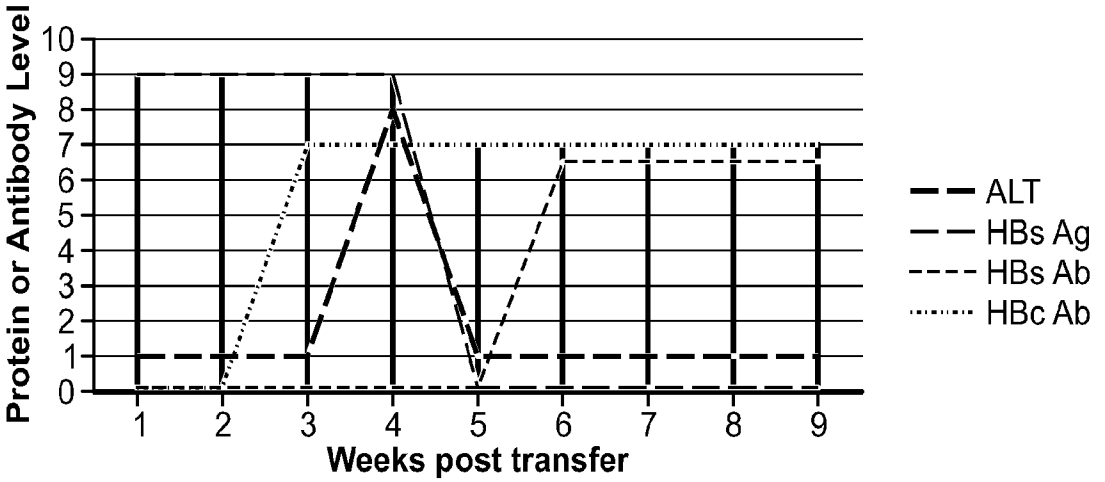


FIG. 10

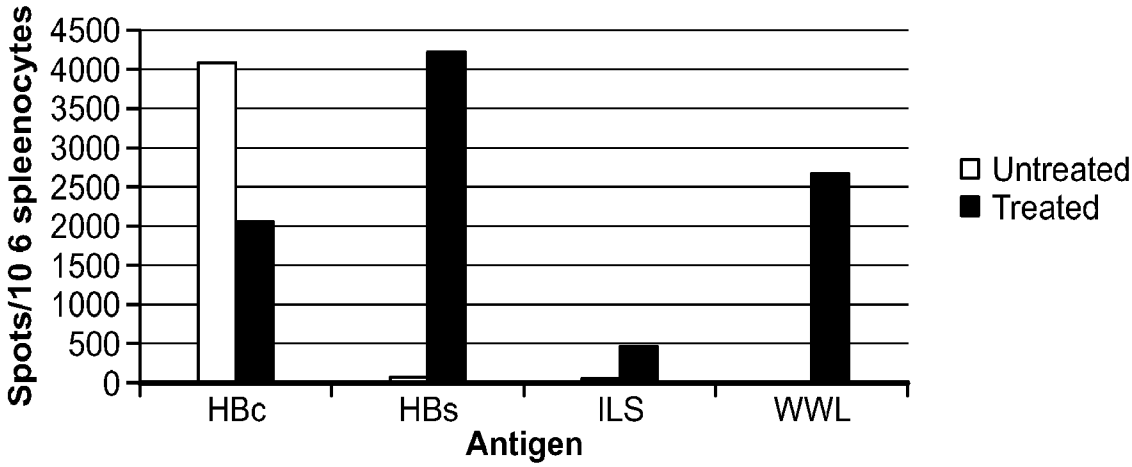


FIG. 11

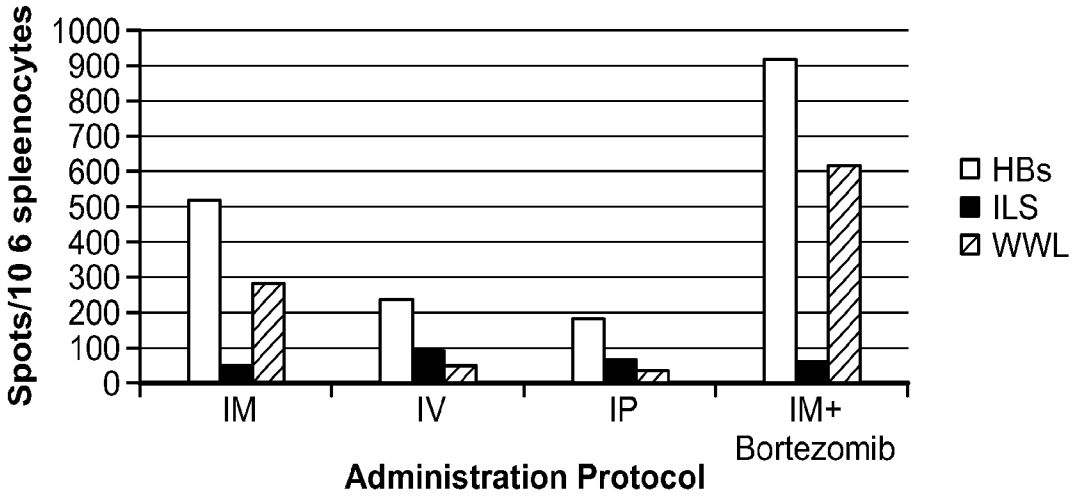


FIG. 12

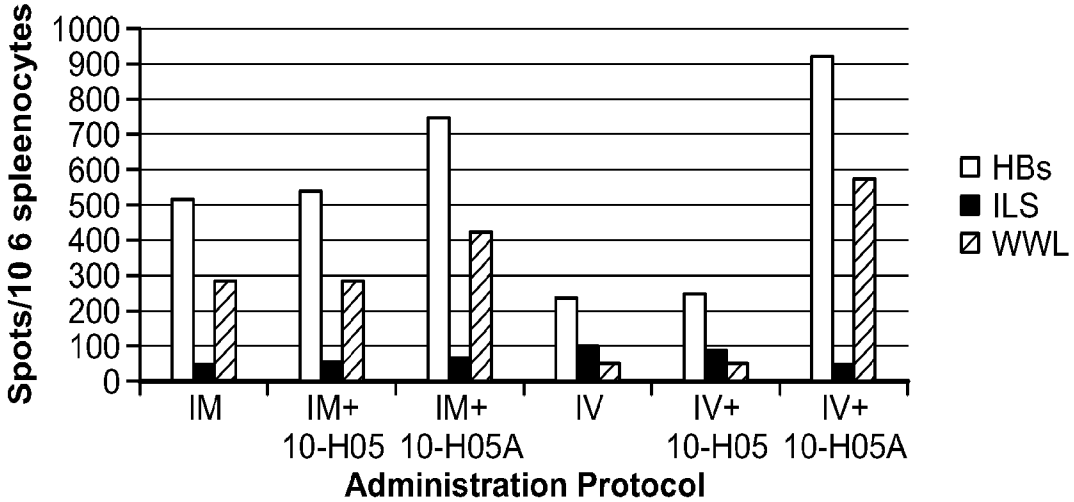


FIG. 13

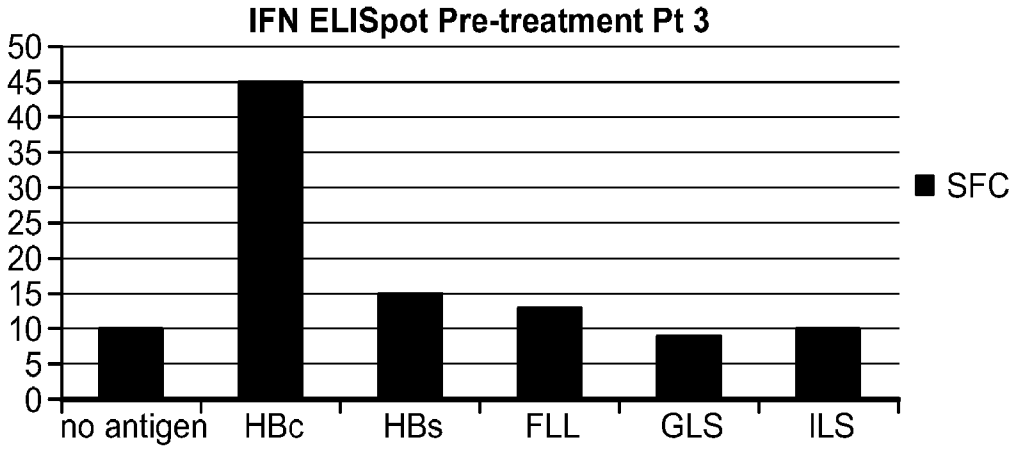

IFN ELISpot Pre-treatment Pt 3
50
45
40
35
30
25
20
15
10
5
0
SFC
no antigen
HBc
HBs
FLL
GLS
ILS

FIG. 14

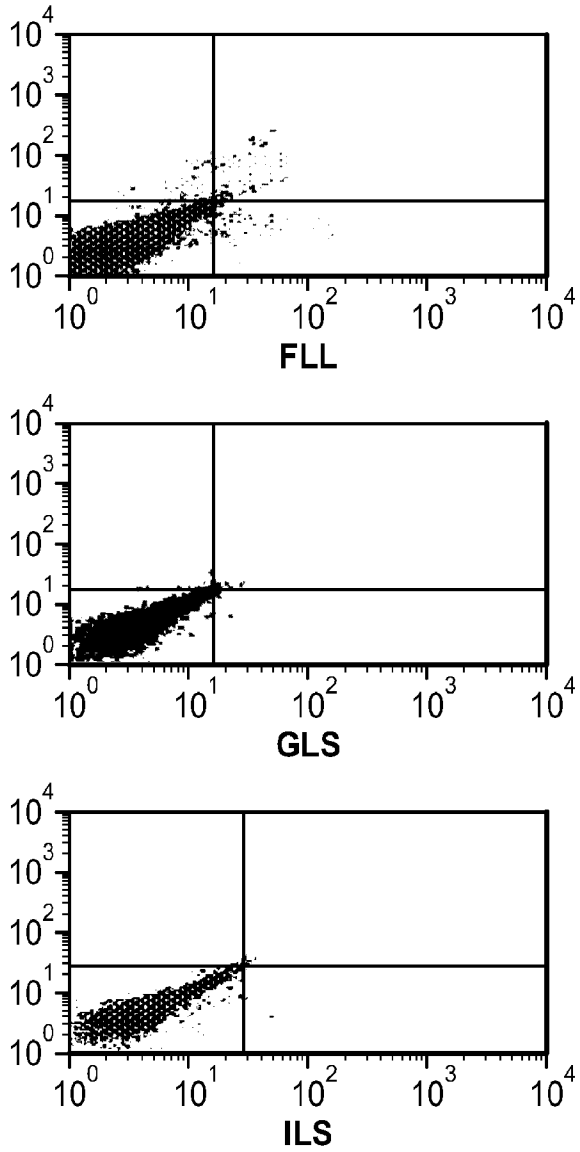

FLL
GLS
ILS

FIG. 15

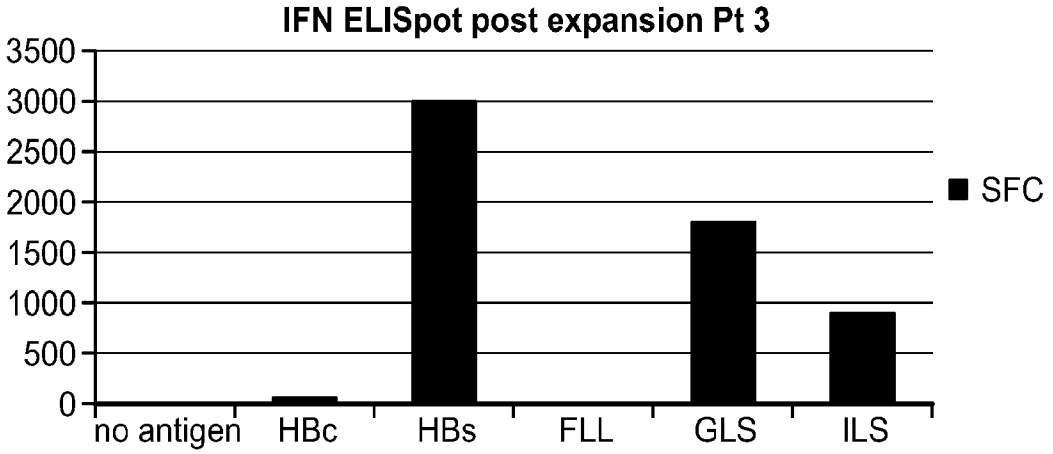


FIG. 16

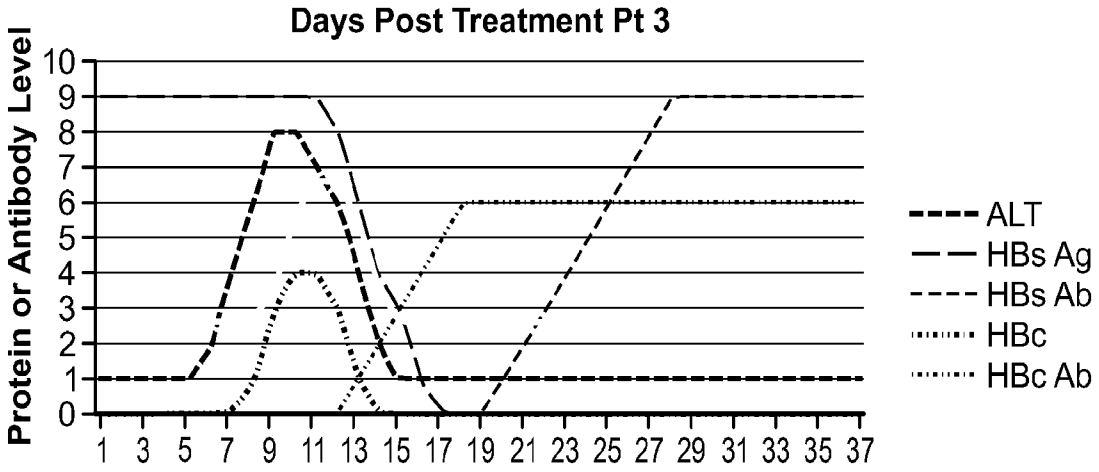


FIG. 17

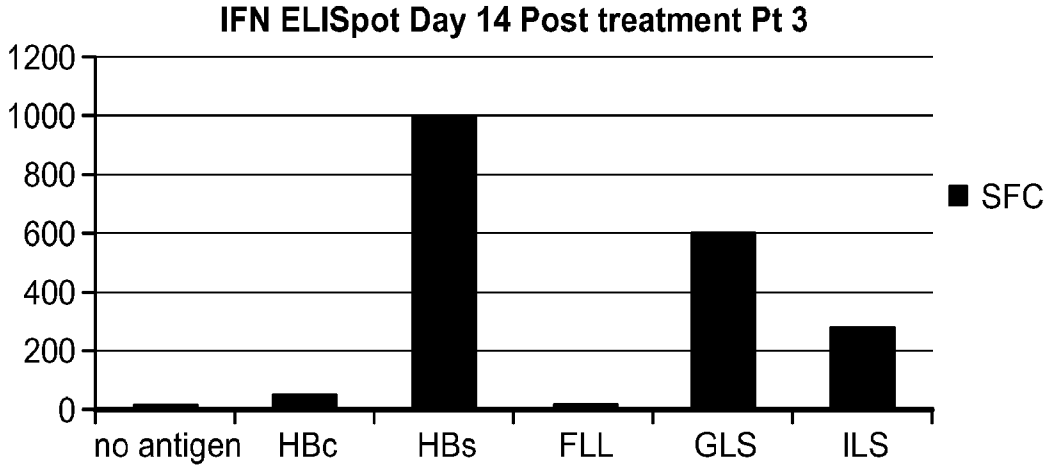


FIG. 18

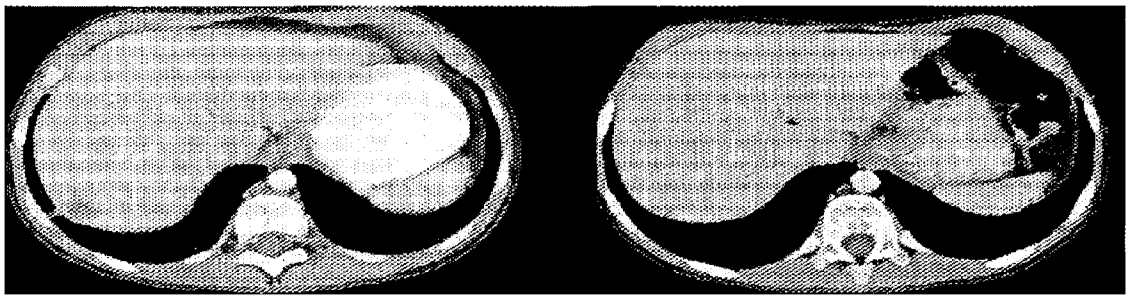


FIG. 19

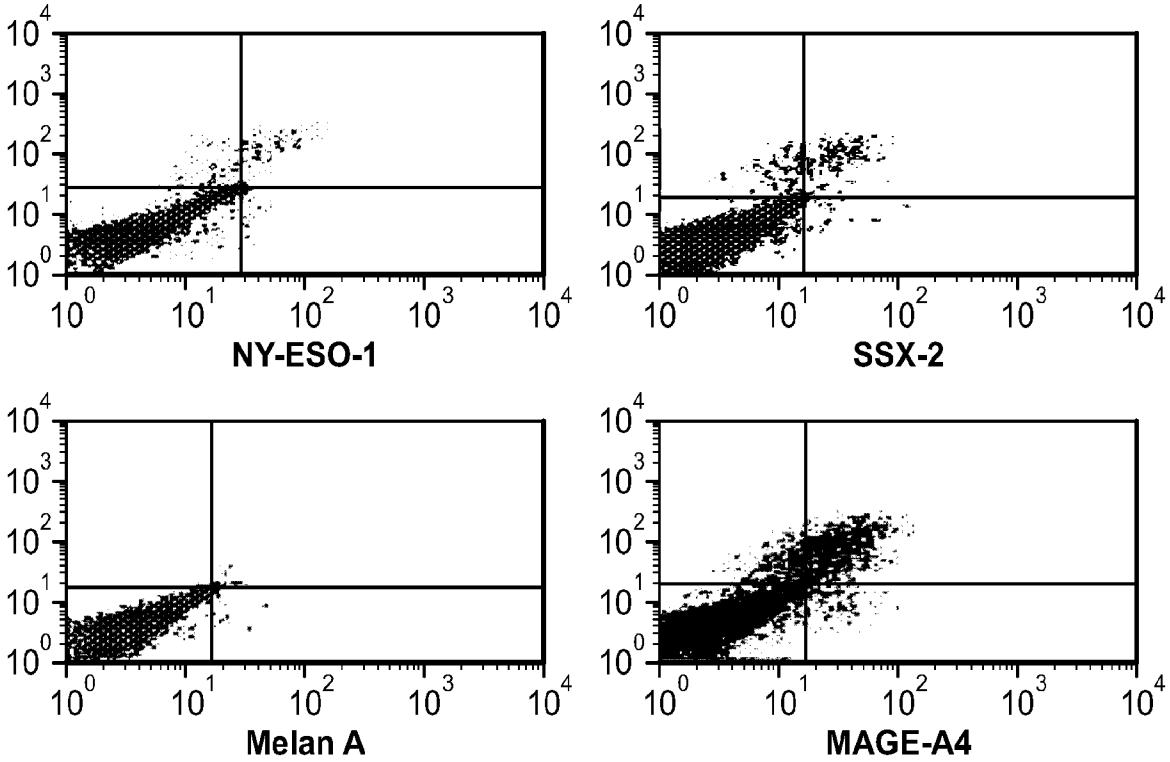


FIG. 20

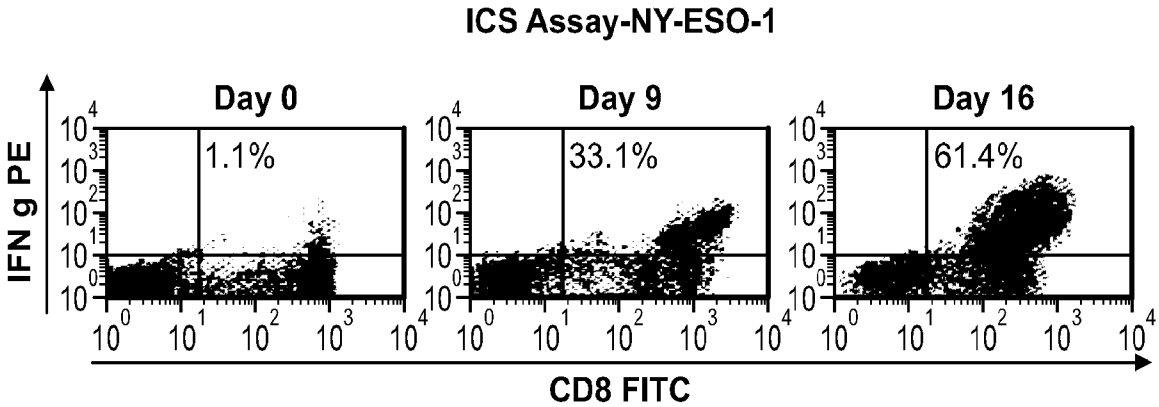


FIG. 21

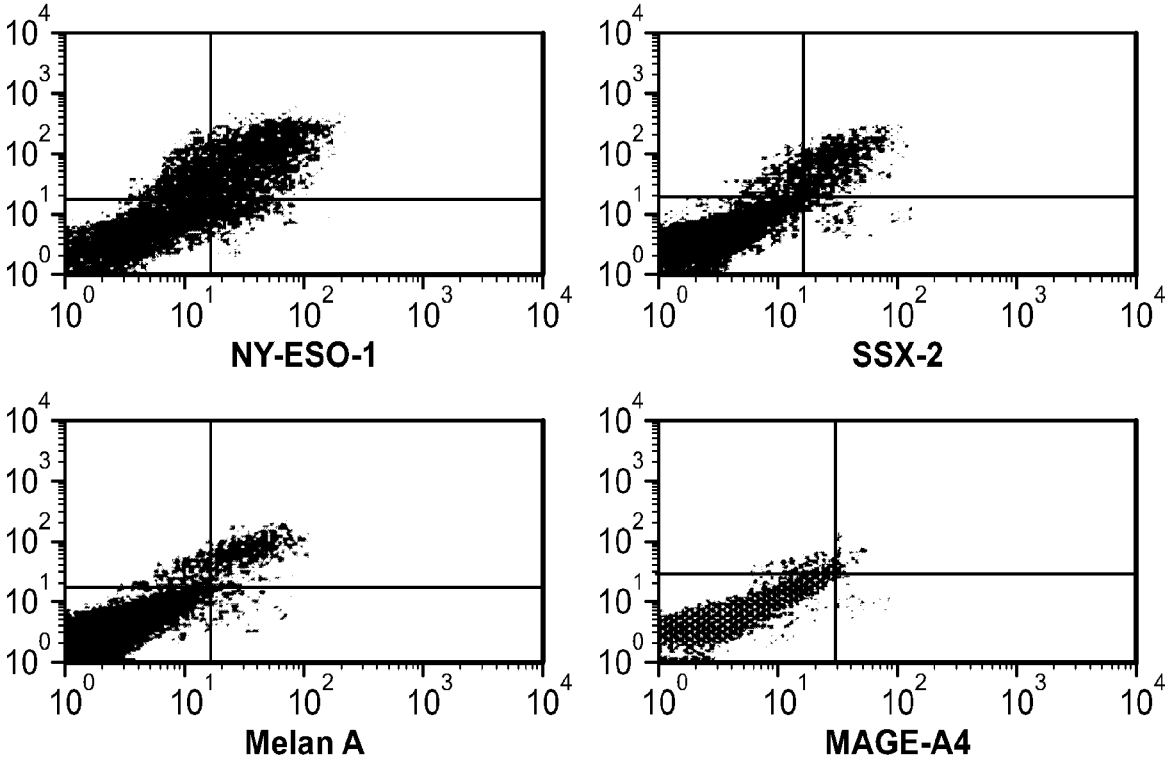

NY-ESO-1
SSX-2
Melan A
MAGE-A4

FIG. 22

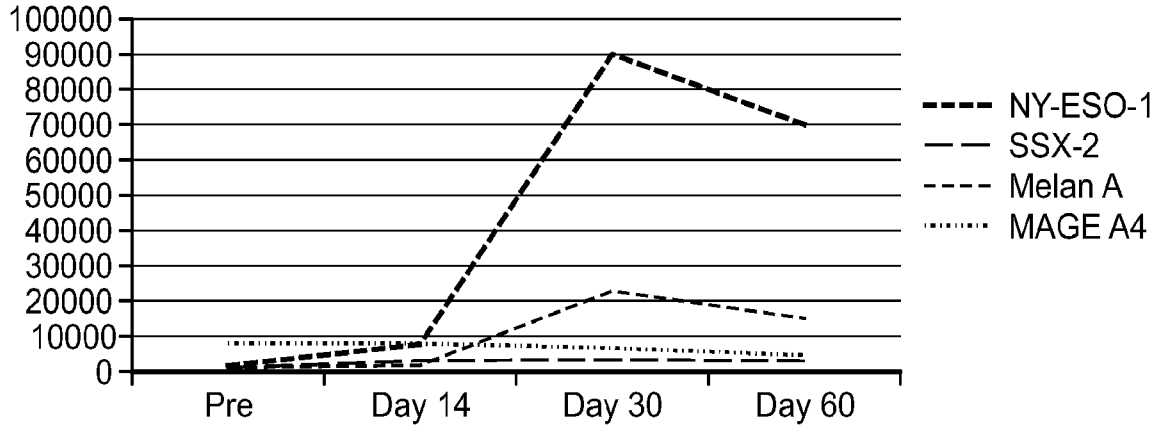
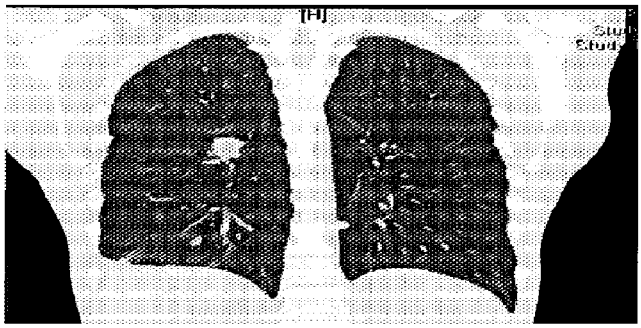
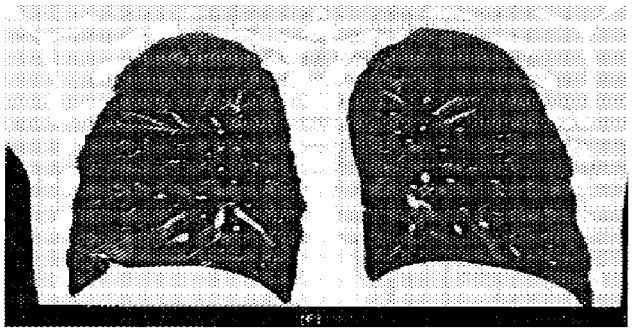

100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
Pre
Day 14
Day 30
Day 60
NY-ESO-1
SSX-2
Melan A
MAGE A4

FIG. 23



Before



6 Mo. Post T Cell Therapy

FIG. 24

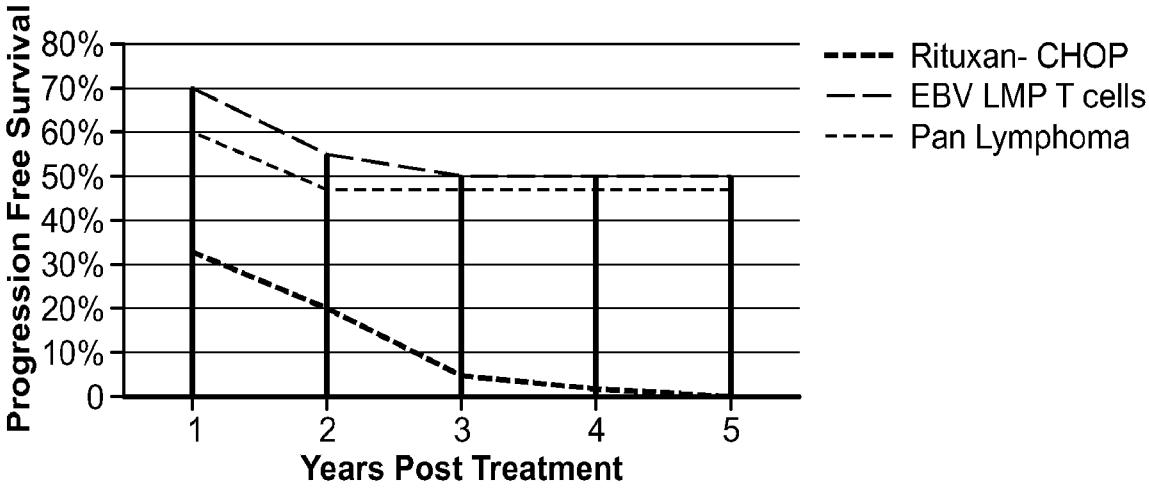


FIG. 25

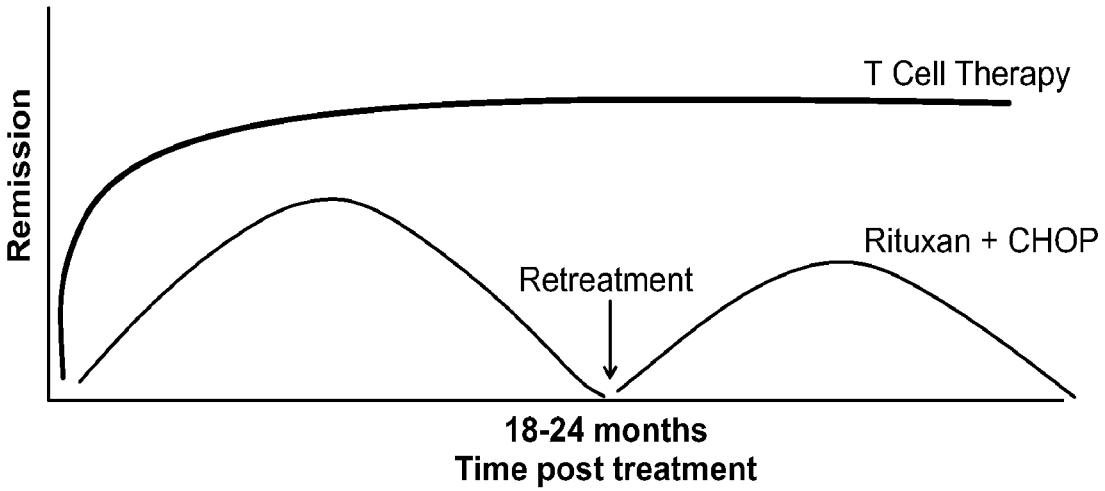

T Cell Therapy
Remission
Rituxan + CHOP
Retreatment
18-24 months
Time post treatment

