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(54) Title: IMMUNE EFFECTOR CELL HYBRIDS

(57) Abstract

This invention provides a substantially pure population of educated, antigen–specific immune effector cells expanded in culture at the expense of hybrid cells, wherein the hybrid cells are antigen presenting cells (APCs) fused to cells that express one or more antigens and methods of using the population of cells.

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IMMUNE EFFECTOR CELL HYBRIDS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority under 35 U.S.C. § 119(e) of U.S. Provisional Application Numbers 60/080,041 and 60/088,357 filed January 26, 1998 and March 31, 1998, respectively, the contents of which are hereby incorporated by reference into the present disclosure.

TECHNICAL FIELD

This invention is in the field of cellular immunology. In particular, the present invention provides populations of antigen-specific immune effector cells and methods of making these cells. Methods of adoptive immunotherapies and identification of the specific antigens recognized by these cells also are provided.

BACKGROUND

Tumor specific T cells, derived from cancer patients, will bind and lyse tumor cells. This specificity is based on their ability to recognize short amino acid sequences (epitopes) presented on the surface of the tumor cells by MHC class I and class II molecules. These epitopes are derived from the proteolytic degradation of intracellular proteins called tumor antigens encoded by genes that are either uniquely or aberrantly expressed in tumor or cancer cells.

The availability of specific anti-tumor T cells has enabled the identification of tumor antigens and subsequently the generation of cancer vaccines designed to provoke an anti-tumor immune response. Anti-tumor T cells are localized within cancer patients, including in the blood (where they can be found in the peripheral blood mononuclear cell fraction), in ascites fluid in

ovarian cancer patients (tumor associated lymphocytes or TALs) or within the tumor itself (tumor infiltrating lymphocytes or TILs). Of these, TILs have been the most useful in the identification of tumor antigens and tumor antigen-derived peptides recognized by T cells.

Conventional methods to generate TILs involve mincing tumor biopsy tissue and culturing the cell suspension *in vitro* in the presence of the T cell growth factor IL2. Over a period of several days, the combination of the tumor cells and IL2 can stimulate the proliferation of tumor specific T cells at the expense of tumor cells. In this way, the T cell population is expanded. The T cells derived from the first expansion are subsequently mixed with irradiated tumor cells and cultured *in vitro* with IL2 to promote further proliferation and enrichment of tumor reactive T cells. After several rounds of *in vitro* expansion, a potent anti-tumor T cell population can be recovered and used to identify tumor antigens via conventional but tedious expression cloning methodology. Kawakami Y. et al. (1994) *PNAS* **91(9)**:3515-19.

The currently employed methodology to generate tumor specific T cells *in vitro* is unreliable. For most other solid tumors, it has been difficult to generate anti-tumor T cells. Only when the tumor biopsy tissue is melanoma does the success rate approach 70%. Due to the comparative ease of generating melanoma-recognizing T cells, the vast majority of tumor antigens identified to date are associated with this disease. Only a handful of non-melanoma tumor antigens have been identified.

Thus, a dilemma one faces in trying to generate T cell responses *in vitro* is that although the tumor cell may present an antigenic target (the tumor antigen) to T cells, they may also elaborate immunosuppressive factors that can thwart one's ability to generate a potent T cell response.

Thus a need exists for a simple and reliable method to generate antigen-specific immune effector cells to assist in the identification and characterization of antigens. This invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

This invention provides a substantially pure population of educated, antigen-specific immune effector cells expanded in culture at the expense of hybrid cells, wherein the hybrid cells are antigen presenting cells (APCs) fused to cells that express one or more antigens.

Also provided by this invention is a method of producing antigen-specific immune effector cells, methods of adoptive immunotherapies and a method of identifying a gene encoding an antigen specifically recognized by the immune effector cells.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Fusion of DCs and MC-38/MUC1 cells. DCs, MC38/MUC1 and fused cells (FC) were analyzed by flow cytometry for the indicated antigens. *See*, Figure 1 of Gong J. et al., *Nature Medicine* (1997) **3**(5):558-561, 558.

Figure 2: Induction of MUC1-specific CTLs by FC/MUC1. Naive lymph node cells isolated from unimmunized MUC1.Tg mice or $CD8^+$ T cells isolated from FC/MUC1-immunized MUC1.Tg mice were incubated at the indicated effector:target ratios with ^{51}Cr-labeled MC-38 (Φ), MC-38/MUC1 (M), MB49 (Θ), and MB49/MUC1 (O) target cells. CTL activity was determined by ^{51}Cr-release. *See*, Figure 2c of Gong J. et al., *PNAS* (1998) **95**:6279-6283, 6281.

MODES FOR CARRYING OUT THE INVENTION

Various publications, patents and published patent specifications are referenced within the specification by an identifying citation. The disclosures of these publications, patents and published patent specifications are hereby incorporated by reference into the present disclosure to more fully describe the state of the art to which this invention pertains.

Definitions

The practice of the present invention employs, unless otherwise indicated, conventional techniques of molecular biology, microbiology, cell biology and recombinant DNA, which are within the skill of the art. See, *e.g.,* Sambrook, Fritsch and Maniatis, MOLECULAR CLONING: A LABORATORY MANUAL, 2nd edition (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F. M. Ausubel et al. eds., (1987)); the series METHODS IN ENZYMOLOGY (Academic Press, Inc.): PCR 2: A PRACTICAL APPROACH (M.J. MacPherson, B.D. Hames and G.R. Taylor eds. (1995)) and ANIMAL CELL CULTURE (R.I. Freshney, ed. (1987)).

As used herein, certain terms have the following defined meanings.

As used in the specification and claims, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a cell" includes a plurality of cells, including mixtures thereof.

The term "immune effector cells" refers to cells that specifically recognize an antigen present, for example on a neoplastic or tumor cell. For the purposes of this invention, immune effector cells include, but are not limited to, B cells, monocytes, macrophages, NK cells and T cells such as cytotoxic T lymphocytes (CTLs), for example CTL lines, CTL clones, and CTLs from tumor, inflammatory sites or other infiltrates. "T-lymphocytes" denotes lymphocytes that are phenotypically CD3+, typically detected using an anti-CD3 monoclonal antibody in combination with a suitable labeling technique. The T-lymphocytes of this invention are also generally positive for CD4, CD8, or both. The term "naïve" immune effector cells refers to immune effector cells that have not encountered antigen and is intended to by synonymous with unprimed and virgin. "Educated" refers to immune effector cells that have interacted with an antigen such that they differentiate into an antigen-specific cell.

The terms "antigen presenting cells" or "APCs" includes both intact, whole cells as well as other molecules which are capable of inducing the presentation of one or more antigens, preferably with class I MHC molecules. Examples of suitable APCs are discussed in detail below and include, but are not limited to, whole cells such as macrophages, dendritic cells, B cells; purified

MHC class I molecules complexed to β2-microglobulin; and foster antigen presenting cells.

Dendritic cells (DCs) are potent APCs. DCs are minor constituents of various immune organs such as spleen, thymus, lymph node, epidermis, and peripheral blood. For instance, DCs represent merely about 1% of crude spleen (Steinman et al. (1979) *J. Exp. Med.* **149**: 1) or epidermal cell suspensions (Schuler et al. (1985) *J. Exp. Med.* **161**:526; and Romani et al. *J. Invest. Dermatol.* (1989) **93**: 600), and 0.1-1% of mononuclear cells in peripheral blood (Freudenthal et al. *Proc. Natl. Acad. Sci. USA* (1990) **87**: 7698). The following references describe methods for isolating DCs from peripheral blood or bone marrow progenitors. Inaba et al. (1992) *J. Exp. Med.* **175**:1157; Inaba et al. (1992) *J. Exp, Med.* **176**: 1693-1702; Romani et al. (1994) *J. Exp. Med.* **180**: 83-93; and Sallusto et al. (1994) *J. Exp. Med.* **179**: 1109-1118). The preferred methods for isolation and culturing of DCs are described in Bender et al. (1996) *J. Immun. Meth.* **196**:121-135 and Romani et al. (1996) *J. Immun. Meth.* **196**:137-151.

"Foster antigen presenting cells" refers to any modified or naturally occurring cells (wild-type or mutant) with antigen presenting capability that are utilized in lieu of antigen presenting cells ("APC") that normally contact the immune effector cells they are to react with. In other words, they are any functional APCs that T cells would not normally encounter *in vivo*.

It has been shown that DCs provide all the signals required for T cell activation and proliferation. These signals can be categorized into two types. The first type, which gives specificity to the immune response, is mediated through interaction between the T-cell receptor/CD3 ("TCR/CD3") complex and an antigenic peptide presented by a major histocompatibility complex ("MHC") class I or II protein on the surface of APCs. This interaction is necessary, but not sufficient, for T cell activation to occur. In fact, without the second type of signals, the first type of signals can result in T cell anergy. The second type of signals, called costimulatory signals, are neither antigen-specific nor MHC-

restricted, and can lead to a full proliferation response of T cells and induction of T cell effector functions in the presence of the first type of signals.

Thus, the term "cytokine" refers to any of the numerous factors that exert a variety of effects on cells, for example, inducing growth or proliferation. Non-limiting examples of cytokines include, IL-2, stem cell factor (SCF), IL-3, IL-6, IL-12, G-CSF, GM-CSF, IL-1α, IL-11, MIP-1α, LIF, c-kit ligand, TPO, and flt3 ligand. Cytokines are commercially available from several vendors such as, for example, Genzyme Corp. (Framingham, Mass.), Genentech (South San Francisco, CA), Amgen (Thousand Oaks, CA) and Immunex (Seattle, WA). It is intended, although not always explicitly stated, that molecules having similar biological activity as wild-type or purified cytokines (*e.g.,* recombinantly produced) are intended to be used within the spirit and scope of the invention and therefore are substitutes for wild-type or purified cytokines.

