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(54) **LYMPHOCYTES T DECLENCHANT UNE REACTION IMMUNITAIRE ET METHODES D'UTILISATION**

(54) **T CELLS MEDIATING AN IMMUNE RESPONSE AND METHODS OF USE**

(57) Cette invention a trait à des lymphocytes T, à des compositions ainsi qu'à des méthodes permettant de les obtenir et de les utiliser, y compris mais sans se limiter à ceci, des méthodes thérapeutiques anticancéreuses. Au nombre des méthodes selon l'invention figurent, une technique de production de lymphocytes T capable de pénétrer dans une tumeur humaine et de participer à une réaction immunitaire contre cette dernière, une méthode thérapeutique anticancéreuse consistant à administrer à un être humain souffrant d'une tumeur une quantité efficace du point de vue thérapeutique de lymphocytes T entraînant la régression de la tumeur. Ces lymphocytes T auront été, soit isolés d'une tumeur humaine après immunisation du sujet à l'aide d'une composition contenant des cellules tumorales modifiées au moyen d'un haptène, soit développés in vitro à partir de lymphocytes T ainsi isolés ou bien encore auront été soumis aux deux processus.

(57) The present invention is directed to T cells, compositions, and methos of obtaining and using the same, including and not limited to methods of treating cancer. The methods of the present invention include inter alia: a method of generating T cells capable of infiltrating a tumor of a human and participating in an immune response against the tumor; and a method of treating cancer comprising administering to a human afflicted with a tumor a therapeutically effective amount of T cells that mediate tumor regression, the T cells having been either isolated from the tumor of the human after immunization of the human with a composition comprising hapten-modified tumor cells and/or expanded in vitro from T cells thus isolated.

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(54) Title: T CELLS MEDIATING AN IMMUNE RESPONSE AND METHODS OF USE

(57) Abstract

The present invention is directed to T cells, compositions, and methos of obtaining and using the same, including and not limited to methods of treating cancer. The methods of the present invention include *inter alia*: a method of generating T cells capable of infiltrating a tumor of a human and participating in an immune response against the tumor; and a method of treating cancer comprising administering to a human afflicted with a tumor a therapeutically effective amount of T cells that mediate tumor regression, the T cells having been either isolated from the tumor of the human after immunization of the human with a composition comprising hapten–modified tumor cells and/or expanded *in vitro* from T cells thus isolated.

T CELLS MEDIATING AN IMMUNE RESPONSE AND METHODS OF USE

REFERENCE TO GOVERNMENT GRANTS

The invention described herein was made in the course of work under a grant from the National Institutes of Health/National Cancer Institute, grant no. CA39248. The United States Government may have certain rights in this invention.

BACKGROUND OF THE INVENTION

Tumor specific T cells are alleged to have been generated *in vitro* by a number of investigators. Many of the T cells may be described as "artifactual," i.e., they appear to react to antigens that do not induce an immune response in the patient. For example, T cells have been generated *in vitro* to the protein, tyrosinase. Tyrosinase is an enzyme expressed by all pigmented cells, both benign and malignant. If there were significant immunity to tyrosinase *in vivo*, the result would be destruction of normal melanocytes in the skin, eye, and possibly brain. In contrast, the present invention comprises T cells that infiltrate a tumor mass and mediate tumor regression, and particularly a subset of such T cells having a specific T cell receptor (TCR) structure. These T cells are elicited by administration to a patient of a hapten-modified composition or by cloning the isolated T cells thus elicited.

Particular TCR families infiltrating tumors following various types of immunotherapy have been identified. However, a TCR family may contain hundreds or thousands of TCR structures, each with a different antigen specificity. Therefore, the predominance of a family is only preliminary evidence that there may be novel T cell clones that may be tumor-specific. There are about 30 families identified for the β

chain and about 25 for the α chain of the T cell receptor. The present invention on the other hand, identifies T cells that are involved in tumor regression; some of these T cells express a TCR within a particular family and, more particularly within a particular family subset.

The molecular definition of melanoma (and other tumor-specific) antigens recognized by T cells (Boon, T. and P. Van der Bruggen, *J. Exp. Med.* **1996***183*:725-730) and the possibility of studying the composition of the T cell receptor (TCR) involved in the MHC-restricted recognition of such epitopes, including epitopes expressed within the tumor mass (Sensi, M. and G. Parmiani, *Immunol. Today* **1995** *16*:588-595.) now allow a more precise evaluation of the immune response in patients treated with immunomodulatory agents. Several groups of investigators have documented overexpression, also known as clonal expansion, of T cells expressing particular T cell receptor β chain variable region (hereafter Vβ) gene families in primary or, less frequently, metastatic tumors of different histologies; in some cases, such clonal expansion has been shown to be driven by the recognition of specific tumor antigens (for review see Sensi *et al., supra.*).

The effect of therapy with biological response modifiers on the TCR repertoire in tumor lesions has also been assessed in melanoma or renal carcinoma patients treated with IL-2 alone, IL-2 with IFN-α or IL-2, cisplatin with/without IFN-α (Farace, F., *et al.*, *International Journal of Cancer* **1993** *54*:741-747, Puisieux, I., *et al.*, *J. Immunol.* **1994** *153*:2807-2818, Willhauck, M., *et al.*, *Clin. Cancer Res.* **1994** *2*:767-772, Kumar, A., *et al.*, *J. Clin. Invest* **1996** *97*:1219-1226). Oligoclonal infiltrations in metastases responding to cytokine treatment but not in progressing lesions of the same patient have been described. In contrast, analysis of the effect of tumor cell vaccination has been limited to a single renal carcinoma patient (Weidmann, E., *et al.*, *Cancer Res.* **1993***53*:4745-4749.). This patient was first injected with irradiated neoplastic cells and was subsequently re-infused with *in vitro* sensitized T-lymphocytes. A lung metastasis isolated from that patient exhibited an oligoclonal population of T cells expressing Vβ13, which was overexpressed compared to T cells isolated from both pre- and post-vaccine peripheral blood lymphocytes (PBL) and T cells isolated from a non-responding renal metastasis of the same patient. However, clonal expansion of T cells in the patient was not demonstrated. In all of these studies, direct evidence that

Vβ repertoire changes occur in response to therapy was not adduced because pre-vaccination tumor specimens were not analyzed. Furthermore it has not been determined whether T cells overexpressing particular Vβ-gene families in responsive lesions could mediate specific antitumor immunity.

The present invention evaluates Vβ-gene family usage by T cells obtained from infiltrates of metastatic lesions of six patients who developed tumor inflammatory responses after treatment with autologous, irradiated DNP-modified tumor cells. For all patients, pre- and post-vaccination metastases and PBL (peripheral blood lymphocytes) were studied. The availability of a series of specimens taken at different times after administration of an immuno-modulatory composition from a clinically responding patient rendered possible the identification of dominant T cell clonotypes and the search for anti-tumor cytotoxic effectors.

The present invention provides the first evidence, to Applicant's knowledge, that selectively expanded T cell clones infiltrate a tumor site after immunization with tumor cells. This finding is general and does not depend on the tumor type, as long as the same tumor is used for immunization as the tumor from which the patient suffers. These expanded T cell clones mediate regression of the patient's tumor, i.e. are responsible directly or indirectly for the mounting of an effective anti-tumor immune response.

SUMMARY OF THE INVENTION

In a first embodiment, the present invention is directed to an isolated human T cell capable of mediating regression of a tumor or another specific immune response directed against a tumor (as evidenced, for example, by T cell expansion *in vivo* or T cell cytotoxicity). The T cell may be elicited *in vivo* by immunization of the patient with a composition comprising haptenized cells of the same tumor type or may be produced from such T cells by cloning *in vitro*. In a preferred embodiment, the isolated human T cell expresses a Vβ receptor, which Vβ receptor may be Vβ1, Vβ5, Vβ13, or Vβ14. A composition comprising a therapeutically effective amount of a T cell as described above and a pharmaceutically acceptable carrier is also contemplated by the present invention.

Methods of using the T cell capable of mediating regression of a tumor are

also provided by the present invention. Additionally, a method of generating a T cell having the property of infiltrating a tumor and mediating regression of a tumor is provided. The method comprises administering to a human a composition comprising a hapten modified syngeneic human tumor cell substantially in a no growth phase (i.e., killed or in G0 phase) and an adjuvant wherein the human suffers from a malignant tumor of the same type as said tumor cell thereby eliciting *in vivo* a T cell having the property of infiltrating a tumor and mediating an immune response against the tumor; and isolating said T cell from the tumor. Alternatively, or additionally, the then isolated T cells can be cloned or expanded *ex vivo*, producing additional such T cells.

The present invention is also directed to a method of treating cancer comprising administering to a human a composition comprising a therapeutically effective amount of a T cell having the property of mediating regression of a tumor, thereby treating cancer.

In another aspect, the invention is directed to a method of monitoring the effectiveness of cancer therapy involving administration of an immunomodulatory agent to a patient. The monitoring method comprises obtaining a tumor sample from the patient under treatment comparing the number of T cells that have the property of infiltrating and/or mediating regression of a tumor before and after administration of the therapy involving the immunomodulatory agent, thereby monitoring the effectiveness of the therapy. The number of such infiltrating and/or type of T cell can be measured directly in the sample, or indirectly, e.g. by assessing cytotoxicity of T cells collected from the tumor sample of the patient under therapy. For a conclusion that the therapy is effective, the post-therapy infiltrating T cells should be at least about two standard deviations more frequent than pretherapy T cells (if any) infiltrating said tumor.

Yet another method of the present invention is a method of screening for antigens having the property of stimulating a specific T cell response directed against a human malignant tumor type comprising exposing a T cell having the property of infiltrating the primary or a metastatic tumor to a sample to be tested, where the sample contains at least one antigen under conditions whereby said at least one antigen is presented to the T cell, and assessing at least one of the following: proliferation of the T cell, cytotoxicity of the T cell against the tumor or cytokine profile of the T cell.

The methods of the present invention may include expanding T cells *in vitro*

by exposing T cells isolated from a patient's tumor to a T cell stimulatory substance such as a cytokine, a superantigen and an antibody to a T cell receptor protein. Tumors treatable with the present invention include solid and nonsolid tumors, malignant and benign or non-malignant tumors such as leukemia, including acute myelogenous leukemia, melanoma, lymphoma, adenocarcinoma, sarcoma, ovarian, colon, breast, rectal, lung, kidney, and prostate tumors. The tumor cells of the composition comprising a hapten modified syngeneic human tumor cell may be autologous. The hapten may be selected from the group of haptens that bind to lysine on the surface of cells through ϵ-amino groups, or that bind to -COOH groups on the cell surface such as and not limited to dinitrophenyl, trinitrophenyl, N-iodoacetyl-N'-(5-sulfonic 1-naphtyl) ethylene diamine, trinitrobenzenesulfonic acid, fluorescein isothiocyanate, arsenic acid, benzene isothiocyanate, trinitrobenzenesulfonic acid, and dinitrobenzene-S-mustard. An adjuvant may be mixed with the tumor cell composition prior to administration. The adjuvant may be *Bacille Calmette-Guerin*, QS-21, or detoxified endotoxin.

The immunomodulatory agent useful in assessing the effectiveness of a cancer therapy may comprise a therapeutically effective amount of a hapten modified syngeneic human tumor cell, killed or in G0 phase, and an adjuvant, wherein said human suffers from a malignant tumor of the same type as said hapten-modified syngeneic human tumor cell. Detecting T cells in the method of assessing the effectiveness of a cancer therapy may comprise detecting expression of the T cell receptor of said T cell, which may be measured by amplification of nucleic acids encoding T cells and identification of T cells with a monoclonal antibody, or detecting the appearance of complementarity determining region 3 of T cell receptors, such as the sequences of SEQ ID NOS: 1-19.

The method of screening for antigens may include a nucleic acid sequence encoding the antigen in said sample has been used to transfect a cell, which cell does not react with a T cell without being transfected with the nucleic acid sequence for an antigen. Stimulation of an isolated T cell in a method of screening for antigens may comprise observing release of a cytokine, and the cytokine may be selected from the group consisting of tumor necrosis factor, gamma interferon, and an immunostimulatory interleukin.