FIG. 26

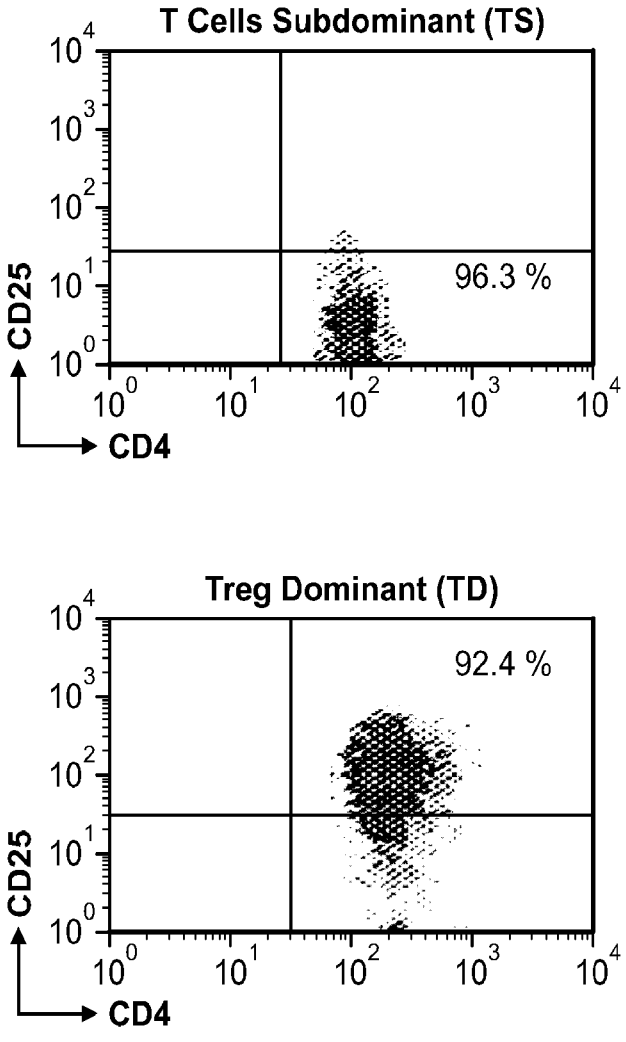

T Cells Subdominant (TS)
96.3 %
CD25
CD4
Treg Dominant (TD)
92.4 %
CD25
CD4

FIG. 27

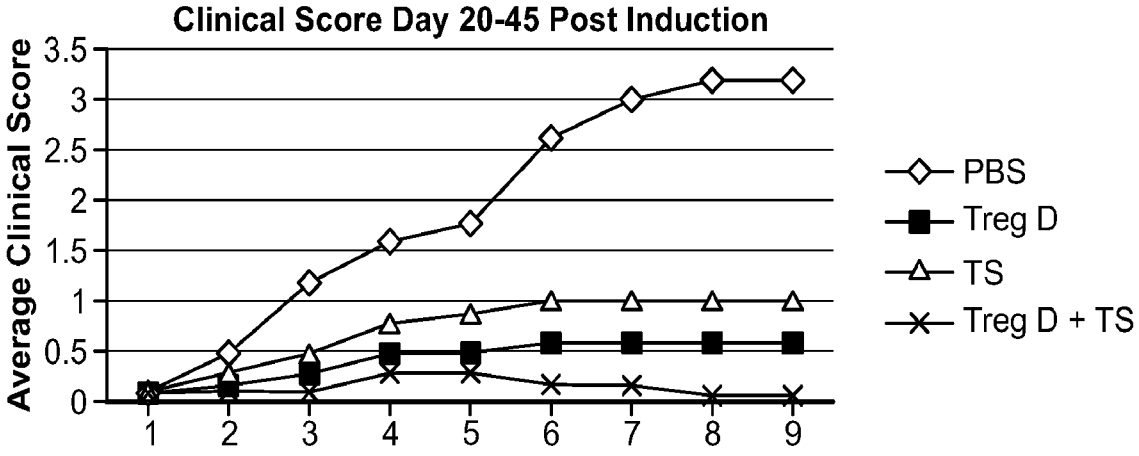

Clinical Score Day 20-45 Post Induction
Average Clinical Score
3.5
3
2.5
2
1.5
1
0.5
0
1
2
3
4
5
6
7
8
9
PBS
Treg D
TS
Treg D + TS

FIG. 28

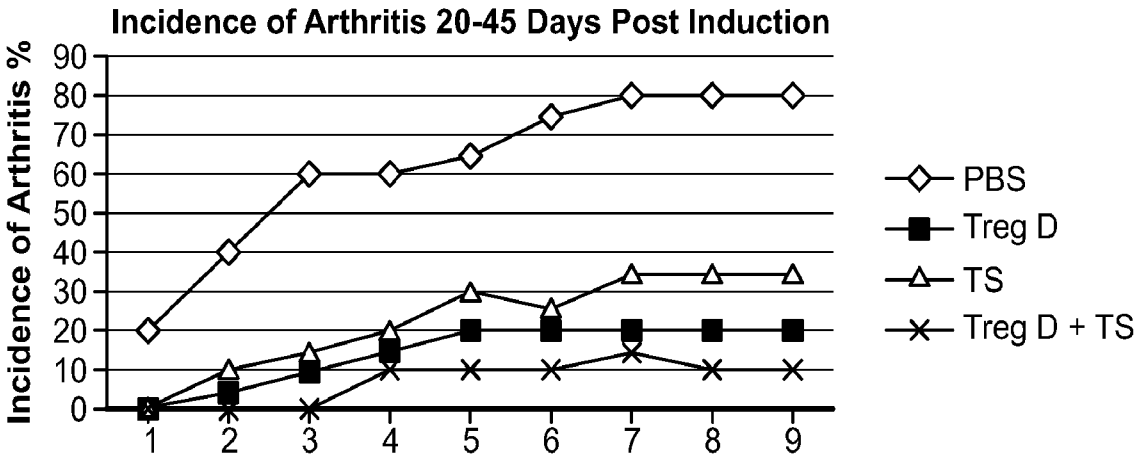
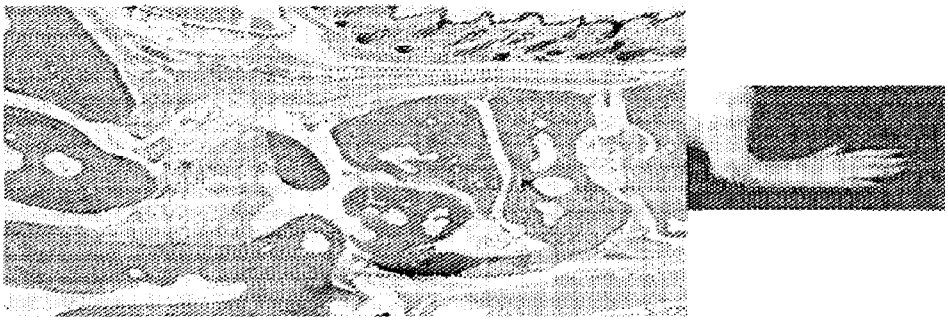
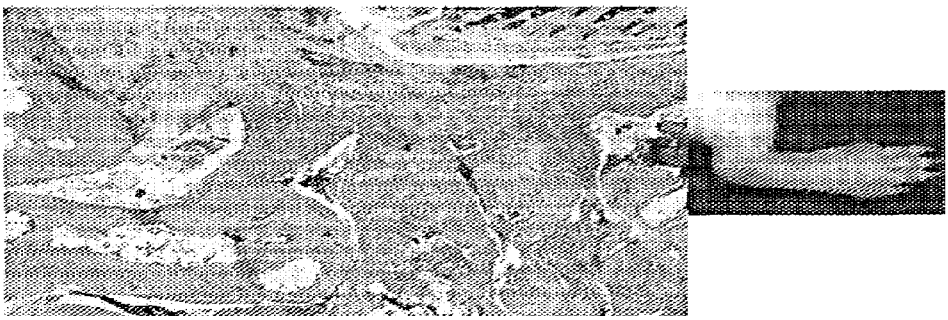

Incidence of Arthritis 20-45 Days Post Induction
Incidence of Arthritis %
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
PBS
Treg D
TS
Treg D + TS

FIG. 29



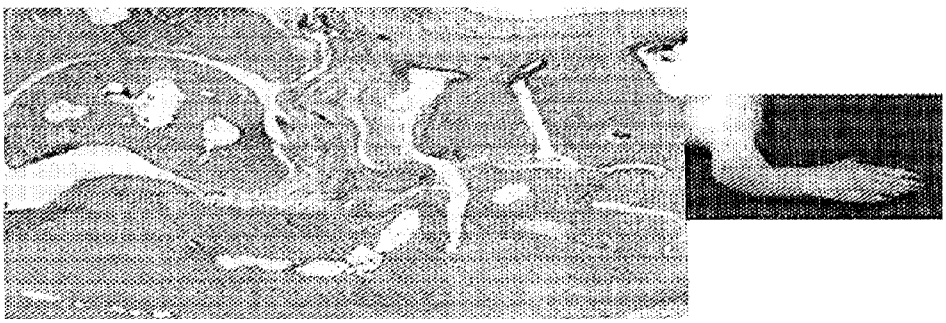
Normal

FIG. 30A



Immunized with human proteoglycan

FIG. 30B



Treated with T cells

FIG. 30C

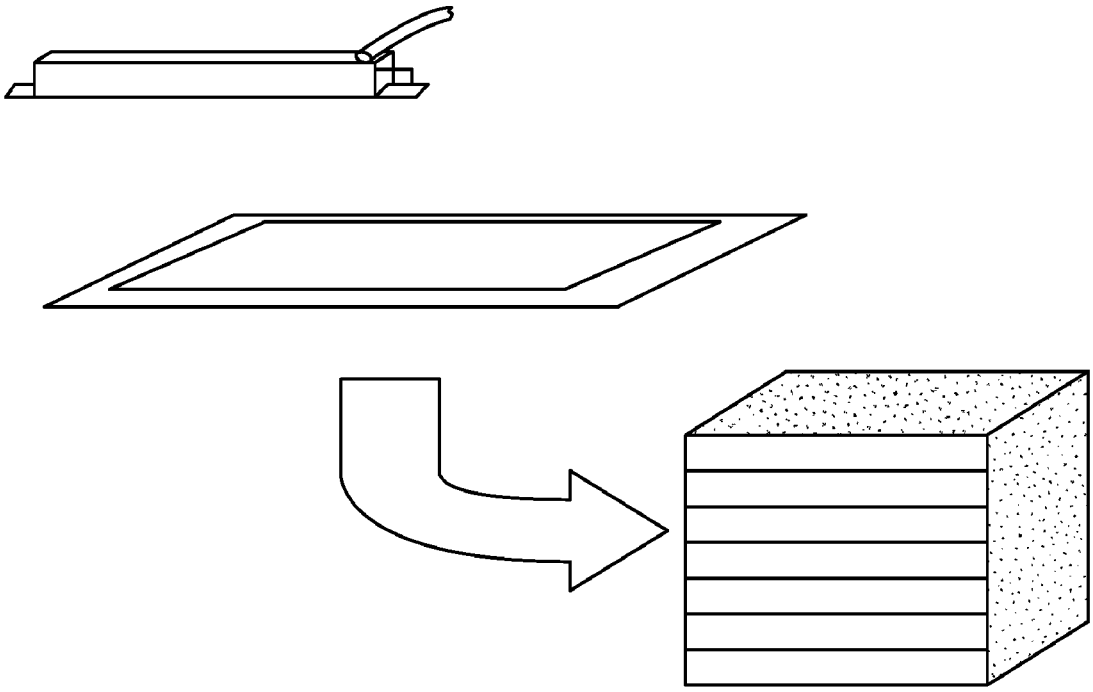


FIG. 31

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2012/039605

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/00 A61K39/245 A61K39/29
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SUBKLEWE MARION ET AL: "Dendritic cells expand Epstein Barr virus specific CD8+ T cell responses more efficiently than EBV transformed B cells.", HUMAN IMMUNOLOGY SEP 2005 LNKD-PUBMED:16360833, vol. 66, no. 9, September 2005 (2005-09), pages 938-949, XP27857154, ISSN: 0198-8859 the whole document ----- -/--	1-19

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 November 2012	15/11/2012

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

1

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

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HASAN AISHA N ET AL: "A panel of artificial APCs expressing prevalent HLA alleles permits generation of cytotoxic T cells specific for both dominant and subdominant viral epitopes for adoptive therapy.", JOURNAL OF IMMUNOLOGY (BALTIMORE, MD. : 1950) 15 AUG 2009 LNKD- PUBMED:19635907, vol. 183, no. 4, 15 August 2009 (2009-08-15), pages 2837-2850, XP55042907, ISSN: 1550-6606 the whole document	1-19
X	GOTTSCHALK STEPHEN ET AL: "Generating CTLs against the subdominant Epstein-Barr virus LMP1 antigen for the adoptive immunotherapy of EBV-associated malignancies.", BLOOD 1 MAR 2003 LNKD- PUBMED:12411306, vol. 101, no. 5, 1 March 2003 (2003-03-01) , pages 1905-1912, XP3011971, ISSN: 0006-4971 the whole document	1-19
X	BOLLARD CATHERINE M ET AL: "Complete responses of relapsed lymphoma following genetic modification of tumor-antigen presenting cells and T-lymphocyte transfer.", BLOOD 15 OCT 2007 LNKD- PUBMED:17609424, vol. 110, no. 8, 15 October 2007 (2007-10-15), pages 2838-2845, XP55042942, ISSN: 0006-4971 the whole document	1-19

(19)中华人民共和国国家知识产权局

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代理人 杜艳玲 李进
(51)Int.Cl.
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A61K 39/245(2006.01)
A61K 39/29(2006.01)

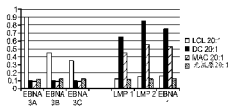
权利要求书2页 说明书25页 附图21页

(54)发明名称

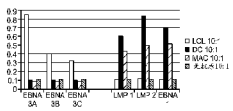
调节的免疫优势疗法

(57)摘要

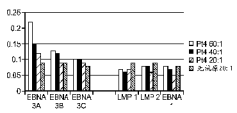
本发明涉及产生针对亚优势抗原的T细胞应答和使用细胞来治疗性改变细胞稳态和免疫应答的性质。在优选的实施方案中，细胞在患者体外产生，以避免患者的免疫环境的影响。通过在组织培养中将来自患者的T细胞针对一种或多种亚优势抗原刺激和培养，并将它们移植入患者中，如果扩增并移植了足够的细胞，则在破坏或诱导免疫耐受或另外修饰针对表达该抗原的细胞或生物的免疫应答的应答中，移植的细胞压倒了内源优势T细胞。当建立记忆细胞时，它们随后反映了该新的免疫优势等级，从而使所需的治疗效果长期持续。实际上，对亚优势抗原具有反应性的外源产生的T细胞移植重现引发并重新平衡患者的靶向细胞或生物中之前亚优势抗原的免疫应答，以产生治疗益处。



A



B



C

CN 103906531 A

1. 能够识别患者中亚优势抗原或表位的 T 细胞，其用于改变患者的免疫优势等级的方法中。

2. 权利要求 1 的 T 细胞，其中所述 T 细胞通过以下来获得：

鉴定获自所述患者的样品中至少一个亚优势抗原或表位，和

培养能够识别所述亚优势抗原或表位的 T 细胞。

3. 权利要求 1 或 2 的 T 细胞，其中改变免疫优势等级治疗 / 预防 / 减缓所述患者中的感染、癌症、炎症、器官移植排斥或移植物抗宿主病。

4. 能够识别患者中亚优势抗原或表位的 T 细胞用于制造改变所述患者的免疫优势等级的药物中的用途。

5. 权利要求 4 的用途，其中所述 T 细胞通过以下来获得：

鉴定获自所述患者的样品中至少一个亚优势抗原或表位，和

培养能够识别所述亚优势抗原或表位的 T 细胞。

6. 权利要求 4 或 5 的用途，其中改变免疫优势等级治疗 / 预防 / 减缓所述患者中的感染、癌症、炎症、器官移植排斥或移植物抗宿主病。

7. T 细胞群，其用于改变患者的免疫优势等级，所述 T 细胞群通过鉴定获自所述患者的样品中至少一个亚优势抗原或表位并且培养能够识别所述亚优势抗原或表位的 T 细胞来获得。

8. 权利要求 7 的 T 细胞群，其用于治疗。

9. 方法，其包括步骤：

鉴定患者样品中的优势抗原或表位及亚优势抗原或表位；

培养能够识别所述亚优势抗原或表位的 T 细胞；和

用治疗有效量的所述 T 细胞治疗患者，以改变所述患者的免疫优势等级。

10. 方法，其包括步骤：

鉴定患者样品中至少一个亚优势抗原或表位；

培养能够识别所述亚优势抗原或表位的 T 细胞；和

用治疗有效量的所述 T 细胞治疗患者，以改变所述患者的免疫优势等级。

11. 权利要求 9 或 10 的方法，其中所述亚优势抗原或表位是对其无法检测到细胞或体液免疫应答或者仅能以低水平检测到的抗原或表位。

12. 权利要求 9 或 10 的方法，其中所述亚优势抗原是病毒抗原、其他传染物抗原、肿瘤抗原、或者与自身免疫、变态反应、炎症、器官移植排斥或移植物抗宿主病相关的抗原。

13. 权利要求 9 或 10 的方法，其中步骤 (b) 进一步包括在优势抗原或表位存在的情况下培养 T 细胞。

14. 权利要求 9 或 10 的方法，其中步骤 (b) 进一步包括在用于富集抑制性 T 细胞或应答性 T 细胞的试剂存在或不存在的情况下培养 T 细胞。

15. 权利要求 9 或 10 的方法，其中步骤 (c) 进一步包括经皮内施用来施用有效数目的 T 细胞。

16. 权利要求 9 或 10 的方法，其进一步包括在步骤 (c) 之前的步骤，其中用调节剂预治疗所述患者以降低内源 T 细胞的数目。

17. 权利要求 9 或 10 的方法，其中所述 T 细胞是抑制性 T 细胞，并且其中步骤 (c) 进一

步包括在所述患者中诱导耐受以治疗或预防自身免疫疾病、变态反应、炎症、器官移植排斥或移植物抗宿主病。

18. 权利要求 9 或 10 的方法，其中所述 T 细胞是应答性 T 细胞，并且其中步骤(c)进一步包括诱导细胞毒性免疫应答以治疗或预防感染或癌症。

19. 权利要求 9 或 10 的方法，进一步包括对耐受或针对亚优势抗原或表位的体液或细胞免疫应答进行概况分析，以确定所述治疗是否成功地将所述患者的免疫应答重新平衡。

调节的免疫优势疗法

[0001] 相关申请

本申请涉及在 2011 年 5 月 26 日提交的美国临时申请序号 61/490,505,其内容通过引用整体并入本文。

[0002] 发明背景

本发明涉及基于免疫优势等级(hierarch)的修饰通过细胞的稳态的治疗性操作,用于癌症、慢性感染、自体免疫疾病和的移植的新疗法。

[0003] 癌症的临床管理对于各种起源位置是特异性的,并且最重要的是依赖于疾病的阶段(即,肿瘤已经在何种程度上局部侵袭或通过转移传播至其他器官)。通常,手术和/或局部放疗是用于原发肿瘤的治疗选择,而化疗、单克隆抗体或细胞因子疗法或全身照射是用于转移疾病的治疗。最近,首个树突细胞疗法,Provenge,被批准用于前列腺癌,其具有延缓进展 4 个月的益处。诊断仍基于活组织检查的组织学分析。分子标志物有时是标准,如果它们将有助于药物(例如,赫赛汀)的选择。然而,尚未在常规临床基础上完成免疫应答概况的生成。

[0004] 在初次免疫应答期间,T 细胞看到抗原的环境决定了后续回忆应答的性质。初始识别事件和该初级细胞周边的微环境可以产生很多结果。如果抗原通过非专业 APC 呈递,在加工期间仅可释放表位的子集。如果共刺激信号,例如 CD28 或 TNF 丢失,则 T 细胞可能不做出反应。取决于微环境,T 细胞可分化成调节性 T 细胞、分泌 Th1 细胞因子的 T 辅助细胞(驱动更多的具有 $CD8^+$ CTL 效应物的增殖的细胞免疫应答),或分泌 Th2 细胞因子的 T 辅助细胞(驱动更多的具有 B 细胞的增殖和成熟以及抗体产生的体液免疫应答)。此外,免疫应答将发展,从而使对环境中的抗原的某些表位或某些抗原做出应答的 T 细胞生长,这是以牺牲群体中对环境中的其他表位或抗原为反应性的 T 细胞为代价的。因为指数型细胞生长,随着初次免疫应答的减退,这些 T 细胞的比例进一步增强并以记忆的形式贮存,使得在二次刺激后,个体内的免疫应答集中在可能表位的小子集上。尽管有多种机制发挥作用,作为有效的模型,生长成在群体中占优势的 T 细胞是对成为支配免疫应答的表位或抗原做出应答的那些。在初次免疫应答中,它们以牺牲应答亚优势表位的 T 细胞为代价进行生长,并且,由于记忆作用而支配后续的免疫应答。

[0005] 在个体的免疫系统首次发现抗原后数天的时期中,产生出应答有限数量的优势表位的 T 细胞的优势群体,并且这些 T 细胞决定了此后对该抗原的应答的性质。尽管有多种类型的细胞参与,但与本发明相关的工作模型是,如果对优势抗原上的优势表位做出应答的 T 细胞生长成为反应性 T 细胞(例如,$CD4^+$:TH1、TH2、Treg、滤泡 T 辅助细胞、TH17、TH22、TH9 ;$CD8^+$ CTL),则产生细胞或体液免疫应答。然而,如果在优势群体中的 T 细胞是抑制性 T 细胞(例如,Treg、TH17、无反应 T 细胞),则诱导耐受。对亚优势抗原做出应答的 T 细胞被对优势抗原做出应答的 T 细胞的克隆群体淹没。

[0006] 在癌症、慢性或潜伏感染的情况下,局部抗原加工/呈递和共刺激环境影响对优势抗原的初次免疫应答,使得对肿瘤或传染物中的优势抗原的 T 细胞应答的平衡趋向于耐受或无效应答,而不是强的效应物应答。由于抗原加工和共刺激 (CD28 和细胞因子)中的

差异，这会在其中树突细胞 (DCs) 不是普遍的抗原呈递细胞 (APCs)（这不同于其中树突细胞是主要的抗原呈递细胞的身体表面）的器官中加重。还熟知，肿瘤和传染物产生的免疫抑制性环境对于强的初次免疫应答不是最佳的。或者，如果在细胞中产生的优势抗原对与其本身反应，则耐受性被破坏且继而发生自身免疫性。这样的耐受性可被存在的导致应答（对弱的亚优势抗原乃至是慢性的）的慢性或潜伏病毒破坏。病毒与自身免疫性有多重联系。在所述部位的炎症导致其他抗原的释放，同时病毒抗原为应答所述器官的 T 细胞提供帮助，导致自身免疫性。在初次应答中确立优势等级（hierarchy）且通过记忆强化后，患者中的免疫系统将在每次抗原存在时有效地复制相同的应答。

[0007] 正在进行的针对优势表位的免疫应答能够减少对亚优势表位的应答 (Wolpert EZ 1998, Kedl RM 2003)。优势 / 亚优势等级在一定程度上是可改变的。例如，消除或沉默针对优势表位的 T 细胞应答，可以导致出现此前无法检测的针对亚优势表位的应答 (VanderMost RG 等人，1997, Andreansky SS 等人，2005)。类似地，删除表位中的优势序列没有消除对所述抗原的应答，而是导致宿主对此前的亚优势表位的更强应答 (Allan JE and Doherty PC 1985, Mylin LM 等人，2000)。

发明内容

[0008] 本发明涉及基于免疫优势等级的修饰通过细胞的稳态的治疗性操作，用于癌症、慢性感染、自体免疫疾病和移植的新疗法。

[0009] 本发明公开了用于重新平衡对抗原的免疫应答的新方法，以特别是在癌症、慢性和潜伏感染、自身免疫性和移植中提供显著的治疗益处。通过对受控微环境中的亚优势表位和亚优势抗原产生免疫应答，本发明从根本上将对疾病的免疫应答的性质改变为提供治疗益处的那种。它能够在对抗原已经发生的在先免疫应答之前或之后，或者甚至在免疫应答正在进行的情况下，改变免疫应答的平衡。

[0010] 本发明的特征在于一种方法，其包括鉴定在患者样品中的优势抗原或表位和亚优势抗原或表位，培养能够识别所述亚优势抗原或表位的 T 细胞，和使用有效数量的 T 细胞治疗患者以改变所述患者的免疫优势等级。

[0011] 本发明的特征还在于改变患者的免疫优势等级的方法，其包括鉴定患者样品中的至少一种亚优势抗原或表位，培养能够识别所述亚优势抗原或表位的 T 细胞，和使用有效数量的那些 T 细胞治疗所述患者以提供治疗益处。

[0012] 在一些方面，本发明进一步包括在不存在优势抗原或表位的情况下培养 T 细胞。在其他方面，本发明进一步包括在不存在或存在富集抑制性 T 细胞或应答性 T 细胞的试剂的情况下培养 T 细胞。此类试剂抗原可以，但不限于，生长因子、激素或其他免疫细胞。

[0013] 在一些方面，本发明进一步包括通过皮内施用来施用有效量的 T 细胞。

[0014] 在其他方面，本发明进一步包括在使用培养的 T 细胞治疗患者之前，使用调节剂预先治疗所述患者以减少内源 T 细胞的数量。所述调节剂可以是，但不限于化疗剂。

[0015] 在一些方面，所述 T 细胞从患者先体外后体内 (*ex vivo*) 提供。

[0016] 所述亚优势抗原或亚优势表位是，例如，针对该抗原或表位的细胞或体液免疫应答不可检测或仅低水平可测的抗原或表位。或者，所述亚优势抗原或亚优势表位是与优势抗原或优势表位相比，激发较弱的耐受或免疫应答的抗原或表位。所述亚优势抗原是，例

如，病毒抗原、真菌抗原、细菌抗原、寄生虫抗原、朊病毒抗原、肿瘤抗原或与自身免疫性、变态反应、炎症、器官移植排斥或移植物抗宿主病相关的抗原。所述病毒抗原是，例如，慢性或潜伏病毒抗原。所述病毒抗原来自 EBV、HPV、HSV、VZV、乙型肝炎、丙型肝炎、HIV、HTLV、CMV、RSV 或流感。所述肿瘤抗原是，例如，肿瘤相关抗原、肿瘤特异性抗原，或与癌症干细胞或转移相关的抗原。

[0017] 本发明的特征还在于一种方法，其用于鉴定患者样品中的优势抗原或表位和 / 或亚优势抗原或表位，培养能够识别所述亚优势抗原或表位的 T 细胞，其中所述 T 细胞是抑制性 T 细胞，和使用有效量的所述 T 细胞治疗患者以改变所述患者的免疫优势等级，由此在所述患者中诱导耐受性以治疗或预防自体免疫疾病、变态反应、炎症、器官移植排斥或移植物抗宿主病。

[0018] 本发明的特征还在于一种方法，其用于鉴定患者样品中的优势抗原或表位和 / 或亚优势抗原或表位，培养能够识别所述亚优势抗原或表位的 T 细胞，其中所述 T 细胞是应答性 T 细胞，和使用有效量的所述 T 细胞治疗患者以改变所述患者的免疫优势等级，从而在所述患者中诱导细胞毒性免疫反应以治疗或预防感染或癌症。所述感染是，例如，细菌性、病毒性、寄生虫或朊病毒感染。

[0019] 在任意的本发明的方法中，疾病、感染、癌症或医疗状况的治疗或预防包括缓解或改善疾病、感染、癌症或医疗状态的至少一种症状。治疗益处包括疾病、感染、癌症或医疗状态的至少一种症状的任何缓解、改善、改进、预防或治疗。

[0020] 在一些方面，所述患者样品是血液样品。

[0021] 本发明的特征还在于一种方法，其进一步包括通过测定耐受性或对所述亚优势抗原或表位响应的体液或细胞免疫应答而重新确定患者的概况，从而确定所述疗法是否成功地使免疫应答重新平衡。

[0022] 除非另有定义，否则本文使用的所有技术和科学术语具有与本发明所属领域的普通技术人员通常理解相同的含义。下文描述了适合的方法和材料，尽管与本文所述那些类似或等效的方法和材料可以用于本发明的实施。本文提到的所有出版物、专利申请、专利和其他参考文献均明确地通过引用整体并入。在冲突的情况下，以本说明书，包括定义为准。此外，本文所述的材料、方法和实施例仅为说明性并且不意图限定。