"Costimulatory molecules" are involved in the interaction between receptor-ligand pairs expressed on the surface of antigen presenting cells and T cells. One exemplary receptor-ligand pair is the B7 co-stimulatory molecules on the surface of DCs and its counter-receptor CD28 or CTLA-4 on T cells (Freeman et al. (1993) *Science* **262**:909-911; Young et al. (1992) *J. Clin. Invest.* **90**: 229; and Nabavi et al. *Nature* **360**:266). Other important costimulatory molecules are CD40, CD54, CD80, and CD86. These are commercially available from vendors identified above.

A "hybrid" cell refers to a cell having both antigen presenting capability and also expresses one or more specific antigens. In one embodiment, these hybrid cells are formed by fusing, *in vitro*, APCs with cells that are known to express the one or more antigens of interest.

A "control" cell refers to a cell that does not express the same antigens as the population of antigen-expressing cells.

The term "culturing" refers to the *in vitro* propagation of cells or organisms on or in media of various kinds. It is understood that the descendants of a cell grown in culture may not be completely identical (i.e., morphologically,

genetically, or phenotypically) to the parent cell. By "expanded" is meant any proliferation or division of cells.

An "effective amount" is an amount sufficient to effect beneficial or desired results. An effective amount can be administered in one or more administrations, applications or dosages. For purposes of this invention, an effective amount of hybrid cells is that amount which promotes expansion of the antigenic-specific immune effector cells, *e.g.,* T cells.

An "isolated" or "enriched" population of cells is "substantially free" of cells and materials with which it is associated in nature. By "substantially free" or "substantially pure" is meant at least 50% of the population are the desired cell type, preferably at least 70%, more preferably at least 80%, and even more preferably at least 90%.

The term "autogeneic", or "autologous", as used herein, indicates the origin of a cell. Thus, a cell being administered to an individual (the "recipient") is autogeneic if the cell was derived from that individual (the "donor") or a genetically identical individual. An autogeneic cell can also be a progeny of an autogeneic cell. The term also indicates that cells of different cell types are derived from the same donor or genetically identical donors. Thus, an effector cell and an antigen presenting cell are said to be autogeneic if they were derived from the same donor or from an individual genetically identical to the donor, or if they are progeny of cells derived from the same donor or from an individual genetically identical to the donor.

Similarly, the term "allogeneic", as used herein, indicates the origin of a cell. Thus, a cell being administered to individual (the "recipient") is allogeneic if the cell was derived from an individual not genetically identical to the recipient; in particular, the term relates to non-identity in expressed MHC molecules. An allogeneic cell can also be a progeny of an allogeneic cell. The term also indicates that cells of different cell types are derived from genetically non-identical donors, or if they are progeny of cells derived from genetically non-identical donors. For example, an APC is said to be allogeneic to an effector cell if they are derived from genetically non-identical donors.

A "subject" is a vertebrate, preferably a mammal, more preferably a human. Mammals include, but are not limited to, murines, simians, humans, farm animals, sport animals, and pets.

As used herein, a "genetic modification" refers to any addition, deletion or disruption to a cell's endogenous nucleotides.

A "viral vector" is defined as a recombinantly produced virus or viral particle that comprises a polynucleotide to be delivered into a host cell, either *in vivo*, *ex vivo* or *in vitro*. Examples of viral vectors include retroviral vectors, adenovirus vectors, adeno-associated virus vectors and the like. In aspects where gene transfer is mediated by a retroviral vector, a vector construct refers to the polynucleotide comprising the retroviral genome or part thereof, and a therapeutic gene.

As used herein, "retroviral mediated gene transfer" or "retroviral transduction" carries the same meaning and refers to the process by which a gene or a nucleic acid sequence is stably transferred into the host cell by virtue of the virus entering the cell and integrating its genome into the host cell genome. The virus can enter the host cell via its normal mechanism of infection or be modified such that it binds to a different host cell surface receptor or ligand to enter the cell.

Retroviruses carry their genetic information in the form of RNA; however, once the virus infects a cell, the RNA is reverse-transcribed into the DNA form that integrates into the genomic DNA of the infected cell. The integrated DNA form is called a provirus.

In aspects where gene transfer is mediated by a DNA viral vector, such as a adenovirus (Ad) or adeno-associated virus (AAV), a vector construct refers to the polynucleotide comprising the viral genome or part thereof, and a therapeutic gene. Adenoviruses (Ads) are a relatively well characterized, homogenous group of viruses, including over 50 serotypes. (see, *e.g.*, WO 95/27071) Ads are easy to grow and do not integrate into the host cell genome. Recombinant Ad-derived vectors, particularly those that reduce the potential for recombination and generation of wild-type virus, have also been constructed. (see, WO 95/00655; WO 95/11984). Wild-type AAV has high infectivity and specificity integrating

into the host cells genome. (Hermonat and Muzyczka (1984) *PNAS USA* **81**:6466-6470; Lebkowski *et al.* (1988) *Mol. Cell. Biol.* **8**:3988-3996).

Vectors that contain both a promoter and a cloning site into which a polynucleotide can be operatively linked are well known in the art. Such vectors are capable of transcribing RNA *in vitro* or *in vivo*, and are commercially available from sources such as Stratagene (La Jolla, CA) and Promega Biotech (Madison, WI). In order to optimize expression and/or *in vitro* transcription, it may be necessary to remove, add or alter 5' and/or 3' untranslated portions of the clones to eliminate extra, potential inappropriate alternative translation initiation codons or other sequences that may interfere with or reduce expression, either at the level of transcription or translation. Alternatively, consensus ribosome binding sites can be inserted immediately 5' of the start codon to enhance expression. Examples of vectors are viruses, such as baculovirus and retrovirus, bacteriophage, cosmid, plasmid, fungal vectors and other recombination vehicles typically used in the art which have been described for expression in a variety of eucaryotic and prokaryotic hosts, and may be used for gene therapy as well as for simple protein expression.

Among these are several non-viral vectors, including DNA/liposome complexes, and targeted viral protein DNA complexes. To enhance delivery to a cell, the nucleic acid or proteins of this invention can be conjugated to antibodies or binding fragments thereof which bind cell surface antigens, e.g., TCR, CD3 or CD4. Liposomes that also comprise a targeting antibody or fragment thereof can be used in the methods of this invention. This invention also provides the targeting complexes for use in the methods disclosed herein.

Polynucleotides are inserted into vector genomes using methods well known in the art. For example, insert and vector DNA can be contacted, under suitable conditions, with a restriction enzyme to create complementary ends on each molecule that can pair with each other and be joined together with a ligase. Alternatively, synthetic nucleic acid linkers can be ligated to the termini of restricted polynucleotide. These synthetic linkers contain nucleic acid sequences that correspond to a particular restriction site in the vector DNA. Additionally, an

oligonucleotide containing a termination codon and an appropriate restriction site can be ligated for insertion into a vector containing, for example, some or all of the following: a selectable marker gene, such as the neomycin gene for selection of stable or transient transfectants in mammalian cells; enhancer/promoter sequences from the immediate early gene of human CMV for high levels of transcription; transcription termination and RNA processing signals from SV40 for mRNA stability; SV40 polyoma origins of replication and ColE1 for proper episomal replication; versatile multiple cloning sites; and T7 and SP6 RNA promoters for *in vitro* transcription of sense and antisense RNA. Other means are well known and available in the art.

As used herein, "expression" refers to the process by which polynucleotides are transcribed into mRNA and translated into peptides, polypeptides, or proteins. If the polynucleotide is derived from genomic DNA, expression may include splicing of the mRNA, if an appropriate eucaryotic host is selected. Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. For example, a bacterial expression vector includes a promoter such as the *lac* promoter and for transcription initiation the Shine-Dalgarno sequence and the start codon AUG (Sambrook et al. (1989), *supra*). Similarly, an eucaryotic expression vector includes a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal., the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors can be obtained commercially or assembled by the sequences described in methods well known in the art, for example, the methods described above for constructing vectors in general.

The terms “major histocompatibility complex” or “MHC” refers to a complex of genes encoding cell-surface molecules that are required for antigen presentation to immune effector cells such as T cells and for rapid graft rejection. In humans, the MHC complex is also known as the HLA complex. The proteins encoded by the MHC complex are known as “MHC molecules” and are classified into class I and class II MHC molecules. Class I MHC molecules include

membrane heterodimeric proteins made up of an α chain encoded in the MHC associated noncovalently with β2-microglobulin. Class I MHC molecules are expressed by nearly all nucleated cells and have been shown to function in antigen presentation to CD8+ T cells. Class I molecules include HLA-A, -B, and -C in humans. Class II MHC molecules also include membrane heterodimeric proteins consisting of noncovalently associated αand β chains. Class II MHCs are known to function in CD4+ T cells and, in humans, include HLA-DP, -DQ, and DR. The term "MHC restriction" refers to a characteristic of T cells that permits them to recognize antigen only after it is processed and the resulting antigenic peptides are displayed in association with either a class I or class II MHC molecule. Methods of identifying and comparing MHC are well known in the art and are described in Allen M. et al. (1994) *Human Imm.* **40**:25-32; Santamaria P. et al. (1993) *Human Imm.* **37**:39-50; and Hurley C.K. et al. (1997) *Tissue Antigens* **50**:401-415.

The term "sequence motif" refers to a pattern present in a group of molecules (*e.g.,* amino acids or nucleotides). For instance, in one embodiment, the present invention provides for identification of a sequence motif among peptides present in an antigen. In this embodiment, a typical pattern may be identified by characteristic amino acid residues, such as hydrophobic, hydrophilic, basic, acidic, and the like.

The term "peptide" is used in its broadest sense to refer to a compound of two or more subunit amino acids, amino acid analogs, or peptidomimetics. The subunits may be linked by peptide bonds. In another embodiment, the subunit may be linked by other bonds, *e.g.* ester, ether, etc.

As used herein the term "amino acid" refers to either natural and/or unnatural or synthetic amino acids, including glycine and both the D or L optical isomers, and amino acid analogs and peptidomimetics. A peptide of three or more amino acids is commonly called an oligopeptide if the peptide chain is short. If the peptide chain is long, the peptide is commonly called a polypeptide or a protein.