The present invention is also directed to the peptides having an amino acid

sequence selected from the group consisting of SEQ ID NOS: 1-19.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A and 1B are photomicrographs of subcutaneous metastases before (A) or after (B) administration of a haptenized melanoma cell vaccine. Figure 1B shows extensive lymphocyte infiltration of melanoma.

Figures 2A - 2F are bar graphs comparing expression of various Vβ genes before and after vaccination of six melanoma patients (respectively, ED, FC, CB, JB, LG, RS), with a vaccine exploiting T cells according to the invention. The X axis is marked in months. The specimen identification for these figures is set forth in Table 1.

Figure 3A and 3B are autoradiographs of HR-PAGE (A) and SSCP (B) analysis of Vβ14 rearrangements in all specimens of patients JB and FC. The dominant clone Vβ14J1S5 (patient JB) is shown, for comparison, in the last lane.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to isolated T cells that infiltrate tumors and mediate specific immune responses against the tumor (eventually, such responses may lead to tumor regression) to a method for generating such T cells, and to various methods of using such T cells for therapeutic, analytical or investigative purposes. Thus, a novel T cell, compositions containing novel T cells and methods of using the same, including and not limited to, methods of treating cancer, are included in the scope of the invention.

The present invention is for use in treating cancer, including metastatic and primary cancers. Cancers treatable with the present invention include solid tumors, including carcinomas, and non-solid tumors, including hematologic malignancies. Solid malignancies include without limitation, sarcomas, lymphomas, melanomas, and epithelial carcinomas such as adenocarcinomas and squamous carcinomas. Nonsolid malignancies include leukemias, and multiple myelomas. The following are non-limiting examples of the cancers treatable with the composition and methods of the present invention: ovarian, including advanced ovarian, leukemia, including and not limited to acute myelogenous leukemia, colon, including colon metastasized to liver, rectal, melanoma, breast, lung, breast, kidney, and prostate cancers.

The ovarian cancers may be epithelial carcinomas such as adenocarcinomas. Colon and prostate cancer may be adenocarcinomas. Leukemias may originate from myeloid cells of the bone marrow or lymphocytic cells of the bone marrow or lymph nodes. Leukemias may be acute, exhibited by maturation arrest at a primitive stage of development, or chronic, exhibited by excess accrual of mature lymphoid or myeloid cells. The present invention can be applied to therapy of primary and metastatic cancer, as well as to therapy of localized, regionally metastatic, or disseminated metastatic cancer. Similarly, the analytical and screening aspects of the present invention are not limited to the type or stage of cancer to which they can be applied.

TUMOR CELLS

The T cells of the present invention may be generated using tumor cells to prepare the initial immunogen. Included within the definition of tumor cell for purposes of the present invention are whole cells, disrupted tumor cells, and combinations thereof.

The tumor cells used in the present invention may be live, attenuated, or killed cells. Tumor cells which do not grow and divide after administration into the subject, such as a human, such that they are substantially in a state of no growth are preferred. It is to be understood that the term "cells in a state of no growth" means live or killed, whole or disrupted (or both whole and disrupted) cells that will not divide *in vivo*. Cells in a state of no growth typically include cells that are killed and cells substantially in G0 phase of the cell cycle. Conventional methods of ensuring that cells are in a state of no growth are known to skilled artisans and may be useful in the present invention. For example, cells may be irradiated prior to use such that they do not grow. Tumor cells may be irradiated at 2500 cGy, for example, to prevent the cells from growing after administration.

The tumor cells are preferably of the same type as, and are preferably syngeneic to, the cancer which is to be treated. For purposes of the present invention, syngeneic refers to tumor cells that are genetically identical. For example, genetic identity may be determined with respect to antigens or immunological reactions, and any other methods known in the art. Preferably the cells originate from the type of cancer which is to be treated, and most preferably, from the same patient who is to be treated.

The tumor cells may but need not be, autologous cells isolated from biopsy specimens or tissue culture. In the case of non-solid tumors, leukemia cells for example, a blood or bone marrow sample is obtained and cells isolated therefrom. Nonetheless, allogeneic cells and stem cells are also within the scope of the present invention.

The compositions of the present invention may be employed in the method of the invention singly or in combination with other compounds, including and not limited to other compositions of the invention. Accordingly, tumor cells, tumor cell extracts (such as disrupted tumor tissue or cells), and T cells each may be used alone or co-administered. For purposes of the present invention, co-administration includes administration together and consecutively. Further, the tumor cells may be co-administered with other biological response modifiers including but not limited to cytokines: e.g., interleukin-2, interleukin-4, gamma interferon, interleukin-12, GM-CSF, beta-interferon, and the like.

The compositions may be administered in a mixture with a pharmaceutically-acceptable carrier, selected with regard to the intended route of administration and the standard pharmaceutical practice. Dosages may be set with regard to weight, and clinical condition of the patient. The proportional ratio of active ingredient to carrier naturally depend on the chemical nature, solubility, and stability of the compositions. as well as the dosage contemplated. Amounts of the tumor cells to be used depend on such factors as the affinity of the compound for cancerous cells, the amount of cancerous cells present and the solubility of the composition. The compounds of the present invention may be administered by any suitable route, including inoculation and injection, for example, intradermal, intravenous, intraperitoneal, intramuscular, and subcutaneous.

In a preferred embodiment of the invention, the composition comprises about 2.5×10^5 to about 25×10^6, more preferably about 5×10^6 to about 10×10^6, even more preferably about 2.5×10^6 live, irradiated, tumor cells suspended in a pharmaceutically acceptable carrier or diluent, such as and not limited to Hanks solution, saline, phosphate-buffered saline, and water. The composition may be administered by intradermal injection into 3 contiguous sites per administration on the upper arms or legs, excluding limbs ipsilateral to a lymph node dissection. The composition is preferably administered weekly for six (6) weeks; optional booster injections may be

administered at six months and twelve months.

HAPTEN

The tumor cells for the present invention may be employed as modified, or a combination of modified and unmodified tumor cells can be employed. For purposes of the present specification "modified" includes and is not limited to modification with a hapten (any other modification that increases immune response of the host against the tumor is also contemplated). For purposes of the present specification, virtually any small protein or other small molecule that does not alone induce an immune response (but that enhances immune response against another molecule to which it is conjugated or otherwise attached) may function as a hapten. A variety of haptens of quite different chemical structure have been shown to induce similar types of immune responses: TNP (Kempkes *et al.*, *J. Immunol.* **1991** *147*:2467); phosphorylcholine (Jang *et al.*, *Eur. J. Immunol.* **1991** *21*:1303); nickel (Pistoor *et al.*, *J. Invest. Dermatol.* **1995** *105*:92); arsenate - Nalefski and Rao, *J. Immunol.* **1993** *150*:3806). Conjugation of a hapten to a cell to elicit an immune response against the tumor may preferably be accomplished by conjugation via ϵ-amino groups of lysine or via -COOH groups. This group of haptens include a number of chemically diverse compounds: dinitrophenyl, trinitrophenyl, N-iodoacetyl-N'-(5-sulfonic 1-naphthyl) ethylene diamine, trinitrobenzenesulfonic acid, fluorescein isothiocyanate, arsenic acid benzene isothiocyanate, trinitrobenzenesulfonic acid. and dinitrobenzene-S-mustard (Nahas and Leskowitz, *Cellular Immunol.* **1980** *54*:241). Once armed with the present disclosure, skilled artisans, would be able to choose haptens for use in the present invention.

Dinitrophenyl, a representative of haptens in general, may but need not be used to sensitize patients to the chemical dinitrophenyl (DNP) by application of dinitrofluorobenzene (DNFB) to the skin prior to immunization. Subsequently, (about two weeks later, for example) the patients may then be injected with a tumor cell composition. The composition may be administered (such as by reinjection) every 4 weeks for a total of eight treatments or weekly for a total of six treatments. The immune response of the patient may be augmented with additional therapy, such as an not limited to drugs. For example, low dose cyclophosphamide (CY) may be administered prior to each administration of a tumor cell composition.

ADJUVANT

In another preferred embodiment, a tumor cell composition may be administered with an immunological adjuvant. While commercially available pharmaceutically acceptable adjuvants are limited, representative examples of adjuvants include *Bacille Calmette-Guerin, BCG*, or the synthetic adjuvant, QS-21 comprising a homogeneous saponin purified from the bark of *Quillaja saponaria, Corynebacterium parvum*, McCune *et al.*, *Cancer* **1979** *43*:1619, IL-12, and detoxified endotoxin (Ribi Immunochem., Hamilton, Montana) (Mitchell, M.S., *et al.*, *J. Clin. Oncol.* **1990** *8*:856-869, and Mitchell, M.S., *et al.*, *Cancer Res.* **1988** *48*:5883-5893).

It will be understood that the adjuvant is subject to optimization. That is, the skilled artisan can engage in no more than routine experimentation and determine the best adjuvant to use.

METHODS OF PREPARING A COMPOSITION FOR USE IN THE PRESENT INVENTION

The tumor cells for use in the present invention may be prepared as follows. Tumors are processed as described by Berd et al. (1986), *supra*, incorporated herein by reference in its entirety. The cells are extracted by dissociation, such as by enzymatic dissociation with collagenase and DNase, or by mechanical dissociation such as with a blender, teasing with tweezers, mortar and pestle, cutting into small pieces using a scalpel blade, and the like.

The dissociated cells may be stored frozen, such as in a controlled rate freezer or in liquid nitrogen until needed. The cells are ready for use upon thawing. Preferably, the cells are thawed shortly before the cells are to be administered to a patient. For example, on the day that a patient is to be skin tested or treated, the cells may be thawed. Optionally the cells may be washed, and optionally irradiated to 2500 R. They may be washed again and then suspended in Hanks balanced salt solution without phenol red.

Modification of the prepared cells with DNP or another hapten may be performed by known methods, for example, by the method of Miller and Claman, *J. Immunol.*, **1976**, *117*, 1519, incorporated herein by reference in its entirety, which involves a 30 minute incubation of tumor cells with DNFB under sterile conditions,

followed by washing with sterile saline.

In the methods of the present invention, a method of generating T cells according to the invention comprises administering to a patient diagnosed with having cancer of a certain type, a pharmaceutically acceptable amount of a composition selected from the group consisting of hapten-modified tumor cells. The composition may be mixed with an immunological adjuvant and/or a pharmaceutically acceptable carrier. A pharmaceutically acceptable amount of a low-dose cyclophosphamide or another low-dose chemotherapy, such as and not limited to melphalan, about 5 to about 10mg/M^2, may optionally be administered preceding the administration of the first tumor cell composition. The haptenized composition may optionally be followed by administration of a pharmaceutically acceptable amount of a non-haptenized vaccine. A non-haptenized composition may also be administered in accordance with the methods of the present invention.

Tumor cells may be obtained following surgery. The tumor may be optimally or sub-optimally debulked. Optimally debulked refers to excising the tumor while small tumor pieces may remain in the patient. Sub-optimally debulked refers to excising the tumor while large pieces remain in the patient. In the case of ovarian cancer, for example, a tumor may be optimally debulked such that about less than 1 cm of tumor remains in the patient. Sub-optimal debulking results in tumor of about 1 cm or greater remaining in the patient. In the case of melanoma, tumors are completely resected grossly, while microscopic tumor may remain in the patient. In the case of non-solid tumors, an appropriate blood or bone marrow sample can be collected, and cancer cells are isolated by known techniques.

The haptenized tumor cell vaccine elicits inflammatory responses in tumors. The tumor becomes reddened, warm and tender. Microscopically, infiltration of T lymphocytes into the tumor mass is observed. Within a period of about one to about four months after the first vaccination, a tumor specimen (preferably of at least a size of about 0.5 grams to about 5 grams, although, theoretically, one T cell from the tumor could be sufficient), may be collected by biopsy.