[0023] 本发明的其他特征和优点将体现于并包含于下文详细的说明书和权利要求书。

[0024] 附图简述

图 1A、1B 和 1C 显示了 ^{51}Cr 释放测定的结果。

[0025] 图 2 显示了 ^{51}Cr 释放测定的结果。

[0026] 图 3A 和 3B 显示了 ^{51}Cr 释放测定的结果。图 3C 显示了活 APCs 的百分比。

[0027] 图 4 显示了通过 ELISPOT（酶联免疫斑点法）测量的产生 IFNγ 的细胞。

[0028] 图 5、6A、6B、7 和 8 显示了 ^{51}Cr 释放测定的结果。

[0029] 图 9 显示了慢性乙型肝炎的小鼠模型。

[0030] 图 10 显示了小鼠模型的治疗。

[0031] 图 11 显示了对 HBs 和 HBc 的 T 细胞应答。

[0032] 图 12 和 13 显示了通过施用方法的应答。

[0033] 图 14 显示了抗原的等级。

[0034] 图 15 显示了 ICS 的应答。

[0035] 图 16 显示了抗原的等级。

[0036] 图 17 显示了在急性发作(flair),随后清除肝炎后的免疫应答。

[0037] 图 18 显示了在清除之后的抗原的等级。

[0038] 图 19 显示了 T 细胞完全溶解了患者的肝细胞癌(治疗前—左图;治疗后 8 周—右图)。

[0039] 图 20 显示了存在于患者肿瘤中的抗原。

[0040] 图 21 显示了对 NY-ESO-1 抗原的细胞应答。

[0041] 图 22 显示了在根据本发明的治疗后存在的抗原。

[0042] 图 23 显示了免疫优势等级的重新平衡。

[0043] 图 24 是在根据本发明的 T 细胞治疗之前和之后的 CT 扫描。

[0044] 图 25 显示了治疗后的存活,无进展。

[0045] 图 26 显示了通过本发明的治疗与利妥昔单抗 (Rituxan)+ CHOP 的比较。

[0046] 图 27 显示了施用给动物的 T 细胞的表征。

[0047] 图 28 显示了对于试验小鼠的临床疾病得分。

[0048] 图 29 显示了在试验小鼠中的关节炎发生率。

[0049] 图 30A、30B 和 30C 显示了正常大鼠、接种人蛋白聚糖的大鼠和用 T 细胞治疗的大鼠的组织病理学。

[0050] 图 31 是用于本发明的治疗的生物反应器的示意图。

[0051] 发明详述

A. 定义

术语“抗体”是以最广的含义使用,并且具体是涵盖人、非人(例如小鼠)及人源化单克隆抗体(包括全长单克隆抗体)、多克隆抗体、多特异性抗体(例如双特异性抗体)和抗体片段,只要它们显示期望的生物活性。

[0052] 术语“抗原”是指能够刺激抗体产生或动物中的 T 细胞应答的化合物、组合物或物质,包括注射或吸收到动物中的组合物。抗原与特异性体液或细胞免疫的产物(包括由异源免疫原诱导的那些)反应。术语“抗原”包括所有相关的抗原性表位。

[0053] “抗原呈递细胞”或“APCs”是用于向 T 细胞呈递抗原的免疫系统的细胞。APC 包括树突细胞、单核细胞、巨噬细胞、边缘区 Kupffer 细胞、小胶质神经细胞、朗氏细胞、T 细胞和 B 细胞(参见,例如 Rodriguez-Pinto and Moreno (2005) Eur. J. Immunol. 35:1097-1105)。

[0054] “自体免疫”、“自体免疫疾病”、“自体免疫状况”或“自体免疫失调”是指与改变的免疫稳态相关的一组持续性的器官特异性或系统性临床症状或征候,所述改变的免疫稳态通过表达的自体免疫组库 (autoimmune repertoires) 的定性和/或定量缺陷来表现。自体免疫疾病的病理学的表现是由自体免疫应答诱导的结构或功能性破坏的结果。自体免疫疾病的特征在于体液(例如,抗体介导的)、细胞(例如,细胞毒性 T 淋巴细胞介导的),或对自体抗原上的表位的两种类型的免疫应答的组合。受影响的个体的免疫系统活化指向呈递这些特异性自体抗原的细胞和组织的炎性级联。受攻击的抗原、组织、细胞类型或器官的破坏引起疾病的症状。

[0055] 术语“癌症”是指通过不受控的细胞生长表征的疾病或病变。癌症的实例包括，但不限于癌、淋巴瘤、母细胞瘤和肉瘤。具体癌症的实例包括，但不限于，肺癌、结肠癌、乳腺癌、睾丸癌、胃癌、胰腺癌、卵巢癌、肝癌、膀胱癌、结直肠癌和前列腺癌。对于本领域技术人员已知的其他癌症也在考虑之内。

[0056] “优势抗原”或“优势表位”是指激发强的耐受性或免疫应答的抗原或表位，其可表征为对该抗原或表位特异性的 T 细胞的存在量大于应答性 T 细胞的总数的 70%。

[0057] 术语“表位”是指参与具体免疫球蛋白的识别的一组氨基酸残基，或者在 T 细胞的背景下，是指对于 T 细胞受体蛋白和 / 或主要组织相容性复合体 (MHC) 受体的识别所必需的那些残基。在体外或体内的免疫系统的背景中，表位是共同形成由免疫球蛋白、T 细胞受体或 HLA 分子识别的位点的分子的集合的特征，例如初级、二级和三级肽结构和电荷。

[0058] “肝炎”是指由肝的炎症定义的医学状况。

[0059] “人白细胞抗原”或“HLA”是人 I 型或 II 型主要组织相容性复合体 (MHC) 蛋白 (参见，例如Stites等人，IMMUNOLOGY, 8TH ED., Lange Publishing, Los Altos, Calif. (1994))。

[0060] “免疫应答”是指免疫系统的细胞，例如 B 细胞、T 细胞或单核细胞，对刺激物的应答。在一个实施方案中，所述应答对于具体抗原是特异性的 (“抗原特异性应答”)。在一个实施方案中，免疫应答是 T 细胞应答，例如 CD4+ 应答或 CD8+ 应答。在另一个实施方案中，所述应答是 B 细胞应答，并且导致特异性抗体的产生。

[0061] 免疫优势是以下观察结果 :尽管在抗原中有大量的可能的表位 (抗原片段)，但免疫系统将其应答集中于有限数量的表位，并且能够安排为可重现的等级 (Sercarz 等人，1993)。对于人工抗原、人病毒，包括流感和牛痘，以及细胞内细菌的免疫应答均呈免疫优势 ((Chen WS 1994, Belze GT等人，2000, Chen W 2000, Tscharke DC 2005)。免疫优势的最后结果由多个步骤决定，包括 MHC 结合亲和力、细胞加工以产生适当的 MHC 结合肽的效率、识别 MHC 结合肽与 MHC 之间的复合物的 TCRs 的可得性、随后是细胞免疫调节机制 (Yewdell JW 2006, Sette A 等人，2009)。

[0062] “淋巴细胞”是指参与机体的免疫防御的一类白细胞。有两种主要类型的淋巴细胞 :B 细胞和 T 细胞。

[0063] “主要组织相容性复合体”或“MHC”是旨在包含在不同物种中描述的组织相容性抗原系统的通用名称，包括人白细胞抗原 (“HLA”)。

[0064] “亚优势抗原”或“亚优势表位”是指与优势抗原或优势表位相比，激发较弱的耐受或免疫应答的抗原或表位。

[0065] 术语“治疗”是指响应患者表现的或有待于在患者中预防的疾病、病变或生理状况而进行的临床干预。治疗的目的包括缓解和 / 或预防症状，以及减缓、阻止或逆转疾病、病变或状况的进展。“治疗”是指治疗性治疗和预防性或防御性措施二者。需要治疗的那些包括已经受到疾病或病变或不良的生理状况影响的那些，以及其中，有待于预防所述疾病或病变或不良生理状况的那些。

[0066] “肿瘤”是指所有的瘤细胞生长和增殖，无论是恶性的还是良性的，以及所有的癌症前期和癌细胞和组织。

[0067] “_- 聚体”是指在目标抗原中发生的 _ 个氨基酸的线性序列。

[0068] B. 识别 / 区分亚优势表位与优势表位的测定

首先，对于患者的肿瘤或感染，测定一组肿瘤相关性、病毒性或其他抗原的存在。这通常通过对肿瘤活组织切片上的免疫组织化学完成，或在恶性血液病中，通过 FACS 完成。对于患者的肿瘤或感染，测定一组肿瘤相关性、病毒性或其他抗原的存在。这通常通过对肿瘤活组织切片上的免疫组织化学完成，或在恶性血液病中，通过 FACS 完成。抽取患者的血液，并测试存在的对抗原的体液和细胞免疫应答二者。主动选择对该抗原的免疫应答不可检测或在低水平检测的抗原，以在体外生长 T 细胞。在这些 T 细胞生长后，测试它们对重新输注的抗原的应答，并且可测定患者的血液对所述抗原的应答。通过这种方式，可有效地重新平衡患者的免疫应答以提供治疗益处。在优选的实施方案中，体液免疫性的测试可包括但不限于 ELISA 测定。在优选的实施方案中，细胞免疫性的测定可包括但不限于对于细胞因子的细胞内细胞因子染色 (ICS)，包括但不限于干扰素 γ (IFNγ) 和肿瘤坏死因子 α (TNFα)。应答性 T 细胞的亚群 (例如，CD8、CD4、Treg) 也可在此测定中进行测定。或者，细胞免疫性的测定可包括但不限于用于 IFNγ 或 TNFα 的 ELISPOT 测定。在可选的实施方案中，ELISPOT 或 ICS 可测定 IL-4、IL-12 (TH2 和 TH1)、IL-10 (Treg) 或 IL-21(滤泡 T 辅助细胞亚群)。在又一个实施方案中，细胞免疫分析测定可以是用于这些或其他细胞因子的细胞内染色 (ICS)。所测试的抗原可以是全抗原、表位删除的抗原，或优势或亚优势表位。在表位的情况下，可使用生物信息学软件预测可与患者的 MHC 结合的表位，并且，随后测定这些表位。在一个实施方案中，这种软件是 Net MHCpan 或在测定部分描述的共有表位免疫信息学软件。在表位的情况下，四聚体结合可用作量化 CTL 的可选测定，前提是 HLA 类型是已知的。包括肽的四聚体与细胞组合且将细胞染色，并且使用 FACS 测定识别每种四聚体的细胞 %。如果患者具有已知的 HLA 类型，例如 HLA A2，这是有用的。然而，优选的方法是用于体液应答的 ELISA 和用于细胞应答的 ICS 或 ELISPOT。在优选的实施方案中，从外周血产生 CTL。或者，从肿瘤浸润淋巴细胞 (TIL) 或从注射部位周围的 DTH 产生 CTL。

[0069] 可以通过在包含 10% FCS (CSL) 的 RPMI 培养基中切碎的 4 mm 从皮肤钻取的活组织，制备 DTH 浸润淋巴细胞。使用 1 μg/ml 植物凝集素 (Sigma) 刺激单细胞悬液，并与经过辐照的自体 PBMC 以及 10 IU/ml IL-2 (Cetus) 和 10 ng/ ml IL-7 (Peprotech, Rocky Hill, NJ) 共培养。每 2-3 天补充培养基。

[0070] 对于体液免疫应答概况分析，对来自患者的血清进行从 1/100 至 1/100000 的 1:4 连续稀释，并且用于使用纯化重组肿瘤抗原 (通常在大肠杆菌中制备) 的标准 ELISA 中。可测试 2 中至 10000+ 种抗原。将 1 微克的每种纯化蛋白吸附到微孔板 (Nunc)，在 4° C 过夜。使用 PBS 清洗平板并使用 2% FCS/PBS 封闭。将患者血清在 2% FCS/PBS 中稀释并添加 2 小时。清洗平板并添加山羊抗人 IgG-AP (Southern Biotechnology Assoc)。清洗平板，并与 Attophose 底物 (JBL Bioscientific) 温育 25 分钟，并立即读取 (CytoFluor 2350, Millipore)。读取的是 UV 吸光度。

[0071] 有两种用于细胞应答概况分析的方法。第一种方法包括用于 IFNγ 的酶联免疫吸附斑点 (“ELISPOT”) 测定。在 4° C，使用 5-15 μg/ml 的抗 IFNγ 单克隆抗体 1-DIK (MABTECH, Stockholm, Sweden) 包被聚偏氟乙烯后背的 96 孔板 (Millipore, Bedford, MA) 过夜。清洗孔，并使用 5% 人 AB 血清 (Valeant Pharm) 封闭。每孔添加 5×10^6 个 PBMCs (或测试在体外扩增后进行时，添加 5x 10^5 个 CTL) 和来自每种抗原的肽混合物各 2 μM。

在37° C,5% CO_2下温育过夜(18小时)。弃去细胞,并使用包含0.05% Tween 20的PBS清洗孔。将1 μg/ml生物素化的抗IFNγ单克隆抗体7-B6-1 (MABTECH)在室温下温育2-4小时,随后再与链霉亲和素缀合的碱性磷酸酶(MABTECH或Sigma Aldrich)温育2小时。随后,与来自alk-phos底物试剂盒(Bio-Rad Richmond, CA)的5-溴-4-氯-3-吲哚磷酸酯和硝基蓝四唑反应30分钟。使用解剖显微镜(SZ CTV Olympus microscope)进行斑点计数。也可以在AIDELISPOT计数器(Autoimmun Diagnostika, Strassberg, 德国)上进行斑点计数。每个斑点是一个细胞,报告为斑点形成细胞(SFC)/10^5个PBMC。可使用10 μg/ml PHA作为阳性对照;没有肽的单独细胞可充当阴性对照。

[0072] 第二种方法包括用于IFNγ和TNFα的细胞内细胞因子染色。对于每种所研究的表位或抗原,将5×10^6个PBMCs(或测试在体外扩增后进行时,5x 10^5个CTL)与肽一起(终浓度10^{-5}至10^{-9} M)平铺到96孔板的100 μl PBS 1% FCS中。在IL-2 (150 U/Ml)、50 μM β巯基乙醇和布雷菲德菌素A (1 μg/ ml)或Golgi Plug (BD Biosciences, San Diego, CA)(后两种组分用于增加IFNγ或TNFα在应答性细胞中的累积)的存在下温育6小时后,将细胞沉淀,在200 ml PBS 1% FCS中清洗,并且随后在4° C使用对于表面抗原的染料(CD4异硫氰酸荧光素和CD8别藻蓝蛋白0.25 μg/ml (Pharmingen, Becton Dickinson))标记30分钟(在冰上30分钟)。清洗后,使用Cytofix/Cytoperm将细胞在冰上透化20分钟,并且随后使用藻红蛋白缀合的抗IFNγ (0.4 μg/ml)或抗TNFα (0.8 μg/ml)抗体(Pharmingen, Becton Dickinson)染色。随后,清洗细胞,固定并悬浮在PBS 1% FCS中,并且在FACScan流式细胞仪上测试,并使用Cell Quest软件分析。或者,可使用FACS Canto (Becton Dickinson)。可测试其他细胞因子,包括但不限于IL-12和IL-4,以测量TH1或TH2亚群。给出T细胞的较宽测定的细胞因子组可以测量IL-12、IFN g、IL-4、IL-10和IL-17。滤泡T辅助细胞可作为$CD4^+$, $CXCR5^+$, $ICOS^+$细胞测量。B细胞可作为$CD19^+$和$B220^+$细胞测量。T细胞中的IL-21应当与B细胞活化和抗体的亲和力成熟相关,因此,这也可以用于本研究。作为分析IFNγ、IL-4 (BD Biosciences) IL-12、IL-10、IL-17 (R&D Systems)和IL-21抗体(R&D systems)概况的备选方案,也可使用ELISPOT。ICS实际上分析出对不同抗原或表位做出应答的CD8或CD4 T细胞的%概况。也可分析其他细胞亚群,包括Treg。可以使用细胞色素标记的CD25单克隆抗体作为大多数Treg的表面标记。或者,使用来自BD Biosciences的细胞色素标记的人FoxP3单克隆抗体克隆259D/C7,对预透化后的细胞进行染色以测量Treg细胞%及其状态。还可测定IL-10。

[0073] NetMHCpan是用于与HLA-A和-B结合的肽的定量预测的生物信息学方法(Nielsen M 2007)。还开发出共有表位预测方法(Mouaftsi M 2006)。这些方法可用于分选对于抗原的所有的潜在的MHC I表型,并排序前1%的肽,并由此预测表位。随后,可将这些预测的表位合成为9-10聚体的肽并测试(例如,对于患者的PBMC,或在转基因小鼠中对于目标的HLA类型)。

[0074] 具有特异性MHC (例如HLA A A2)的四聚体与8聚体的肽表位一起合成(在I型MHC的情况下),且在II型MHC的情况下,与15聚体肽表位一起合成。在室温下使用1/200稀释的四聚体将培养的细胞染色20分钟;随后,添加抗CD8抗体并进一步染色30分钟。随后,清洗细胞并在FACS Calibur (BD Biosciences)。

[0075] 治疗后,通过测定耐受性和对亚优势抗原或表位的体液或细胞免疫应答而重新分

析所述患者概况，以确定治疗是否成功地使免疫应答重新平衡。

[0076] C. 重新平衡免疫应答

在本发明的一个实施方案中，可通过对亚优势抗原或亚优势表位先体外后体内培养来自患者的 T 细胞，随后将这些 T 细胞输注或施用到所述患者中，而重新平衡免疫应答。针对亚优势抗原或表位的 T 细胞可在组织培养物中先体外后体内生长（脱离患者的免疫调节环境）。在培养足够的细胞以淹没此前的优势细胞后，将细胞重新输注到患者体内以改变细胞平衡并治疗性地转变优势等级。在进一步的优选实施方案中，作为治疗从头引入的该数量的 T 细胞的这个数量大于应答所述抗原、传染物、肿瘤或器官的 T 细胞的 5%。可通过使用减少内源 T 细胞数量的调节剂对所述患者进行预治疗（即，化疗）而进一步改变这个比例以有利于输注的细胞。

[0077] 本发明包括优化针对组织培养中的亚优势抗原的 T 细胞的生长的方法。在一个实施方案中，所述细胞在不存在优势抗原的情况下生长。这通过选择未曾暴露于优势抗原的专业抗原呈递细胞和将抗原修饰以消除优势表位或限制抗原加工能力的其他组分而完成。

[0078] 由于治疗方法需要富集对于亚优势抗原 / 表位且理想地对亚优势抗原 / 表位完全反应性的 T 细胞，所以用于此类 T 细胞的生长的有效方法是重要的。针对优势表位和抗原的其他 T 细胞的生长增加了在培养物中产生足够的特异性细胞以改变此比例所需的时间。此外，生长出的对于优势表位的细胞妨碍在重新输注后正确比例的实现。因此，已开发出特异性地限制优势抗原或表位的引入的 T 细胞培养方法。例如，尽管该方法已广泛应用于所有肿瘤，但它对于 EBV 恶性肿瘤具有明显的优势，因为它不使用 EBV 转化的 B 细胞（其表达 EBV 优势抗原 / 表位）。在施用其中显著 % 的 CTL 是应答亚优势抗原 / 表位的细胞时，其也更为可靠。

[0079] 在体外，从来自患者的外周血单核细胞 (PBMCs) 产生单核细胞衍生的树突细胞。在优选的实施方案中，将 PBMC 在组织培养烧瓶中铺板 2 小时以允许单核细胞粘附。在可选的实施方案中，可使用 $CD14^+$ 磁珠从 PBMC 分离树突细胞 (Miltenyi Biotec, Auburn, CA)。此时，除去未粘附的细胞，并在 -80° C 冷冻以供后续作为 T 细胞的来源使用。使用白介素 -4 (IL-4) 和粒细胞 - 巨噬细胞集落刺激因子 (GM-CSF) 处理粘附的单核细胞，导致在约 1 周内分化成不成熟的树突细胞。随后使用肿瘤坏死因子 (TNF) 或巨噬细胞条件培养基处理 2 天，使 iDC 进一步分化成为成熟的树突细胞。随后，使用包含亚优势抗原的肽或质粒对这些细胞进行脉冲处理 2 小时，并且，随后将 PBMC 解冻并添加到脉冲处理的树突细胞。数小时后，将细胞汇集并重新悬浮在包含 IL-2 或 IL-15 （优选 IL-15）的培养基中，以在体外产生已识别所述抗原的 T 细胞的扩增。对于某些方案，添加 IL-7 和 IL-15 以提高 T 细胞存活。对于其他方案，调整培养条件以优化 T 细胞的某些亚群的生长。例如，可添加 IL-12 以极化成 TH1 细胞。或者，可添加 IL-4 极化成 TH2。在某些方案中，可添加 IL-6 以阻止 Treg 的生长。在可用于自体免疫或器官移植应用中的又一个变化中，可添加低水平的 $IL-2^+$ 雷帕霉素以加速 Treg 的生长。更详细的方案列出在多个实施例中，并且实施例 3、图 3 中描述了使用将细胞极化成某些 T 细胞亚群的方案产生的细胞的体内比较。如果 T 细胞生长在组织培养烧瓶中，则必须在第 14 日和 21 日更换培养基。然而，在优选实施方案中，可使用生物反应器以减少这种需求，例如，气体通透性生物反应器，例如 Grex (Wilson Wolf) 或 Hyperstack (Corning)。通常，与使用传统方法的 12-24 周相比，可在 2-6 周内产生向患者

施用的足够的细胞。

[0080] 在本发明的另一个实施方案中，本发明人已开发了用于过继转移针对其优势表位已经被删除的抗原的 T 细胞，并且证实产生更多的针对亚优势表位的 T 细胞。可使用此类蛋白或相应的 DNA 疫苗以产生对亚优势表位具有广泛免疫应答的 T 细胞。这种方法应广泛地适用于多种类型的疾病以实现对亚优势表位的免疫反应的平衡，包括但不限于 EBV、癌症、HIV 或肝炎。在另一个实施方案中，使用抗原或编码它的质粒 / 重组疫苗对患者进行免疫接种以诱导对亚优势抗原的新的广泛免疫反应。在另一个实施方案中，可以在使用亚优势抗原的免疫接种后施用亚优势反应性 T 细胞以强化所述应答。在又一个实施方案中，可治疗性或预防性地使用所述方法，其中可以确定患者的免疫概况以测量患病的风险，并且随后可以使用本文公开的任何方法以适当的亚优势抗原对所述患者进行初免 (prime)。

[0081] 在可选的实施方案中，生长的 T 细胞诱导耐受性以预防或治疗自体免疫疾病、变态反应、炎症、器官移植排斥或移植物抗宿主病。取决于期望的 T 细胞的类型，可调整培养条件以优先生长或富集相关亚群，包括但不限于 CD8、CD4、TH1、TH2 或 Treg。例如，T 细胞可在存在或不存在某些生长因子、细胞因子、药物、小分子或其他免疫细胞的情况下生长。在优选的实施方案中，在存在经刺激的专业抗原呈递细胞（例如，单核细胞衍生的树突细胞、巨噬细胞或 EBV 永生化 B 细胞）的情况下，从组织培养物中的外周血单核细胞 (PBMC) 产生亚优势抗原反应性 T 细胞。

[0082] 在另一个实施方案中，使用各种技术以调整抗原加工，从而有利于亚优势表位。在本发明的一个实施方案中，这通过修饰抗原以消除优势表位、抑制抗原加工的区域，或限制同时呈递至抗原呈递细胞的优势或亚优势表位的数量而实现。这些修饰提高了识别的亚优势表位的应答和多样性（实施例 1，图 5）。在可选的实施方案中，可使用病毒载体，例如腺病毒或牛痘苗，将修饰的 LMP1、LMP2 和 EBNA-1 序列递送至 APC。在其他抗原 (LMP1 和 EBNA-1) 的情况下，消除导致不良抗原加工的蛋白区域极大地增强了对这些抗原上的亚优势表位的免疫应答（实施例 1，图 6）。可使用肽、蛋白、质粒或病毒载体，例如腺病毒或牛痘苗，将修饰的 LMP1、LMP2 和 EBNA-1 序列递送至 APC。

[0083] 在可选的实施方案中，可在 CTL 产生期间向 APC 和抗原添加蛋白酶体拮抗剂以增加识别的亚优势表位的数量并且增强对亚优势抗原的应答（实施例 2，图 7）。有具有不同机制的很多可得的蛋白酶体拮抗剂（例如，硼替佐米、氯碘羟喹、乳胞素、环氧霉素 (epoxomycin)、MG-132、MLN9708、卡非佐米 (carfilzomib, PR-171)。

[0084] 在可选的实施方案中，可施用与抗体在复合物中的抗原，所述抗体对于针对亚优势决定簇的应答具有不同的同种型。与抗原一起注射的抗体结合期望的 T 细胞表位侧翼的决定簇以靶向专业抗原呈递细胞，并引导对侧翼表位的抗原加工。

[0085] 在本发明的另一个实施方案中，使用包含亚优势表位或抗原的质粒产生所施用 T 细胞，或者将包含亚优势表位或抗原的质粒直接通过各种施用途径与 IFNγ、IL-21 或其他细胞因子组合而直接施用，或在经脉冲处理的树突细胞上直接施用，以诱导对亚优势抗原的应答。抗原在 T 细胞刺激之前诱导 IFNγ 或其他细胞因子，以增强对亚优势表位的 T 细胞反应性，并改变免疫优势等级。

[0086] 在另一个实施方案中，调整施用途径以改变免疫优势等级。牛痘苗应答的施用途径决定了优势决定簇的程度。已发现，在腹膜内施用时，优势决定簇仅占应答的四分之

一，相比之下，在皮内施用的情况下，优势决定簇占应答的一半（Tscharke DC 等人，2006，Tscharke DC 等人，2005）。如实施例 3、图 11 中所示，与 IP 或 IV 途径相比，通过 IM 途径施用抗原产生了对亚优势表位的更强的应答和更广的应答。因此，调整施用途径是本发明人主张的用于调节免疫优势等级的另一个体内机制。在优选的实施方案中，通过皮内施用递送培养的 T 细胞。通过靶向不同的 APC（例如，巨噬细胞、树突细胞），施用途径改变了优势等级（实施例 3，图 12）。

[0087] 在优选的实施方案中，所述抗原是病毒抗原，特别是其上带有亚优势表位的潜伏病毒抗原或慢性病毒抗原。例如，所述病毒抗原来自选自包含以下的组的病毒：EBV、HSV、VZV、乙型肝炎和丙型肝炎、HIV 和 HTLV。病毒抗原是，例如 EBV LMP1、LMP2、EBNA-1、HPV E6 或 HPV E7。例如，所述病毒抗原与 EBV、HSV、VZV、乙型肝炎和丙型肝炎、HIV，和 HTLV、CMV、RSV 或流感相关。在另一个实施方案中，所述抗原是在其他慢性和潜伏传染物（例如与细菌、真菌、寄生虫或朊病毒相关的试剂）上的抗原。在又一个实施方案中，所述抗原是肿瘤抗原，包括但不限于：肿瘤相关抗原、肿瘤特异性抗原、与癌干细胞或转移相关的抗原。在其他实施方案中，所述抗原与自体免疫性、变态反应、炎症或器官抑制排斥或移植物抗宿主病相关。

[0088] 在一个实施方案中，通过鉴定患者样品中的优势抗原或表位和亚优势抗原或表位，培养能够识别所述亚优势抗原或表位的 T 细胞，并使用有效量的所述 T 细胞治疗患者，而改变所述患者的免疫优势等级。

[0089] 在一个实施方案中，通过鉴定患者样品中的至少一种亚优势抗原或表位，培养能够识别所述亚优势抗原或表位的 T 细胞，和使用有效数量的那些 T 细胞治疗所述患者来改变患者的免疫优势等级，从而提供治疗益处。

[0090] 在另一个实施方案中，所述 T 细胞是反应性 T 细胞，并且使用有效量的所述 T 细胞治疗患者以改变所述患者的免疫优势等级，由此诱导所述患者中的细胞毒性免疫应答以用于治疗或预防感染或癌症。所述感染是，例如，细菌性、病毒性、寄生虫或朊病毒感染。

[0091] 在任意的本发明的方法中，疾病、感染、癌症或医疗状况的治疗或预防包括缓解或改善所述疾病、感染、癌症或医疗状态的至少一种症状。

[0092] D. 治疗方法

1. 癌症

优势和亚优势抗原的免疫概况分析的临床使用的工作流程

步骤 1：肿瘤活检（免疫组织化学）或血液（IHC、FACS 或 ELISA）抗原 1、2、3、4、5、6、7、8、10

结果：抗原 1、2 和 6 在肿瘤上，组中的其他则不在肿瘤上

步骤 2：免疫应答概况分析

体液概况分析

对血清的 ELISA

细胞概况分析

对由每种抗原刺激的 PBMC 进行 ELISPOT 或 ICS（至少对于 IFNg，还有 IL-10、IL-4 和 IL-12、IL-21 以测定 T 细胞亚群）

结果：抗原 1：强应答（优势）；抗原 2 和 6：无 / 中度应答（亚优势）

步骤 3 :在体外培养对亚优势抗原的 T 细胞 (CD8 和 CD4)

结果 :T 细胞对抗原 2 和 6 应答

步骤 4 （一旦治疗充分确定,则为任选的）:使用细胞免疫概况分析 (ELISPOT 或 ICS) 确定 >5% 的生长的 T 细胞对亚优势抗原应答

结果 :25% 的 T 细胞对抗原 2 和 6 应答

在继续进行步骤 5 之前,提供至少 5% 的对亚优势抗原应答的 T 细胞

步骤 5 :在进行或不进行预先调节（例如,环磷酰胺）的情况下,将细胞 IV 输注到患者中

步骤 6（一旦治疗充分确定,则成为任选的）:在输注后 2-3 周从血液分离 PBMC 并分析免疫应答概况

结果 :细胞概况分析 :抗原 1 :无 / 中度应答 ;抗原 2& :对至少一个抗原的强应答（优势）

步骤 7 :评估临床应答

RECIST (CR, PR) ;存活或无进展存活

结果 :改进的应答率和存活

在实施例 4 中,使用这个系统性方法治疗黑色素瘤。在实施例 5 中,将该方法应用于用多质粒 LMP2 治疗淋巴瘤。实际上,T 细胞治疗改变了淋巴瘤的天然过程,从复发 - 缓解的过程改变为持久缓解的过程。这种抗原以及删除的 LMP1 和 EBNA-1 也可以用于治疗包括 EBV 抗原的其他肿瘤,包括鼻咽癌、伯基特淋巴瘤、CLL、霍奇金病和除此之外的一些胃癌等。在实施例 3 中,使用类似的方法治疗肝细胞癌。这些实施例通过引用并入本发明,并且证实了免疫应答向亚优势抗原的重新平衡可广泛地应用于所有的癌症形式。