As used herein, "solid phase support" is used as an example of a "carrier" and is not limited to a specific type of support. Rather a large number of supports

are available and are known to one of ordinary skill in the art. Solid phase supports include silica gels, resins, derivatized plastic films, glass beads, cotton, plastic beads, alumina gels. A suitable solid phase support may be selected on the basis of desired end use and suitability for various synthetic protocols. For example, for peptide synthesis, solid phase support may refer to resins such as polystyrene (*e.g.*, PAM-resin obtained from Bachem Inc., Peninsula Laboratories, etc.), POLYHIPE® resin (obtained from Aminotech, Canada), polyamide resin (obtained from Peninsula Laboratories), polystyrene resin grafted with polyethylene glycol (TentaGel®, Rapp Polymere, Tubingen, Germany) or polydimethylacrylamide resin (obtained from Milligen/Biosearch, California). In a preferred embodiment for peptide synthesis, solid phase support refers to polydimethylacrylamide resin.

The term "aberrantly expressed" refers to polynucleotide sequences in a cell or tissue which are differentially expressed (either over-expressed or under-expressed) when compared to a different cell or tissue whether or not of the same tissue type, i.e., lung tissue versus lung cancer tissue.

A "tag" or "SAGE tag" is a short polynucleotide sequence, generally under about 20 nucleotides, that occur in a certain position in messenger RNA. The tag can be used to identify the corresponding transcript and gene from which it was transcribed. A "ditag" is a dimer of two sequence tags.

"Host cell" or "recipient cell" is intended to include any individual cell or cell culture which can be or have been recipients for vectors or the incorporation of exogenous nucleic acid molecules, polynucleotides and/or proteins. It also is intended to include progeny of a single cell, and the progeny may not necessarily be completely identical (in morphology or in genomic or total DNA complement) to the original parent cell due to natural, accidental, or deliberate mutation. The cells may be procaryotic or eucaryotic, and include but are not limited to bacterial cells, yeast cells, animal cells, and mammalian cells, e.g., murine, rat, simian or human.

An "antibody" is an immunoglobulin molecule capable of binding an antigen. As used herein, the term encompasses not only intact immunoglobulin

molecules, but also anti-idiotypic antibodies, mutants, fragments, fusion proteins, humanized proteins and modifications of the immunoglobulin molecule that comprise an antigen recognition site of the required specificity.

An "antibody complex" is the combination of antibody (as defined above) and its binding partner or ligand.

A native antigen is a polypeptide, protein or a fragment containing an epitope, which induces an immune response in the subject.

The term "isolated" means separated from constituents, cellular and otherwise, in which the polynucleotide, peptide, polypeptide, protein, antibody, or fragments thereof, are normally associated with in nature. As is apparent to those of skill in the art, a non-naturally occurring polynucleotide, peptide, polypeptide, protein, antibody, or fragments thereof, does not require "isolation" to distinguish it from its naturally occurring counterpart. In addition, a "concentrated", "separated" or "diluted" polynucleotide, peptide, polypeptide, protein, antibody, or fragments thereof, is distinguishable from its naturally occurring counterpart in that the concentration or number of molecules per volume is greater than "concentrated" or less than "separated" than that of its naturally occurring counterpart. A polynucleotide, peptide, polypeptide, protein, antibody, or fragments thereof , which differs from the naturally occurring counterpart in its primary sequence or for example, by its glycosylation pattern, need not be present in its isolated form since it is distinguishable from its naturally occurring counterpart by its primary sequence, or alternatively, by another characteristic such as glycosylation pattern. Although not explicitly stated for each of the inventions disclosed herein, it is to be understood that all of the above embodiments for each of the compositions disclosed below and under the appropriate conditions, are provided by this invention. Thus, a non-naturally occurring polynucleotide is provided as a separate embodiment from the isolated naturally occurring polynucleotide. A protein produced in a bacterial cell is provided as a separate embodiment from the naturally occurring protein isolated from a eucaryotic cell in which it is produced in nature.

A "composition" is intended to mean a combination of active agent and another compound or composition, inert (for example, a detectable agent , carrier, solid support or label) or active, such as an adjuvant.

A "pharmaceutical composition" is intended to include the combination of an active agent with a carrier, inert or active, making the composition suitable for diagnostic or therapeutic use *in vitro*, *in vivo* or *ex vivo*.

As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents. The compositions also can include stabilizers and preservatives. For examples of carriers, stabilizers and adjuvants, see Martin, REMINGTON'S PHARM. SCI., 15th Ed. (Mack Publ. Co., Easton (1975)).

As used herein, the term "inducing an immune response in a subject" is a term well understood in the art and intends that an increase of at least about 2-fold, more preferably at least about 5-fold, more preferably at least about 10-fold, more preferably at least about 100-fold, even more preferably at least about 500-fold, even more preferably at least about 1000-fold or more in an immune response to an antigen (or epitope) can be detected (measured), after introducing the antigen (or epitope) into the subject, relative to the immune response (if any) before introduction of the antigen (or epitope) into the subject. An immune response to an antigen (or epitope), includes, but is not limited to, production of an antigen-specific (or epitope-specific) antibody, and production of an immune cell expressing on its surface a molecule which specifically binds to an antigen (or epitope). Methods of determining whether an immune response to a given antigen (or epitope) has been induced are well known in the art. For example, antigen-specific antibody can be detected using any of a variety of immunoassays known in the art, including, but not limited to, ELISA, wherein, for example, binding of an antibody in a sample to an immobilized antigen (or epitope) is detected with a detectably-labeled second antibody (e.g., enzyme-labeled mouse anti-human Ig antibody). Immune effector cells specific for the antigen can be detected any of a variety of assays known to those skilled in the art, including, but not limited to,

FACS, or, in the case of CTLs, ^{51}CR-release assays, or ^{3}H-thymidine uptake assays.

This invention provides a population of educated, antigen-specific immune effector cells expanded in culture at the expense of hybrid cells, wherein the hybrid cells comprise antigen presenting cells (APCs) fused to cells that express one or more antigens. In one embodiment, the APC are dendritic cells (DCs) and the hybrid cells are expanded in culture. In another embodiment, the cells expressing the antigen(s) are tumor cells and the immune effector cells are cytotoxic T lymphocytes (CTLs). The DCs can be isolated from sources such as blood, skin, spleen, bone marrow or tumor. Methods for preparing the cell populations also are provided by this invention.

In an alternate embodiment, any or all of the antigen-specific immune effector cells or the hybrid cells can be or have been genetically modified by the insertion of an exogenous polynucleotide. As an example, the polynucleotide introduced into the cell encodes a peptide, a ribozyme or an antisense sequence.

In a separate embodiment, the cells expressing the antigen(s) and the immune effector cells have been enriched from a tumor. In a further embodiment, the immune effector cells are cytotoxic T lymphocytes (CTLs). The method also provides the embodiment wherein the APCs and the antigen-expressing cells are derived from the same subject or from different subjects (autologous or allogeneic).

In a further modification of this method, the immune effector cells are cultured in the presence of a cytokine, e.g., IL2 or GM-CSF and/or a costimulatory molecule.

Further provided by this invention is adoptive immunotherapy comprising administering an effective amount of the antigen-specific immune effector cells described herein, effective to induce an immune response.

This invention also provides use of the population of antigen-specific immune effector cells prepared by the above method to further identify a polynucleotide fragment of a gene that encodes an antigen recognized by the

population of antigen-specific immune effector cells. The method comprises the steps of: a) obtaining a set of polynucleotides fragments or "tags" representing gene expression in an antigen-expressing population of first cells recognized by the immune effector cells of this invention; b) obtaining a set of polynucleotides fragments or "tags" representing gene expression in a second set of cells lacking the antigen of the first cells; and c) identifying a unique tag between the polynucleotides obtained from the first and second cells, the unique tag representing a fragment of a gene that is differentially or aberrantly expressed in the population of antigen-expressing cells as compared to the second cells. In a further embodiment, the gene corresponding to the unique polynucleotide or "tag" is isolated and cloned.

The method of step, (c) (above) may, in one embodiment, be performed prior to step (b). The first and second cells are animal cells that include, but are not limited to human, murine, rat or simian cells. They can be antologous or allogeneic as defined above.

Many methods are known in the art to identify differentially expressed polynucleotides and each can be used to provide the polynucleotides in the above method. As used herein, the term "polynucleotide fragment" includes SAGE tags (defined above) as well as any other nucleic acid obtained from any methods that yield quantitative/comparative gene expression data. Such methods include, but are not limited to cDNA subtraction, differential display and expressed sequence tag methods. Techniques based on cDNA subtraction or differential display can be quite useful for comparing gene expression differences between two cell types (Hedrick et al. (1984) *Nature* **308**:149 and Lian and Pardee (1992) *Science* **257**:967). The expressed sequence tag (EST) approach is another valuable tool for gene discovery (Adams et al. (1991) *Science* **252**:1651), like Northern blotting, RNase protection, and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis (Alwine et al. (1977) *PNAS* **74**:5350; Zinn et al. (1983) *Cell* **34**:865; and Veres et al. (1987) *Science* **237**:415). A futher method is differential display coupled with real time PCT and representational difference analysis (Lisitisyn and Wigler (1995) *Meth. Enzymol.* **254**:291-304). Another approach

requires the steps of: (a)providing complementary deoxyribonucleic acid (cDNA) polynucleotides from an antigen expressing cell recognized by the immune effector cells of this invention; (b) providing cDNA polynucleotides from cells having a compatible major histocompatability complex (MHC) to the cells of step (a) but which do not express antigen; (c) determining and analyzing the cDNAs that are aberrantly expressed by the first cells as compared to the second cells. The cDNA polynucleotides may in one embodiment, be obtained using a method identified herein as SAGE and described in U.S. Patent No. 5,695,937.