The biopsied tissue will contain the T cells of interest within it. Alternatively, such as in the case of non-solid tumors, such as leukemia, for example, blood or marrow T cells are collected or biopsies of the bone marrow are studied

similar to a solid tumor sample. The T cells can be isolated by known techniques, such as preparation of single cell suspension, filtration, depletion of monocytes and isolation of a subset expressing a particular TCR type by causing that subset to expand in the presence of TCR-subtype specific antibody and/or in the presence of IL-2 and/or in the presence of a superantigen. The T cells of interest will be expanded *in vivo* since they are collected from infiltrates from or within the tumor which are already enriched in the T cells of interest.

The effectiveness of the T cell response may be improved by adding various biological response modifiers. These agents work by directly or indirectly stimulating the immune response. Biological response modifiers of the present invention include and are not limited to interleukins, such as interleukin-12, and gamma interferon. In this embodiment, IL12 may be administered to a patient following each vaccine injection. Administration of IL12 to patients with inflammatory responses causes the T lymphocytes within the tumor mass to proliferate and become more active. The increased T cell numbers and functional capacity leads to immunological destruction of the tumors. Dosages for IL12 will be prepared in accordance with the dosage indications set forth above. The preferred IL12 dosage is about 50 ng to about 50 ng/M^2, administered intravenously or subcutaneously, for example. and optionally repeated, once or twice weekly, for example, following each vaccine injection.

The T cells isolated by the present invention, may be cytotoxic T lymphocytes (CTL), or more generally, tumor infiltrating lymphocytes (TIL), i.e., a type of effector lymphocyte associated with cell mediated immunity directed against the tumor. The T cells specifically isolated from patients in the examples below, were CD8+ T cells which are MHC class I specific. CTLs and TILs may also produce CD8+ cells which are also MHC class I specific.

Thus, cancer patients treated with an autologous hapten modified tumor cell composition develop inflammatory responses in primary or metastatic tumors characterized by infiltration of T cells. Expansion of a restricted set of TCRVβ-gene families has been observed following therapy with the hapten modified composition compared to pre-vaccine metastases. The T cell receptor families expanded in investigations with the present invention described herein include and are not limited to members of the Vβ family, including and not limited to Vβ1, Vβ5, Vβ13, and Vβ14. It

is determined that expansion of an infiltrating T cell expressing a particular receptor (not necessarily one of the foregoing receptors) has taken place when the number of T cells expressing the same receptor after a given event is greater than the number before such event by at least two standard deviations.

These T cells can be used as an early marker of the effectiveness of cancer therapy. Any cancer therapy in which an immune response is mounted against the tumor, i.e., any cancer therapy that involves administration of an immunomodulatory agent and results in T cell infiltration of the tumor, can be the target of this method. The T cells directed specifically against the tumor, i.e. T cells involved in an immune response against the tumor, can be identified as follows:

(1) Directly, by measuring e.g., T cell nucleic acid (DNA or RNA) in a biopsy specimen both before and after therapy and comparing the two, or if the TCR subtype(s) of the T cells of interest is (are) known by reacting a tumor specimen with antibodies to the TCR subtype(s) and detecting the binding to such antibodies.

(2) Indirectly, by providing T cells from the tumor specimen, expanding same, and assessing cytotoxicity, cytokine profile, or proliferation of T cells *in vitro* against reporter cells (before and after therapy).

Monoclonal antibodies against various TCR subtypes are commercially available. Binding can be quantitated via a method known in the art such as fluorescence, activated cell sorting (FACS) and flow cytometry without limitation.

Alternatively, nucleic acid encoding for a portion of the T cell receptor may be amplified and identified with a probe or sequenced and compared to a known sequence, for example.

In the case of amplification of nucleic acids encoding TCRVβ, nucleic acids such as DNA (including cDNA) and RNA (including mRNA), may be obtained from a human tumor sample before and after administration of an immunomodulatory agent-type cancer therapy. Nucleic acid can be obtained in accordance with any method known in the art, such as described in Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989), incorporated herein by reference in its entirety, or by modification of known methods

routine to the skilled artisan.

Nucleic acid extraction is followed by amplification of the same by any technique known in the art. Amplification may include the use of at least one primer sequence which is complementary to a portion of a TCRVβ specific sequence. TCRVβ specific sequences of the present invention to include, but are not limited to the sequences set forth in Salvi, S., *et al., Cancer Research*, **1995**, *55*:3374-3379, including nucleic acid sequences encoding the amino acid sequences of SEQ ID NOS: 1-19. Additional such sequences can be identified using the protocols described herein.

Total extraction of RNA may be carried out by use of the LiCl-urea method (Auffray *et al., Euf. J. Biochem 107*:303-314). As used herein, the term "amplification" refers to template-dependent processes and vector-mediated propagation which result in an increase in the concentration of a specific nucleic acid molecule relative to its initial concentration, or in an increase in the concentration of a detectable signal. As used herein, the term template-dependent process is intended to refer to a process that involves the template-dependent extension of a primer molecule. The term template-dependent process refers to nucleic acid synthesis of an RNA or a DNA molecule wherein the sequence of the newly synthesized strand of nucleic acid is dictated by the well-known rules of complementary base pairing (see, for example, Watson, J. D. *et al.*, In: Molecular Biology of the Gene, 4th Ed., W. A. Benjamin, Inc., Menlo Park, Calif. (1987) incorporated herein by reference in its entirety). Typically, vector mediated methodologies involve the introduction of the nucleic acid fragment into a DNA or RNA vector, the clonal amplification of the vector, and the recovery of the amplified nucleic acid fragment. Examples of such methodologies are provided by Cohen *et al.* (U.S. Pat. No. 4,237,224), Maniatis, T. et al., Molecular Cloning (A Laboratory Manual), Cold Spring Harbor Laboratory, 1982, each incorporated herein by reference in its entirety.

A number of template dependent processes are available to amplify the target nucleic acid sequences of interest present in a sample. One of the best known amplification methods is the polymerase chain reaction (PCR) which is described in detail in U.S. Patents 4,683,195, 4,683,202 and 4,800,159, and in Innis *et al., PCR Protocols*, Academic Press, Inc., San Diego CA, 1990, each of which is incorporated herein by reference in its entirety. Briefly, in PCR, two primer sequences are prepared

which are complementary to regions on opposite complementary strands of the target sequence. An excess of deoxynucleoside triphosphates are added to a reaction mixture along with a DNA polymerase (e.g., *Taq* polymerase). If the target sequence is present in a sample, the primers will bind to the target and the polymerase will cause the primers to be extended along the target sequence by adding on nucleotides. By raising and lowering the temperature of the reaction mixture, the extended primers will dissociate from the target to form reaction products, excess primers will bind to the target and to the reaction products and the process is repeated. Preferably a reverse transcriptase PCR amplification procedure may be performed in order to quantify the amount of mRNA amplified. Polymerase chain reaction methodologies are well known in the art.

Other methods for amplification are: the ligase chain reaction (referred to as LCR), disclosed in EPA No. 320,308, Qbeta Replicase, described in PCT Application No. PCT/US87/00880, and an isothermal amplification method for amplification of target molecules that contain nucleotide 5'-[alpha -thio]triphosphates in one strand of a restriction site (Walker, G. T., *et al.*, *Proc. Natl. Acad, Sci. (U.S.A.)* **1992**, *89*:392-396, each of which is hereby incorporated herein by reference in its entirety.

Strand Displacement Amplification (SDA) is another method of carrying out isothermal amplification of nucleic acids which involves multiple rounds of strand displacement and synthesis, i.e. nick translation. A similar method, called Repair Chain Reaction (RCR) is another method of amplification which may be useful in the present invention and is involves annealing several probes throughout a region targeted for amplification, followed by a repair reaction in which only two of the four bases are present. The other two bases can be added as biotinylated derivatives for easy detection. A similar approach is used in SDA.

TCRVβ specific sequences can also be detected using a cyclic probe reaction (CPR). In CPR, a probe having a 3' and 5' sequences of non-TCRVβ specific DNA and a TCRβV specific RNA is hybridized to DNA which is present in a sample. Upon hybridization, the reaction is treated with RNaseH, and the products of the probe identified as distinctive products generating a signal which are released after digestion. The original template is annealed to another cycling probe and the reaction is repeated. Thus, CPR involves amplifying a signal generated by hybridization of a probe to a

TCRβV specific expressed nucleic acid.

Still other amplification methods are described in GB Application No. 2 202 328, and in PCT Application No. PCT/US89/01025, transcription-based amplification systems (TAS) (Kwoh D., *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)* **1989**, *86*:1173, Gingeras T. R., *et al.*, PCT Application WO 88/10315,a nucleic acid amplification process involving cyclically synthesizing single-stranded RNA ("ssRNA"), ssDNA, and double-stranded DNA (dsDNA) Davey, C., *et al.*, European Patent Application Publication No. 329,822, a nucleic acid sequence amplification scheme based on the hybridization of a promoter/primer sequence to a target single-stranded DNA ("ssDNA") followed by transcription of many RNA copies of the sequence of Miller, H. I., *et al.*, PCT Application WO 89/06700, "race" amplification method disclosed by Frohman, M. A., In: *PCR Protocols: A Guide to Methods and Applications* **1990**, Academic Press, N.Y.) "one-sided PCR" (Ohara, O., *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)* **1989**, *86*:5673-5677), and methods based on ligation of two (or more) oligonucleotides in the presence of nucleic acid having the sequence of the resulting "di-oligonucleotide", thereby amplifying the di-oligonucleotide (Wu, D. Y. *et al.*, *Genomics* **1989**, *4*:560) each of which is incorporated herein by reference in its entirety.

Following amplification, the presence or absence of the amplification product may be detected. The amplified product may be sequenced by any method known in the art, including and not limited to the Maxam and Gilbert method and the dideoxy method, see Sambrook, *supra*. The sequenced amplified product is then compared to a sequence known to be in a TCRVβ specific sequence. Alternatively, the nucleic acids may be fragmented into varying sizes of discrete fragments. For example, DNA fragments may be separated according to molecular weight by methods such as and not limited to electrophoresis through an agarose gel matrix. The gels are then analyzed by Southern hybridization. Briefly, DNA in the gel is transferred to a hybridization substrate or matrix such as and not limited to a nitrocellulose sheet and a nylon membrane. A labelled probe is applied to the matrix under selected hybridization conditions so as to hybridize with complementary DNA localized on the matrix. The probe may be of a length capable of forming a stable duplex. The probe may have a size range of about 200 to about 10,000 nucleotides in length, preferably about 1000 nucleotides in length, and more preferably about 200 nucleotides in length. Mismatches

which permit substantial similarity hereto, such as and not limited to sequences with similar hydrophobicity and hydrophilicity, will be known to those of skill in the art once armed with the present disclosure. In addition, sequences with substantially similar activity to the sequences of Salvi *et al.*, 1995, *supra.*, are also contemplated by the present invention.

Various labels for visualization or detection are known to those of skill in the art, such as and not limited to fluorescent staining, ethidium bromide staining for example, avidin/biotin, radioactive labeling such as ^{32}P labeling, and the like. Preferably, the product, such as the PCR product, may be run on an agarose gel and visualized using a stain such as ethidium bromide. See Sambrook *et al.*, *supra.* The matrix may then be analyzed by autoradiography to locate particular fragments which hybridize to the probe.

Particular complementarity determining region 3 (CDR3) sequences of Vβ14 receptors were measurable following vaccine therapy as compared to pre-vaccine. The amino acid sequences of the CDR3 that resulted following therapy are set forth in Table 3 and are identified by SEQ ID NOS: 1-19. While not intending to be bound by any particular theory of operation, it is believed that T cells expressing Vβ14 receptor often become enriched following therapy such that the quantity of the Vβ14 receptor greatly increases. As a result, the sequences identified above become available in a quantity that is measurable.

T cells according to the invention, i.e., T cells that infiltrate a tumor after therapy (or immunization) but not before, or T cells that are expanded after therapy but not before, may also be clonally expanded and identical T cell clono-types could be detected. The TCR Vβ14+ is one such type described herein. Two other major recurring clones are biased toward TCRβJ1S5 and have SEQ ID NOS: 1, 2, and 12 . Furthermore, T cell lines derived from two such infiltrated skin lesions and enriched in TCRβV14+ T cells, displayed HLA-class I restricted lysis of the autologous melanoma cells. This indicates that cytotoxicity would be a good measure for assessing the relative abundance of the T cells of interest before and after therapy.