[0093] 2. 一般使用肿瘤上的大量亚优势抗原以产生泛（pan）肿瘤类型的治疗

在另一个实施方案中,本发明人提出,如果能够鉴定对于具体类型的肿瘤的足够的亚优势抗原,则可以使用对于多种亚优势抗原的 T 细胞治疗该类型的肿瘤,而无需对患者进行测试。类似地,靶向相同肿瘤上的多种抗原将降低抗性,例如组合化疗。例如,开发靶向亚优势抗原 EBV LMP2 （其在 40% 的淋巴瘤上）和存活（其在 50% 的淋巴瘤上）的单一 T 细胞系的能力将允许使用对这两种抗原特异性的 T 细胞系靶向约 80% 的淋巴瘤。实施例 5 中证实了泛淋巴瘤产品的临床测试。经过 3 年,使用泛淋巴瘤产品治疗的患者的无进展存活,与治疗前测试抗原的产品相当。这可以是因为,与组合化疗类似,CTL 对在相同肿瘤上的多种抗原的应答可减少逃脱的可能性。使用单一 T 细胞产物治疗所有淋巴瘤的能力（不是必须仅治疗对单个抗原阳性的亚群）是新的产品构思。在本发明的另一个实施方案中,本发明人还主张用于实际上任何癌症的泛癌症产品。

[0094] 3. 慢性感染

肝炎和肝细胞癌

在慢性肝炎患者中,不产生 HBs 抗体而产生 HBc 抗体 (Ganem D 等人,2004)。在急性肝炎患者中,产生对两种抗原的抗体。>90% 的新生儿和 30% 的 1-5 岁的儿童发展出慢性形式,而 >90% 的成人此时剧烈地清除所述病毒。95% 的肝细胞癌与乙肝病毒的慢性感染相关。HBsAg 通常在 HCC 的细胞表面。基于这些观察结果,本发明人选择研究 HBs 在肝炎和肝细胞癌患者中是否是亚优势的,以及 T 细胞的生长是否能够产生针对 HBs 抗原的 CTL,其能够重

新平衡对于亚优势抗原的免疫应答，并且可以是治疗性的。

[0095] 基于这个发现，本发明人施用针对 HBs Ag 培养的 CTL，以治疗肝炎动物模型中的慢性肝炎，并且最终治疗与 HBV 相关的 HCC 患者中的慢性肝炎。在动物模型中，施用并测试了 HBVtgRAG 细胞。在接受对照细胞的动物中，慢性肝炎发展。此外，当动物发展肝炎并且接受针对亚优势抗原的 CTL 时，动物发展出急性肝炎但清除了肝炎病毒（实施例 3、图 9-11）。

[0096] 基于所述振奋人心的动物数据，使用 T 细胞重新平衡疗法治疗肝炎患者。

[0097] 优势和亚优势抗原的免疫概况分析的临床使用的工作流程

步骤 1 ：肝炎表面抗原和肝炎核心抗原的免疫应答概况分析

体液分析 细胞分析

对血清的 ELISA 对由抗原刺激的 PBMC 进行 ELISPOT 或 ICS （至少对于 IFNγ，还有 IL-10、IL-4 和 IL-12、IL-21 以测定 T 细胞亚群）

结果 ：Hbc 强应答（优势）；Hbs 无 / 中度应答（亚优势）

步骤 2 ：在体外培养针对亚优势抗原的 T 细胞 (CD8 和 CD4)

结果 ：T 细胞对 HBs 抗原应答

步骤 3 （一旦治疗充分确定，则为任选的）：使用细胞免疫概况分析 (ELISPOT 或 ICS) 确定 >5% 的生长的 T 细胞对亚优势抗原应答

结果 ：25% 的 T 细胞对 HBs 抗原应答

在继续进行步骤 4 之前，提供至少 5% 的对亚优势抗原应答的 T 细胞

步骤 4 ：在进行或不进行预先调节（例如，环磷酰胺）的情况下，将细胞 IV 输注到患者中

步骤 5 （一旦治疗充分确定，则成为任选的）：在输注后 2-3 周从血液分离 PBMC 并分析免疫应答概况

结果 ：细胞分析 ：HBc 无 / 中度应答 ；HBs 强应答（优势）

步骤 6 ：评估临床应答

结果 ：感染的清除

对具有 HBV 和 HCC 的 5 名患者进行免疫概况分析。如所预期的，ELISA 证实对高滴度的针对乙型肝炎核心抗原 (HBc Ag) 的抗体，但没有针对乙型肝炎表面抗原 (HBs Ag) 的抗体（实施例 3，图 17）。然而，在将 PBMC 诱导成树突细胞，使用乙型肝炎核心抗原 (HBc Ag) 和乙型肝炎表面抗原 (HBs Ag) 进行脉冲处理，并用于从来自相同患者的 PBMC 生长 CTL 时，观察到预想不到的结果。通过产生 IFN 的 T 细胞在 ELISPOT 上的频率，观察到等级如下 ：无抗原 (10 sfc)<HBsAg(15 sfc)<HBcAg(45 sfc) （实施例 3，图 14）。这表明，相对于肝炎核心抗原，乙型肝炎表面抗原的确是亚优势的。此外，通过 ELISPOT （实施例 3、图 14）且通过 ICS （实施例 3、图 15），显示 HBs 的一个表位 (FLL) 是优势的。将包含这个表位的肽从用于培养来自患者细胞的肽混合物中排除。

[0098] 在 HCC 患者中，接受针对亚优势 HBs （实施例 3，图 16）的 T 细胞的 3/5 个患者清除了 HBs 抗原，并且证实在肝功能测试中丙氨酸转氨酶 (ALT) 瞬时提高之后，对 HCC 的 CR（实施例 3，图 17 和 19）。当测试在施用后 2 周来自这些患者的 PBMC 时，优势应答针对 HBs 而不是针对 HBc，并且针对此前 HBs 的亚优势表位（实施例 3，图 18）表明患者已经重新平衡了他们的免疫优势等级。作为治疗和免疫优势等级转化的结果，提供了治疗益处（实施

例 3，图 17 和 19)。

[0099] 因此，针对亚优势抗原的 T 细胞过继免疫治疗可以用于根除慢性病毒并治疗其上存在慢性病毒的癌症。此外，指导针对亚优势抗原和表位的 T 细胞的产生的免疫分析为多种疾病开拓了新的治疗道路。

[0100] 实际上，可以治疗任何病毒、细菌、真菌、朊病毒、寄生虫或其他传染病，包括但不限于丙型肝炎病毒和 HIV 病毒。使用重新平衡，还可以治疗其他慢性传染病。例如，结核分枝杆菌是很多患者中的慢性感染，但只有在免疫系统受到压制时恢复活动。在其潜伏状态中，其包含在肉芽肿中，通常在肺中。最近，分枝杆菌吞噬体的蛋白质组研究表明，MTB 抑制树突细胞中抗原呈递的程度大于巨噬细胞 (Li 等人，2011)。为此，本发明人提出重新平衡免疫系统以应答亚优势表位是用于实际上任何癌症或传染病的广泛治疗方法。

[0101] 4. 自体免疫性

流行病学研究证实，患者发生多发性硬化的风险与 EBV 抗体滴度相关。通过在其感染 EBV 之前跟踪几十万个体，并且在初始感染后对其数年随访，Ascherio 等人得以研究发生 MS 的 305 名患者。他们的风险在 EBV 感染后剧烈提高 (Ascherio A 等人，2010)。在与同样是 EBV 携带者的健康个体相比时，相对于其他 EBV 抗原，MS 患者中针对 EBNA-1 400-641 应答的记忆 CTL 增加 (Lunemann JD 等人，2006)。看起来，EBNA-1 特异性 Th1 细胞能够通过自体抗原的交叉识别或旁观者机制维持自体免疫性。此外，转基因小鼠研究表明，表达 LMP2a 的 B 细胞绕过正常耐受性检查点，并强化自体免疫疾病的发展 (Swanson-Mungerson M 2007)。因此，尽管本发明人已经研究了对 EBV 亚优势表位反应性的 T 细胞作为对 EBV 和 EBV 相关癌症的治疗，但对 EBV 亚优势表位反应性的 T 细胞也可用于恢复自体免疫性中的平衡。通过培养并引入针对 EBV 潜伏抗原 (EBNA-1、LMP1 和 LMP2) 应答的 T 细胞，本发明人提出，可以重新平衡免疫应答，在具有 EBV 相关性的自体免疫疾病中重新引入耐受性。对于与其他病毒相关的其他自体免疫疾病也是如此。例如，小核糖核酸病毒，例如库克萨基病毒 B3 导致心肌炎 / 扩张型心肌病、I 型糖尿病、脑炎，肌炎，睾丸炎，肝炎。

[0102] 在又一个实施方案中，可以产生针对与器官相关的抗原的 T 细胞，在所述器官中发生了器官特异性自身免疫。由于免疫优势现象涉及所有在任一时刻加工的抗原的表位，因此知晓在那一时刻驱动自身免疫的准确的自身抗原并不是完全必需的需求。例如，产生针对胶原的反应性亚优势表位的 T 细胞并将其引入至类风湿关节炎患者的发炎关节中，正在进行的免疫应答将重新平衡并且将恢复耐受。相反且意料不到的是认为引入有活性的 T 细胞至炎性部位将是有利的，但根据我们的操作模型，在该部位上免疫应答的重新平衡将对异常的免疫应答恢复适合的控制。自身免疫疾病的治疗是本发明的另一个实施方案。

[0103] 5. Treg 体外扩增

免疫调节原则之一是 Tregs 与 T 细胞的其他亚群的平衡。本发明的另一个实施方案是使先体外后体内 T 细胞生长向 Treg 细胞极化。在这种情况下，代替靶向亚优势表位，产生对优势表位的 Treg 作为另一种重新平衡免疫应答的方式。在一个实施方案中，将这样产生的 Treg T 细胞用作单独的治疗产品。在另一个实施方案中，将 Treg 亚群与来自对亚优势表位或亚优势抗原生长的其他亚群的 T 细胞组合使用。尽管已经使用 FACS 分选随后用抗 CD23 和抗 CD28 包被的珠扩增将 Tregs 以非抗原特异性方式扩增 (Putnam 等 2009)，但到目前为止还未有人将 Tregs 以抗原特异性方式扩增用于过继免疫治疗。本文描述了用于建立

特异性对于不同抗原，尤其是在自身免疫疾病、移植物抗宿主病或移植排斥中观察到的免疫应答中优势的那些抗原的 Tregs 的方法。

[0104] 通过 FACS 分选 (BD FACS Aria II 高速细胞分选仪）使用无菌技术在 GMP 清洁房间内用 CD4-PerCP(SK3)、CD127-PE (hIL-7R-M21)、CD25 APC (2A3)、CD45RA-PE.Cy7 (L48) 和 CD45RO-PE.Cy5 (UCHLI) 从自身免疫或移植患者的 PBMC 中分离 Tregs。分选 $CD4^+CD127^{lo/-}CD25^+$ 和 $CD4^+CD127^{lo/-}$ T 细胞并收集于 3ml 含有 10％人热失活混合 AB 血清 (Valley Biomedical, Winchester, VA) 的 X-Vivo 15 培养基 (Lonza, Walkersville, MD) 中。(或者，可以使用包被有相同抗体的磁珠分离 Tregs(Miltenyi Biotec, Auburn, CA))。将这些细胞以 2.5×10^5 Tregs/ 孔铺于 24 孔板 (Costar, Cambridge, MA) 中，每孔含有从 PBMC 制备的树突细胞(如上文对 T 细胞刺激所述)，所述 PBMC 已经用以 Treg:APC 比为 1:5 的优势抗原预先脉冲。18 小时孵育后，将细胞在第 1 － 7 天在培养中接受雷帕霉素 (100ng/ml; Wyeth, Madison, NJ)。在第 2 天，将培养体积加倍并加入 300 单位 /ml IL-2(Chiron, Emeryville, CA)。将细胞重悬于新鲜培养基中并在第 2、5、7、9 和 12 天加入 IL-2。在第 9 天用肽脉冲的树突细胞再刺激细胞。或者，抗 CD23/ 抗 CD28 包被的微珠 (Invitrogen, Carlsbad, CA) 可以用于该第二次刺激。此外，气体渗透生物反应器(诸如 Grex) 可以用于进行该细胞生长，并在封闭系统内有更少操作，并且改善生长的动力学。在另一种变化中，将 IL-10 与包被的珠在刺激时一同加入以驱动 Treg 向 Treg1(其分泌高水平的 IL-10 并调节 TH1 和 Th2 应答) 的进一步分化。

[0105] 自身免疫中优势和亚优势抗原的免疫概况分析的临床使用的工作流程

步骤 1 :免疫应答概况分析

体液概况 细胞概况

ELISA 或血清 在用每一种抗原刺激的 PBMCs 上的 ELISPOT 或 ICS （至少对于 IFNγ，还有 IL-10、IL-4I 和 L-12、IL-21 以测定 T 细胞亚群）

结果 :抗原 1 强应答(优势);抗原 2 无 / 中度应答(亚优势)

步骤 2 :体外生长针对亚优势抗原的 T 细胞(CD8 和 CD4);体外生长针对优势抗原的 Treg

结果 :T 细胞对亚优势抗原应答 ;Treg 对优势抗原应答

步骤 3 (一旦良好建立了治疗，则任选):使用细胞免疫概况分析 (ELISPOT 或 ICS) 确定 >5% T 细胞对亚优势抗原应答生长，并且 Treg 对优势抗原生长

结果 :25％的 T 细胞对抗原应答

提供对亚优势抗原的至少 5％的 T 细胞应答和 / 或对优势抗原的至少 5％的 Treg 细胞应答，进行步骤 4

步骤 4 :IV 将细胞输注入具有或不具有在前调节(例如环磷酰胺) 的患者中

步骤 5 (一旦良好建立了治疗，则可以成为任选):从输注后 2-3 周的血液中分离 PBMC 并进行免疫应答概况分析

结果 :细胞概况抗原 1 无 / 中度效应物应答 ;抗原 2 和强 Treg 应答(优势)

步骤 6 :评估临床应答

在自身免疫中(MS- 发作下降，类风湿关节炎 - 关节肿胀减少，哮喘 - 发作数目下降，早期 I 型糖尿病，胰维持)，在移植中－器官排斥率和移植物抗宿主病下降

结果 :改善的临床结果

6. 在自身免疫或移植中施用针对优势抗原的 Tregs

在又一个实施方案中,针对优势抗原的 Treg 能够与 TH1、TH2 或 CTL 亚群的 T 细胞(其自身对亚优势抗原应答)组合。此类组合将更加充分转换应答向亚优势表位的平衡。本发明人已经证明关节炎中两种类型的 T 细胞之间的协同作用(实施例 6)。

[0106] 7. 移植

在可选实施方案中,使用上述 Treg 培养条件从健康供体的 PBMC 用来自其他健康供体的树突细胞(或受照射的 PBMC)培养 Treg。以这种方式,建立了每一种 MHC 的同种异体反应性 Treg 系并储存。如上我们所讨论,用针对 20 — 50 种 MHC 单元型产生的 Treg 系能够覆盖 80%的 MHC。这些系的每一种能够以单次剂量的等分试样冷冻于 -80 摄氏度。当进行器官或 BMT 时,还将 5×10^7 细胞 $/m^2$ 的对错配的 MHC 有反应性的 Treg 移植入患者中。以这种方式,减缓了同种异体排斥或移植物抗宿主病。

[0107] 8. 自动免疫概况分析测定和封闭系统细胞培养设备

气体渗透膜设备是培养技术的优选实施方案。由于膜是气体可渗透的,因此培养规模由培养细胞所需的膜的表面积和培养基的体积所决定。这些气体渗透设备的实例包括 Hyperstack (Corning) 或 Grex (Wilson Wolf)。这类生物反应器的有用特色之一是细胞培养过程是线性比例可变的。作为我们的方法标准化的部分,我们已经设计了在生产套件中使用的可滑入标准 CO_2 培养箱内的生物反应器版本。在独立的实施方案中,我们已经设计了适合于标准组套的用于在自动生产设施中温暖房间的生物反应器。旨在自动化的该生物反应器制成两种大小 :一种用于对单个患者的自体细胞生长,而第二种更大的版本用于同种异体 T 细胞系的商品化生产。在改进的方法中,将细胞培养设备为了自动化而修饰成矩形形状,以滑入标准 CO_2 培养箱中的槽内。这是明显的优点,因为气体渗透膜在瓶的底部并如果它位于架上则会被堵塞。通过使瓶成为架,存在对膜的更好的气流。关键属性是瓶侧面的凸缘,其允许它们托住生物反应器的重量。在一个实施方案中,生物反应器放置入不锈钢框内以形成插入架支撑物中的板。在另一个实施方案中,将它们塑成塑料部分。在实施方案之一中,它们具有完整架的底座。在另一个实施方案中,它们包含架的 ½、¼ 或 1/3 或 1/5。在优选的实施方案中,培养箱由 New Brunswick、Forma、ThermoElectron、Nuaire、ESCO 制造。在优选的实施方案中,培养箱可以是空气套或水套的或者是其他设计。在又一个实施方案中,它们适合于金属框的架内或在温暖房间的平面上。在另一个优选的实施方案中,该平面可移动并由机器处理。在又一个实施方案中,生物反应器适用于多种商品化处理设备,包括但不限于在收集细胞前使细胞回到悬浮状态的振荡器。

[0108] 每一生物反应器是封闭系统,其具有接触接口以引入培养基、组分和细胞,并且从该接口收获细胞用于冷冻和质量控制。在优选的实施方案中,这些接口是使流体和细胞进出生物反应器的管,所述接口的盖具有达到生物反应器的底部的一体的固体管。在可选实施方案中,接口是无菌的橡胶片,针或其他探针可以经其插入生物反应器中以手动或使用自动化机器来注射或移除流体细胞或其他试剂。将该设备用条形码编号以对其追踪,从而每一患者和每一细胞系将具有其自身指定的生物反应器。当该生物反应器使用后即可将其丢弃。还将生物反应器大小设计为适合自动化细胞培养中的标准机器人自动化设备,包括但不限于组套、运输器和摇动搅拌器。图 31 是此类生物反应器的实例。

[0109] 如同生物反应器，还将商品化多重免疫概况分析测定设计为能够测量抗体的效价和针对抗原组的 T 细胞应答。为了此目的，使用 ELISPOT 测定，这是由于可以应用标准 96 孔板形式。在优选的实施方案中，使用 AIDELISPOT 读数器 (Autoimmun Diagnostika, Strassberg, Germany) 来计数点。或者，使用 96 孔板来输入细胞至 FACS 进行 ICS 测定。在任何情况下，每一患者具有它们自身指定的 96 孔板，其经条形码编号并且所有板均是一次性的。这些商品化测定和制造方法是本发明的实施方案。

[0110] 实施例

1. 实施例 1 :EBV 潜在感染、淋巴瘤和鼻咽癌

如同由血液中的抗体所测量的，全世界人口的 90％已经暴露于 EBV(单核细胞增多症中的致病性病毒)。EBV 在 B 细胞中变为潜伏的并且关闭了大部分它的蛋白，但表达非常低水平的潜伏抗原 LMP1、LMP2 及有时表达 EBNA-1。这些蛋白是弱免疫原性的，但对于维持病毒甚至在它的潜伏状态中是必需的。由于它们主要来源于 B 细胞，因此 40％的淋巴瘤对 EBV 潜伏抗原测试为阳性。因此，这些抗原可以用作在过继细胞治疗中产生 CTL 应答的靶标。此外，鼻咽癌也如同其他肿瘤(例如，-10% 的胃癌) 一样表达 EBV 潜伏抗原。尽管 CTL 已经用于治疗 EBV 淋巴瘤，但目前使用转染有 EBV 作为重复刺激的 B 细胞的产生方法是耗时(3-6 个月) 且繁琐的。此外，以这种方式产生的针对 LMP2 的 CTL 产生 T 细胞批次的仅一半在产生后对 LMP2 具有可检测的应答。本发明人相信对此的原因是由于来自 EBV 蛋白的优势表位存在于 EBV 转化的产生 CTL 的 LCL 细胞中，其在 50％的时间过度生长针对 LMP2 的细胞。在患有巨大肿瘤的患者中，52％用通过传统方法产生的 CTL 治疗的患者具有完全应答。尽管现有技术认为所有 CTL 无论存在或缺少对 LMP2 的应答均是等效的，但本发明人认为这可以是可变的临床应答的一种原因。此外，本发明的操作模型预测这是真正的原因。因此，增加针对 LMP2 的 CTL 产生效率可以在临床上是重要的。

[0111] 尽管 CTL 制备的其他方法也包含在本发明内，但使用以下方法来产生以下实验数据。从患者收集 40 ml-100 ml 的外周血在 Vacutainer 管中。将外周血单核细胞 (PBMC) 通过在 Lymphoprep (Nycomed, Oslo, Norway) 上离心分离，在包含 2mM L 谷氨酰胺、100 IU/ml 青霉素、100 μg 链霉素 /ml 及 10％胎牛血清 (FCS) (5×10^6 细胞 /ml) 的 RPMI 1640 (Gibco, Grand Island, NY) 中重悬，并以 10^7 细胞 / 孔接种于 6 孔板 (Costar Corp, Cambridge, MA) 中。在 37 摄氏度下 2 小时后，将未粘附的细胞去除并重悬于置于试管内含有 10％聚乙二醇(PEG) 的 FCS 中，在干冰上冷冻并储存于 -80 冰箱中。将仍在 6 孔板中的粘附细胞在添加有每 ml 50 ng 的 GM-CSF 和 1000 U 的 IL-4 的 RPMI + 10％ FCS 中培养。在第 2 天和第 4 天用包含上述相同生长因子的新鲜培养基更换一半培养基。在第 6 天，用所述培养基将培养基完全更换，并加入 25％体积的巨噬细胞条件培养基以刺激成熟。巨噬细胞条件培养基由粘附于免疫球蛋白包被的板(由将 PBS 中的免疫球蛋白铺板并在 4 摄氏度下孵育过夜制备) 上的 PBMCs 在 RPMI 10％ FCS 中 37 摄氏度下 24 小时，收集上清液，经 0.2 mm 孔径膜 (Acrodisc, Gelman Sciences) 过滤来制备，并且使用前可以在 -20 摄氏度下储存至多 8 周。未粘附的细胞在 2 天后收集并用作树突细胞的来源。进行用单克隆抗体对表面标志物包括 CD54、CD80、CD83 和 CD86 的免疫荧光染色以确保树突细胞质量 (>50% 的细胞 +)。

[0112] 将 DC 刺激细胞在 37 摄氏度下预先暴露于在添加有 3 μg/ml 的人 β2 微球蛋白

的无血清 RPMI 1640 中的浓度为 μg/ml 的蛋白（肽为 50）中 2 小时。随后将它们洗涤并以 10^5 细胞 / 2 ml 孔接种于添加有 IL-7 5 ng/ml 的 RPMI 10% FCS 中。将 2 ×10^6 PBMC 加入每一孔中作为应答细胞，其与刺激细胞比例为 20:1。在第 14 天和第 21 天将培养物用在添加有 20 U/ml 的 IL-2 的 RPMI 10% FCS 中的负荷有自体肽的树突细胞再刺激（如需要，则分至额外的孔中）。

[0113] 用于治疗患者的 CTL 释放测试包括生存力 >70%、7 天后对细菌和真菌的阴性培养、低于 5 EU/ml 的内毒素检测、支原体的阴性结果、在 ^{51}Cr 释放测定中以 20:1 比例的受体淋巴母细胞的杀死低于 20%、低于 2% 的 $CD19^+$ B 细胞、低于 2% 的 $CD14^+$ 单核细胞和 HLA 特性。

[0114] 将多克隆 T 细胞群收集并用作 5 小时铬释放测定的效应物。对于铬释放测定，从 CTL 供体的皮肤活组织检查中建立成纤维细胞的单层培养，并且暴露于重组牛痘病毒（2x 10^6 细胞 /9 cm 培养皿）。转染后 18 小时收集细胞并用 $^{51}CrO_4$ 标记 1 小时，洗涤三次，并用作 5 小时铬释放测定中的靶标。在 γ 计数器上计数前，将测定的上清液收集入 1% 甲醛中。

[0115] a 实验 1：当树突细胞或活化的巨噬细胞代替 LCL 用作抗原呈递细胞时，对亚优势表位应答的 T 细胞的相对频率增加。

[0116] 使用 3 种不同的抗原呈递细胞作为刺激细胞从 10 位患者中制备 T 细胞系：EBV 转化的淋巴母细胞细胞系（LCL）呈递和扩增；树突细胞（DC）呈递及细胞因子扩增；IFNγ 巨噬细胞 (MAC) 呈递及细胞因子扩增。所述 3 种中的每一种在抗原呈递过程中用 3 种质粒的混合物刺激，所述 3 种质粒表达 EBNA-1（具有 aa 90-325 缺失的 EBNA-1）、LMP1（具有 aa 1-43 和 aa 260-315 缺失的 LMP1）和 LMP2（LMP2A 在 2 种质粒中，一种表达 aa 1-399 并且第二种质粒表达 aa 400-497）的亚优势表位。将使用本发明的方案这样产生的 T 细胞系随后在 ^{51}Cr 释放测定中研究。

[0117] 图 1A 和 1B：分别以 20:1 和 10:1 的两种不同的效应物：靶标（E/T）（CTL 系：HLA 匹配成纤维细胞）比例的 ^{51}Cr 释放，HLA 匹配的成纤维细胞用来自所示抗原的肽混合物预先脉冲，使用不同的指示的 APC 从代表性患者中扩增 CTL 以培养 CTL 系。

[0118] 结论：树突细胞（DC）和巨噬细胞（MAC）比 EBV 转化的 B 细胞 (LCL) 更选择性地刺激针对亚优势抗原的 CTL 的生长。

[0119] 图 1C: 以三种不同的效应物：靶标 (E/T) 比（CTL: HLA 匹配的成纤维细胞）的 ^{51}Cr 释放，HLA 匹配的成纤维细胞用来自血液采集后直接得自相同患者的 PBMC（培养前）的指示的抗原 CTL 的肽混合物预先脉冲。

[0120] 结论：患者对于 EBV 优势抗原具有某些应答，但相比于 T 细胞培养后具有明显更高的 E/T 比。

[0121] 图 2：以 20:1 的效应物：靶标 (E/T) 比（CTL 系：HLA 匹配成纤维细胞）的 ^{51}Cr 释放，HLA 匹配的成纤维细胞用代表来自 LMP2 的特定 HLA A2 限定亚优势表位的肽预先脉冲，并且 CTLs 使用上述三种不同方法从相同患者中制成。

[0122] 结论：树突细胞和巨噬细胞均比 LCL 导致针对更大数目的亚优势表位的应答和更高水平的 CTL 活性，并且对不同表位的应答强度在树突细胞和巨噬细胞之间不同。

[0123] 图 3A 和 3B：以 20:1 的效应物：靶标 (E/T) 比（CTL 系：HLA 匹配成纤维细胞）的 ^{51}Cr 释放，HLA 匹配的成纤维细胞用来自 LMP2 的肽预先脉冲：LCL 刺激在图 3A 中，并且 DC/MAC 刺激在图 3B 中。

[0124] 结论：相比于 LCL 其仅导致 50％的来自患者的 CTL 系具有可检测的 LMP2 应答，树突细胞和巨噬细胞导致 90％的患者中的 CTL 细胞具有可检测的 LMP2 应答。因此，DC/MAC 处理更有效且更可重复地产生针对亚优势抗原和表位的 T 细胞。

[0125] 图 3C：使用巨噬细胞、树突细胞或 LCL 细胞作为抗原呈递细胞培养的 CTL 系中为 $CD4^+$、$CD8^+$ 和 $CD25^+$ 的存活 $CD3^+$ 细胞％。将系用针对 CD3、CD4、CD8 和 CD25 的抗体染色并通过流式细胞术分析。

[0126] 结论：尽管所有方法建立 $CD8^+$ 细胞而非 Tregs，但使用树突细胞和巨噬细胞作为 APC 似乎相对于用 LCLs 产生的增加了 $CD4^+$ 细胞的％。

[0127] ELISpot 测定：

进行 ELISPOT 测定以确定针对特定抗原产生的 T 细胞数目。用 15 μg/ml 的抗 IFNγ 单克隆抗体 1-DIK (MABTECH, Stockholm, Sweden) 包被 ELISPOT γ IFN 96 孔聚亚乙烯二氟衬板 (Millipore, Bedford, MA)。将 5×10^6 PBMCs 及每种蛋白各 2 μM 的肽混合物加入每孔中，并在 37 摄氏度 5% CO_2 下孵育过夜。丢弃细胞，并将 1 μg/ml 生物素化的 IFNγ 单克隆抗体 7-B6-1 (MABTECH) 在室温下孵育 2-4 小时，随后加入链霉抗生物素缀合的碱性磷酸酶 (MABTECH) 再孵育 2 小时。用来自 alk-phos 底物试剂盒 (Bio-Rad Richmond, CA) 的 5- 溴 -4- 氯 -3- 吲哚磷酸酯和硝基蓝四唑反应 30 分钟后，根据样品的数目，使用解剖显微镜或 AIDELISPOT 读取器 (Autoimmun Diagnostika, Strassberg, Germany) 对点计数。每一个点为报道为斑点形成细胞 (SFC)/ 10^5 PBMC 的细胞。在这些测定中，阳性对照为细胞 + 10 μg/ml PHA，并且阴性对照为单独的不含肽的细胞