The polynucleotides identified in steps (b) and (c) are compared to identify those polynucleotides or the polynucleotides corresponding to the genes, or fragments of the genes, that are common to the polynucleotides of the first and second cells. The common polynucleotides represent fragments of the genes that encode antigens recognized by the immune effector cells of this invention. The biological activity of the peptides encoded by the invention polynucleotides can be confirmed using methods described herein.

This method identifies polynucleotides that have the potential to encode the peptide sequences or motifs that are antigenic or a fragment of the antigenic protein or polypeptide. Thus, the method further encompasses confirmation that the expression product encodes the antigen of interest by introducing into a cell the polynucleotide under conditions that it is expressed and presented by an APC by a compatible MHC. Methods for recognition by immune effector cells are well known in the art.

Alternatively, the genes may be identified by providing one or more immune effector cells having an identified major histocompatibility and identifying a peptide sequence motif in the antigen recognized by an immune effector cells of this invention. The polynucleotide that encodes the gene is then identified. In a further embodiment, the gene encoding the antigen that contains or comprises the peptide sequence motif is isolated and cloned. The method comprises:

(a) providing a first cell that expresses an antigen recognized by the immune effector cell of this invention and having an identified major

histocompatibility complex (MHC) restriction and one or more second cells having a compatible major histocompatibility complex (MHC) to the first cell but which does not express antigen;

(b) identifying polynucleotides encloding a peptide, a sequence motif in the antigen displayed by antigen presenting cells and recognized by the immune effector cell of this invention;

(c) identifying polynucleotides which are aberrantly expressed by the first cells as compared one or more to second cells; and

(d) comparing the polynucleotides identified in step (c) with the polynucleotides encoding the peptide sequence motifs identified in step (b) to identify the fragment of the gene encoding the antigen recognized by the immune effector cell of this invention. The method of step, (c) (above) may, in one embodiment, be performed prior to step (b). The first and second cells are animal cells that include, but are not limited to human, murine, rat or simian cells. They can be autologous or allogeneic as defined above.

This method identifies polynucleotides that have the potential to encode the peptide sequences or motifs that are antigenic or a fragment of the antigenic protein or polypeptide. Thus, the method further encompasses confirmation that the expression product encodes the antigen of interest by introducing into a cell the polynucleotide under conditions that it is expressed and presented by an APC by a compatible MHC. Methods for recognition by immune effector cells are provided below.

The "first cell" must satisfy two criteria: 1) it must express an antigen recognized by an immune effector cell; and 2) it must have an identified major histocompatibility complex restriction. The first and second cell populations are pre-selected to have compatible MHC restriction. Methods of identifying and comparing MHC are well known in the art and are described in Allen M. et al. (1994) *Human Imm.* **40**:25-32; Santamaria P. et al. (1993) *Human Imm.* **37**:39-50 and Hurley C.K. et al. (1997) *Tissue Antigens* **50**:401-415. Methods of determining whether the antigen is recognized by an immune effector cell are well known in the art, and include methods such as ^{3}H-thymidine incorporation;

metabolic activity detected by conversion of MTT to formazan blue; increased cytokine mRNA expression; increased cytokine protein production; and chromium release by target cells.

Any cell or population of cells that presents antigen recognized by immune effector cells is useful and within the scope of this invention. Such cells include, but are not limited to antigen presenting cells (defined above), cells having a purified MHC class I molecule complexed to a β_2-microglobulin, dendritic cells, intact antigen presenting cells or foster antigen presenting cells. Methods for isolating and culturing these cells are well known in the art.

Immune effector cells (defined above) recognize the APCs. Immune effector cells for the purpose of are prepared by the method of this invention. These methods may utilize CTLs, and cells isolated from a site of viral infection, a site of autoimmune infiltration, a site of transplantation rejection, a site of inflammation, a site of lymphocyte infilitration and a site of leukocyte infiltration. Suitable CTLs include, but are not limited to polyclonal T cells isolated from one individual, polyclonal T cells isolated from two or more individuals sharing the same MHC restriction, two or more CTLs or any combination thereof. The second cell that does not express antigen can be in one embodiment, a foster antigen presenting cell that lacks antigen processing activity and expresses MHC molecules free of bound peptides.

After preselection of the first and second cell(s), the polynucleotides that encode a peptide sequence motif in the antigen displayed by the antigen presenting cells (the first cell population) is then identified. In one embodiment, the peptide sequence motif is first identified, from which the polynucleotide is then derived. Any of the various methods that identify peptide sequence motifs in antigens recognized by immune effector cells are useful to perform this step of the invention. Briefly, such methods include, but are not limited to the "phage method" (Scott and Smith (1990) *Science* **249**:386-390; Cwirla et at. (1990) *PNAS* **87**:6378-6382; and Devlin et al. (1990) *Science* **249**:404-406), the Geysen method (Geysen et al. (1986) *Molecular Immunology* **23**:709-715; and Geysen et al. (1987) *J. Immunologic Method* **102**:259-274), the method of Fodor et al. (1991)

Science **251**:767-773), methods to test peptides that are agonists or antagonists as described in Furka et al. (1988) 14th International Congress of Biochemistry, Volume 5. Abstract FR:013; Furka, (1991) *Int. J. Peptide Protein Res.* **37**:487-493); Houghton (U.S. Patent No. 4,631,211 issued December 1986); and Rutter et al. (U.S. Patent No. 5,101,175, issued April 23, 1991), the method utilizing synthetic libraries (Needels et al. (1993) *PNAS* **90**:10700-4; Ohlmeyer et al. (1993) *PNAS* **90**:10922-10926; and Lam et al., International Patent Publication No. WO 92/00252), the method that utilizes indexed combinatorial peptide displays (Ohlmeyer et al. (1993) *PNAS* **90**:10922-26) and the pepscan technique by Van der Zee (1989) *Eur. J. Immunol.* **19**:43-47. In one embodiment, the method utilizes SPHERE (described in PCT WO 97/35035). Briefly, SPHRE is an empirical screening method for the identification of MHC Class I-restricted CTL epitopes that utilizes peptide libraries synthesized on a solid support (*e.g.*, plastic beads) where each bead contains approximately 200 picomoles of a unique peptide that can be released in a controlled manner. The synthetic peptide library is tailored to a particular HLA restriction by fixing anchor residues that confer high-affinity binding to a particular HLA allele (*e.g.*, HLA-A2) but contain a variable TCR epitope repertoire by randomizing the remaining positions. Roughly speaking, 50 96-well plates with 10,000 beads per well will accommodate a library with a complexity of approximately 5×10^7. In order to minimize both the number of CTL cells required per screen and the amount of manual manipulations, the eluted peptides can be further pooled to yeild wells with any desired complexity. Based on experiments with soluble libraries, it should be possible to screen 10^7 peptides in 96-well plates (10,000 peptides per well) with as few as 2×10^6 CTL cells. After cleaving a percentage of the peptides from the beads and incubating them with ^{51}Cr-labeled APCs (*e.g.*, foster antigen presenting cells or T2 cells) and the CTL line(s), peptide pools containing reactive species can be determined by measuring ^{51}Cr-release according to standard methods known in the art. Alternatively, cytokine production (*e.g.*, interferon-γ) or proliferation (e.g., incorporation of ^{3}H-thymidine) assays may be used. After identifying reactive 10,000-peptide mixtures, the beads corresponding

to those mixtures are separated into smaller pools and distributed to new 96-well plates (*e.g.*, 100 beads per well). An additional percentage of peptide is released from each pool and reassayed for activity by one of the methods listed above. Upon identification of reactive 100-peptide pools, the beads corresponding those peptide mixtures are redistributed at 1 bead per well of a new 96-well plate. Once again, an additional percentage of peptide is released and assayed for reactivity in order to isolate the single beads containing the reactive library peptides. The sequence of the peptides on individual beads can be determined by sequencing residual peptide bound to the beads by, for example, N-terminal Edman degradation or other analytical techniques known to those of skill in the art.

Degenerate polynucleotide sequences that encode the peptide motif or motifs are then determined.

As described above, an alternate embodiment further comprises identifying the gene that encodes an antigen that is specifically recognized by the immune effector cell population. Expression cloning of genes expressed in the antigen expressing cells is one means to identify the gene. In this approach (described in Kawakami Y. et al. (1994) *PNAS* **91(9)**:3515-3519) mRNA is isolated from the cells that bear a given antigen. The mRNA is converted into cDNA. The resulting cDNA fragments are inserted into plasmids or other appropriate expression vectors. The cDNA is amplified in eucaryotic (yeast, mammalian or insect cells) or procaryotic (e.g., bacteria) or another appropriate host cell. The DNA is then introduced or transfected into host cells such as COS cells (a permanent cell culture derived from African green monkey kidney cells) together with DNA encoding the appropriate HLA molecule. The tumor-specific immune effector cell clone is then added to the transfected host cells. If some of the host cells express the antigen (because they received the right cDNA), the CTL will be stimulated to produce an identifying cytokine such as IFN-γ or tumor necrosis factor (TNF), which can be detected in the culture medium. In order to screen all the mRNA molecules present in the sample cells such as tumor, approximately 10^5 DNA containing vectors have to be tested, in pools of 100 different molecules. The pool of DNA found to be positive for T-cell stimulation

can then be divided and the transfection procedure repeated until the preparation of a single species of DNA is found that can transfer the expression of the antigen.

The isolated polynucleotides and the genes corresponding to the isolated polynucleotides are also provided by this invention. As used herein, the term "polynucleotide" encompasses DNA, RNA and nucleic acid mimetics. In addition to the polynucleotides and their complements, this invention also provides the anti-sense polynucleotide stand, e.g. antisense RNA to these sequences or their complements. One can obtain an antisense RNA using the sequences provided by this invention and the methodology described in Vander Krol et al. (1988) *BioTechniques* **6**:958.