Clonal expansion of T cells was demonstrated in T cell-infiltrated, post-vaccine metastases. These results indicate that vaccination with autologous, DNP-modified melanoma cells can expand selected clones of T cells at the tumor site and that

such clones are potentially destructive to the tumor. This *in vivo* clonal expansion of T cells that mediate immune responses against the tumor and ultimately tumor regression has not been previously observed.

Histopathological examination of the inflamed lesions, demonstrated intense infiltration of T lymphocytes, mostly CD8+ (Berd, D., *et al.*, *Cancer Res.* **1991** *51*:2731-2734; and Berd, D., *et al.*, *Cancer Immunol. Immunother.* **1994** *39*:141-147) which are ordinarily not seen in metastases from non-vaccinated patients (Berd. D., *et al.*, 1991, *supra.*). The same type of treatment administered to patients with stage III melanoma, free of disease after resection of palpable, large (>3cm) regional lymph node metastases, has resulted in 50% and 60% 4-year relapse-free and overall survival, respectively (Berd, D. and G. Parmiani. *Cancer Immunol. Immunother.* **1995** *41*:199-200.).

The molecular definition of melanoma antigens recognized by T cells (Boon, T. and P. Van der Bruggen, *J. Exp. Med.* **1996***183*:725-730) and the possibility of studying the composition of the TCR involved in the MHC-restricted recognition of such epitopes, including epitopes expressed within the tumor mass (Sensi, M. and G. Parmiani, *Immunol. Today* **1995***16*:588-595.) 16:588-595, allow a more precise evaluation of the immune response in vaccinated patients.

The number of T cells infused into a patient may be ascertained and adjusted by routine experimentation. It is anticipated that in most cases the initial number of T cells infused will be from about 10^6 to about 10^{12}, more preferably about 10^8 to about 10^{11}. (Care should be taken not to infuse the patient with too many T cells at any one time such as would produce toxic amounts of lymphokines but that is within the skill of the art.) The frequency of reinfusion into the patient depends in part on the number of T cells available, although the available T cells may be further expanded as provided herein. Preferably, the T cells may be administered at least once or twice, from about a few to about several weeks apart. Infusions can continue as long as T cells are available and benefits persist.

The following is a more detailed protocol for T cell isolation and *in vitro* expansion:

Preparation of T Lymphocyte Suspensions. T lymphocytes will be prepared from tumors as follows. Single cell suspensions will be prepared from tumors by

digestion with a mixture of 0.14% collagenase, 0.03% Dnase and optionally 2.5 U/ml hyaluronidase (Sigma Chemical CO., St. Louis, MO) for 3 hours at room temperature. The cells will be filtered through a layer of no. 100 nylon mesh then washed and resuspended in buffer, e.g., Hanks Buffered Saline. The mixture of cells will be depleted of monocytes by panning of the mixture over plastic dishes in a final volume of 2ml RPMI-1640 supplemented with 10% pooled human serum and cultured for one week. T cells can be expanded by exposure to anti-Vβ antibodies and/or an immunostimulatory cytokine (such as, IL-2) and/or superantigens as disclosed e.g., in PCT/US93/05213. Activity of the T cells will be measured after four to five weeks of *in vitro* stimulation.

T cells can also be purified using Dynabeads (DYNAL, Lake Success, New York) coated with various antibodies, e.g., anti-BV14 or anti-CD8+, to enhance the degree and speed of purification of the T cells prior to or after expansion of the T cells. Alternative methods of T cells expansion *in vitro* involve use of superantigens, or monoclonal antibody to a T cell receptor expressed by the tumor infiltrating cells or use of an immunostimulatory cytokines such as TNF, IFN-γ, an interleukin (IL-1, IL-2, IL-12 etc.).

Another method according to the invention involves the use of the T cells of the invention to screen for tumor-specific antigens recognized by T cells that infiltrate the tumor. These antigens are useful as vaccine candidates and as diagnostic markers to detect early metastases. Peptide antigens can also be constructed from such tumor antigens and also used in preparing vaccines. This method can be implemented as follows:

The gene encoding the antigen recognized by the T cell clone(s) will be isolated by methods known in the art, such as the following which are illustrated below.

Construction of the cDNA Library - Poly(A+) RNA will be extracted from melanoma tumor cells using mRNA extraction kit Fastrack (Invitrogen Corporation, Oxon, United Kingdom). mRNA will be converted into cDNA using random primers, ligated to adaptors as described in the SuperScript plasmid system kit (GIBCO-BRL, Gaithersburg, Maryland), and inserted into the EcoRI site of expression vector pCDNA-AI/Amp (Invitrogen Corporation, Carlsbad CA), which places the recombinant insert under control of the cytomegaloviral (CMV) promoter. Recombinant plasmids will be

electroporated into *Escherichia coli* JM 101 and transformants selected with ampicillin (50 mg/ml). The library will be divided into pools of bacteria, each pool containing multiple recombinant inserts. Each pool of bacteria will be amplified to saturation and plasmid DNA will be extracted by the alkaline lysis method.

Transfection of COS-7 Cells - Plasmid DNA prepared as described above will be used to transfect COS-7 cells. Transfection of mammalian cells will be performed using the DEAE-dextran-chloroquine method (Seed et al., *Proc. Natl. Acad. Sci. USA* 84, 3365-3369, 1987; Brichard et al, *J. Exp. Med.* 178, 489-495, 1993; Coulie et al., *J. Exp. Med.* 180, 36-42, 1994). COS-7 cells (1.5×10^4) will be transfected with 100 ng of plasmid pcDNAI/Amp, containing cDNA from an HLA class I gene (Wolfel et al. *Int. J. Cancer* 5:237-244, 1993) and 100 ng of DNA of a pool of the cDNA library in duplicate microtiter wells. Transfected COS-7 cells will be tested in a cytotoxic T cells (CTL) stimulation assay after 24 or 48 hr growth of the cells. Single clones expressing the gene of interest will be isolated by limiting dilution of the plasmid pools.

CTL Stimulation Assay - Transfectants will be tested for their ability to stimulate the production of cytokines, such as Tumor Necrosis Factor (TNF) (Traversari et al., *J. Exp. Med.* 176 1453-1457; Traversari et al. *Immunogenetics* 35 145-152, 1992), interferon, such as gamma interferon, interleukins, such as interleukin 2 (IL2), and granulocyte macrophage colony stimulating factor (GM-CSF). In the case of TNF, for example, microtiter wells containing target cells, 1500 CTL will be added in 100ul of medium (GIBCO-BRL) containing 10% human serum and 20 U/ML r-human IL-2. After 24 hrs, the supernatant will be collected and the TNF content of the supernatant determined by testing its cytotoxic affect on cells of WEHI-164 clone 13 (Espovik et al., *J. Immunol. Meth.* 95, 99-105, 1986), in a MTT colorimetric assay (Hansen et al., *J. Immunol. Meth.* 119, 203-210, 1989; Travorsari et al., *Immunogenetics* 35, 145-152, 1992).

Alternatively, cytotoxicity may be assayed. The tumor cells will be labeled with a fluorescent or radioactive (^{51}Cr or ^{125}I-iododeoxyuridine, for example) label. The T cells will be added in various numbers. After incubating for about four to about twenty-four hours, release of isotope by the tumor cells will be assayed as a measure of tumor cell lysis. Optionally, retention of fluorescent label may be assayed as a measure

of survival of tumor cells. Other assays include simply counting surviving tumor cells before and after incubation with T cells or counting surviving T cells by ability to retain a dye, such as and not limited to MTT.

Yet another assay is for T cell proliferation. The tumor cells are inactivated by irradiation and mixed to varying ratios. After about one to about ten days of incubation, preferably about five to about six days a radioisotope that labels T cells DNA is added. Such radioisotopes include and are not limited to ^{125}I-iododeoxyuridine and ^{3}H-thymidine. The cells are incubated for about four to about eighteen hours and radioactivity is counted in a gamma counter.

DNA Sequencing and Homology Search - cDNA clones expressing cytotoxic activity after transfection will be isolated and sequenced. DNA sequencing analysis will be performed by specific priming with synthetic oligonucleotides. The sequencing reactions will be performed by the dideoxy-chain termination method (T7 Sequencing Kit, Pharmacia, Uppsala, Sweden, Taq Cycle-Sequencing Kit, United States Bio-chemical, Cleveland, Ohio). The computer search for the sequence homology will be done with programs such as FASTA@EMBL-Heidelberg and blast@ncbi.nim.nth.gov.

Production of Progressive Deletions in the Clone of Interest - cDNA clones activating T cells will be inserted in the EcoRl site of cDNA/Amp (Invitrogen Corporation, San Diego, California). The plasmid will be opened with Natl and Sphl before being digested with exonuclease III. This treatment will be performed with the Erase-a-base System (Promega, Madison, Wisconsin). After ligation, the plasmids will be electroporated into *E. coli* DH5aF'10 and selected with ampicillin (50 mg/ml). Clones will be isolated and plasmid DNA will be extracted from each clone. The resulting clones will be transfected into COS-7 cells together with an expression vector for the HLA gene isolated as described above. The ability of the transfected cells to stimulate CTL activity will be assayed as described above.

Antigenic Peptides and CTL Assay - Peptides will be synthesized on solid phase using F-moc for transient NH2-terminal protection as described by Atherton et al. (*J. Chem. Soc. Lond. Perkin Trans.*1: 538, 1981) and characterized by mass spectrometry. All peptides will be >90% pure as indicated by analytical high pressure liquid chromatography. Lyophilized peptides will be dissolved at 20 mg/ml in DMSO,

diluted at 2 mg/ml in 10 mM acetic acid, and stored at -80°C. Peptides will be tested in a CTL stimulation assay with COS-7 cells transfected with an HLA cDNA clone and incubated with the peptides. This is one example of conditions under which the COS-7 cells present peptide in conjunction with their MHC, and thus act as antigen presenting cells. The transfectants will also be tested by chromium release assay as previously described (Boon et al., *J. Exp. Med.* 152, 1184-1193, 1980). In this peptide sensitization assay, target cells will be ^{54}Chromium-labeled for 1 hr at 37°C and washed extensively. Then 1000 target cells will be incubated in 96-well microplates in the presence of various concentrations of peptide for 30 min at 37°C before CTL cells isolated as described above will be added. Chromium release will be measured after 4 hr at 37°C.

Alternatively, DNA clones expressing the antigen recognized by the T cell will be cloned using an approach that has been used successfully with mouse and human tumors. This approach is based on transfection of mammalian cells with DNA from cosmid libraries prepared from DNA of cells expressing the relevant antigen. Transfectants are identified by their ability to stimulate the appropriate T cells. (See, Van der Bruggen et al. *Science* 254:1643-1647, 1991: disclosing a method of cloning human melanoma antigens using this approach; and, De Plaen et al. *Proc. Natl. Acad. Sci.* 85:2274-2278, 1988: disclosing a method of cloning murine antigens using this approach).

The invention is further illustrated by means of the following examples which are meant to be illustrations only and are not intended to limit the present invention to these specific embodiments.

EXAMPLES

Patients and clinical specimens.

The patients studied and the characteristics of their melanoma samples are shown in Table 1. The clinical protocol for the DNP-vaccine administration has been previously described (Berd *et al.*, 1991, *supra.*, Berd, *et al.*, 1994, *supra.*). Briefly, patients were contact sensitized to the hapten by topical application of dinitrofluorobenzene 3 days following low-dose (300 mg/M^2) cyclophosphamide. Two weeks later patients were injected i.d. on the upper arm with irradiated, DNP-modified

melanoma cells mixed with BCG as adjuvant. Two schedules of administration were tested: 1) DNP-vaccine administered every 28 days with cyclophosphamide (Cy) administered 3 days before the first two vaccine injections (patient ED); and 2) DNP-vaccine administered weekly for 6 weeks with Cy given 3 days before the 6 week series (patients FC, CB, JB, RS, LC).