图 4：由 ELISPOT 测量的体外培养的 IFN 产生细胞 (SFC) /105 T 细胞。

[0128] 结论：树突细胞和巨噬细胞相比于 LCL 更选择性地扩增针对亚优势抗原的 T 细胞。

[0129] 优化抗原以建立针对亚优势表位的应答。

[0130] 选择 LMP2 用于优化，这是由于它是相对于 EBNA-3 的亚优势抗原，并且如使用共同软件和 PBMC 测试所鉴定的，所识别的 EBV 亚优势表位的大部分似乎均在该蛋白质上。为了增加识别的亚优势表位数目，将 LMP2 分开至两个或更多个质粒上。以下质粒构建于 p 穿梭或 pUC19 质粒中，并在 CMV 启动子和人工 ATG 和多聚 A 的控制下：

质粒 1 LMP2B (aa 1-497)

质粒 2 LMP2A 第一外显子 (aa 1-119)

质粒 3 LMP2A 第二外显子 (aa 120-497)

质粒 4 LMP2A (aa 120-399)

质粒 5 LMP2A (aa 400-497)

质粒 6 LMP2A (aa 120-440)

质粒 7 LMP2A (aa440-497)

质粒 8 LMP2A (aa 1-399)

质粒 9 LMP2A (aa400-497)

使用标准程序在 SCS110 细菌菌株 (Stratagene, La Jolla, CA) 中产生所有质粒，并且用 Endo free Plasmid Maxi 试剂盒 (Qiagen, Hilden, Germany) 纯化。在成熟后使用 Amaxa DC Nucleofection Kit (Amaxa, Koeln, Germany) 用 2-20 μg 质粒 DNA/10^6 细胞转染抗原呈递细胞 24 小时。

[0131] 在 LMP2 上的 T 细胞免疫亚优势表位包括但不限于：

LLW 329-337 LLWTLVVLL HLA A 2.01

CLG 426-434 CLGGLLTMV HLA A 2.01

IED 200-208 IEDPPFNSL HLA B 40.01

SSC 340-350 SSCSSCPLSKI HLA A11.01

TYG 419-427 TYGPVFMCL HLA A24.02

LLS 447-455 LLSAWILTA HLA A2

LTA 453 461 LTAGFLIFL HLA A2

FLY 356-364 FLYALALL HLA A2

合成这些表位的肽并用于在 ^{51}C 释放测定中测试 CTL 对从 PMBC 供体的皮肤分离的表达 HLA-A2 的成纤维细胞的应答。

[0132] 图 5：以 20:1 的效应物：靶标 (E/T) 比（CTL 系：HLA 匹配成纤维细胞）的 51Cr 释放，HLA 匹配的成纤维细胞用来自 LMP2 的肽预先脉冲：CTL 系用分至 1、2、3 或 4 个质粒上的 LMP2 建立（1P: 497 aa LMP2B; 2P: 第 1 质粒上的 LMP2A 第一外显子 (119 aa), LMP2A 第二外显子 (378 aa); 3P: 在第 1 质粒上的 LMP2A 第一外显子 (119 aa), 在第 2 质粒上的 LMP2A (120-399 aa) 和在第 3 质粒上的 LMP2A (400-497 aa); 4P: 在第 1 质粒上的 LMP2A 第一外显子 (119 aa), 在第 2 质粒上的 LMP2A (120-399 aa)，在第 3 质粒上的 LMP2A (400-440 aa)，在第 4 质粒上的 LMP2A (440-497 aa); 2P 2: 质粒 1 (aa 120-440), 质粒 2 (aa 440-497))。

[0133] 结论：将 LMP2 分至至少两个质粒上（一个含有 aa 400-497，另一个含有 399 之前的残基）导致针对更大数目的亚优势表位的更强应答。

[0134] 从 EBNA-1 序列中删除对应于 Gly Ala 重复结构域的 EBNA-1 氨基酸 90-325，并插入 p 穿梭质粒中在 CMV 启动子的控制下。选择该序列删除是由于证明它抑制肽加工。将以下 HLA-A2 限制肽用于评估应答：VLK 574-582 HLA A2。

[0135] 此外，制备删除 aa 1-43（以避免它的聚集 / 保护影响蛋白酶体加工）和删除 260-315（5 个拷贝的 11 个氨基酸串联重复）的 LMP1 序列。将这些序列构建在 p 穿梭质粒中在 CMV 启动子控制下，并与野生型 LMP1 比较。为了评估应答的广度，制备并测试以下 LMP1 的 HLA-A2 限制表位：

YLL 125-133 YLLEMLWRL HLA A2

YLQ 159-167 YLQQNWWTL HLA A2

TLL 166-174 TLLVDLLWLL HLA A2

LLV 167-175 LLVDLLWLL HLA A2

LLL 92-100 LLLIALWNL HLA A2

RLG 132-140 RLGATIWQL HLA A2

图 6A 和 6B：以 20:1 的效应物：靶标 (E/T) 比（CTL 系：HLA 匹配的成纤维细胞）的 ^{51}Cr 释放，HLA 匹配的成纤维细胞用来自 EBNA-1 或 LMP1 的肽预先脉冲：比较用 EBNA-1 野生型与 EBNA-1 删除所建立的 CTL 系，以及比较用 LMP1 野生型与 LMP1 删除所建立的 CTL 系。

[0136] 结论：删除蛋白的避免抗原加工的某些区域导致针对更大数目的亚优势表位的更强应答。

[0137] b. 实施例 2

在以下实验中，将 T 细胞如上文实施例 1 中所概述的相同方式培养，并且设计不同变化以增强抗原加工，以利于 T 细胞与亚优势表位的产生发生反应。

[0138] 图 7 :以 20:1 的效应物 :靶标 (E/T) 比 (CTL 系 :HLA 匹配成纤维细胞) 的 (^{51}Cr 释放)Cr 释放，HLA 匹配的成纤维细胞用代表优势抗原 (EBNA-3A)、亚优势抗原 (LMP2) 和来自 LMP2 的特定 HLA A2 限制性亚优势表位的肽预先脉冲，并且 CTLs 使用两种不同方法从相同患者中制成 :一种在抗原呈递过程中加入 100nM-300nM 的硼替佐米，另一种则不加入。

[0139] 结论 :在 CTL 抗原呈递过程中加入蛋白酶体拮抗物硼替佐米修饰了抗原加工以产生针对更广泛数目的亚优势表位的 CTL，并且在针对优势表位的应答中适度降低。

[0140] 图 8 :以 20:1 的效应物 :靶标 (E/T) 比 (CTL 系 :HLA 匹配的成纤维细胞) 的 (^{51}Cr 释放)Cr 释放，HLA 匹配的成纤维细胞用代表优势抗原 (EBNA-3A)、亚优势抗原 (LMP2) 和来自 LMP2 的特异性 HLA A2 限制性亚优势表位的肽预先脉冲，并且 CTLs 使用两种不同方法从相同患者中制成 :一种在抗原呈递之前 12 小时和呈递过程中加入 10 ng/ml 的干扰素 γ (IFNγ)，另一种则不加入。

[0141] 结论 :在 CTL 抗原呈递过程中加入 IFNγ 修饰了抗原加工以产生针对更广泛数目的亚优势表位的 CTL，并且在针对优势表位的应答中适度降低。该效应在巨噬细胞中比在树突细胞中更显著。

[0142] c. 实施例 3 :肝炎和肝细胞癌

近来已开发了慢性肝炎的小鼠模型，其允许研究乙肝中的原发性和继发性免疫应答 (Publicover J 等 2011)。该 HBVtgRAG 小鼠是在 C57BL/6 背景下 HBV 复制 15 代的转基因小鼠 (HBVRpl) (其组成性地允许病毒复制和病毒体的释放) 与 RAG-1 缺失小鼠 (使其无法产生 T 细胞和 B 细胞) 的杂交小鼠。当从 C57BL/6 小鼠转移 10^8 脾细胞时，免疫系统重建并且针对肝炎感染的原发性免疫应答建模。如果施用脾细胞至年幼 (3-4 周龄) 小鼠，则动物 (与感染有乙肝的幼童类似) 会发展慢性肝炎。和人一样，它们清除血清中的 HBc，但血清中的 HBs 保留高水平 (图 1)。将该慢性肝炎的模型用于以下实验中。

[0143] 使用 ALT-L3K 试剂盒 (Diagnostic Chemicals Ltd) 在 Cobas Miras Plus 分析仪 (Roche diagnostics) 上测量丙氨酸转氨酶。使用 ETI-MAX 2 Plus (Diasorin) 测量 HBs Ag ;使用 ETI-AB-AUK-PLUS 和 ABAU 标准组 (Diasorin) 测量 HBs 抗体。使用 ETI-AB-COREK-PLUS (Diasorin) 测量 HBcAB。测定在 ELx800 (Biotek Instruments) 上波长 450nm 和 630nm 处读取。

[0144] 图 9 :慢性乙肝的 HBVtgRAG 小鼠模型。

[0145] 使用我们的方案先体外后体内将 T 细胞培养为 HBs，并在原始脾细胞转移后 3-4 周通过尾静脉注射。从 1×10^5 — 1×10^8 细胞进行滴定，在 1×10^6 细胞时达到平台。如下文可见，我们的 T 细胞重新平衡治疗清除了 HBVtgRAG 模型中的慢性肝炎。

[0146] 图 10 :用对 HBs Ag 反应性的 T 细胞处理 HBVtgRAG 模型导致急性炎症和之前的慢性感染的清除。

[0147] 当在抗原呈递过程中将 IL-4 或 IL-21 用作向培养基中的添加物时，达到平台所需的 T 细胞的量分别为 5×10^5 和 7×10^4。该细胞数低于在标准 IL-15 方案中扩增的 T 细胞多于一个对数，并且表明在培养过程中向滤泡 T 辅助细胞和 TH2 细胞的极化是有利的。另

一方面，IL-12 的极化增加达到平台所需的细胞数至 1×10^7，并且用 IL-2 和雷帕霉素的对 Treg 的极化消除了对治疗的应答。

[0148] 在清除 HBVtgRAG 小鼠中的肝炎中不同的培养条件导致对不同 T 细胞亚群的极化和不同数目的 T 细胞达到平台。

培养条件	达到平台的 T 细胞数目
IL-15	1×10^6
IL-15+IL-4	5×10^5
IL-15+IL-12	1×10^7
IL-15+IL-21	7×10^4
IL-2+ 雷帕霉素	无应答

[0149] 因此，T 细胞对不同 T 细胞亚群的极化具有重要的治疗作用。

[0150] 为了确定 T 细胞治疗对针对不同表位的 T 细胞频率的作用，通过 ELISPOT 测定针对不同表位的 IFNγ 产生表位的%。在移植后第 6 周，从 HBVtgRAG 小鼠中收集脾细胞，所述 HBVtgRAG 小鼠未用或者用平台水平的针对 HBs Ag 生成的 T 细胞处理。用肝炎核心抗原 (HBc)、肝炎表面抗原 (HBs) 或 HBs 的两种 Kb 限制肽对脾细胞脉冲：ILS 或 WWL。

[0151] HBs Ag 190-197 VWLSVIWM K^b

HBs Ag 208-215 ILSPFLPL K^b

观察到以下结果：

图 11：用 HBs T 细胞处理将免疫系统重新平衡至新的优势等级。

[0152] 如可见的，使用我们的方案制备的 T 细胞系产生明显更高的针对 HBs 的 T 细胞应答和略微降低的针对 HBc 的应答。HBs 应答主要通过针对之前的亚优势表位的从新应答所驱动 (WWL)。因此，HBs 内的优势等级由 T 细胞重新平衡治疗所转换，这是由于存在于未处理的动物中的应答 (尽管微弱) 是针对优势表位 (ILS)，其相对于之前优势的 HBc 抗原转换 HBV 应答的总体平衡以利于之前的亚优势 HBs 抗原。

[0153] 还使用 HBVtgRAG 小鼠模型以研究体内方法是否还能够将免疫优势等级重新平衡。在一种方法中，本发明人通过尾静脉注射 (IV)、肌内 (IM) 或腹腔内 (IP) 注射弗氏佐剂中的 HBs 抗原。或者，每只小鼠施用 100μg 质粒 DNA (数据未显示)。

[0154] 图 12：在免疫接种过程中用蛋白酶抑制剂的剂量处理时，IM 途径施用导致针对亚优势抗原的更广泛的应答。

[0155] 如可见的，相比于另外两种，通过 IM 施用产生了 4 倍更强的应答，并且这与针对亚优势表位的应答相关。此外，当用蛋白酶体拮抗物硼替佐米处理该小鼠并且抗原经 IM 施用时，针对该亚优势表位的应答进一步增强。

[0156] 在第二组实验中，单独以及与不同同种型 (IgG1 对 IgG2a) 的两种 HBs 反应性小鼠抗体复合进行应答。

[0157] 针对 HBs Ag 的单克隆抗体

10-H05 小鼠 IgG1 HBsAg (Fitzgerald, Concord, MA)

10-H05A 小鼠 IgG2a HBsAg (Fitzgerald, Concord, MA)

图 13：对 HBs Ag 特异性的 IgG2a 单克隆抗体而非 IgG1 抗体导致不同的优势等级和通过 IV 途径的明显更好的抗原呈递。

[0158] 当将抗原抗体复合物静脉内和 IM 施用时，IgG2a 复合物产生更多的针对亚优势表位的应答。此外，该方案产生了通过 IV 施用的与通过 IM 施用获得的几乎相等的免疫应答

水平，表明 IgG2a 免疫复合物的 IV 施用可以能够重新平衡体内免疫优势等级。

[0159] 本发明人假设可能该观察的机制是由于 IgG2a 结合了优先表达于树突细胞中的高亲和力 FcR，并因此将 HBs 抗原靶向树突细胞。此外，由于 HBs Ag a 决定簇第一环 (aa 124-147) 是由中和抗体识别的主要表位，并且 ILS 表位离得较远，因此可能是对于 ILS 的抗原加工得到增强。

[0160] T 细胞重新平衡治疗在 HBV 相关的肝细胞癌中的临床试验

对 5 位具有 HBV 和 HCC 的为 HLA-A *0201 的患者进行它们的体液和细胞免疫应答的免疫概况分析。治疗前的临床实验室测试证明针对乙肝核心抗原 (HBc Ag) 而非针对乙肝表面抗原 (HBs Ag) 的高效价抗体(图 10)。

[0161] 将 PBMC 诱导成为树突细胞，用乙肝核心抗原 (HBc Ag) 和乙肝表面抗原 (HBs Ag) 脉冲，并用于培养来自 PBMC 的 CTL，其通过 ELISPOT 测试 IFNγ。

[0162] 图 14：来自患者 3 的 10^5 PBMCs 中斑点形成细胞的数目

通过在 ELISPOT 中产生 IFNγ 的 T 细胞的频率，观察到等级：无抗原 (10 sfc)<HBsAg(15 sfc)<HBcAg(45 sfc)。这表明乙肝表面抗原相对于乙肝核心抗原是亚优势的。此外，研究了 3 种 HBs 肽 (FLL、GLS 和 ILS)：

HBs Ag 20-28 FLLTRILTI HLA-A*201

HBs Ag 185-194 GLSPTVWLSV HLA-A*201

HBs Ag 208-216 ILSPFLPLL HLA-A*201

就针对 HBs 的微弱应答是可检测到的而言，FLL 肽似乎稍微是优势的，但由于应答微弱，因此通过 ICS 测定来确定优势等级：

胞内细胞因子染色(ICS)

将 5×10^5 CTL 重悬并在 100 μl 的含有肽 (10^{-5}-10^{-9} M 终浓度) 的 1XPBS 1% FCS 中孵育 1 小时。随后加入 Golgi Plug (BD Biosciences, San Diego, CA)，将细胞在 37 摄氏度 5% CO_2 下孵育 5 小时，沉淀，用 200μl PBS 1%FCS 洗涤，并在 4 摄氏度下对表面抗原 (CD4 异硫氰酸荧光素和 CD8 别藻蓝蛋白 (Pharmingen, Becton Dickinson) 染色 30 分钟。在固定和透性溶液中重悬后，将细胞在冰上用 1/20 稀释的抗人 IFNγ 藻红蛋白 (Pharmingen, BD) 染色 30 分钟，洗涤一次，并在 PBS 1% FCS 中重悬并在 FACS Canto (Becton Dickinson) 上分析。

[0163] 图 15：针对 HBs 表位的应答的 ICS 分析

尽管通过 ICS 可检测到的应答并不强，但 FLL 表位的确表现出相对于其他两种表位为优势的。

[0164] 将 5×10^6 PBMC 从每一患者中分离，并将 T 细胞按照我们的方案使用 HBs 15 聚体肽混合物作为刺激抗原进行培养，其中所述肽排除了肽 15-30 (以消除 FLL)。在扩增过程中使用 IL-4 + IL-15。将 1×10^{10} T 细胞获取并冷冻，并取一等分试样用于 ELISPOT 测定。

[0165] 图 16：HBs 细胞是大部分向患者施用的 T 细胞，并且这些细胞对之前亚优势的抗原应答。

[0166] 如可见的，产生的 CTL 系几乎仅对之前亚优势的 HBs 具有反应性，其是主要由之前的亚优势表位驱动的应答。将 CTL 经 IV 输注以 5×10^7-1×10^8 细胞 /m^2 给药，并监测患者。

[0167] 图 17：患者具有急性发作并随后清除了肝炎。

[0168] 在发展成急性肝炎后，患者缓解并完全清除了肝炎。在第 14 天，再次收集 PBMC 并且再次确定细胞免疫应答的概况。

[0169] 图 18 :患者的免疫优势等级已经重新平衡为之前亚优势抗原（HBs）和该抗原上之前亚优势的表位。

[0170] 通过由我们的方法培养的 T 细胞已成功地将免疫优势等级重新平衡。除了清除慢性肝炎之外，患者还具有针对他的肝细胞癌的完全应答。

[0171] 图 19 :T 细胞完全缓解了患者的肝细胞癌。

[0172] d. 施例 4 :治疗癌症的系统方法

癌症的临床诊断和治疗涉及肿瘤的活组织检查和成像，以确定它的组织的来源、分化及局部和全身转移的程度。尽管有时进行对癌基因或肿瘤抑制基因中的缺陷的诊断以及确定对化学治疗剂或生物制剂的敏感性的测定，但通常根据患者的具体癌症和阶段，使他们用手术、化学治疗和放射治疗的组合进行治疗。类似地，尽管已经使用了不同技术研究了多种患者中的免疫系统，但这一信息尚无法用于患者的临床处理中。随着他们的细胞治疗以重新平衡免疫系统的来临，本发明人可以建立免疫概况分析在癌症患者的处理中的用途。本发明人已经开发了标准化的免疫概况分析方法学，其用于选择在该患者中是亚优势的抗原并且可以用于体外培养 T 细胞，所述 T 细胞能够重新输注入患者中以重新平衡针对肿瘤的免疫应答。治疗后，对患者进行免疫概况分析以确定是否该治疗成功地将免疫应答重新平衡。这样的治疗方法是新颖的，并且能导致临床应答和增进存活。同样的方法可用于传染病和自身免疫和器官移植。

[0173] 第一步是鉴定哪一种与肿瘤相关的抗原存在于患者的肿瘤中。这通常通过对来自患者肿瘤的活体样本上进行免疫组织化学来完成。检测抗原组将依赖于肿瘤类型。例如，在黑素瘤中，抗原包括 NY-ESO-1、SSX-2、Melan A、gp100、MAGE A4、MAGE A1、酪氨酸酶，并且当描述了新的肿瘤相关抗原时将被补充。并非所有肿瘤都将表达组内的所有抗原。对于患者肿瘤中具有的抗原，使用如上所述对体液和细胞免疫应答的系统性概况分析来确定针对不同抗原的基线免疫概况。使用 Elisa 来确定血清中针对每一抗原的抗体滴度，并且通过 IFNγ ICS 或 ELISPOT 测定对抗原相应的 T 细胞的％。以这种方式，确定基线概况。

[0174] 图 20 :患者 1 中对肿瘤抗原组的 IFNγ ICS

在患者 1 中，患者的肿瘤具有 NYESO-1、SSX-2、Melan A 和 MAGE A4。ICS 证明对 MAGE A4 的强应答，但对 NYESO-1、SSX-2 和 Melan A 的中度 / 无应答。因此选择 NYESO-、SSX-2 和 Melan A 抗原使用以下方案来在体外培养中生长并扩增 CTL :

从血液中分离 5×10^6 PBMC。通过将 PBMCs 铺于组织培养瓶中 2 小时以允许单核细胞的粘附，从患者的外周血单核细胞 (PBMCs) 体外产生单核细胞来源的树突细胞。此时，将未粘附的细胞去除并冷冻于 -80 中后期用作 T 细胞来源。用白细胞介素 4(IL-4)和粒细胞 - 巨噬细胞菌落刺激因子（GM-CSF）处理粘附的单核细胞导致在约 1 周内分化为未成熟的树突细胞 (iDCs)。用肿瘤坏死因子（TNF)的后续处理进一步将 iDCs 分化为成熟的树突细胞。随后将这些细胞分至 3 个单独的瓶（或者无论多少希望用于培养 T 细胞的抗原数量）中，其含有添加有 45% Click 培养基 (Irvine Scientific, Santa Ana, CA)、2mM Glutamax 1 和 5% 人血清的 RPMI 1640 培养基。用包含目标抗原的编码序列（在该患者 1 中含有 NY-ESO-1，1 位患者含有 SSX-2，并且第三位含有 Melan A）的 3 种质粒（或 pepmix）之一将每一瓶脉冲。

将细胞储存在37摄氏度下2小时。同时，将PBMC融化并以PBMC对树突细胞比例1:20-1:100加入经脉冲的树突细胞，并在37摄氏度下孵育18小时。随后合并三瓶细胞并在含有IL-15(5ng/ml)相同的培养基中重悬，以产生已识别抗原的T细胞的体外扩增。使用Grex气体渗透生物反应器(Wilson Wolf Manufacturing Minneapolis, MN)，从而不需更换培养基并实现指数生长动力学。通常在3-6周内获得3×10^{8}-1.5×10^{10}细胞的足够细胞以向患者施用。

[0175] T细胞在气体渗透生物反应器中生长。

[0176] T细胞在气体渗透膜上生长为单层以优良的气体交换，并具有足以生长至所需密度的培养基体积。

[0177] 在体外培养结束时，用相同抗原在ICS测定中测定T细胞：NY-ESO-1、SSX-2、Melan A、gp100、MAGE A4、MAGE A1、酪氨酸酶。

[0178] 图21：患者1的ICS测定

基于测定，在该情况下确定针对每一亚优势抗原/表位(NY-ESO-1、SSX-2和Melan A)应答的细胞的%。对每一亚优势抗原或表位应答细胞总数可以使用该%和培养物中CTLs的数目来计算。在患者1的情况下，61.4%的细胞对NY-ESO-1应答(图21)。

[0179] 基于这一点，可以将对亚优势抗原应答的CTLs的已知剂量向患者施用。将CTL以5×10^{6} — 2×10^{8}细胞/m^{2}给药。

[0180] 向患者输注后2-3周，再次收集PBMC，并通过ICS再次确定细胞免疫应答的概况：

图22：患者1—治疗后通过ICS的细胞免疫概况分析

图23：患者1—通过ELISA的体液免疫概况分析(体液免疫应答的倒数效价在下文作图)。

[0181] 如同从免疫等级测定的细胞和体液概况中可见，免疫优势等级被重新平衡以利于之前的亚优势抗原。

[0182] 临床发现：

临床上，患者为52岁男性，其被诊断患有IV期转移性黑素瘤。他对组合有IL-2的达卡巴嗪(DTIC)和Temodar(替莫唑胺(Temolzolomide))化学治疗剂的组合方案无应答。

[0183] T细胞治疗两年后，患者存活并且已进行完全应答。如胸部CT扫描中可见，在免疫重新平衡治疗的6个月内肺中的转移完全缓解并且已保持稳定。

[0184] 图24：T细胞治疗前和后的CT扫描

e. 实施例5：淋巴瘤的T细胞治疗

将150位患有复发性侵袭性非霍奇金淋巴瘤的成年患者随机分为3组：A:Rituxan + CHOP; B:测试肿瘤中的EBV LMP1和LMP2抗原，随后为EBV LMP1和LMP2 T细胞重新平衡和C：泛淋巴瘤：不测定抗原；用对EBV LMP1、LMP2、存活、MAGE A3应答生长的T细胞治疗。40%的淋巴瘤活组织检查对EBV LMP2测试为阳性，50%的测试对存活为阳性，并且15%的测试对MAGE A3为阳性。使用Care R-CHOP方案的标准，并以5×10^{7}-2×10^{8} T细胞/m^{2}给药。

[0185] 图25：无疾病进展的存活

如可见的，对肿瘤应答的T细胞提供了优于目前的护理标准的应答率。尽管在第1年中发生失败，但第1年后CTL使患者维持减轻。这是免疫系统重新平衡后正常行使功能的

证据。最后，尽管最初 EBV LMP T 细胞产生了较好的应答，在到第 3 年，无疾病进展的存活已达到泛淋巴瘤产品的应答。此外，如今的淋巴瘤是复发性 - 减轻性疾病，患者通常在 18 个月至 2 年内复发。CTL 重新平衡治疗改变了这一过程：一旦患者的免疫系统重新平衡，患者会发展出维持长期减轻的记忆应答。因此，与淋巴瘤的其他治疗不同，T 细胞治疗提供了持久的减轻。

[0186] 图 26：T 细胞治疗改变了疾病的自然史

f. 实施例 6：自身免疫疾病的 T 细胞治疗

胶原诱导的关节炎模型 (CIA) 是类风湿关节炎（RA）模型，其能够由用异源胶原 II（CII）在 DBA/1 小鼠中免疫来诱导。

[0187] DBA/1 雄性 6-8 周龄的小鼠获自 Jackson Laboratories (Bar Harbor, ME)。将 100 μg 含有 4 mg/ml 结核分枝杆菌 (Chondrex) 在 CFA 中乳化的牛 CII (Chondrex, Redmond, WA) 经皮下注射入尾中。到注射后第 5 周，80-100% 的未处理的小鼠显示出完全发生的疾病。

[0188] 使用对 T 细胞的体外生长所述的方案将 T 细胞以下肽培养。

[0189] II 型胶原

用于刺激 Treg 生长的 263-270 免疫优势肽（IL-2 + 雷帕霉素）

用于刺激 T 细胞生长的 286-300 亚优势肽（IL-15）

在诱导后第 20 天将 5×106 细胞单独或与相等部分组合向每一动物施用。见图 27。CD25 是 Treg 细胞的标志物。对照动物接受 PBS。见图 28。

[0190] 每周三次对小鼠进行临床疾病评分，使用每肢 0-3 分，最大可能总分为 12 分：0-1 正常；1 单趾轻微发红或肿胀；2 踝或腕明显肿胀并有红斑；3 多个关节的严重肿胀及红斑。组中具有关节炎损伤的动物的百分比代表关节炎的发生率。组中平均临床分数反映了疾病的严重性。参见图 28 和图 29。

[0191] 关节的组织病理学

图 30A 显示了正常大鼠，图 30B 显示了用人蛋白聚糖免疫的大鼠，并且图 30C 显示了用 T 细胞处理的大鼠。

[0192] 如可见的，T 细胞治疗明显降低了动物关节中炎症的发生率、严重性和量。在重新平衡针对亚优势表位生长的 T 细胞和针对优势表位生长的 Treg 之间的免疫应答中似乎存在协同效应。

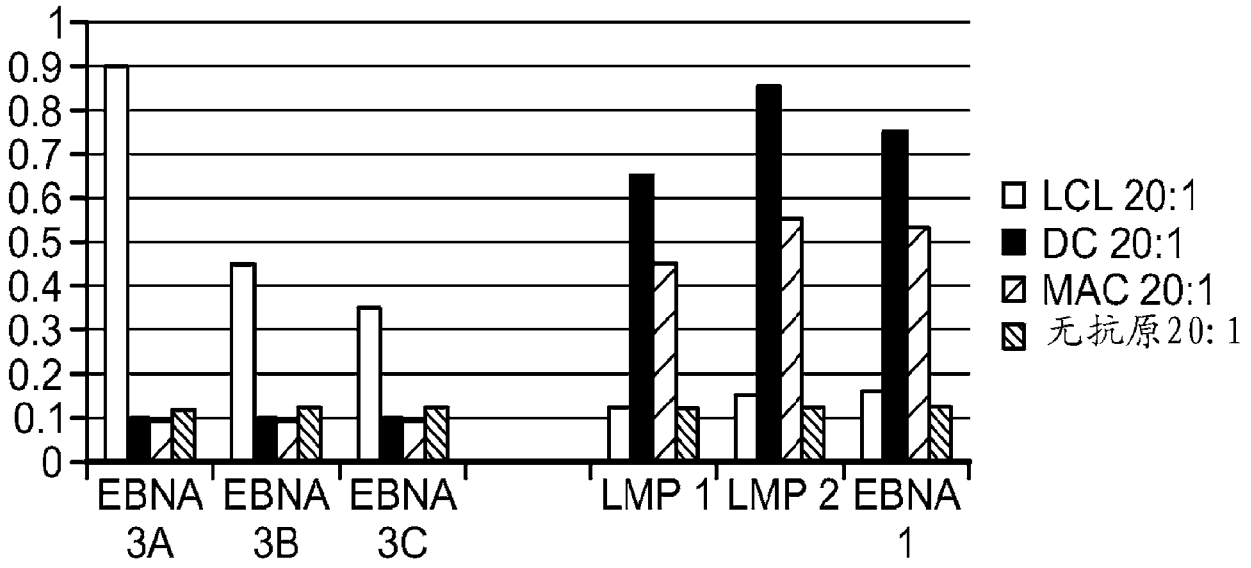

1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
EBNA 3A
EBNA 3B
EBNA 3C
LMP 1
LMP 2
EBNA 1
LCL 20:1
DC 20:1
MAC 20:1
无抗原20:1