The polynucleotides can be conjugated to a detectable marker, e.g., an enzymatic label or a radioisotope for detection of nucleic acid and/or expression of the gene in a cell. A wide variety of appropriate detectable markers are known in the art, including fluorescent, radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of giving a detectable signal. One of skill in the art can employ a fluorescent label or an enzyme tag, such as urease, alkaline phosphatase or peroxidase, instead of radioactive or other environmental undesirable reagents. In the case of enzyme tags, colorimetric indicator substrates are known which can be employed to provide a means visible to the human eye or spectrophotometrically, to identify specific hybridization with complementary nucleic acid-containing samples. Briefly, this invention further provides a method for detecting a single-stranded or its complement, by contacting target single-stranded polynucleotides with a labeled, single-stranded polynucleotide (a probe) which is at least 4, and more preferably at least 5 or 6 and most preferably at least 10 contingent nucleotides of this invention under conditions permitting hybridization (preferably moderately stringent hybridization conditions) of complementary single-stranded polynucleotides, or more preferably, under highly stringent hybridization conditions. Hybridized polynucleotide pairs are separated from un-hybridized, single-stranded polynucleotides. The hybridized polynucleotide pairs are detected using methods well known to those of skill in the art and set forth, for example, in Sambrook et al. (1989) *supra*. The

polynucleotides can be provided in kits with appropriate reagents and instructions for their use as probes or primers.

The polynucleotides of this invention can be replicated using PCR. PCR technology is the subject matter of United States Patent Nos. 4,683,195, 4,800,159, 4,754,065, and 4,683,202 and described in PCR: THE POLYMERASE CHAIN REACTION (Mullis et al. eds, Birkhauser Press, Boston (1994)) and references cited therein.

Alternatively, one of skill in the art can use the sequences provided herein and a commercial DNA synthesizer to replicate the DNA. Accordingly, this invention also provides a process for obtaining the polynucleotides of this invention by providing the linear sequence of the polynucleotide, appropriate primer molecules, chemicals such as enzymes and instructions for their replication and chemically replicating or linking the nucleotides in the proper orientation to obtain the polynucleotides. In a separate embodiment, these polynucleotides are further isolated. Still further, one of skill in the art can insert the polynucleotide into a suitable replication vector and insert the vector into a suitable host cell (procaryotic or eucaryotic) for replication and amplification. The DNA so amplified can be isolated from the cell by methods well known to those of skill in the art. A process for obtaining polynucleotides by this method is further provided herein as well as the polynucleotides so obtained.

RNA can be obtained by first inserting a DNA polynucleotide into a suitable host cell. The DNA can be inserted by any appropriate method, e.g., by the use of an appropriate gene delivery vehicle (e.g., liposome, plasmid or vector) or by electroporation. When the cell replicates and the DNA is transcribed into RNA; the RNA can then be isolated using methods well known to those of skill in the art, for example, as set forth in Sambrook et al. (1989) *supra.* For instance, mRNA can be isolated using various lytic enzymes or chemical solutions according to the procedures set forth in Sambrook et al. (1989) *supra* or extracted by nucleic acid-binding resins following the accompanying instructions provided by manufactures.

The polynucleotides can be used as probes or primers. Host cells containing polynucleotides of this invention also are within the scope of this invention. It is known in the art that a "perfectly matched" probe is not needed for a specific hybridization. Minor changes in probe sequence achieved by substitution, deletion or insertion of a small number of bases do not affect the hybridization specificity. In general, as much as 20% base-pair mismatch (when optimally aligned) can be tolerated. Preferably, a probe useful for detecting the aforementioned mRNA is at least about 80% identical to the homologous region of comparable size contained in the polynucleotides of this invention. More preferably, the probe is 85% identical to the corresponding gene sequence after alignment of the homologous region; even more preferably, it exhibits 90% identity.

These probes can be used in radioassays (*e.g.* Southern and Northern blot analysis) to detect or monitor various cells or tissue containing these cells. The probes also can be attached to a solid support or an array such as a chip for use in high throughput screening assays for the detection of expression of the gene corresponding to one or more polynucleotide(s) of this invention. Accordingly, this invention also provides at least one probe as defined above of the transcripts or the complement of one of these sequences, attached to a solid support such as a chip for use in high throughput screens.

In a further embodiment, the polynucleotide or gene sequence can also be compared to a sequence database, for example, using a computer method to match a sample sequence with known sequences. Sequence identity can be determined by a sequence comparison using, i.e., sequence alignment programs that are known in the art, such as those described in CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F.M. Ausubel et al., eds., 1987) Supplement 30, section 7.7.18, Table 7.7.1. A preferred alignment program is ALIGN Plus (Scientific and Educational Software, Pennsylvania), preferably using default parameters, which are as follows: mismatch = 2; open gap = 0; and extend gap = 2. Another preferred program is the BLAST program for alignment of two nucleotide sequences, using default parameters as follows: open gap = 50; extension gap - 2 penalties; gap x

dropoff = 0; expect = 10; word size = 11. The BLAST program is available at the following Internet address: http://www.ncbi.nlm.nih.gov. As noted above, alternatively, hybridization under conditions of high, moderate and low stringency can also indicate degree of sequence identity.

The polynucleotides of the present invention also can serve as primers for the detection of genes or gene transcripts that are expressed in APC, for example, to confirm transduction of the polynucleotides into host cells. In this context, amplification means any method employing a primer-dependent polymerase capable of replicating a target sequence with reasonable fidelity. Amplification may be carried out by natural or recombinant DNA-polymerases such as T7 DNA polymerase, Klenow fragment of *E.coli* DNA polymerase, and reverse transcriptase. A preferred length of the primer is the same as that identified for probes, above.

The invention further provides the isolated polynucleotide operatively linked to a promoter of RNA transcription, as well as other regulatory sequences for replication and/or transient or stable expression of the DNA or RNA. As used herein, the term "operatively linked" means positioned in such a manner that the promoter will direct transcription of RNA off the DNA molecule. Examples of such promoters are SP6, T4 and T7. In certain embodiments, cell-specific promoters are used for cell-specific expression of the inserted polynucleotide. Vectors which contain a promoter or a promoter/enhancer, with termination codons and selectable marker sequences, as well as a cloning site into which an inserted piece of DNA can be operatively linked to that promoter are well known in the art and commercially available. For general methodology and cloning strategies, see GENE EXPRESSION TECHNOLOGY (Goeddel ed., Academic Press, Inc. (1991)) and references cited therein and VECTORS: ESSENTIAL DATA SERIES (Gacesa and Ramji, eds., John Wiley & Sons, N.Y. (1994)), which contains maps, functional properties, commercial suppliers and a reference to GenEMBL accession numbers for various suitable vectors. Preferable, these vectors are capable of transcribing RNA *in vitro* or *in vivo*.

Expression vectors containing these nucleic acids are useful to obtain host vector systems to produce proteins and polypeptides. It is implied that these expression vectors must be replicable in the host organisms either as episomes or as an integral part of the chromosomal DNA. Suitable expression vectors include plasmids, viral vectors, including adenoviruses, adeno-associated viruses, retroviruses, cosmids, etc. Adenoviral vectors are particularly useful for introducing genes into tissues *in vivo* because of their high levels of expression and efficient transformation of cells both *in vitro* and *in vivo*. When a nucleic acid is inserted into a suitable host cell, e.g., a procaryotic or a eucaryotic cell and the host cell replicates, the protein can be recombinantly produced. Suitable host cells will depend on the vector and can include mammalian cells, animal cells, human cells, simian cells, insect cells, yeast cells, and bacterial cells constructed using well known methods. See Sambrook et al. (1989) *supra*. In addition to the use of viral vector for insertion of exogenous nucleic acid into cells, the nucleic acid can be inserted into the host cell by methods well known in the art such as transformation for bacterial cells; transfection using calcium phosphate precipitation for mammalian cells; or DEAE-dextran; electroporation; or microinjection. See Sambrook et al. (1989) *supra* for this methodology. Thus, this invention also provides a host cell, e.g. a mammalian cell, an animal cell (rat or mouse), a human cell, or a procaryotic cell such as a bacterial cell, containing a polynucleotide encoding a protein or polypeptide or antibody.

When the vectors are used for gene therapy *in vivo* or *ex vivo*, a pharmaceutically acceptable vector is preferred, such as a replication-incompetent retroviral or adenoviral vector. Pharmaceutically acceptable vectors containing the nucleic acids of this invention can be further modified for transient or stable expression of the inserted polynucleotide. As used herein, the term "pharmaceutically acceptable vector" includes, but is not limited to, a vector or delivery vehicle having the ability to selectively target and introduce the nucleic acid into dividing cells. An example of such a vector is a "replication-incompetent" vector defined by its inability to produce viral proteins, precluding spread of the vector in the infected host cell. An example of a replication-

incompetent retroviral vector is LNL6 (Miller, A.D. et al. (1989) *BioTechniques* **7**:980-990). The methodology of using replication-incompetent retroviruses for retroviral-mediated gene transfer of gene markers is well established (Correll et al. (1989) *PNAS* **86**:8912; Bordignon (1989) *PNAS* **86**:8912-52; Culver K. (1991) *PNAS* **88**:3155; and Rill D.R. (1991) *Blood* **79(10)**:2694-700.

The polynucleotides, genes and encoded peptides and proteins can be further cloned and expressed *in vitro* or *in vivo*. The proteins and polypeptides produced and isolated from the host cell expression systems are also within the scope of this invention. Expression and cloning vectors as well as host cells containing these polynucleotides and genes are claimed herein as well as methods of administering them to a subject in an effective amount. Peptides corresponding to these sequences can be generated by recombinant technology and they may be administered to a subject as a vaccine or alternatively, introduced into APC which in turn, are administered in an effective amount to a subject. The genes may be used to produce proteins which in turn may be used to pulse APC. The APC may in turn be used to expand immune effector cells such as CTLs. The pulsed APC and expanded effector cells can be used for immunotherapy by administering an effective amount of the composition to a subject.

In another preferred embodiment, the methods of this invention are used to monitor expression of the genes which specifically hybridize to the probes of this invention in response to defined stimuli, such as a drug.