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Table 1 Melanoma patients and specimens: identification and description

PATIENTS		SPECIMENS				
(HLA-A type)	Before DNP-vaccine		After DNP-vaccine			
	Tissue*	Code	Tissue*	Code	Inflammation	Interval (months)=
ED	PBL	PBL-0	PBL	PBL-1		15
(A1)	Lymph node	LN-0	Subcutaneous	S-1	Intense	18
FC	PBL	PBL-0	Subcutaneous	S-1	Intense	2
(A1/A1)	Subcutaneous	S-0				
CB	PBL	PBL-0	PBL	PBL-1		6
(A1)	Lymph node	LN-0	Lymph node	LN-1	Intense	5
JB	PBL	PBL-0	PBL	PBL-1		2
(A1/A28)	Lymph node	LN-0	Subcutaneous	S-1	Intense (regressing tumor)	3
			Lung	L-1	Intense	12
			PBL	PBL-2		17
			Subcutaneous	S-2	Intense	18
			PBL	PBL-3		20
			Subcutaneous	S-3	Intense	20
			Subcutaneous	S-4	Minimal	24
RS	PBL	PBL-0	PBL	PBL-1		4
(A2)	Lymph node	LN-0	Dermal	S-1	Intense	4
LC	PBL	PBL-0	PBL	PBL-1		2
(A2/A29)	Bowel	B-0	Subcutaneous	S-1	Intense	2
			Bowel	B-1	Intense	7
			Bowel	B-2	Intense	13

* Except for peripheral blood lymphocytes (PBL), all tissues are metastatic melanomas.

** No inflammation was observed in pre-vaccination specimens

= Interval between the beginning of DNP-vaccine program and the excision of metastases or

collection of blood.

The clinical course of the six patients was as follows: *CB* - The inflamed metastasis was excised, and the patient remains free of melanoma at 30 months after beginning DNP-vaccine treatment. *JB* - Multiple lung metastases underwent >90% regression; the patient survived 35 months before succumbing to metastatic melanoma in other visceral sites. *LC* - Despite histologic evidence of tumor inflammation, there was no evidence of tumor regression, but the patient survived for 22 months. *FC* - The inflamed subcutaneous metastasis remained stable in size and was excised; the patient remained tumor-free until he died suddenly of a hemorrhagic brain metastasis at 12 months. *ED* - This patient had a mixed response (regression of some subcutaneous tumors simultaneously with growth of others); she survived 24 months. *RS* - Despite gross and histologic evidence of tumor inflammation, there was no evidence of tumor regression, and the patient died at 11 months

Quantification of the cDNA and TCR repertoire analysis.

Semi-quantitative PCR was used as recently described (Salvi, S., *et al.*, *Cancer Res.* 1995 *55*:3374-3379. Briefly, total RNA was prepared, with the use of RNAzolB (Cinna/Biotecx, Friendswood, Texas), from thawed cryopreserved PBL and enzymatically digested tumor samples (without *in vitro* culture). First-strand cDNA was synthesized with oligo-dT and reverse transcriptase (Superscript; Gibco BRL, Gaithersburg, Maryland). Serially diluted cDNA from all samples was amplified using TCR constant-region (BC)-specific primers (Salvi, *et al.*, 1995, *supra.*). PCR products were electrophoresed on agarose gels, transferred to nylon membranes (Hybond N+; Amersham International, Buckinghamshire, England), hybridized with an internal [^{32}P]-labeled BC oligonucleotide and scanned with a model 425 PhosphorImager (Molecular Dynamics, Sunnyvale, California). Individual bands were digitized and integrated using the Imagequant software package provided by the manufacturer. Vβ repertoire analysis was then performed on the same amount of TCR β-chain specific cDNA template from PBL and tumor samples using a panel of described oligonucleotide primers (Genevee, C., *et al.*, *Eur. J. Immunol.* **1992** *22*:1261-1269.). All experiments were performed at least twice. The levels of specific amplification were measured by densitometry as described above and each Vβ spot was expressed as a percentage of the sum of all Vβ

signals detected in the repertoire analysis.

Clonality within selected Vβ-gene families was assessed by high resolution-polyacrylamide gel electrophoresis (HR-PAGE) and SSCP analysis as described (Gorski, J., et al., *J. Immunol.* 1994 *152*:5109-5119; and Orita, M., *et al.*, *Proc. Natl. Acad. Sci. U. S. A.* **1989** *86*:2766-2770.). Briefly, Vβ PCRs were performed by ^{33}P-end-labeling the BC-specific antisense primer (0.08 μM). PCR products (5 μl) were heat-denatured, separated both on 5% denaturing sequencing gel and on 6% non-denaturing polyacrylamide gels containing 10% glycerol and visualized by autoradiography. The official nomenclature proposed by the International Union of Immunological Societies subcommittee on nomenclature has been adopted throughout this application (Williams, A. F., *et al.*, *Immunogenetics* **1995** *42*:451-453). Designation of Vβ-gene subfamilies is according to Arden *et al.*, *Immunogenetics* **1995** *42*:455-500.

A series of metastatic melanomas was obtained from six patients that developed an inflammatory response following administration of DNP-vaccine, and compared their Vβ repertoire with that of metastases excised before vaccine and with pre- and post-vaccine PBL (Table 1). For patient JB, five post-vaccine biopsy specimens (S-1, L-1, S-2, S-3, S-4) were collected over a two year period. The last sample (S-4) was obtained when the patient had ceased to clinically respond to the vaccine, and that lesion displayed minimal inflammation. Multiple post-vaccine metastases were also analyzed for patient LC.

The α-chain repertoire could have been compared instead or in addition; thus, the invention is not in principle limited to T cells expressing a Vβ family of TCR but only to T cells (elicited as a result of immunization or other immunomodulatory therapy) participating in the immune response against the tumor.

Figure 1 shows the histology of subcutaneous (sc) metastases (at 100X) excised from patient FC before and after DNP-vaccine treatment. It is representative of the histologies of inflamed tumors observed in other patients. The post-vaccine tumor (Fig. 1B) shows marked infiltration with lymphocytes (shown by flow cytometry to be CD3+ CD8+ T-cells) at two months after beginning DNP injections, while in the pre-vaccine lesion lymphocytes are present, but sparse (Fig. 1A). The development of tumor inflammation following DNP-vaccine in a subcutaneous (sc) metastasis (S-1) of a second patient (JB) has been previously described (Berd *et al.*, 1994 *supra.*). The post-

vaccine sample represents a sample two months after beginning DNP-vaccine injections. It should be noted that the pre-vaccine tumors from patients ED, CB, JB, and RS were lymph node metastases and therefore contained an abundance of T-cells.

Figure 2 shows, for each patient, the relative representation of Vβ-gene families which, in at least one of the studied specimens, was overexpressed, *i.e.*, increased more than about 1.8 fold over pre-vaccine (either PBL or tumor) samples. This threshold was chosen because in these experiments it was always above the mean + 2SD of each corresponding pre-vaccination sample. Each Vβ determination, expressed as a mean percentage of the sum of all Vβ signals, was repeated at least twice, and up to four times. The results are presented as a mean percentage (± SD). PBL 1-3 represent the mean value of Vβ expressed by post-vaccine PBL taken at different time points.

Some pre-vaccine metastases displayed differences in the expression of several Vβ-gene families when compared to matched pre-vaccine PBL. These include: Vβ3 in patients LC, JB, and CB; Vβ5 in patients ED, LC, and CB; Vβ14 in patients ED, FC, and LC; Vβ7 in patients ED, FC, LC, and JB. Several other overexpressed Vβ-gene families were found in similar percentages in pre- and post-vaccine tumors (patient ED: Vβ13, Vβ15; patient JB: Vβ20; patient CB: Vβ16; patient LC: Vβ4, Vβ8, Vβ20; patient RS: Vβ4, Vβ8). These results extend to metastatic lesions the observation previously made in primary tumors: T-cells infiltrating metastases of *untreated* patients may be biased in their Vβ usage (Sensi *et al.*, 1995, *supra.*). Several of these Vβ-gene families maintained high levels of expression in post-vaccine lesions; *ie.*, they were not further modified by vaccination (Figure 2).

Next, Vβ expression was compared in post-vaccine metastases of each patient with the corresponding pre-vaccine tumors (Figure 2 and Table 2). Vβ14 was the only Vβ-gene subfamily overexpressed in post-vaccine sc metastases of patients FC and RS. In patient FC, Vβ14 accounted for over 30% of the total repertoire in the post-vaccine metastasis having undergone a four-fold expansion compared with the pre-vaccine tumor and a seven-fold expansion compared to pre-vaccine PBL. In patient RS, Vβ14 was the only Vβ-gene family expressed by T-cells infiltrating the post-vaccine metastasis. In patient JB there was an increase in Vβ1 and Vβ14 expression in multiple post-vaccine sc metastasis and in the lung metastasis L-1 (Figure 2 and Table

2). Vβ3, one of the families overexpressed in the pre-vaccine tumor, was not increased further in the post-vaccine specimens (Figure 2).

The post-vaccine tumor of patient ED displayed an overexpression of Vβ5, which accounted for more than 30% of the Vβ repertoire; this represented a three-fold expansion of Vβ5 compared with the pre-vaccine metastasis and a seven-fold expansion when compared to PBL. Vβ23 and 24 were also overexpressed in patient ED compared to pre-vaccine tumor, although to a much lesser extent.

Changes in the Vβ repertoire of the remaining two patients - LC and CB - were more complex. In patient LC, from whom multiple metastases were available, Vβ-gene families involved were different from lesion to lesion, with the exception of Vβ15. In the post-vaccine metastasis of patient CB, Vβ23 was overexpressed compared to pre-vaccine tumor although its level accounted for only 2% of the total TCR repertoire. Vβ5, highly represented in pre-vaccine tumor, did not increased following vaccination. These results could be attributed to the histologies of post-vaccine tumors in these two patients: both showed extensive tumor necrosis, which could also be associated with a non-specific (polyclonal) infiltration of mononuclear cells that would mask a more restricted response.

In contrast, differences observed in Vβ expression of peripheral blood lymphocytes before and after vaccination began were minimal even for Vβ-gene families whose levels increased at the tumor site following vaccination (Figure 2). Patient LC displayed the highest number of Vβ-gene family variation in post-vaccine PBL.

These results show that differences in the number of tumor-infiltrating T cells expressing a particular receptor occur as a result of vaccination and this sets the stage for various advantageous uses of such T cells. Moreover, these vaccination-elicited T cells participate in the immune response against the tumor (Vβ4, Vβ7, Vβ8, Vβ15, Vβ20).

Table 2 Vβ-gene families overexpressed in post-vaccine tumors compared to pre-vaccine tumors

Patients	Post-vaccine Specimens	TCRVβ families overexpressed
HLA-A1		
ED	S-1	5 (30.6)*; 23 (2.2); 24 (2.5)
FC	S-1	14 (31.6)
CB	LN-1	23 (2.1)
JB	L-1	1 (12.5);14 (6.7)
	S-1	1 (11.1)
	S-2	14 (7.2)
	S-3	1 (9.73); 14 (8.2)
	S-4	1 (12.6)
HLA-A2 patients		
RS	S-1	14 (100)
LC	S-1	5 (32.9); 15 (3.2)
	B-1	4 (10.4); 15 (5.6)
	B-2	7 (8.4); 15 (3.4)

* In parenthesis is indicated the relative percentage of expression.

All these Vβ gene families were also overexpressed when compared to pre-vaccine PBL, the exception being Vβ1 in patient JB, specimens S-1 and S-3.

Cloning and sequencing of Vβ transcripts.

PCR products derived from at least two different sets of amplifications were cloned into the pCR-Script™ SK(+) vector (Stratagene, La Jolla, California) and random clones sequenced with Sequenase 2.0 (United States Biochemicals, Cleveland, Ohio). The complementarity-determining region (CDR3) has been defined according to Moss and Bell Moss, *Immunogenetics* **1995** *42*:10-18. Their boundaries include one of the serine residues encoded by the 3' end of the Vβ region up but not including the phenylalanine

residue of the conserved joining region (BJ) motif FGXGT, SEQ ID NO: 20. Germline BJ sequences were obtained according to Toyonaga *et al.*, *Proc. Natl. Acad. Sci. USA* **1985** *82*:8624-8628.