图 1A

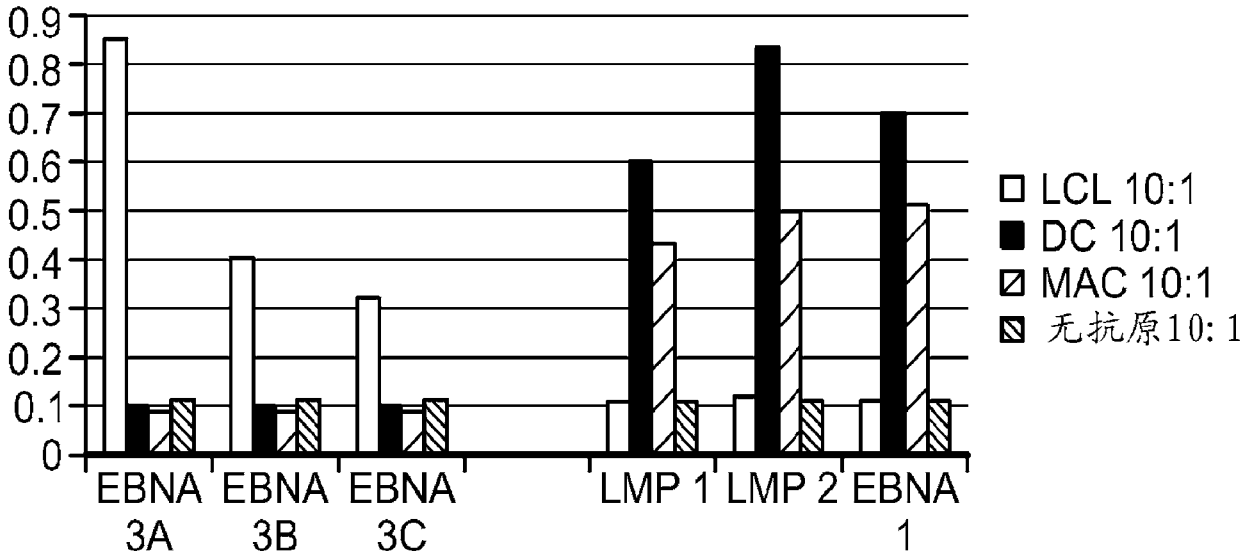

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
EBNA 3A
EBNA 3B
EBNA 3C
LMP 1
LMP 2
EBNA 1
LCL 10:1
DC 10:1
MAC 10:1
无抗原10:1

图 1B

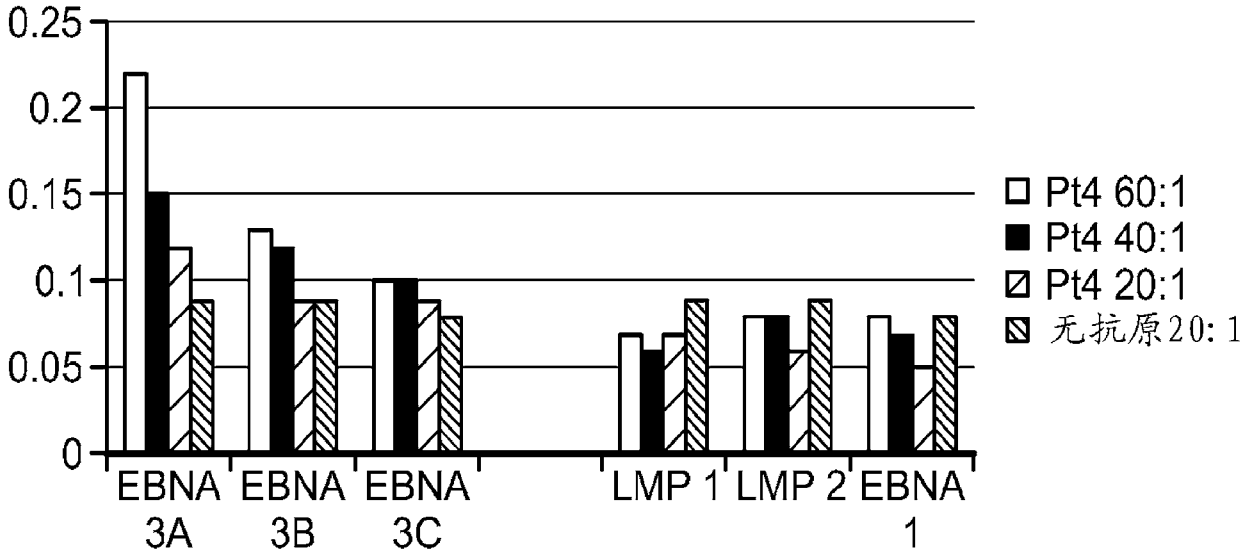

0.25
0.2
0.15
0.1
0.05
0
EBNA 3A
EBNA 3B
EBNA 3C
LMP 1
LMP 2
EBNA 1
Pt4 60:1
Pt4 40:1
Pt4 20:1
无抗原20:1

图 1C

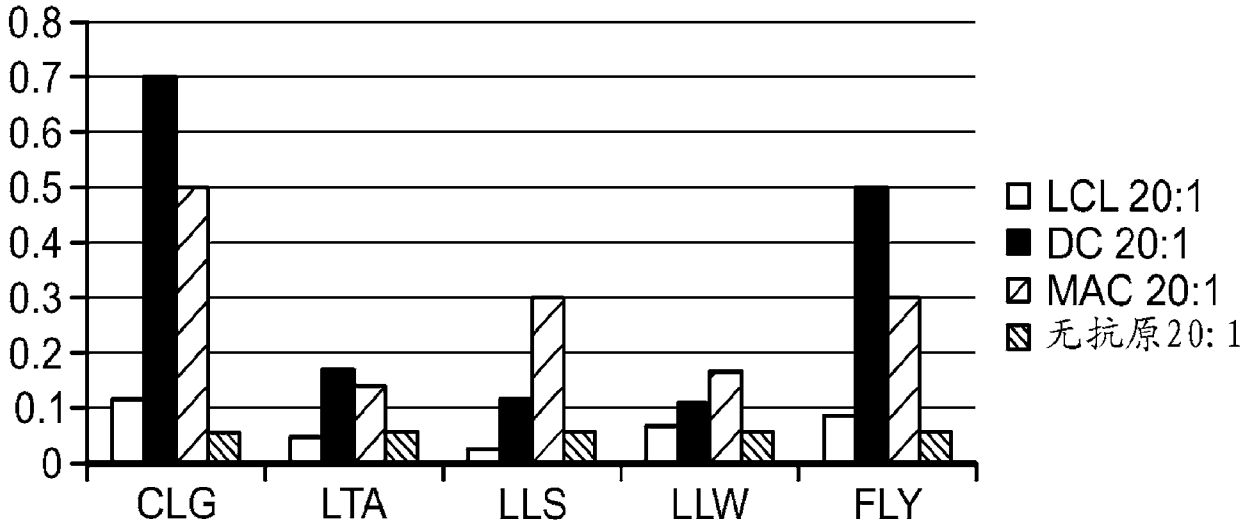

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
CLG
LTA
LLS
LLW
FLY
LCL 20:1
DC 20:1
MAC 20:1
无抗原20:1

图 2

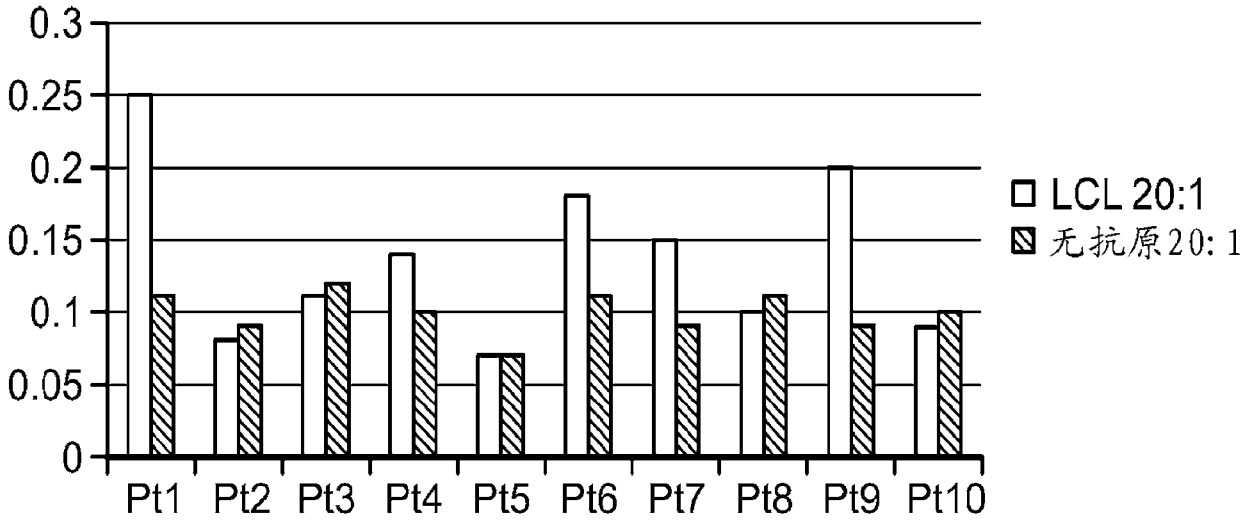

0.3
0.25
0.2
0.15
0.1
0.05
0
Pt1
Pt2
Pt3
Pt4
Pt5
Pt6
Pt7
Pt8
Pt9
Pt10
LCL 20:1
无抗原20:1

图 3A

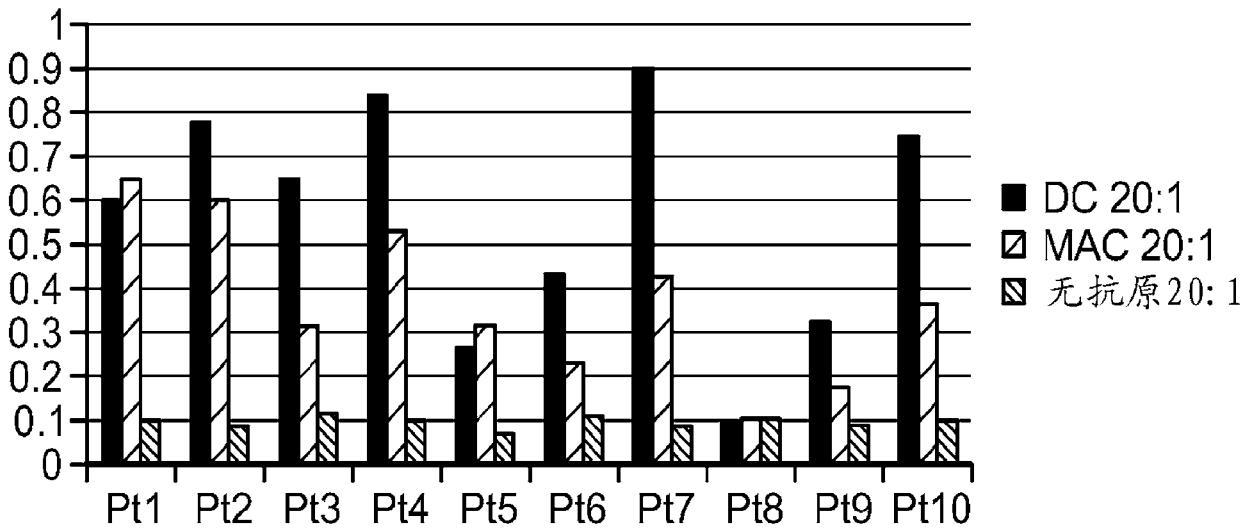

1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Pt1
Pt2
Pt3
Pt4
Pt5
Pt6
Pt7
Pt8
Pt9
Pt10
DC 20:1
MAC 20:1
无抗原20:1

图 3B

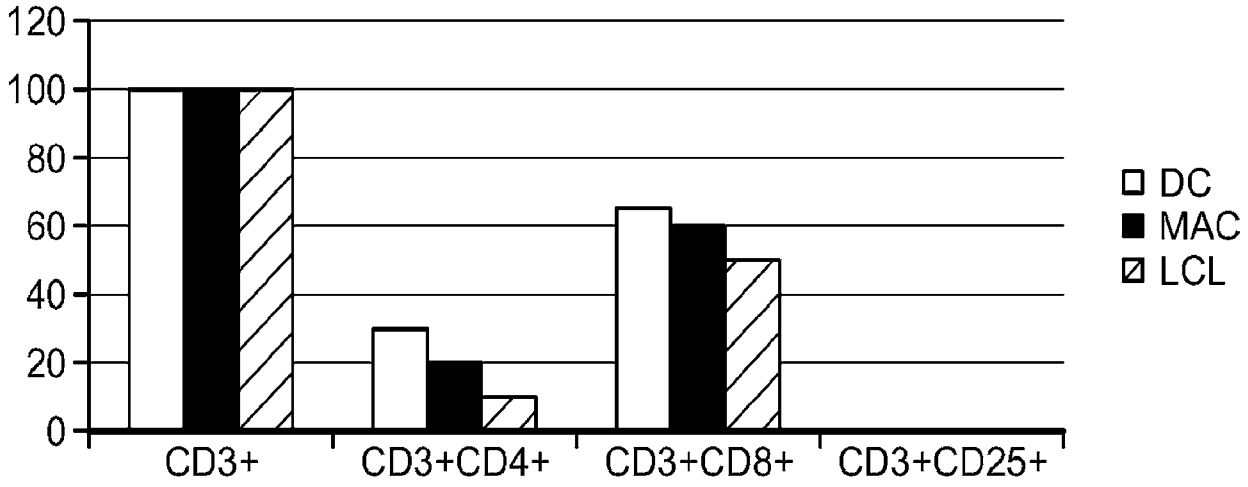

120
100
80
60
40
20
0
CD3+
CD3+CD4+
CD3+CD8+
CD3+CD25+
DC
MAC
LCL

图 3C

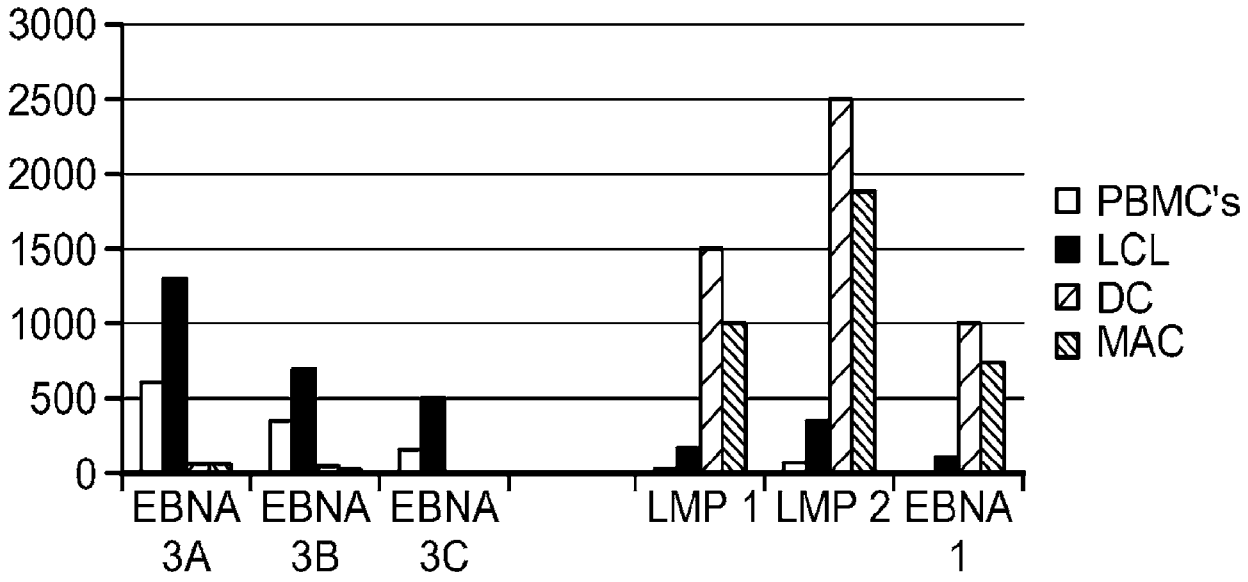

3000
2500
2000
1500
1000
500
0
EBNA 3A
EBNA 3B
EBNA 3C
LMP 1
LMP 2
EBNA 1
PBMC's
LCL
DC
MAC

图 4

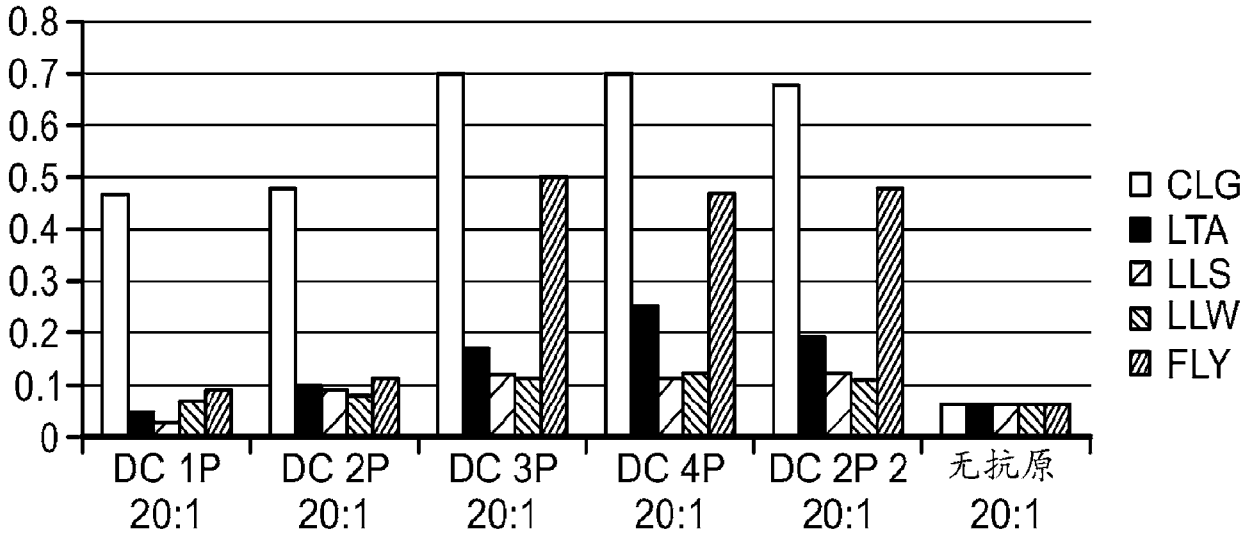

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
DC 1P 20:1
DC 2P 20:1
DC 3P 20:1
DC 4P 20:1
DC 2P 2 20:1
无抗原 20:1
CLG
LTA
LLS
LLW
FLY

图 5

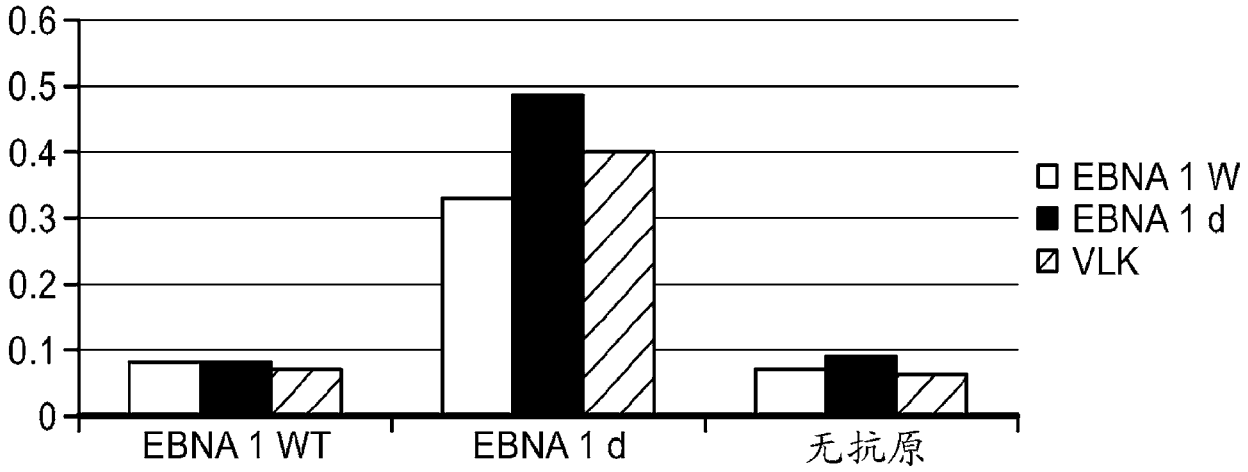

0.6
0.5
0.4
0.3
0.2
0.1
0
EBNA 1 WT
EBNA 1 d
无抗原
EBNA 1 W
EBNA 1 d
VLK

图 6A

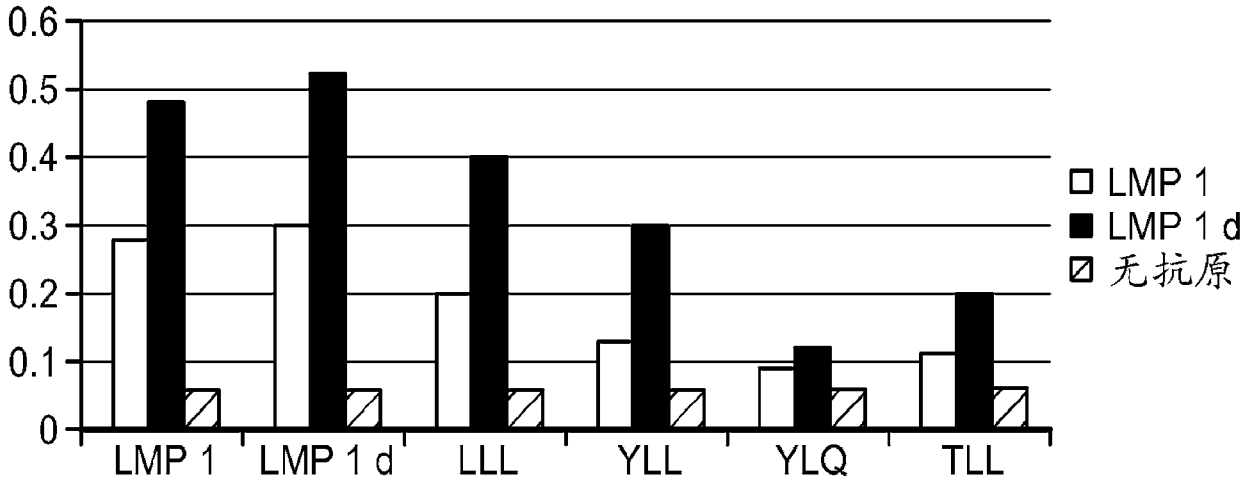

0.6
0.5
0.4
0.3
0.2
0.1
0
LMP 1
LMP 1 d
LLL
YLL
YLQ
TLL
LMP 1
LMP 1 d
无抗原

图 6B

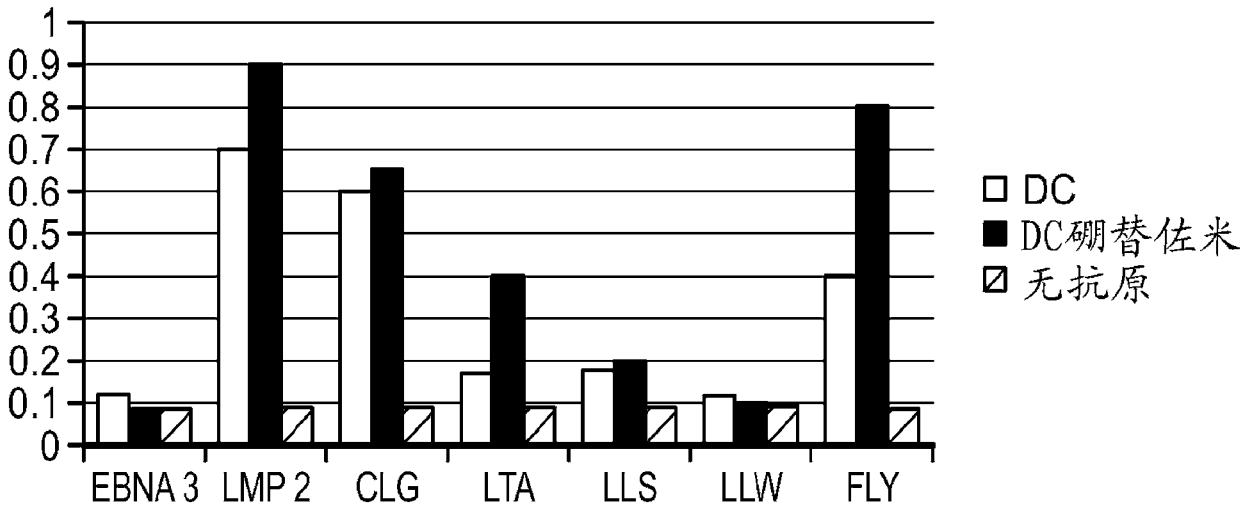

1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
EBNA 3
LMP 2
CLG
LTA
LLS
LLW
FLY
DC
DC硼替佐米
无抗原

图 7

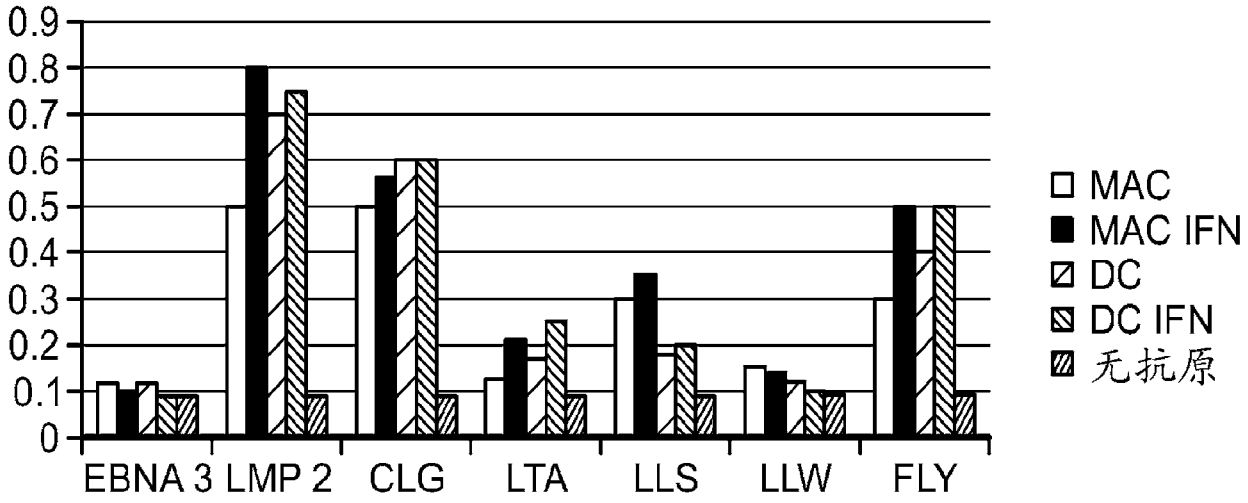

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
EBNA 3
LMP 2
CLG
LTA
LLS
LLW
FLY
MAC
MAC IFN
DC
DC IFN
无抗原

图 8

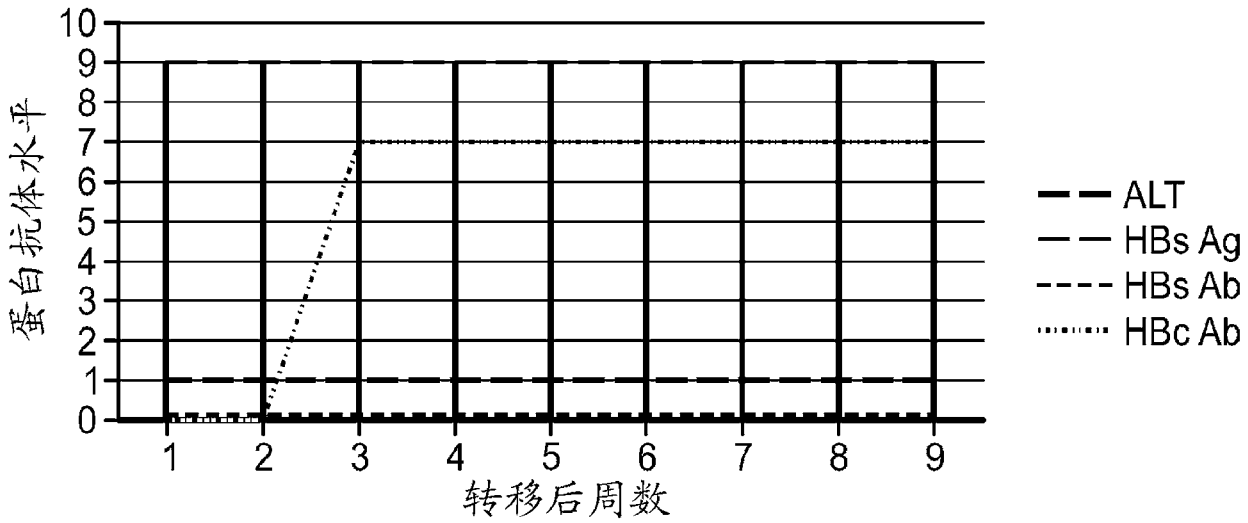

蛋白抗体水平
10
9
8
7
6
5
4
3
2
1
0
1
2
3
4
5
6
7
8
9
转移后周数
ALT
HBs Ag
HBs Ab
HBc Ab

图 9

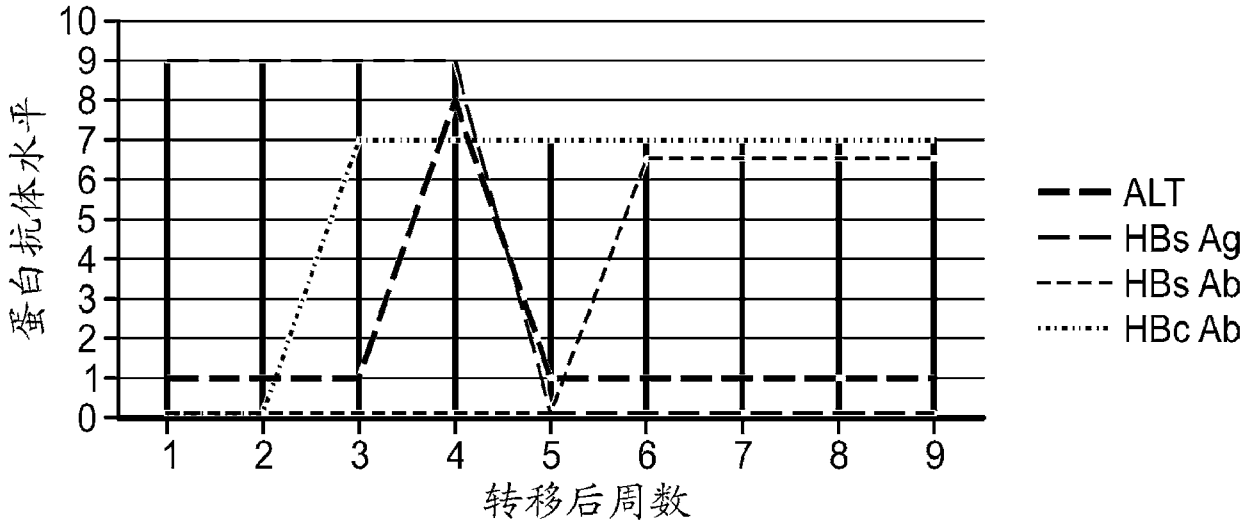

蛋白抗体水平
10
9
8
7
6
5
4
3
2
1
0
1
2
3
4
5
6
7
8
9
转移后周数
ALT
HBs Ag
HBs Ab
HBc Ab

图 10

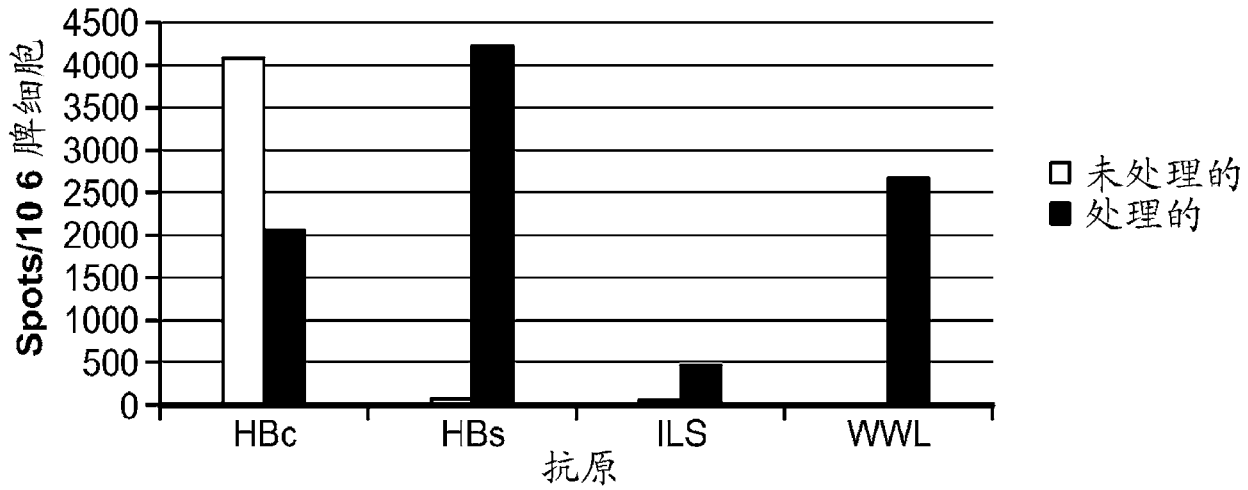

Spots/10 6 脾细胞
4500
4000
3500
3000
2500
2000
1500
1000
500
0
HBc
HBs
ILS
WWL
抗原
未处理的
处理的