In one embodiment, the hybridized nucleic acids are detected by detecting one or more labels attached to the sample nucleic acids. The labels may be incorporated by any of a number of means well known to those of skill in the art. However, in one aspect, the label is simultaneously incorporated during the amplification step in the preparation of the sample nucleic acid. Thus, for example, polymerase chain reaction (PCR) with labeled primers or labeled nucleotides will provide a labeled amplification product. In a separate embodiment, transcription amplification, as described above, using a labeled nucleotide (e.g. fluorescein-labeled UTP and/or CTP) incorporates a label in to the transcribed nucleic acids.

Alternatively, a label may be added directly to the original nucleic acid sample (e.g., mRNA, polyA, mRNA, cDNA, etc.) or to the amplification product after the amplification is completed. Means of attaching labels to nucleic acids are well known to those of skill in the art and include, for example nick translation or end-labeling (e.g. with a labeled RNA) by kinasing of the nucleic acid and subsequent attachment (ligation) of a nucleic acid linker joining the sample nucleic acid to a label (e.g., a fluorophore).

The polynucleotide also can be modified prior to hybridization to a high density probe array in order to reduce sample complexity thereby decreasing background signal and improving sensitivity of the measurement using the methods disclosed in WO 97/10365. They also can be attached to a chip for use in diagnostic and analytical assays. Results from the chip assay are typically analyzed using a computer software program. See, for example, EP 0717 113 A2 and WO 95/20681. The hybridization data is read into the program, which calculates the expression level of the targeted gene(s). This figure is compared against existing data sets of gene expression levels for diseased and healthy individuals.

Also provided by this invention are antibodies that specifically react with the peptides and proteins of this invention. Such antibodies include, but are not limited to polyclonal antibodies, monoclonal antibodies, chimeric antibodies, humanized antibodies and antibody fragments. These can be combined with detectable labels and used to identify antigens and fragments thereof using well known methods. Alternatively, they can be combined with pharmaceutically acceptable carriers and administered therapeutically to a subject in need of such treatment. Kits containing the antibodies, reagents and instructions for use are further provided by this invention.

Thus, it should be understood, although not always explicitely stated, that the compositions of this invention can be combined with a pharmaceutically acceptable carrier prior to administration or combined with a carrier for *in vitro* use. These *in vitro* carriers, include, but are not limited, beads for use in cell separation methodologies.

These host cells containing the polynucleotides of this invention are useful for the recombinant replication of the polynucleotides and for the recombinant production of peptides. Alternatively, the cells may be used to induce an immune response in a subject in the methods described herein. When the host cells are antigen presenting cells, they can be used to expand a population of immune effector cells such as tumor infiltrating lymphocytes which in turn are useful in adoptive immunotherapies.

An effective amount of the cells is administered to a subject to provide adoptive immunotherapy. An effective amount of cytokine or costimulatory molecule also can be coadministered to the subject.

Further provided by this invention is a vaccine comprising antigen-specific immune effector cells according to the present invention. Still further provided by this invention is a vaccine comprising an antigen or a fragment thereof such as an epitope or sequence motif utilizing the antigen specific immune effector cells described herein. Methods of administering vaccines are known in the art and the vaccines may be combined with an acceptable pharmaceutical carrier. An effective amount of a cytokine and/or costimulatory molecule also can be administered.

The following examples are intended to illustrate, but not limit the invention as defined herein.

Materials and Methods

Preparation of Hybrid Cells

The hybrid cells used in the present invention may be formed by any suitable method known in the art. In one embodiment, a tumor biopsy sample is minced and a cell suspension created. Preferably, the cell suspension is separated into at least two fractions -- one enriched for immune effector cells, *e.g.*, T cells, and one enriched for tumor cells. Immune effector cells also can be isolated from bone marrow, blood or skin using methods well known in the art.

In general, it is desirable to isolate the initial inoculation population from neoplastic cells prior to culture. Separation of the various cell types from

neoplastic cells can be performed by any number of methods, including the use of cell sorters, magnetic beads, and packed columns. Other procedures for separation can include, but are not limited to, physical separation, magnetic separation, using antibody-coated magnetic beads, affinity chromatography, cytotoxic agents joined to a monoclonal antibody or used in conjunction with a monoclonal antibody, including, but not limited to, complement and cytotoxins, and "panning" with antibody attached to a solid matrix, *e.g.*, plate, elutriation or any other convenient technique.

The use of physical separation techniques include, but are not limited to, those based on differences in physical (density gradient centrifugation and counter-flow centrifugal elutriation), cell surface (lectin and antibody affinity), and vital staining properties (mitochondria-binding dye rho123 and DNA-binding dye Hoechst 33342). These procedures are well known to those of skill in this art.

Monoclonal antibodies are another useful reagent for identifying markers associated with particular cell lineages and/or stages of differentiation can be used. The antibodies can be attached to a solid support to allow for crude separation. The separation techniques employed should maximize the retention of viability of the fraction to be collected. Various techniques of different efficacy can be employed to obtain "relatively crude" separations. Such separations are up to 10%, usually not more than about 5%, preferably not more than about 1%, of the total cells present not having the marker can remain with the cell population to be retained. The particular technique employed will depend upon efficiency of separation, associated cytotoxicity, ease and speed of performance, and necessity for sophisticated equipment and/or technical skill.

Another method of separating cellular fractions is to employ culture conditions which allow for the preferential proliferation of the desired cell populations. For example, in one aspect, the fraction enriched for antigen-expressing cells is then fused to APCs, preferably dendritic cells. Fusion between the APCs and antigen-expressing cells can be carried out with any suitable method, for example using polyethylene glycol (PEG) or Sendia virus. In a

preferred embodiment, the hybrid cells are created using the procedure described by Gong et al. (1997) *Nat. Med.* **3(5)**:558-561.

DCs can be obtained from bone marrow cultures, peripheral blood, spleen, or other appropriate tissue of a mammal using protocols known in the art. Bone marrow contains DC progenitors, which, upon treatment with cytokines such as granulocyte-macrophage colony-stimulating factor ("GM-CSF") and interleukin 4 ("IL-4"), proliferate and differentiate into DCs. DCs so obtained are relatively immature (as compared to, for instance, spleen DCs). These immature DCs may be more amenable to fusion than the more mature DCs found in spleen.

Peripheral blood also contains relatively immature DCs or DC progenitors, which can propagate and differentiate in the presence of appropriate cytokines such as GM-CSF and which can also be used in fusion.

Precomitted DCs are isolated, for example using metrizamide gradients; nonadherence/adherence techniques (Freduenthal, PS et al. (1990) PNAS **87**:7698-7702); percoll gradient separations (Mehta-Damani et al (1994) *J. Immunol.* **153**:996-1003) and fluorescence-activated cell sorting techniques (Thomas et al. (1993) *J. Immunol.* **151**:6840-6852). In one embodiment, the DCs are isolated essentially as described in WO 96/23060 by FACS techniques. Although there is no specific cell surface marker for human DCs, a cocktail of markers (*e.g.* HLA-DR, B7.2, CD13/33, etc) are known to be present on DCs. In addition, DCs are known to lack CD3, CD20, CD56 and CD14 antigens. Therefore, combining negative and positive FACS techniques provides a method of isolating DCs.

In one embodiment, the APCs and cells expressing one or more antigens are autologous, *i.e.* derived from the same subject from which that tumor biopsy was obtained. In another embodiment, the APCs and cells expressing the antigen are allogeneic, *i.e.,* derived from a different subject since dendritic cells are known to promote the generation of primary immune responses.

Preferably, the ratio of APCs:antigen-expressing cells is between about 1:100 and about 1000:1. Typically, unfused cells will die off after a few days in culture, therefore, the fused cells can be separated from the parent cells simply by

allowing the culture to grow for several days. In this embodiment, the hybrid cells both survive more and, additionally, are only lightly adherent to tissue culture surfaces. The parent cells are strongly adherent to the containers. Therefore, after about 5 to 10 days in culture, the hybrid cells can be gently dislodged and transferred to new containers, while the unfused cells remained attached.

Alternatively, it has been shown that fused cells lack functional hypoxanthine-guanine phosphoribosyl transferase ("HGPRT") enzyme and are, therefore, resistant to treatment with the compound HAT. Accordingly, to select these cells HAT can be added to the culture media. However, unlike conventional HAT selection, hybrid cell cultures should not be exposed to the compound for more than 12 days.

Hybrid cells typically retain the phenotypic characteristics of the APCs. Thus, hybrids made with dendritic cells will express the same MHC class II proteins and other cell surface markers. Moreover, the hybrids will express those antigens expressed on the cells from which they were formed.

Expansion of Antigen-Specific Cells

In one aspect, the present invention makes use of these hybrid cells to stimulate production of an enriched population of antigen-specific immune effector cells. The antigen-specific immune effector cells are expanded at the expense of the hybrid cells, which die in the culture. The process by which naïve immune effector cells become educated by other cells is described essentially in Coulie (1997) *Molec. Med. Today* 261-268.

The hybrid cells prepared as described above are mixed with naïve antigen-specific cells. Preferably, the antigen-specific cells are immune effector cells that specifically recognize tumor cells and have been enriched from the tumor biopsy sample as described above. Optionally, the cells may be cultured in the presence of a cytokine, for example IL2. Because DCs secrete potent immunostimulatory cytokines, such as IL12, it may not be necessary to add supplemental cytokines during the first and successive rounds of expansion. In

any event, the culture conditions are such that the antigen-specific immune effector cells expand (*i.e.* proliferate) at a much higher rate than the hybrid cells. Multiple infusions of hybrid cells and optional cytokines can be performed to further expand the population of antigen-specific cells.

Using the hybrid cells as described, a potent antigen-specific population of immune effector cells can be obtained. In one embodiment, the cells are T cells and are specific for tumor-specific antigens.