Since vaccination with DNP-modified autologous tumor cells elicited an expansion of specific Vβ-gene families in metastatic lesions, whether such expansions were due to T-cell clonal subpopulations and whether identical clonotypes could be found in multiple lesions were examined. To screen the extent of heterogeneity of the T cell infiltrate, molecular analysis of length and sequence composition of the CDR3 region, which is encoded by the V-D-J junctions and is important for MHC/peptide recognition was performed according to the method of Jorgensen, J. L., *et al.*, *Nature* **1992** *355*:224-230, for the Vβ14 transcripts that were overexpressed in 3/5 post-vaccine lesions from patient JB. To score for any possible intratumoral clonal expansions, all of the JB specimens were included in this analysis.

In polyclonal populations, CDR3 lengths for individual Vβ-gene subfamilies are visualized as a ladder pattern of 3 bp-spaced bands on a sequencing gel. In contrast, the post-vaccine metastases, but not the pre-vaccine metastases or the PBL, exhibited predominance of particular CDR3 size bands (Figure 3). This finding could have been due to the expansion of either single clones or of several clones with the same CDR3 size. To distinguish between these alternatives, single strand conformational polymorphism (SSCP) analysis was performed. By this technique, each single band represents a specific T-cell clonotype and it can be detected on a smear if it is expanded in an heterogeneous population. As shown in Figure 3, while pre-vaccine metastases and pre- and post-vaccine PBL of patient JB taken at different time points exhibited a polyclonal pattern, in all post-vaccine lesions limited numbers of expanded clonotypes could be resolved. In particular, two different bands were common to all of the post-vaccine samples.

Nucleotide sequences of random Vβ14 amplification clones derived from post-vaccine sc metastases S-1, S-2, S-3, and S-4 of patient JB confirmed the oligoclonal nature of the expansions (Table 3). In fact, 6/8 clones in tumor S-1 had an identical V-D-J rearrangement, which was identical to that found in 2/12, 6/9 and 2/9 clones in tumors S-2, S-3 and S-4, respectively. When evaluated by SSCP, it corresponded to one of the two recurring clonotypes of Figure 3. A second rearranged transcript was found in 1/8, 1/12 and 1/9 recombinant clones sequenced in S-1, S-2, S-4, respectively. These two clones

were biased toward the use of BJ1S5 and displayed a conserved non germline encoded basic arginine residue (R, in single letter amino-acid nomenclature) at the same position within their junctional regions. A third transcript was shared by S-2 and S-3. All of the remaining sequences occurred only one or twice in single post-vaccine lesions (Table 3). None of these sequences could be found in a similar number of random Vβ14 clones obtained from the pre-vaccine lymph node metastasis (LN-0) or pre- and post-vaccine PBL (PBL-0, 1, 2) and their sequences were never repeated.

The nature of Vβ14 expansion was also studied in patient FC. This Vβ region was already overexpressed in the pre-vaccine tumor which included several distinct clonotypes (Figure 3). The pattern observed in the post-vaccine lesion, both in CDR3 length and SSCP analysis, was consistent with the emergence of two new major clonotypes.

The presence of these clonotypic changes, their frequency, and their persistence in different metastases from the same patient indicate that an immune response elicited by the autologous vaccine was occurring in these tumors.

Peptides having the foregoing amino acid and sequences can be prepared by known techniques and used as reagents, e.g. to determine how restricted the T cells limited by vaccination may be in wider patient population, e.g., by making antipeptide antibodies.

Table 3 Analysis of Vβ14 transcripts expressed in asynchronous metastatic lesions of patient JB

			No. of occurrences/No. of sequences in the following samples			
	BJC	**CDR3***	**S-1**	**S-2**	**S-3**	**S-4**
	Sequences occurring identically in multiple post-vaccine metastases					
	J1S5C1	SPRDRLNQPQH	6/8	2/12	6/9	2/9
		SLSVRPNLQPQH	1/8	1/12		1/9
	J2S1C2	SFGGLNEQF		1/12	1/9	
	Sequences occurring no more than twice in single post-vaccine metastases					
	JIS1C1	SLGGSTEAF		1/12		
		SILRNTEAF		1/12		
		SLLGADTEAF	1/8			
	J1S2C1	SLTGGGGVGYT		2/12		
		SFPGRGYT			1/9	
		SFANGYGYT			1/9	
		SYPQASGYT				1/9
		SLYQGGYGYT				1/9
	J1S5C1	SLLTQPQH				1/9
	J1S6C1	SNLNSPLH		1/12		
	J2S1C2	SYSGTYNEQF		1/12		
		RQTGGNEQF		1/12		
		SLIRLLHEQF				1/9
		SPEWTSGANEQF				1/9
	J2S7C2	SWGRGEQY		1/12		
		SLGAGLVGHEQY				1/9

* Amino acid sequences in the single letter code.

HLA typing.

HLA-class I typing was available for patients JB (A1/28, B8/51), LC (A2/29, B44/51), FC (A1/A1, B8/60) and was determined by serology. Patients ED, CB were typed as HLA-A1 whereas patient LC was HLA-A2 by PCR on cDNA from pre-vaccine PBL using the method and primers described by Browning *et al.*, *Proc. Natl. Acad. Sci. U. S. A.* **1993** *90*:2842-2845.

The availability of a monoclonal antibody (mAb) directed to the single member of Vβ14-gene family gave us the opportunity to determine whether such T-cells could mediate anti-tumor activity. Tumor infiltrating lymphocytes (TIL) were recovered from three different sc metastatic lesions (S-2, S-3, S-4) of patient JB. TIL were not available from the others. Expression of Vβ14 could be assessed by immunofluorescence in S-3 where it accounted for 21% of T-cells confirming that this subpopulation had expanded compared to both pre- (11%) and matched post-vaccine PBL (12%). All T-lymphocyte cultures were initiated at the same time and lymphocytes were grown *in vitro* in the presence of anti-Vβ14 and anti-CD28 mAbs as described in herein. This procedure yielded a population of T-cells highly enriched for CD3+ and Vβ14+ (Table 4) that were tested for cytotoxicity on the autologous target. After four weeks of *in vitro* restimulation Vβ14-enriched T-cell lines derived from the two inflamed metastases (S-2, S-3) but not from the minimally-inflamed metastasis (S-4) lysed the autologous tumor line derived from S-3 (Table 4). Inhibition of lysis by mAbs W6/32 (anti-MHC class I antigen) and CR11.351 which recognizes HLA-A28, in addition to HLA-A2, see Holmes, N., P. *et al.*, *J. Imunnol.***1987** *139*:936-941, indicated recognition of antigens in association with this restriction element (Table 4). Similar results were obtained by testing VB14-enriched T-cell lines after five weeks of *in vitro* restimulation.

The procedures disclosed herein can be used to assay for the increased occurrence of T cells that participate in an immune response against a tumor and can thus be used to assess the effectiveness of cancer therapy involving administration of an immunomodulatory agent.

Table 4 Autologous tumor cytotoxicity by Vβ14+ TIL lines obtained from post-vaccine metastatic lesions of patient JB

CTL	% positive cells*			% Specific lysis on melanoma cell line S-3		
lines	CD3	CD8	Vβ14	no mAB**	+αHLA class 1=	+αHLA-A1/A28=
TIL #2 (S-2)	80	38	51	13	1	0
TIL #3 (S-3)	86	67	81	21	0	0
TIL #4 (S-4)	88	64	59	0	0	0
LAK#				60	51	53

* As detected by indirect immunofluorescence with mAbs OKT3 (anti-CD3), OKT8 (anti-CD8) and CAS.1.1.3 (anti-Vβ14).

**Lysis of the autologous metastatic cell line S-3 by Vβ14+TIL lines was tested in a 4 hr ^{51}Cr-release assay at an E:T ratio of 20:1.

= Inhibition of lysis was tested after pre-incubation of the tumor target with anti-HLA-class I mAb w6/32 or with anti-HLA-A2/A28 mAb wCR11.351.

LAK cells used as control of target lysability were produced by culturing PBL of healthy donors for one week in the presence of 500 U/ml of recombinant IL-2.

Cell lines.

Melanoma lines from sc metastases were established *in vitro* according to described methods of Anichini, A., *et al., J. Immunol.* **1989***142*:3692-3701.

***In vitro* expansion of Vβ14+ TIL.**

TIL from post-vaccine sc metastases of patient JB were expanded ex *vivo* in presence of mAbs to Vβ14 (CAS.1.1.3, Immunotech, Marseille, France) and to CD28 (L293, Becton-Dickinson, Mountainview, CA). Briefly, anti-Vβ14 (0.2 μg/ml) and anti-CD28 (1 μg/ml) were added in 0.5 ml of RPMI-1640 (Bio-Whitaker, Verviers, Belgium) to culture wells (24 well culture plates, 3524, Costar, Cambridge, Massachusetts) precoated

with 10 μg/ml of affinity-purified goat anti-mouse (GAM) IgG (Sigma ImmunoChemicals, St. Louis, Missouri) and incubated for 40 min at 4°C. A cell suspension consisting of T-lymphocytes and tumor cells, recovered from tumor sites and depleted of monocytes after a 2 hr adherence to plastic, was panned over the coated wells in a final volume of 2 ml RPMI-1640 supplemented with 10% pooled human serum (complete medium, CM) and cultured for one week. Cells were then restimulated for an additional week on anti-Vβ14 mAb coated well in CM plus 20 U/ml recombinant IL-2 and then cultured in CM plus 50U/ml recombinant IL-2. Cytotoxic activity on autologous tumor was tested by a standard ^{51}Cr-release assay as described by Anichini, A., *et al.*, *supra.*, after four and five weeks of *in vitro* stimulation. Inhibition of lysis was performed by preincubating tumor targets with 10 μg/ml of purified W6/32 (anti-HLA class-I, HB95, ATCC, Rockville, Maryland according to the methods of Parham, *et al.*, *J. Immunol.* **1979** *123*:342-349) or CR11.351 according to the methods of Russo, C., *et al. Immunogenetics* **1983** *18*:23-35 mAbs for 40 min at 37° C.

This procedure can be used to expand the number of T cells prior to reinfusion of the expanded T cells to a patient for therapeutic purposes, i.e., as a passive immunization.

Phenotypic analysis.

Phenotype of lymphocytes was assessed by indirect immunofluorescence followed by cytofluorimetric analysis with a FACScan cytofluorimeter (Becton Dickinson, Sunnyvale, California) as described by Anichini, A., *et al.*, *supra.* The following mAbs were used: CAS.1.1.3 (anti-Vβ14), OKT3 (anti-CD3, CRL8001, ATCC), OKT8 (anti-CD8, CRL8014, ATCC).

The present invention constitutes the first demonstration of clonal T-cell expansion elicited at the tumor site by a human tumor vaccine. Thus, it provides immunological validation for the therapeutic approach with autologous DNP-modified tumor cells. In addition, the present invention shows that analysis of TCR expression at the tumor site is a powerful tool for tracing the evolution of the immune response to autologous tumor that is induced by immunotherapy.

Various modifications of the invention in addition to those shown and described herein will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the

appended claims.

The disclosures of each patent, patent application and publication cited or described in this document are hereby incorporated herein by reference, in their entirety. In case of conflict the present specification, including its definitions, controls.

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PCT Application No. PCT/US89/01025.