图 11

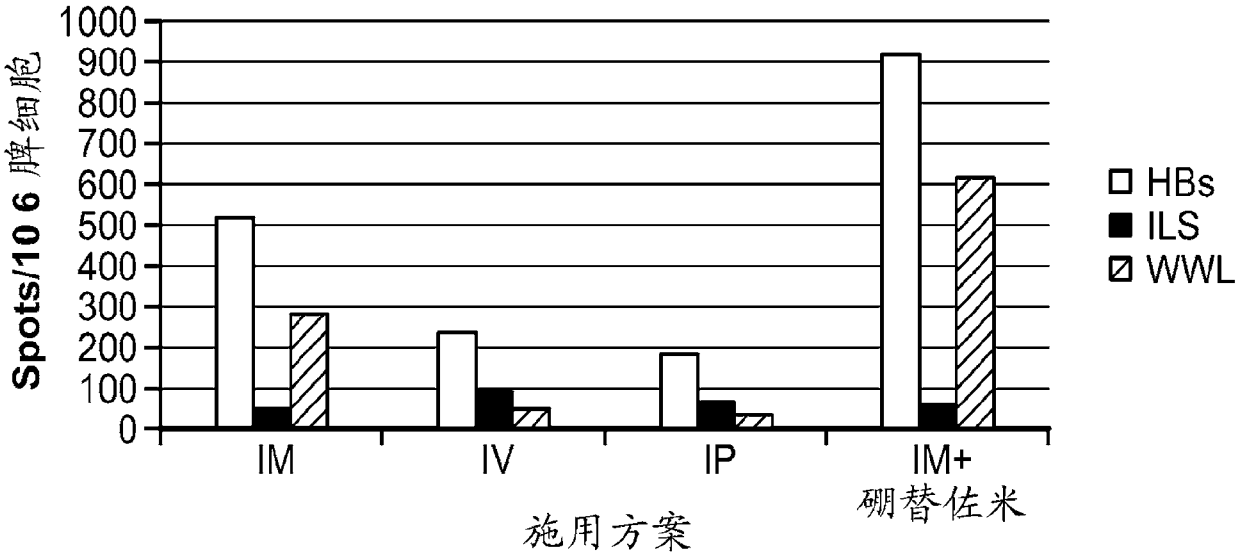

Spots/10 6 脾细胞
1000
900
800
700
600
500
400
300
200
100
0
IM
IV
IP
IM+
硼替佐米
施用方案
HBs
ILS
WWL

图 12

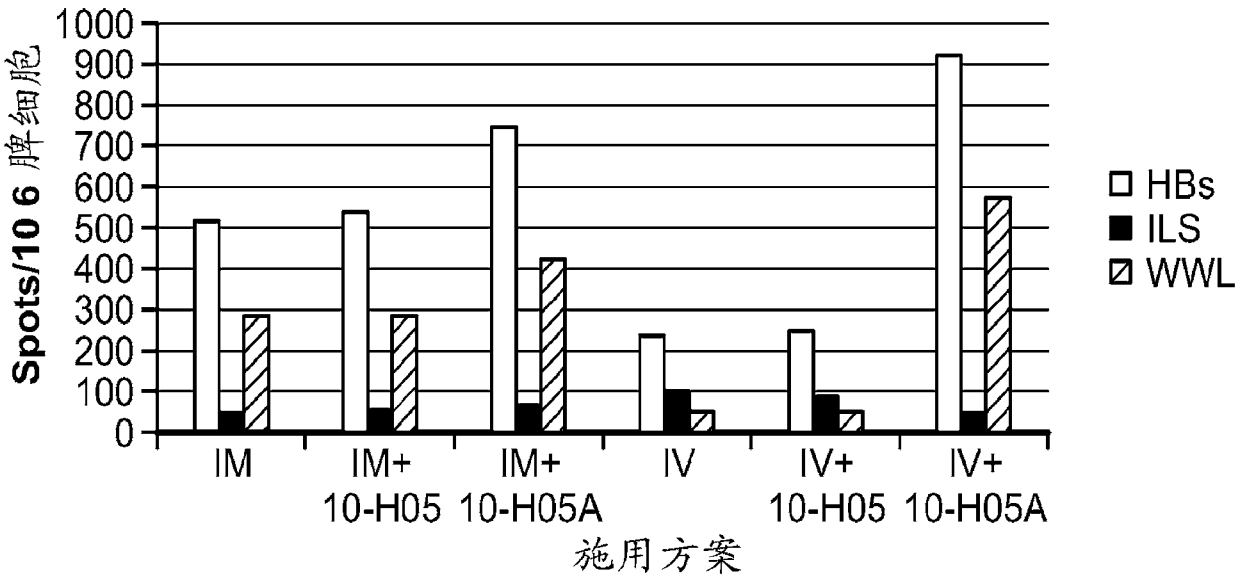

Spots/10 6 脾细胞
1000
900
800
700
600
500
400
300
200
100
0
IM
IM+
10-H05
IM+
10-H05A
IV
IV+
10-H05
IV+
10-H05A
施用方案
HBs
ILS
WWL

图 13

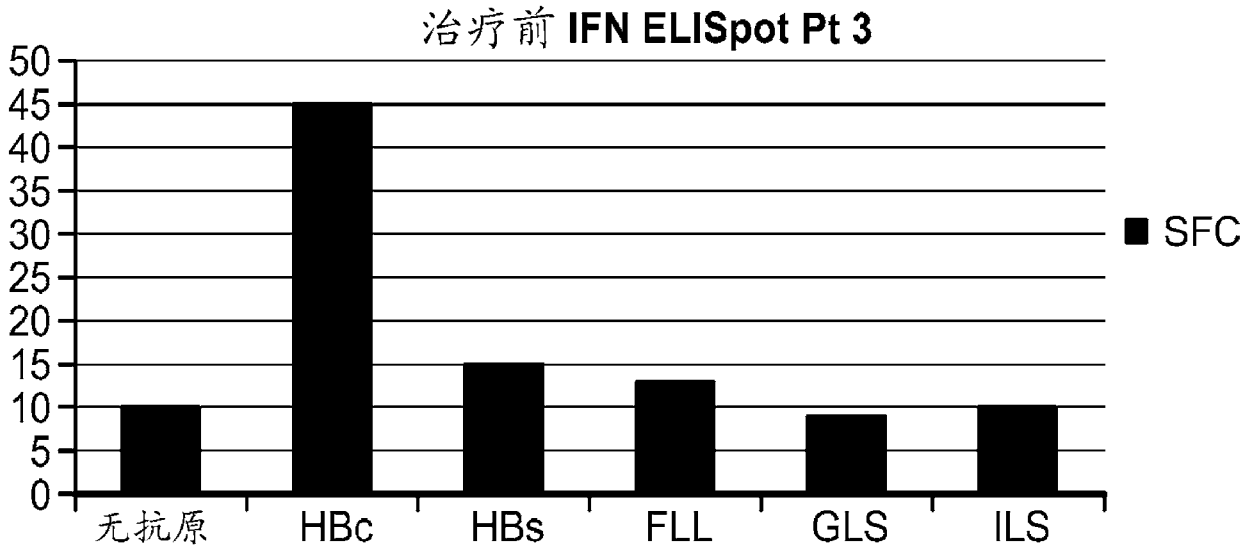
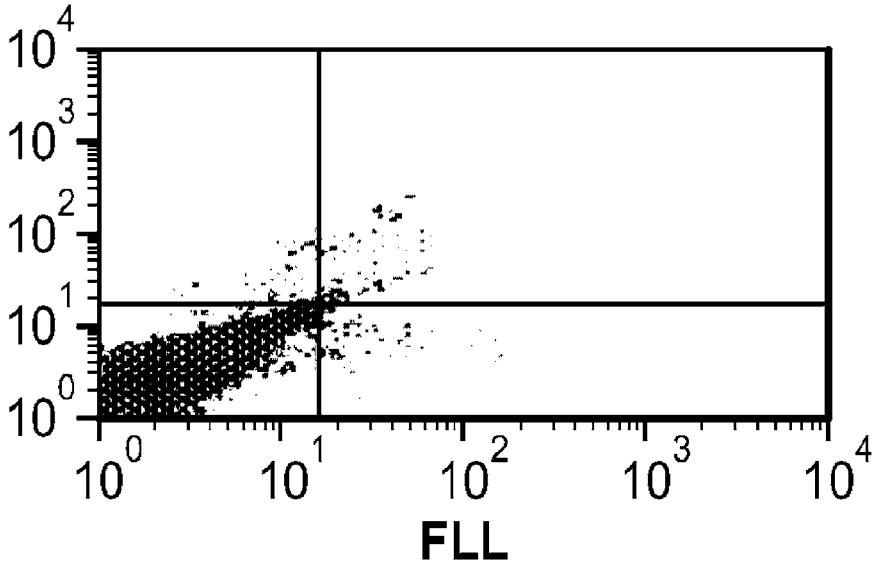
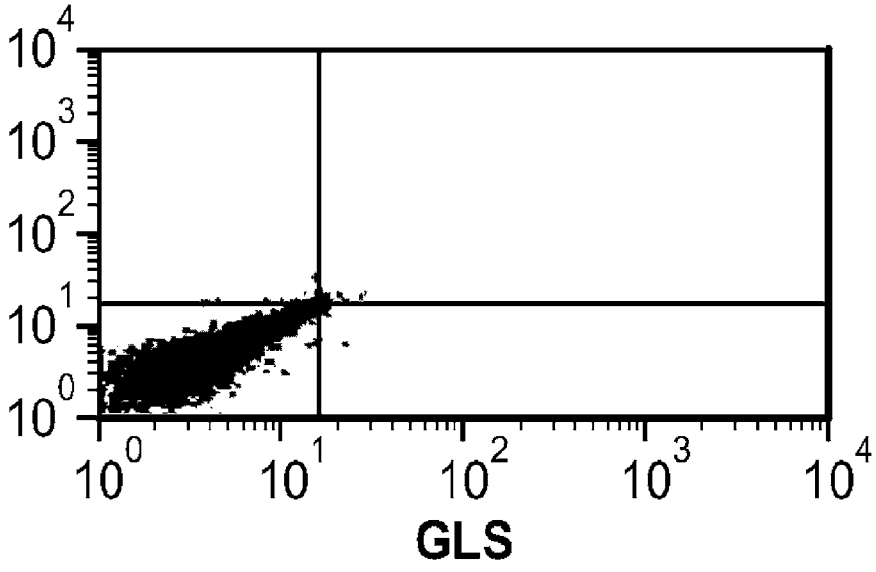

治疗前 IFN ELISpot Pt 3
50
45
40
35
30
25
20
15
10
5
0
无抗原
HBc
HBs
FLL
GLS
ILS
SFC

图 14


10^4
10^3
10^2
10^1
10^0
10^0 10^1 10^2 10^3 10^4
FLL


10^4
10^3
10^2
10^1
10^0
10^0 10^1 10^2 10^3 10^4
GLS

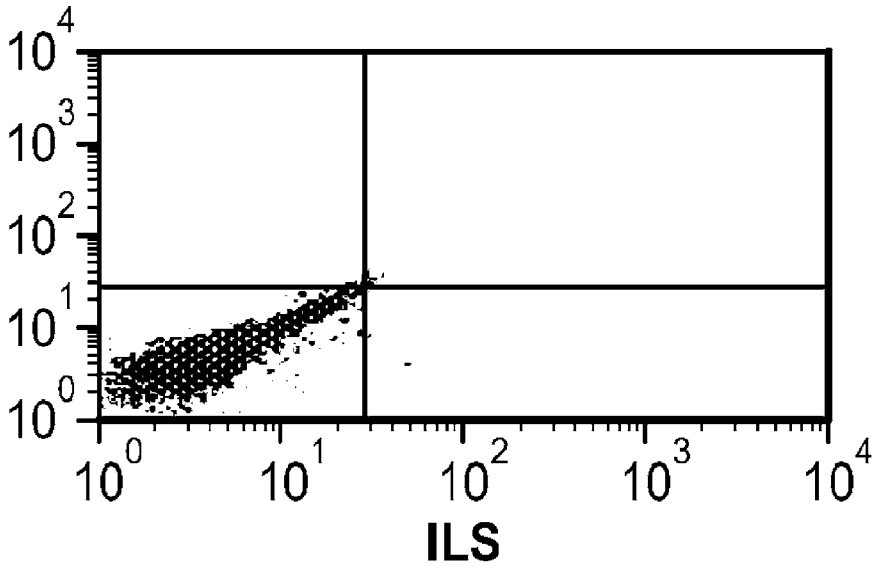

10^4
10^3
10^2
10^1
10^0
10^0 10^1 10^2 10^3 10^4
ILS

图 15

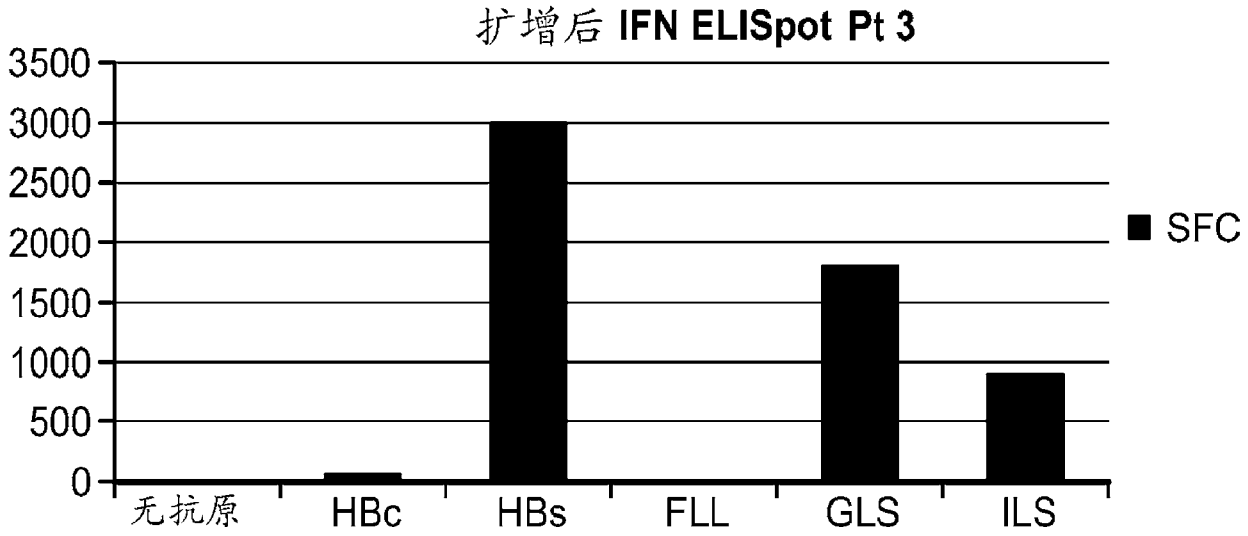

扩增后 IFN ELISpot Pt 3
3500
3000
2500
2000
1500
1000
500
0
无抗原
HBc
HBs
FLL
GLS
ILS
SFC

图 16

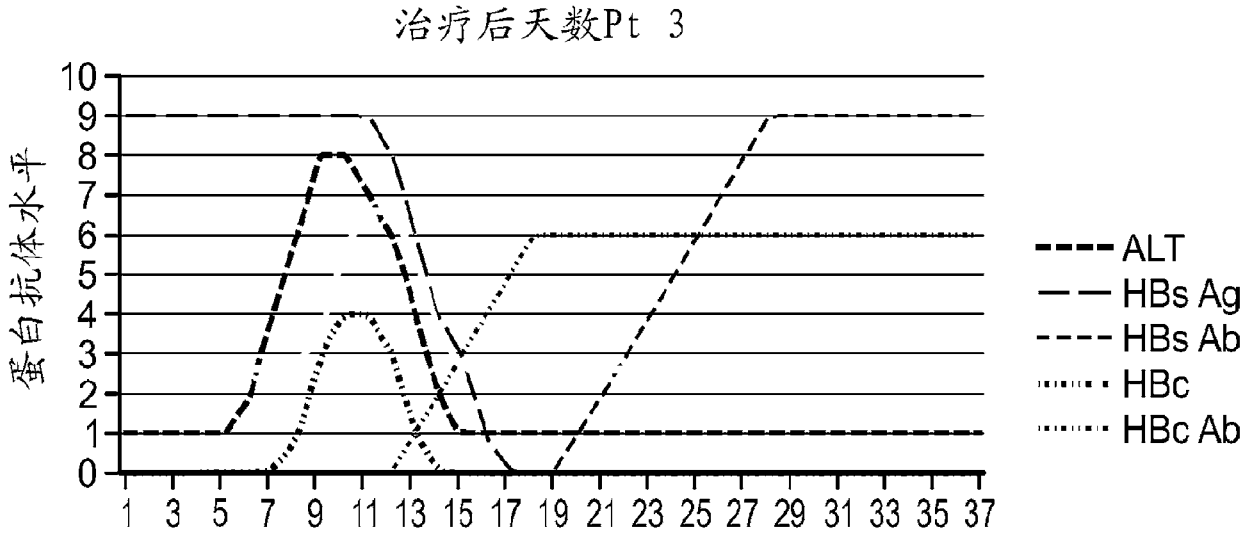

治疗后天数Pt 3
蛋白抗体水平
10
9
8
7
6
5
4
3
2
1
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37
ALT
HBs Ag
HBs Ab
HBc
HBc Ab