Assaying Antigen-Specificity

In a preferred embodiment, the antigen-specific immune effector cells are CTLs. In other words, they actively lyse the cells expressing the specific antigen. Cytolytic activity of the cells can be measured in various ways, including, but not limited to, tritiated thymidine incorporation (indicative of DNA synthesis), and examination of the population for growth or proliferation, *e.g.*, by identification of colonies. (See, *e.g.,* WO 94/21287). In another embodiment, the tetrazolium salt MTT (3-(4,5-dimethyl-thazol-2-yl)-2,5-diphenyl tetrazolium bromide) may be added (Mossman (1983) *J. Immunol. Methods* **65**:55-63 and Niks and Otto (1990) *J. Immunol. Methods* **130**:140-151). Succinate dehydrogenase, found in mitochondria of viable cells, converts the MTT to formazan blue. Thus, concentrated blue color would indicate metabolically active cells. Similarly, protein synthesis may be shown by incorporation of ^{35}S-methionine. In still another embodiment, cytotoxicity and cell killing assays, such as the classical chromium release assay, may be employed to evaluate epitope-specific CTL activation. Other suitable assays will be known to those of skill in the art.

As pointed out above, cytokine production or cytolytic ^{51}Cr-release assays can be used (Coutic et al. (1992*) Int. J. Cancer* **50**:289-291) to identify antigens. Alternatively, antigens can be identified using the method described in PCT WO 97/35035. The following experimental details provide a detailed description of this method.

Strategy 1. The supernatant from each well is distributed to replica plates and 1-2x10^3 irradiated (1500 rads) foster APCs (expressing the proper MHC

allele) are added to each well. Next, the cloned CTLs are added to a total of 10^3-10^4 cells representing equal amounts of 10-20 different clones of the same MHC restriction such that the total final volume per well is 200 µl and the plates are incubated in a humidified CO_2 incubator for 4 days at 37°C. Each well is then pulsed with 18.5 kBq of [^{3}H] dThd to measure CTL proliferation. 16 hours later, the radioactivity incorporated into the DNA of mitotically active CTLs is assayed by scintillation counting (Estaquier et at. (1994) *Eur. J. Immunol.* **24**:2789-2795). The magnitude of the proliferative response may serve as a preliminary screen for crossreacting epitopes. The greater the response the more likely it is that more than one CTL clone was stimulated. While all reactive peptides are of interest, the most efficacious vaccine candidates will be those that crossreact with CTLs derived from independent donors and which are restricted by the most common MHC alleles. Note that identification of epitopes containing the HLA B7-like supermotif would be of great value as vaccine candidates since it will bind to many HLA B alleles which are represented in over 40% of individuals from all major ethnic groups (Sidney et at. (1995) *J. Immunol.* **154**:247-259).

Strategy 2. Alternatively, the first step is to administer ^{51}Cr-labeled T2 cells to the wells of the 2° daughter plates, followed by the addition of the CTLs. After 4 hours the released ^{51}Cr is measured in the standard manner. When a positive well is identified, the 10 wells from the 1° daughter plate that correspond to that well are similarly assayed. At this point, the epitope search is narrowed down to the beads in a single well on one of the master plates.

Wells that register positive will be further analyzed as follows: the beads that correspond to the positive well are manually distributed (1 per well) to new plates and the remaining peptide is released from each. These plates are assayed as before, and in this way the reactive bead(s) are unambiguously isolated. The positive bead(s) can be rapidly and efficiently decoded since the molecular tags that encode the bead's synthesis history has remained on the bead (coupled with a non-photocleavable crosslinker). For example, analysis of the bead(s) by electron capture capillary gas chromatography immediately reveals the peptide sequence that was synthesized on that bead (Ohlmeyer et al., 1993, *supra*). Thus the

unambiguous identification of an epitope can be achieved in approximately ten days using the ^{3}H-thymidine incorporation assay and in as few as two days if a ^{51}Cr-release assay is used.

In another embodiment, application of the library beads to the surface of freshly poured top agar in a standard tissue culture plate, followed by release of a portion of the peptide, will result in a three dimensional concentration gradient of eluted peptide around each bead. Antigen presenting cells could be present in the top agar or applied to the surface after peptide release. Next, the CTL(s) of interest are plated over the top agar/peptide/APCs, followed by incubation at 37°C for 4-12 hours. Reactive beads may be detected by the formation of plaques, where the size of the plaque indicates the magnitude of the response. Positive beads can then be taken from the plate, washed, and sequenced. This assay requires very little manual manipulation of the beads and the entire library can be screened simultaneously (in one step) in as little as four hours. Furthermore, the beads can be recovered, washed in 6M guanidiium, and reused.

In another embodiment, the described method for the identification of $CD8^{+}$ MHC Class I restricted CTL epitopes can be applied to the identification of $CD4^{+}$ MHC Class II restricted helper T-cell (Th) eptitopes. In this case, MHC Class II allele-specific libraries are synthesized such that haplotype-specific anchor residues are represented at the appropriate positions. MHC Class II agretopic motifs have been identified for the common alleles (Rammensee (1995) *Curr. Opin. Immunol.* 7:85-96; Altuvia et al. (1994) *Mol. Immunol.* **24**:375-379, Reay et al. (1994) *J. Immunol.* **152**:3946-3957; Verreck et al. (1994) *Eur. J. Immunol.* **24**:375-379; Sinigaglia and Hammer (1994) *Curr. Opin. Immunol.* **6**:52-56; Rotzschke and Falk (1994) *Curr. Opin. Immunol.* **6**:45-51). The overall length of the peptides will be 12-20 amino acid residues, and previously described methods may be employed to limit library complexity. The screening process is identical to that described for MHC Class I-associated epitopes except that B lymphoblastoid cell lines (B-LCL) are used for antigen presentation rather than T2 cells. In a preferred aspect, previously characterized B-LCLs that are defective in antigen processing (Mellins et al. (1991) *J. Exp. Med.* **174**:1607-

1615); thus allowing specific presentation of exogenously added antigen, are employed. The libraries are screened for reactivity with isolated $CD4^+$ MHC Class II allele-specific Th cells. Reactivity may be measured by ^3H-thymidine incorporation according to the method of Mellins et al. *supra.*, or by any of the methods previously described for MHC Class I-associated epitope screening.

The above methods utilize foster antigen presenting cells. The human cell line 174xCEM.T2, referred to as T2, contains a mutation in its antigen processing pathway that restricts the association of endogenous peptides with cell surface MHC class I molecules (Zweerink et al. (1993) *J. Immunol.* **150**:1763-1771). This is due to a large homozygous deletion in the MHC class II region encompassing the genes TAP1, TAP2, LMP1, and LMP2 which are required for antigen presentation to MHC class I-restricted $CD8^+$ CTLs. In effect, only "empty" MHC class I molecules are presented on the surface of these cells. Exogenous peptide added to the culture medium binds to these MHC molecules provided that the peptide contains the allele-specific binding motif. These T2 cells are referred to as "foster" APCs.

Genetic Modifications

The methods of this invention are intended to encompass any method of gene transfer into either the hybrid cells or the antigen-specific population of cells derived using the hybrid cells as stimulators. Examples of genetic modifications includes, but are not limited to viral mediated gene transfer, liposome mediated transfer, transformation, transfection and transduction, *e.g.*, viral mediated gene transfer such as the use of vectors based on DNA viruses such as adenovirus, adeno-associated virus and herpes virus, as well as retroviral based vectors. The methods are particularly suited for the integration of a nucleic acid contained in a vector or construct lacking a nuclear localizing element or sequence such that the nucleic acid remains in the cytoplasm. In these instances, the nucleic acid or therapeutic gene is able to enter the nucleus during M (mitosis) phase when the nuclear membrane breaks down and the nucleic acid or therapeutic gene gains access to the host cell chromosome. In one embodiment, genetic modification is

performed *ex vivo* and the modified (*i.e.* transduced) cells are subsequently administered to the recipient. Thus, the invention encompasses treatment of diseases amenable to gene transfer into antigen-specific cells, by administering the gene *ex vivo* or *in vivo* by the methods disclosed herein.

The expanded population of antigen-specific cells can be genetically modified. In addition, the hybrid cells can also be genetically modified, for example, to supply particular secreted products including, but not limited to, hormones, enzymes, interferons, growth factors, or the like. By employing an appropriate regulatory initiation region, inducible production of the deficient protein can be achieved, so that production of the protein will parallel natural production, even though production will be in a different cell type from the cell type that normally produces such protein. It is also possible to insert a ribozyme, antisense or other message to inhibit particular gene products or susceptibility to diseases, particularly hematolymphotropic diseases.

Suitable expression and transfer vectors have been described above.

Therapeutic genes that encode dominant inhibitory oligonucleotides and peptides as well as genes that encode regulatory proteins and oligonucleotides also are encompassed by this invention. Generally, gene therapy will involve the transfer of a single therapeutic gene although more than one gene may be necessary for the treatment of particular diseases. In one embodiment, the therapeutic gene is a dominant inhibiting mutant of the wild-type immunosuppressive agent. Alternatively, the therapeutic gene could be a wild-type, copy of a defective gene or a functional homolog.

More than one gene can be administered per vector or alternatively, more than one gene can be delivered using several compatible vectors. Depending on the genetic defect, the therapeutic gene can include the regulatory and untranslated sequences. For gene therapy in human patients, the therapeutic gene will generally be of human origin although genes from other closely related species that exhibit high homology and biologically identical or equivalent function in humans may be used, if the gene product does not induce an adverse

immune reaction in the recipient. The therapeutic gene suitable for use in treatment will vary with the disease.

A marker gene can be included in the vector for the purpose of monitoring successful transduction and for selection of cells into which the DNA has been integrated, as against cells which have not integrated the DNA construct. Various marker genes include, but are not limited to, antibiotic resistance markers, such as resistance to G418 or hygromycin. Less conveniently, negative selection may be used, including, but not limited to, where the marker is the HSV-tk gene, which will make the cells sensitive to agents such as acyclovir and gancyclovir. Alternatively, selections could be accomplished by employment of a stable cell surface marker to select for transgene expressing cells by FACS sorting. The NeoR (neomycin /G418 resistance) gene is commonly used but any convenient marker gene whose sequences are not already present in the recipient cell, can be used.