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SEQUENCE LISTING

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Ser Pro Arg Asp Arg Leu Asn Gln Pro Gln His
1 5 10

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ser Leu Ser Val Arg Pro Asn Leu Gln Pro Gln His
1 5 10

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ser Phe Gly Gly Leu Asn Glu Gln Phe
1 5

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Ser Leu Gly Gly Ser Thr Glu Ala Phe
1 5

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Ser Ile Leu Arg Asn Thr Glu Ala Phe
1 5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids

(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Ser Leu Leu Gly Ala Asp Thr Glu Ala Phe
1 5 10

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Ser Leu Thr Gly Gly Gly Gly Val Gly Tyr Thr
1 5 10

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Ser Phe Pro Gly Arg Gly Tyr Thr
1 5

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Ser Phe Ala Asn Gly Tyr Gly Tyr Thr
1 5

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Ser Tyr Pro Gln Ala Ser Gly Tyr Thr
1 5

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Ser Leu Tyr Gln Gly Gly Tyr Gly Tyr Thr
1 5 10

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Ser Leu Leu Thr Gln Pro Gln His
1 5

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Ser Asn Leu Asn Ser Pro Leu His
1 5

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Ser Tyr Ser Gly Thr Tyr Asn Glu Gln Phe
1 5 10

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Arg Gln Thr Gly Gly Asn Glu Gln Phe
1 5

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Ser Leu Ile Arg Leu Leu His Glu Gln Phe
1 5 10

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Ser Pro Glu Trp Thr Ser Gly Ala Asn Glu Gln Phe
1 5 10

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Ser Trp Gly Arg Gly Glu Gln Tyr
1 5

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Ser Leu Gly Ala Gly Leu Val Gly His Glu Gln Tyr
1 5 10

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Phe Gly Xaa Gly Thr
1 5

WHAT IS CLAIMED IS:

1. A method of generating T cells having the property of infiltrating a malignant tumor of a human and participating in an immune response directed against said tumor comprising the steps of:

(a) immunizing said human with a composition comprising a hapten- modified syngeneic human tumor cell, substantially in a no growth phase, and an adjuvant, wherein said human suffers from a tumor of the same type as said hapten-modified syngeneic human tumor cell, thereby producing T cells having the property of infiltrating said tumor of said human; and

(b) isolating from said tumor obtained from said patient T cells that have been elicited *in vivo* in said patient after the administration of said composition.

2. The method of claim 1 further comprising expanding said T cells *in vitro*.

3. A method of treating a tumor comprising administering to a patient suffering from a tumor a composition comprising T cells that have the property of (i) infiltrating said tumor and (ii) mediating an immune response against the tumor, said T cells selected from the group consisting of

(a) T cells isolated from the tumor of said patient elicited *in vivo* as a result of immunizing said human with a composition comprising a hapten-modified syngeneic tumor cell substantially in a no growth phase, and an adjuvant, wherein said tumor cell is of the same tumor type as the tumor of said human;

(b) *in vitro* expanded clones of said *in vivo* elicited T cells; and

(c) combinations thereof.

4. The method of claim 2 or 3 wherein said *in vitro* expanding step comprises exposing T cells isolated from the tumor of said patient to a T cell stimulatory substance selected from the group consisting of a cytokine, a superantigen and an antibody to a T cell receptor protein.

5. The method of claim 3 wherein said tumor is a malignant tumor selected from the group consisting of melanoma, lymphoma, adenocarcinoma, sarcoma and nonsolid tumors.

6. The method of claim 3 wherein said tumor is a malignant tumor selected from the group consisting of melanoma, ovarian, colon, breast, rectal, lung, kidney, prostate cancer, and leukemia.

7. The method of claim 6 wherein said leukemia is acute myelogenous leukemia.

8. The method of claim 1 or 3 wherein said tumor cells are autologous.

9. The method of claim 1 or 3 wherein said hapten in said hapten-modified tumor cell is selected from the group of haptens that bind to lysine on the surface of cells through ϵ-amino groups, or that bind to -COOH groups on the cell surface.

10. The method of claim 1 or 3 wherein said hapten is selected from the group consisting of dinitrophenyl, trinitrophenyl, N-iodoacetyl-N'-(5-sulfonic 1-naphtyl) ethylene diamine, trinitrobenzenesulfonic acid, fluorescein isothiocyanate, arsenic acid, benzene isothiocyanate, trinitrobenzenesulfonic acid, and dinitrobenzene-S-mustard.

11. The method of claim 3 wherein said composition is mixed with the adjuvant prior to administration.

12. The method of claim 11 wherein said immunological adjuvant is selected from the group consisting of *Bacille Calmette-Guerin*, QS-21 and detoxified endotoxin.

13. A method for assessing the effectiveness of a cancer therapy comprising the administration to a patient suffering from cancer of an immunomodulatory agent, said method comprising

(a) detecting T cells expressing a T cell receptor and having the property of infiltrating the tumor (i) prior to said therapy and (ii) at a point in time after commencement of said therapy; and

(b) determining whether said therapy is effective based on whether the infiltrating T cells expressing the same T cell receptor at said point in time have been expanded compared to their pre-therapy occurrence by at least about 2 standard deviations of their mean pre-therapy occurrence.

14. The method of claim 13 wherein said immunomodulatory agent comprises a composition comprising a therapeutically effective amount of a hapten modified syngeneic human tumor cell, killed or in G0 phase, and an adjuvant, wherein said human suffers from a malignant tumor of the same type as said hapten-modified syngeneic human tumor cell.

15. The method of claim 13 wherein said detecting step comprises detecting expression of the T cell receptor of said T cell.

16. The method of claim 15 wherein said detecting step comprises at least one of amplification of nucleic acids encoding said T cell and identification of said T cell with a monoclonal antibody.

17. The method of claim 13 wherein said detecting step comprises at least one of:

(i) determining the cytokine profile of T cells from said tumor;

(ii) determining the cytotoxicity of T cells from said tumor; and

(iii) determining the proliferation of T cells from said tumor.

18. The method of claim 13 wherein said detecting step comprises detecting the appearance of complementarity determining region 3 of T cell receptors.

19. The method of claim 18 wherein the appearance of complementarity determining region 3 of T cell receptors is selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, and SEQ ID NO: 19.

20. A method of screening for antigens having the property of stimulating a specific T cell response directed against a human tumor type comprising exposing a T cell having the property of infiltrating said tumor to a sample to be tested said sample containing at least one antigen under conditions whereby said at least one antigen is presented to said T cell, and assessing at least one of the following:

(i) production of a cytokine by said T cell;

(ii) cytotoxicity of said T cell against said tumor and

(iii) proliferation of said T cell.

21. The method of claim 18 wherein a nucleic acid sequence encoding the antigen in said sample has been used to transfect a cell, which cell does not react with said T cell without being transfected with said nucleic acid sequence for said antigen.

22. The method of claim 18 wherein said stimulation of said isolated T cell comprises observing release of a cytokine.

23. The method of claim 20 wherein said cytokine is selected from the group consisting of tumor necrosis factor, gamma interferon, and an immunostimulatory interleukin.

24. A method of treating cancer comprising administering to a human afflicted with a malignant tumor, a composition comprising a therapeutically effective amount of a T cell having the property of infiltrating said tumor, and participating in an immune response directed against said tumor.

25. An isolated human T cell capable of infiltrating and participating in an immune response against a tumor, said T cell selected from a group consisting of,

(a) T cells isolated from the tumor or of said patient elicited *in vivo* as a result of immunizing said human with a composition comprising a hapten-modified syngeneic tumor cell substantially in a no growth phase, and an adjuvant, wherein said tumor cell is of the same tumor type as the tumor of said human; and

(b) *in vitro* expanded clones of said *in vivo* elicited T cells.

26. The isolated human T cell of claim 25 capable of mediating regression of a tumor.

27. The isolated human T cell of claim 26 expressing a Vβ receptor.

28. The isolated human T cell of claim 27 may change wherein said T cell has a Vβ14 receptor.

29. A pharmaceutical composition comprising (i) a therapeutically effective amount of a T cell, said T cell having the property of infiltrating a tumor of a human, and being expanded upon immunization of said human with a composition comprising a hapten-modified syngeneic tumor cell and an adjuvant, wherein said tumor cell is of the same tumor type as the tumor of said human; (ii) and a pharmaceutically acceptable carrier.

30. A peptide having an amino acid sequence selected from the group consisting of SEQ ID NOS: 1-19.

31. A method of generating T cells having the property of infiltrating a tumor of a human patient afflicted with said tumor and participating in an immune response against the tumor, comprising the steps of isolating from said tumor malignant T cells that have been elicited in vivo in said patient by immunization of said patient with a composition comprising (i) a hapten-modified syngeneic human tumor cell, wherein said tumor cell is of the same type as the tumor afflicting said patient, said modified tumor cell in a substantially no-growth phase, and (ii) an adjuvant.