图 17

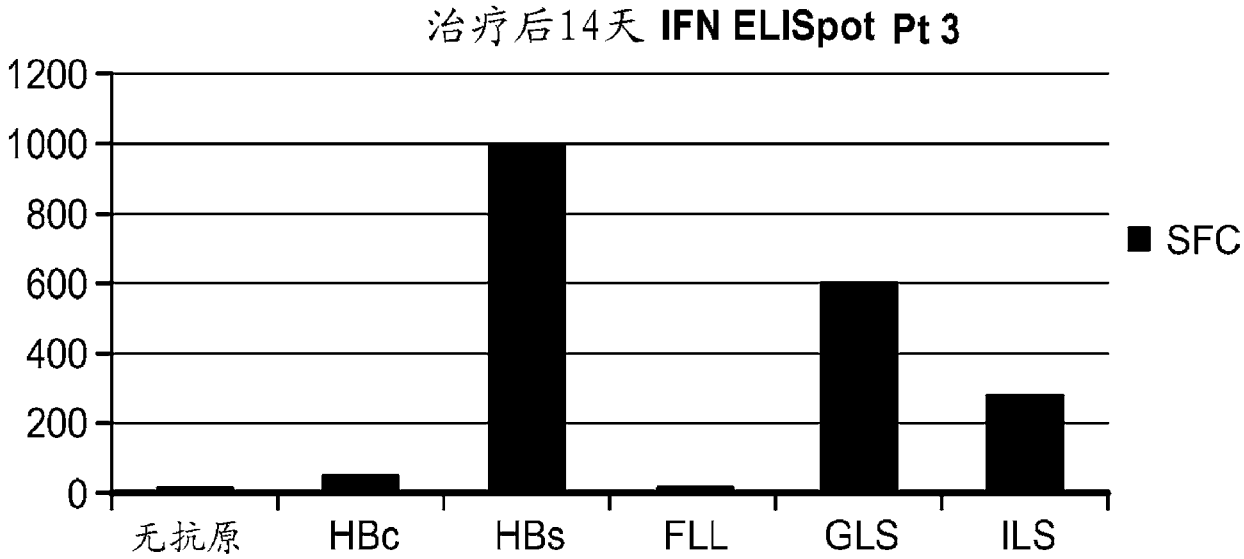

治疗后14天 IFN ELISpot Pt 3
1200
1000
800
600
400
200
0
无抗原
HBc
HBs
FLL
GLS
ILS
SFC

图 18

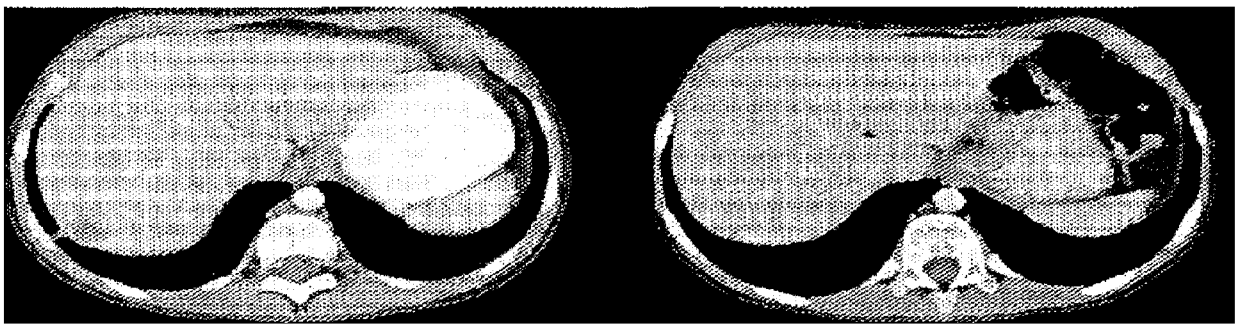


图 19

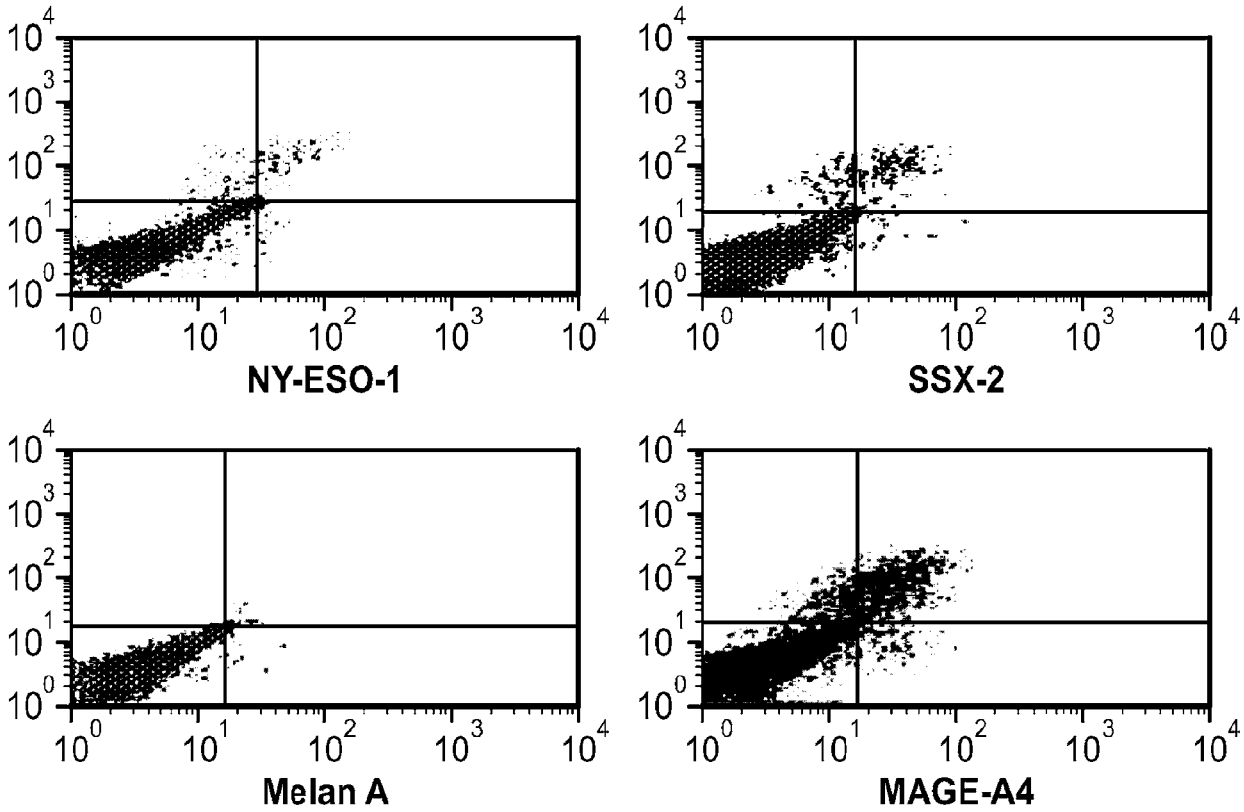

NY-ESO-1
SSX-2
Melan A
MAGE-A4

图 20

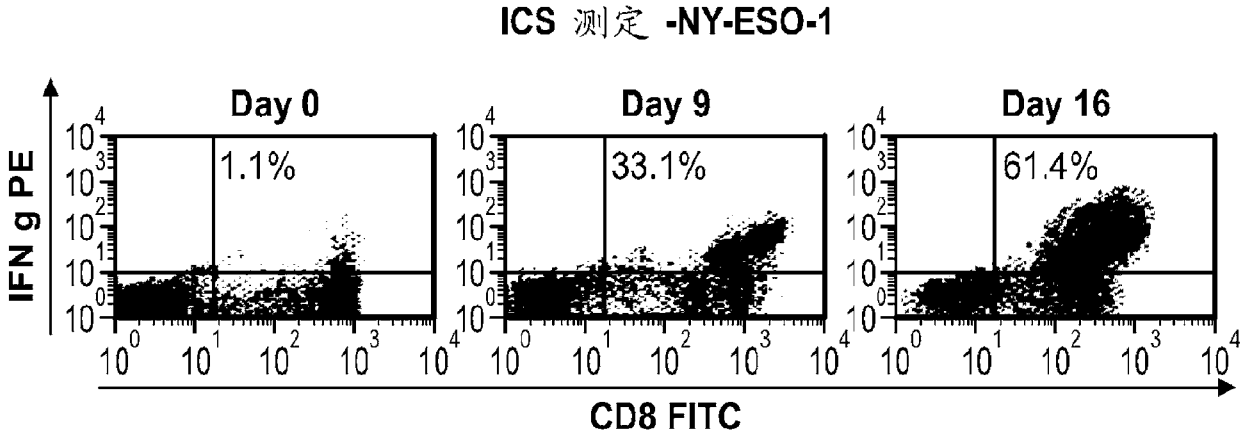

ICS 测定 -NY-ESO-1
Day 0
Day 9
Day 16
1.1%
33.1%
61.4%
IFN g PE
CD8 FITC

图 21

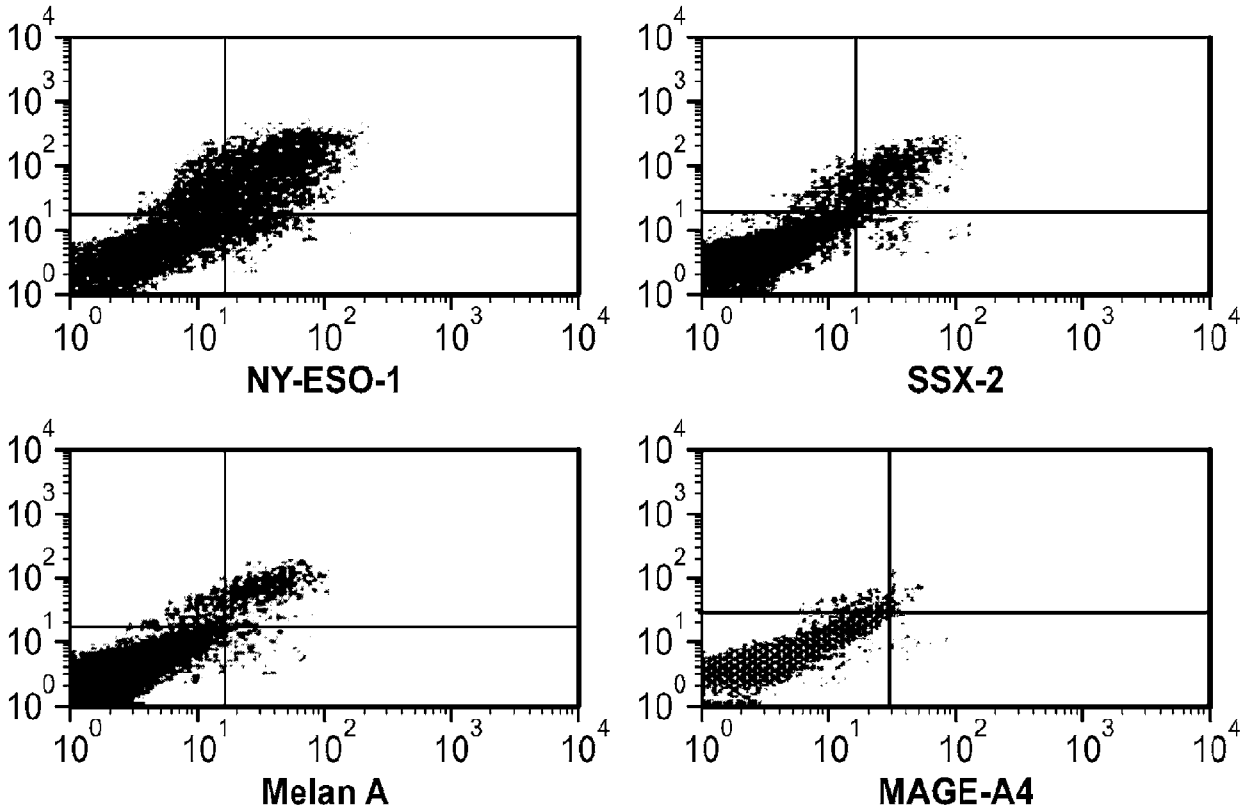

NY-ESO-1
SSX-2
Melan A
MAGE-A4

图 22

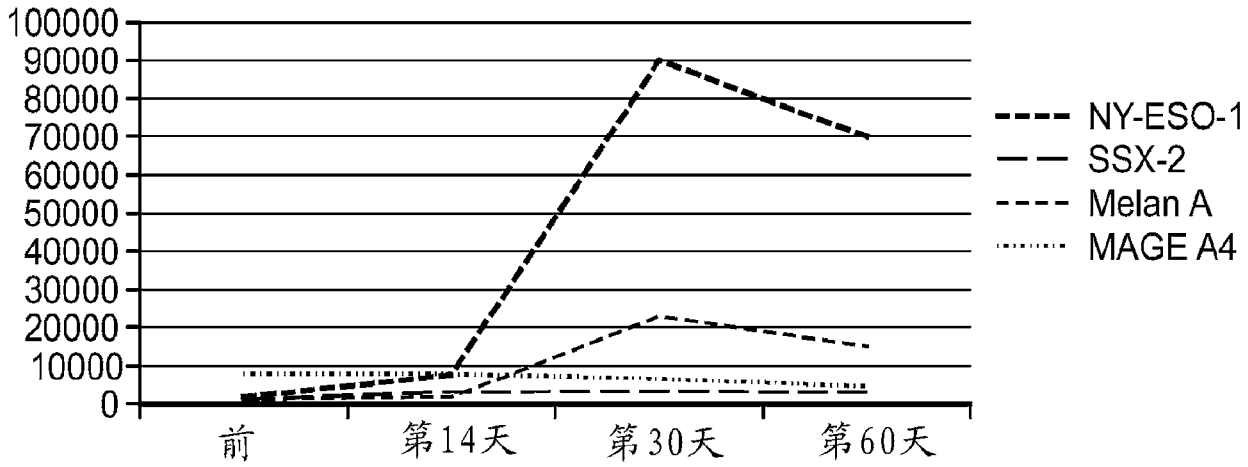
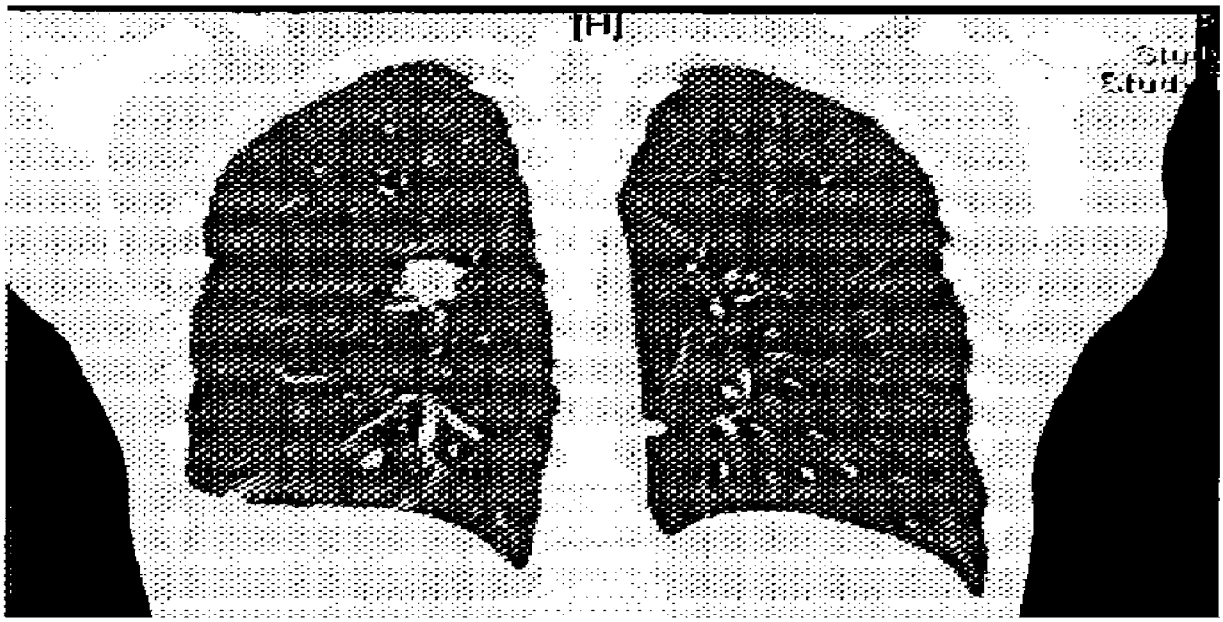
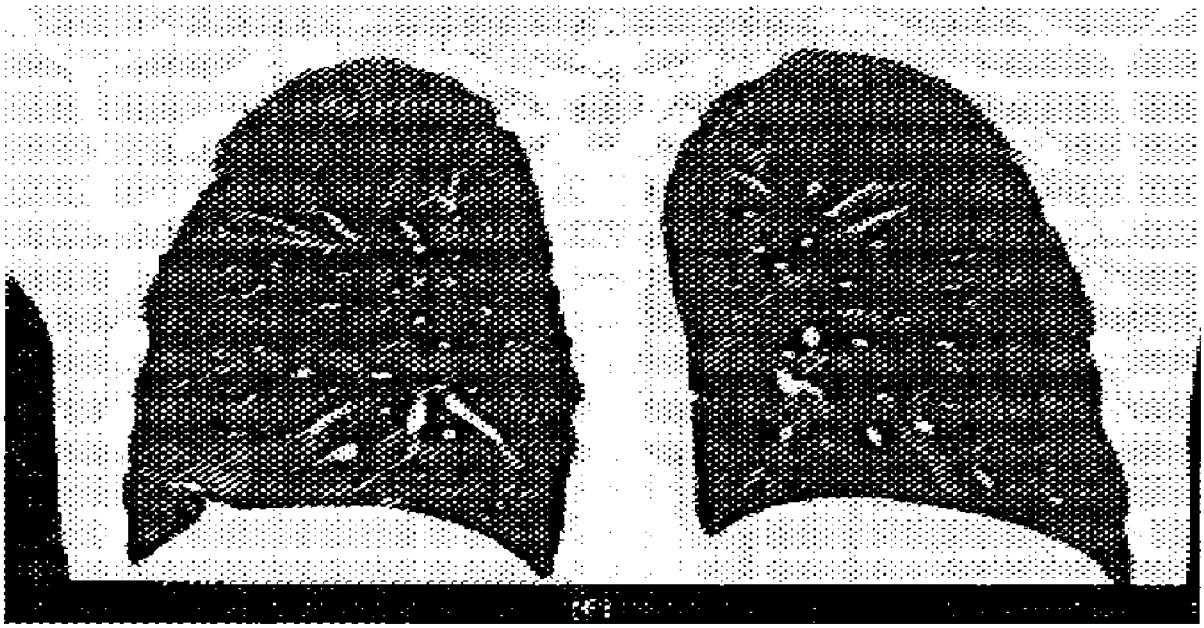

100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
前
第14天
第30天
第60天
NY-ESO-1
SSX-2
Melan A
MAGE A4

图 23



之前



T细胞治疗后6个月

图 24

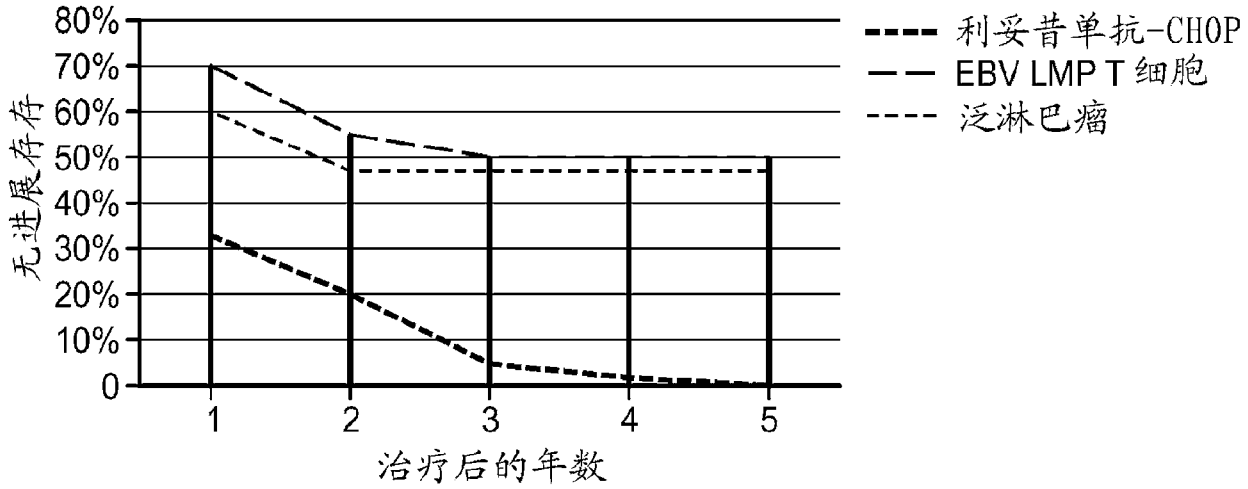

80%
70%
60%
50%
40%
30%
20%
10%
0
无进展存存
1
2
3
4
5
治疗后的年数
利妥昔单抗-CHOP
EBV LMP T 细胞
泛淋巴瘤

图 25

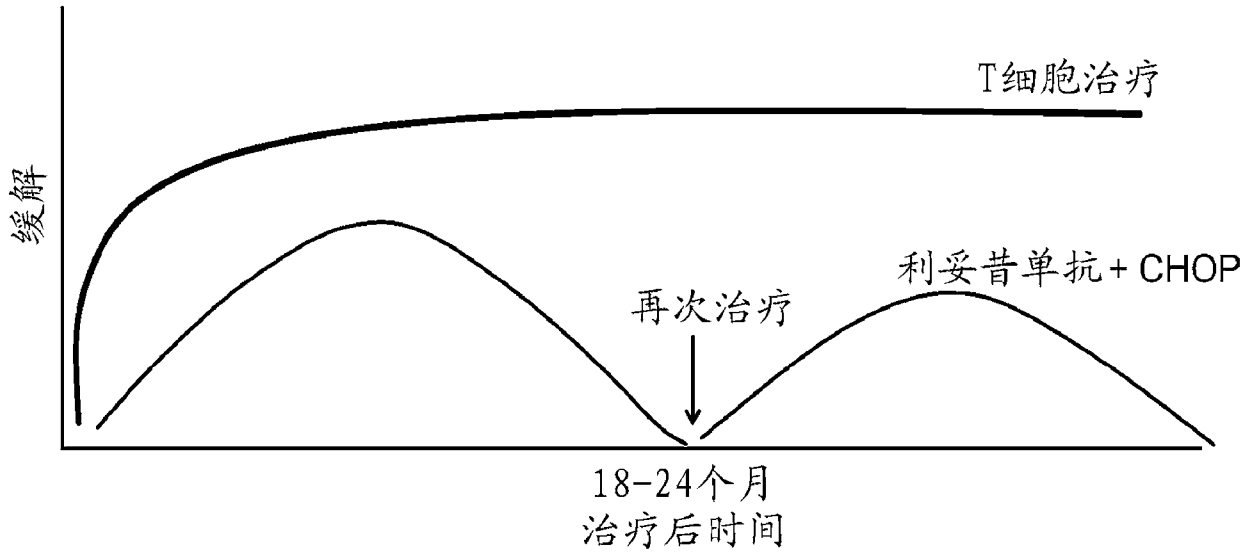

缓解
T细胞治疗
利妥昔单抗+ CHOP
再次治疗
18-24个月
治疗后时间

图 26

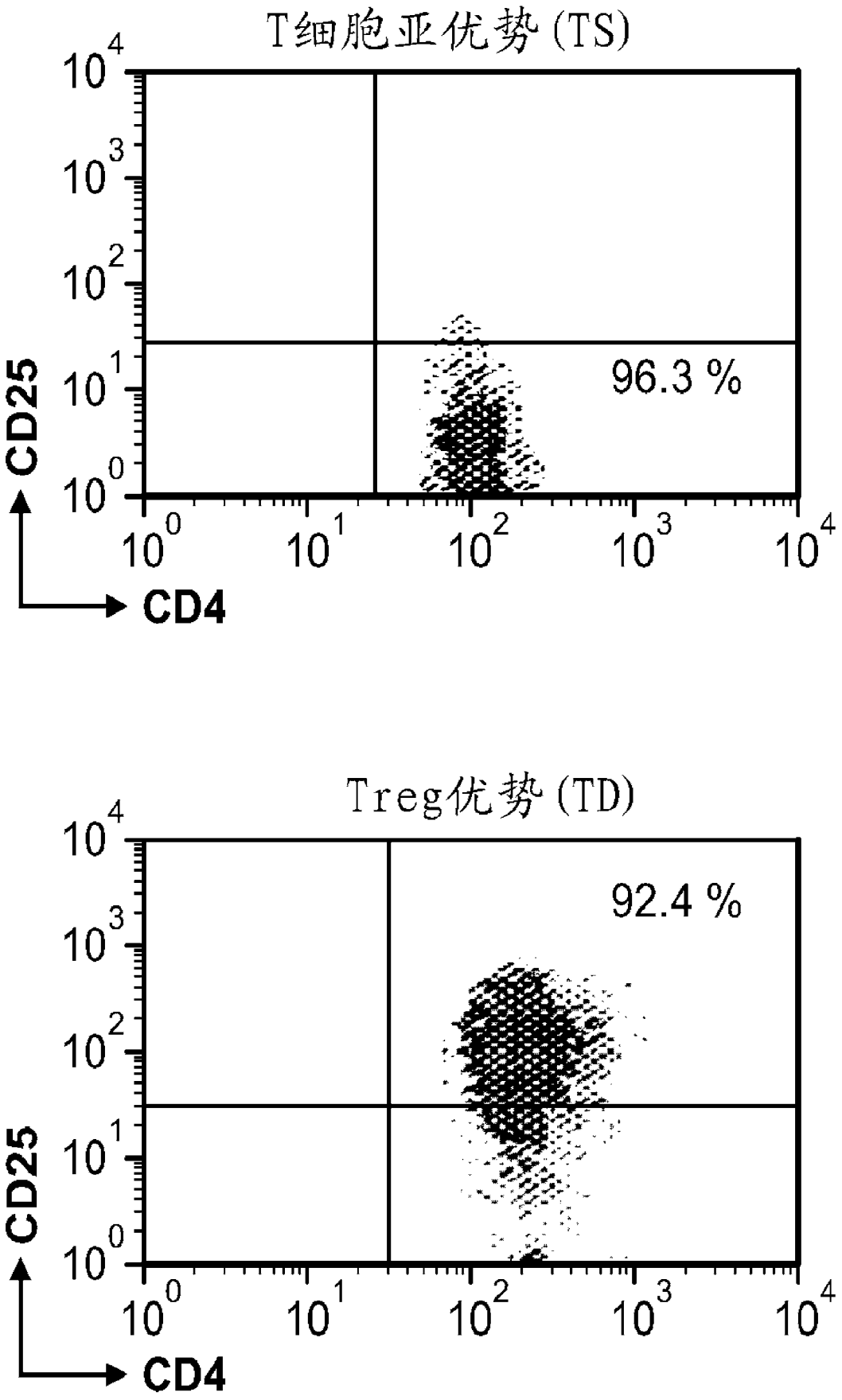


图 27

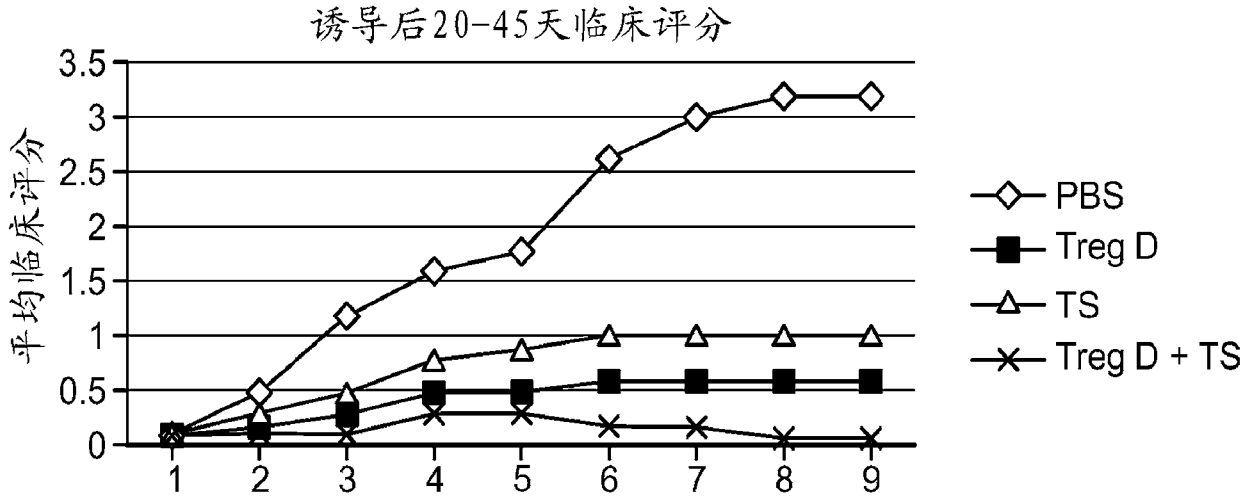

诱导后20-45天临床评分
平均临床评分
3.5
3
2.5
2
1.5
1
0.5
0
1
2
3
4
5
6
7
8
9
PBS
Treg D
TS
Treg D + TS

图 28

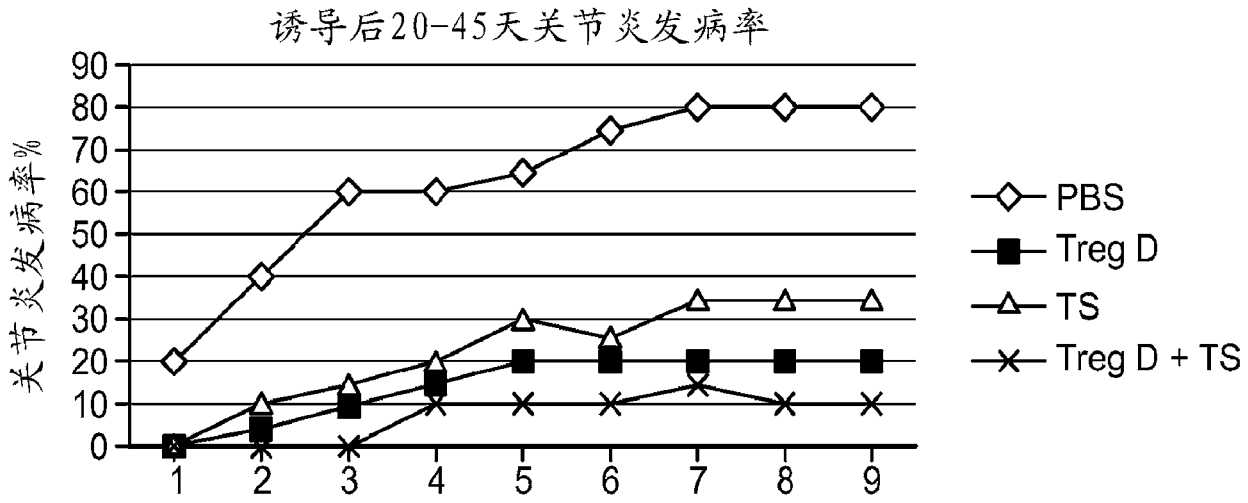
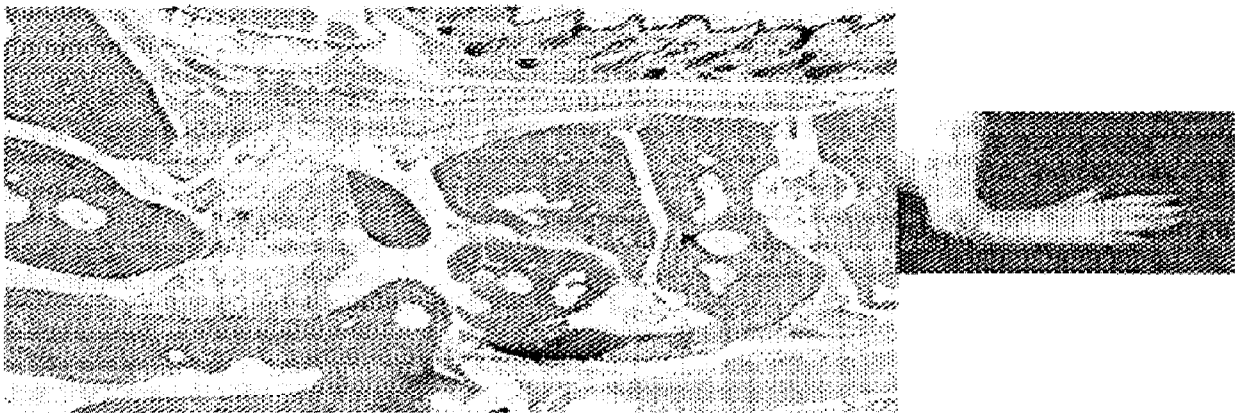
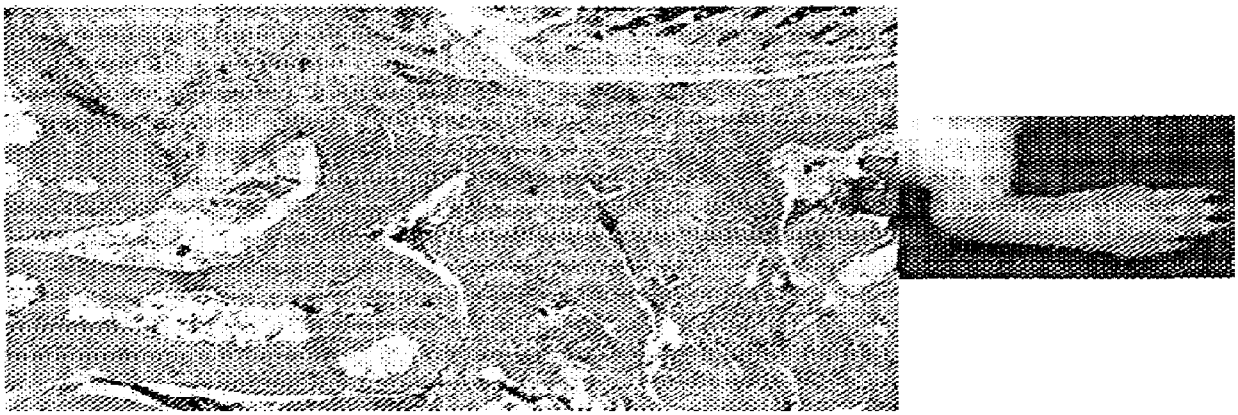

诱导后20-45天关节炎发病率
关节炎发病率%
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
PBS
Treg D
TS
Treg D + TS

图 29



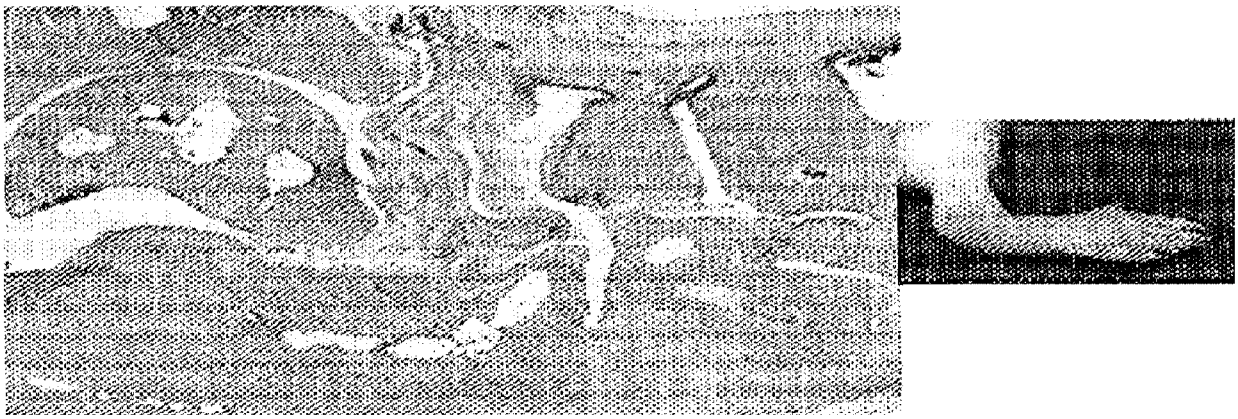
正常

图 30A



用人蛋白聚糖免疫

图 30B



用T细胞治疗

图 30C

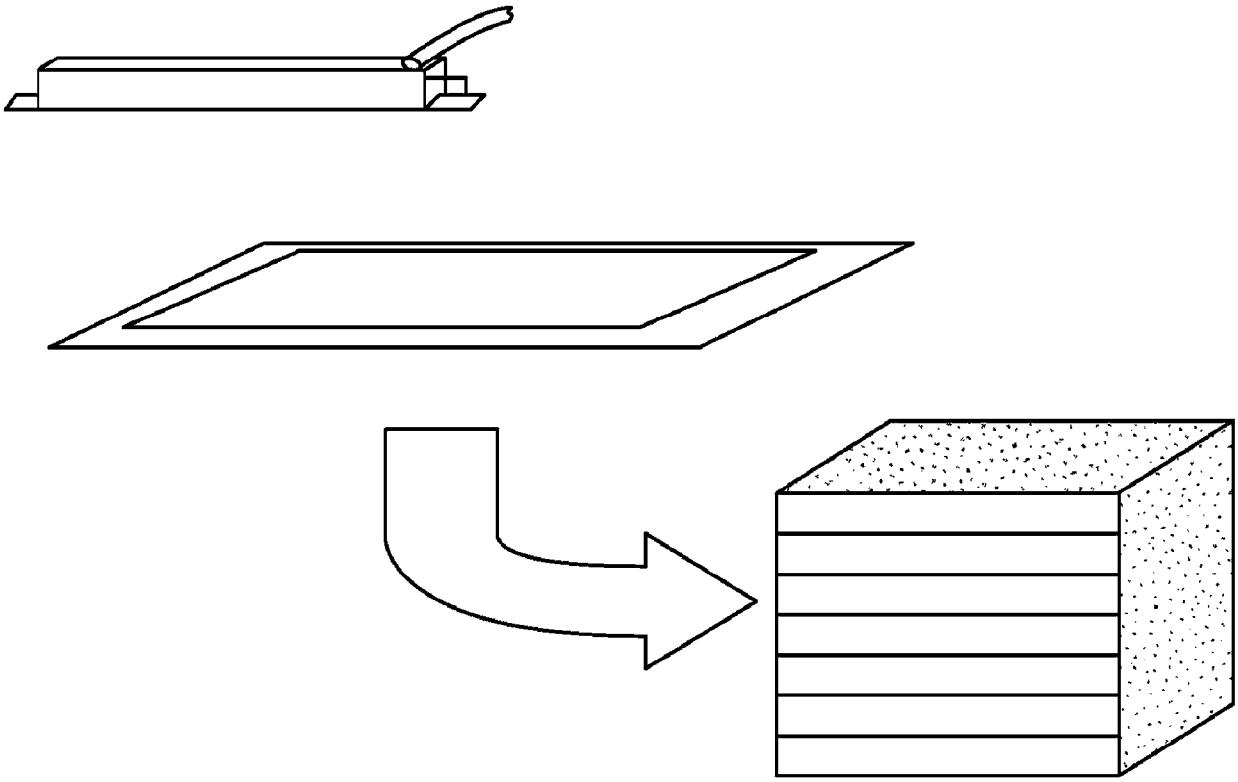


图 31