32. The method of claim 1, 20 or 31 wherein said tumor is a malignant tumor.

33. The all of claim 25 wherein said tumor is a malignant tumor.

34. The composition of claim 29 wherein said tumor is a malignant tumor.

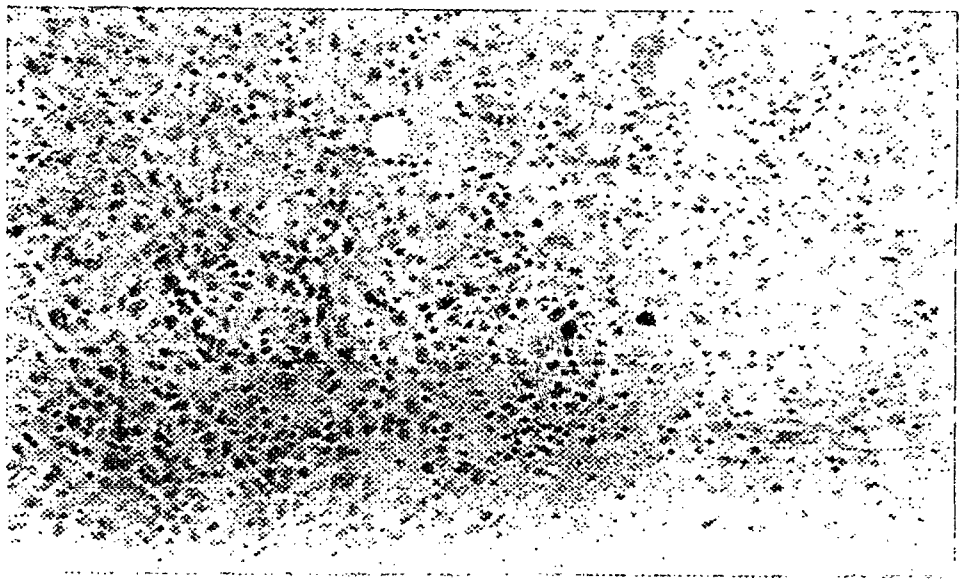


FIG. 1A

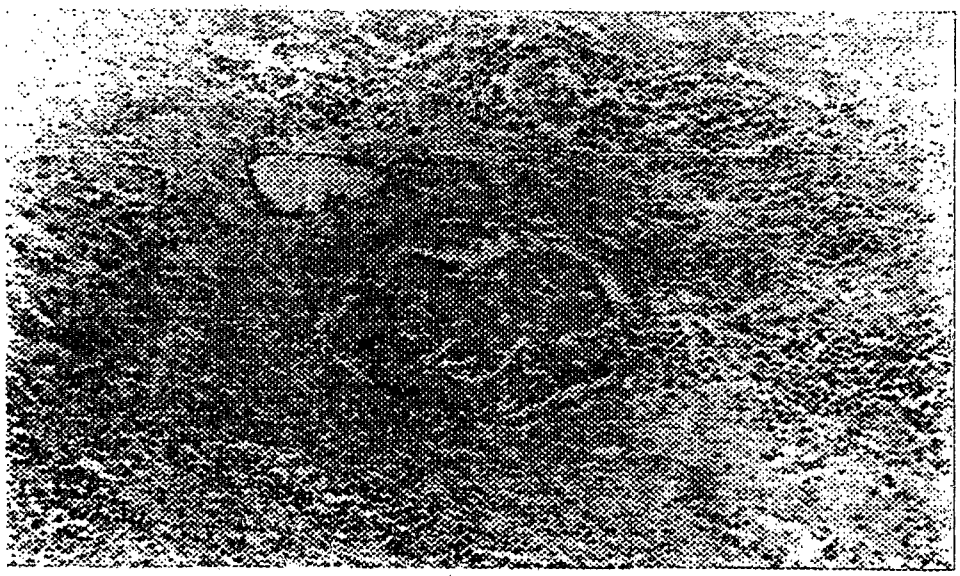


FIG. 1B

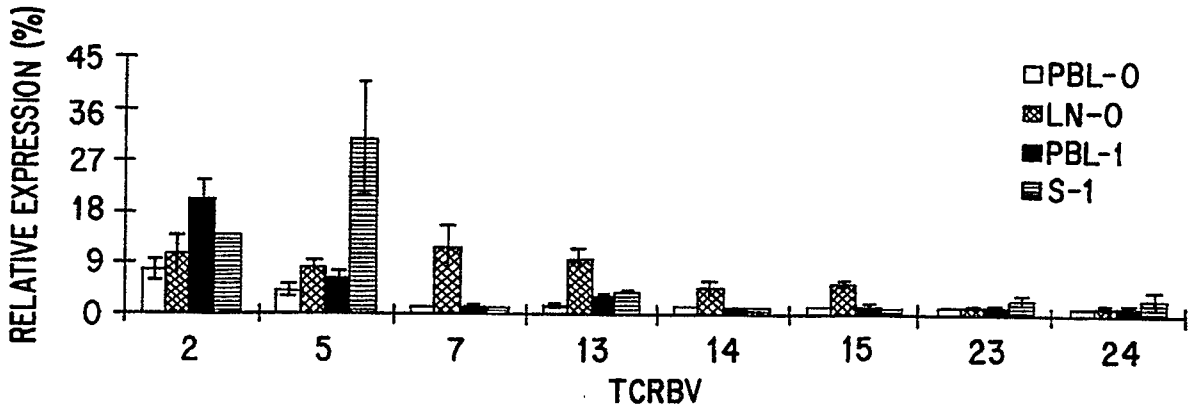


FIG.2A

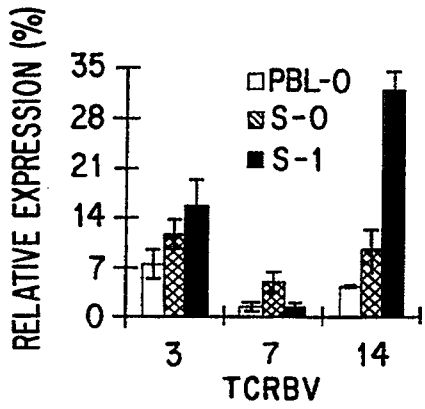
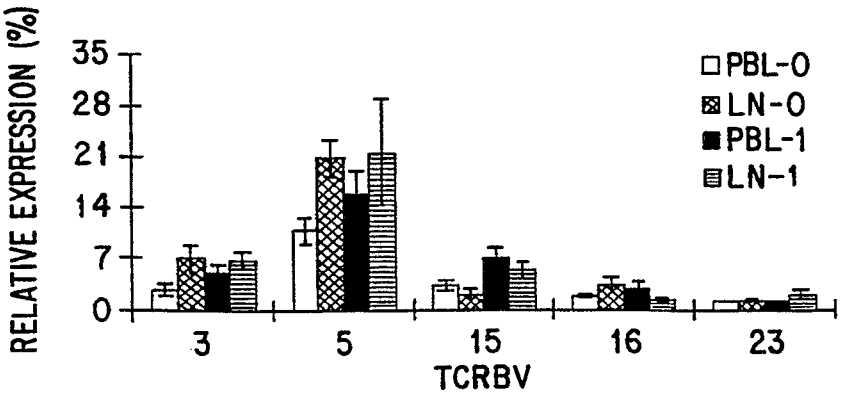


FIG.2B

FIG.2C



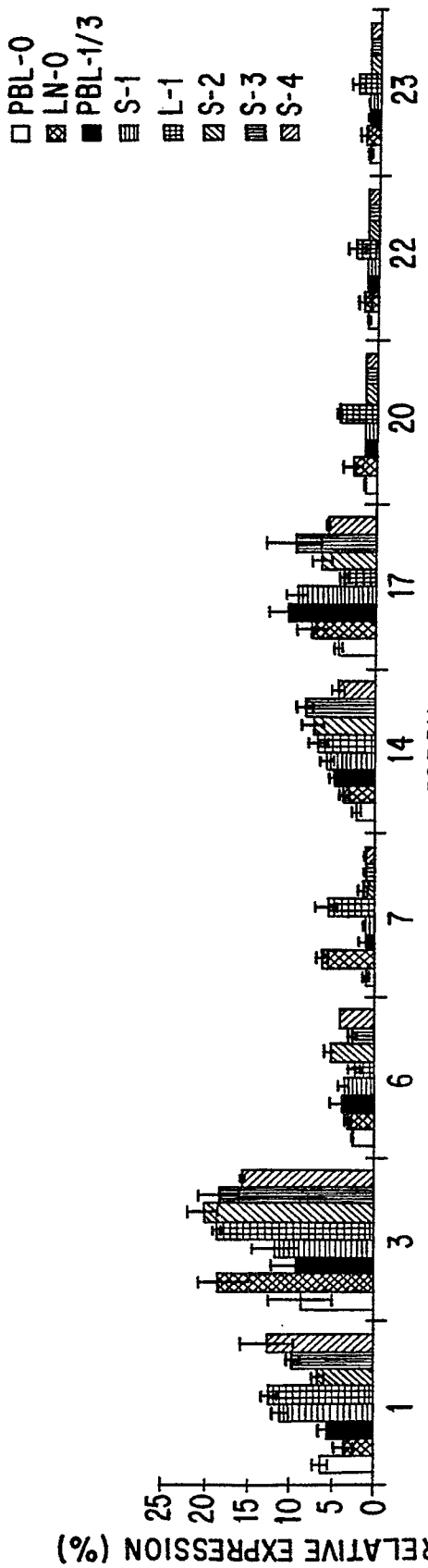

PBL-0
LN-0
PBL-1/3
S-1
L-1
S-2
S-3
S-4
RELATIVE EXPRESSION (%)
0
5
10
15
20
25
1
3
6
7
14
17
20
22
23
TCRBV

FIG. 2D

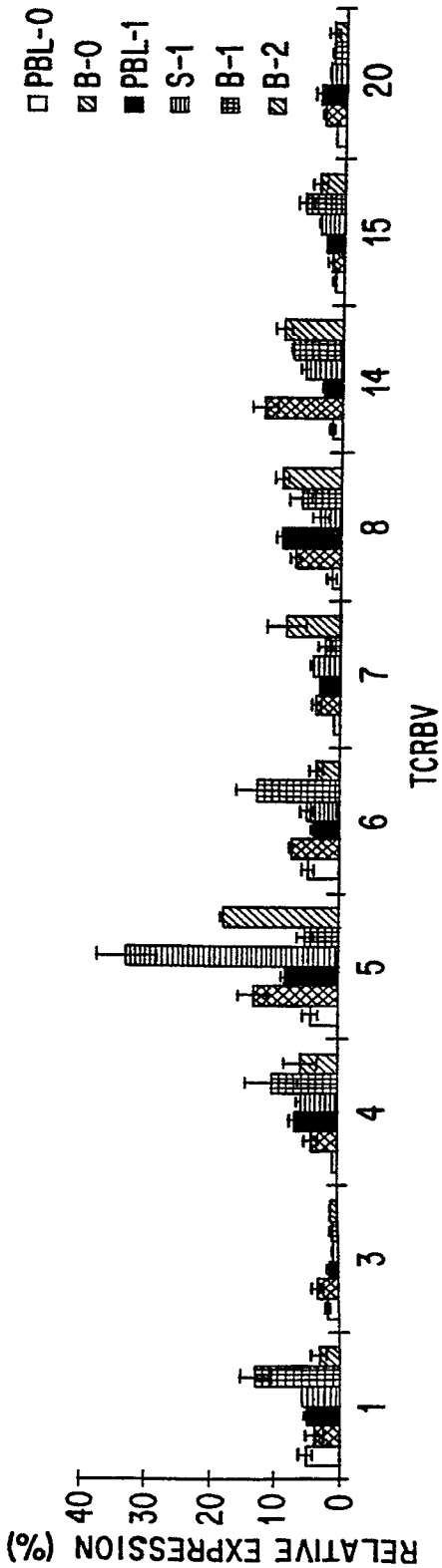

PBL-0
B-0
PBL-1
S-1
B-1
B-2
RELATIVE EXPRESSION (%)
0
10
20
30
40
1
3
4
5
6
7
8
14
15
20
TCRBV

FIG. 2E

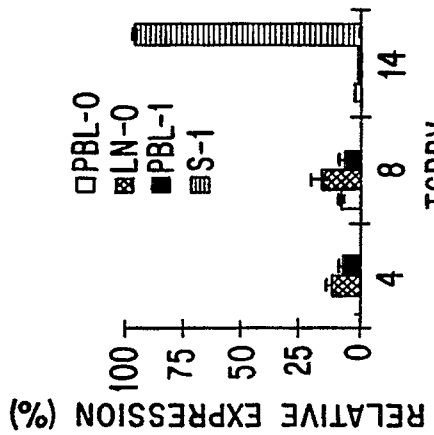

PBL-0
LN-0
PBL-1
S-1
RELATIVE EXPRESSION (%)
0
25
50
75
100
4
8
14
TCRBV

FIG. 2F

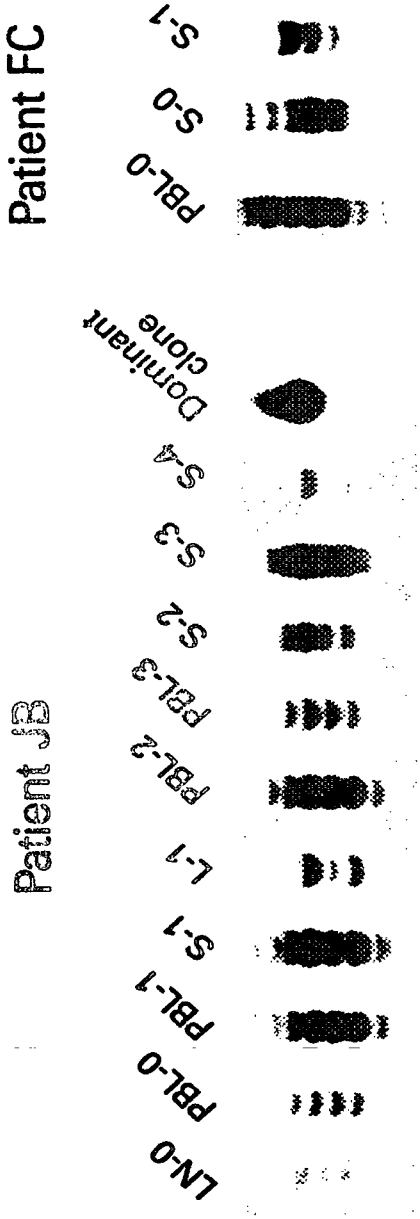

Patient FC
S-1
S-0
PBL-0
Patient JB
Dominant clone
S-4
S-3
S-2
PBL-3
PBL-2
L-1
S-1
PBL-1
PBL-0
LN-0

FIG. 3A

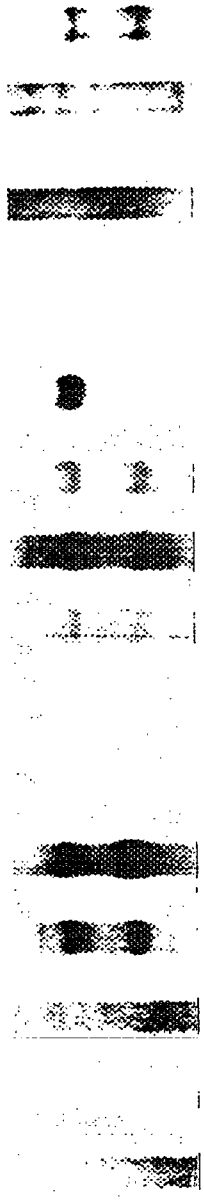


FIG. 3B