The viral vector can be modified to incorporate chimeric envelope proteins or nonviral membrane proteins into retroviral particles to improve particle stability and expand the host range or to permit cell type-specific targeting during infection. The production of retroviral vectors that have altered host range is taught, for example, in WO 92/14829 and WO 93/14188. Retroviral vectors that can target specific cell types *in vivo* are also taught, for example, in Kasahara et al. (1994) *Science* **266**:1373-1376. Kasahara et al. describe the construction of a Moloney leukemia virus (MoMLV) having a chimeric envelope protein consisting of human erythropoietin (EPO) fused with the viral envelope protein. This hybrid virus shows tissue tropism for human red blood progenitor cells that bear the receptor for EPO, and is therefore useful in gene therapy of sickle cell anemia and thalassemia. Retroviral vectors capable of specifically targeting infection of cells are preferred for in vivo gene therapy.

Expression of the transferred gene can be controlled in a variety of ways depending on the purpose of gene transfer and the desired effect. Thus, the introduced gene may be put under the control of a promoter that will cause the

gene to be expressed constitutively, only under specific physiologic conditions, or in particular cell types.

Examples of promoters that may be used to cause expression of the introduced sequence in specific cell types include Granzyme A for expression in T-cells and NK cells, the CD34 promoter for expression in stem and progenitor cells, the CD8 promoter for expression in cytotoxic T-cells, and the CD11b promoter for expression in myeloid cells.

Inducible promoters may be used for gene expression under certain physiologic conditions. For example, an electrophile response element may be used to induce expression of a chemoresistance gene in response to electrophilic molecules. The therapeutic benefit may be further increased by targeting the gene product to the appropriate cellular location, for example the nucleus, by attaching the appropriate localizing sequences.

After viral transduction, the presence of the viral vector in the transduced cells or their progeny can be verified such as by PCR. PCR can be performed to detect the marker gene or other virally transduced sequences. Generally, periodic blood samples are taken and PCR conveniently performed using e.g. NeoR probes if the NeoR gene is used as marker. The presence of virally transduced sequences in bone marrow cells or mature hematopoietic cells is evidence of successful reconstitution by the transduced cells. PCR techniques and reagents are well known in the art, See, generally, PCR PROTOCOLS, A GUIDE TO METHODS AND APPLICATIONS. Innis, Gelfand, Sninsky & White, eds. (Academic Press, Inc., San Diego, 1990) and commercially available (Perkin-Elmer).

Adoptive Immunotherapy and Vaccines

The expanded populations of antigen-specific immune effector cells of the present invention also find use in adoptive immunotherapy regimes and as vaccines.

Adoptive immunotherapies involve, in one aspect, administering to a subject an effective amount of a substantially pure population of educated, antigen-specific immune effector cells made by culturing naïve immune effector

cells with hybrid cells, wherein the hybrid cells are antigen presenting cells (APCs) fused to cells that express one or more antigens and wherein the educated, antigen-specific immune effector cells are expanded at the expense of the hybrid cells. Preferably, the APCs are DCs.

The cells can be autologous or allogeneic. In one embodiment, the adoptive immunotherapy methods described herein are autologous. In this case, the hybrid cells are made using parental cells isolated from a single subject. The expanded population also employs T cells isolated from that subject. Finally, the expanded population of antigen-specific cells is administered to the same patient.

In another embodiment, the adoptive immunotherapy methods are allogeneic. Here, cells from two or more patients are used to generate the hybrid cells, and stimulate production of the antigen-specific cells. For instance, cells from other healthy or diseased subjects can be used to generate antigen-specific cells in instances where it is not possible to obtain autologous T cells and/or dendritic cells from the subject providing the biopsy. The expanded population can be administered to any one of the subjects from whom cells were isolated, or to another subject entirely.

The populations and methods described herein can also be used to develop cell based vaccines.

Additional Utilities

The populations described herein can also be used to identify novel antigens and the genes encoding these antigens using a variety of methods, such as that described in PCT WO 97/35035. In another embodiment, a SAGE analysis (described in U.S. Patent No. 5,695,937) can be employed to identify the antigens recognized by the expanded populations. SAGE analysis involves identifying nucleotide sequences aberrantly or differentially expressed in the antigen-expressing cells. Briefly, SAGE analysis begins with providing complementary deoxyribonucleic acid (cDNA) from (1) the antigen-expressing population and (2) cells not expressing that antigen. Both cDNAs can be linked to primer sites. Sequence tags are then created, for example, using the appropriate

primers to amplify the DNA. By measuring the differences in these tags between the two cell types, sequences which are aberrantly expressed in the antigen-expressing cell population can be identified.

Alternatively, mass-spectrophotometric analysis of the peptides eluted from the tumor cell:MHC complexes can be used. Other techniques of identifying antigens will be known to those of skill in the art.

EXPERIMENTAL

The rationale for immunotherapy is predicated on the observation that non-professional APCs (*e.g.*, tumor cells, virus-infected cells, *etc.*) toward which active specific immune responses are sought, can serve as lytic targets for educated immune effector cells even though they are inefficient at educating immune effector cells *in vivo* and *in vitro*. The molecular basis of this inefficiency is due, at least in part, to the lack of poorly defined costimulatory signals required for T cell priming such as those found in professional APCs (*e.g.*, dendritic cells). Gong *et al.* (*PNAS* (1998) **95**: 6279 and *Nat. Med.* (1997) **3**(5):558), have demonstrated that fusion of murine DCs to syngeneic carcinoma cells results in a hybrid cell that substantially retains the immune effector cell priming capacity of the DCs while endogenously expressing and presenting a spectrum of carcinoma-associated tumor antigens. Given the high degree of morphologic, phenotypic and functional homology that exists between murine and human DCs, the present invention extends the utility of DC/tumor fusions to human DCs fused to human tumor cells for the purpose of educating effector T cells directed against tumor antigens *in vitro*. The experimental method of Gong *et al.*, as it pertains to the murine system, is presented below. There are no significant changes to the fusion protocol that are anticipated in order to adapt the process to human DC fusions.

Materials and Methods

Cell Culture. Murine (C57BL/6) MC-38 and MB49 cells were maintained in DMEM supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-

glutamine, 100 units/ml penicillin, and 100 ug/ml streptomycin. MC-38 and MB49 cells were stably transfected with a MUC1 cDNA to produce the cell lines MC-38/MUC1 and MB49/MUC1.

DC/tumor cell fusions. Murine bone marrow-derived dendritic cells were prepared as described in Gong *et al.* (*Nat. Med.* (1997), **3**(5):558-561). Fusion of the nonadherent DCs with tumor lines was carried out with 50% PEG in Dulbecco's PBS without Ca^{2+} or Mg^{2+} at pH 7.4. The fused cells were plated in 24 well plates in the presence of HAT medium (Sigma) for 10-14 days. The HAT provides a selective growth advantage for the fused cells. Additionally, unfused cells remain firmly attached to the plates whereas the fused cells can be dislodged by gentle pipetting. Fusions of DCs and MC-38 cells are denoted FC/MC-38 and fusions of DCs and MC-38/MUC1 cells are denoted FC/MUC1.

Immunizations. MUC1.Tg mice (transgenic for MUC1, Rowse *et al.*, (1988) *Cancer Res.* **58**:315) were injected subcutaneously on day 0 and day 7 with 1×10^6 MC-38/MUC1 cells exposed to 100 Gy ionizing radiation. FC/MUC1 fusion cells (5×10^5) were administered subcutaneously on day 0 and day 7 for tumor prevention studies.

Generation of $CD8^+$ T cell Lines and Cytotoxicity Assays. Lymph node cells were suspended in complete RPMI 1640 medium containing 5 units/ml MUC1 antigen. Murine IL-2 (10 units/ml) was added after 5 days of culture. On days 10 and 15 the cells were restimulated with 5 units/ml MUC1 antigen and 1:5 irradiated (30 Gy) syngeneic spleen cells as antigen presenting cells. T cell cultures were analyzed by a standard ^{51}Cr-release assay after Ficoll centrifugation to remove dead cells and passage through nylon wool to deplete residual APCs.

Results

FACS analysis of surface marker expression comparing DCs, MC-38/MUC1 tumor cells, and the MC-38/MUC1-DC fusion cells (FC/MUC1) is shown in Figure 1. It is readily apparent that the fusion cells are equipped with all of the DC markers including MHC I, MHC II, B7-1, B7-2, and ICAM-1 whereas, with the exception of MHC I, none of the markers are upregulated in the parental

MC-38/MUC1 cells. This is consistent with the DC-like "veiled" morphology of the fusion cells. In addition, the fusion cells also express the tumor antigen MUC1 at the same high level as the parental tumor cells whereas MUC1 expression is not detected in the parental DCs. Thus, the gene expression pattern observed in fusion cells is a composite of the expression patterns observed in the individual parental cell populations and importantly, the expression levels of the DC markers believed to confer potent APC functionality are maintained.

It was also demonstrated that vaccination of MUC1 transgenic mice (MUC1.Tg) with the fusion cells (FC/MUC1 and FC/MC-38) conferred potent and specific protection against tumor rechallenge whereas mice vaccinated with irradiated MC-38/MUC1 cells developed tumors upon rechallenge (Table 1). This is a remarkable demonstration of the immune stimulating potency of the fusion cells since these animals were tolerized from birth with the MUC1 antigen. This reversal of tolerance and concommitant tumor protection was shown to be specific since the fusion cells provided no protection against MB49 cells.

Table 1 Potency and specificity of antitumor immunity induced with fusion cells

Immunogen	Tumor challenge	Animals with tumor
Irradiated MC38/MUC1 (1×10^6)	MC38/MUC1 (1×10^6)	2/3
	MC38/MUC1 (2×10^6)	3/3
FC/MUC1 (2.5×10^5)	MC38/MUC1 (1×10^6)	0/10
	MC38/MUC1 (2×10^6)	0/10
	MB49 (5×10^5)	6/6
FC/MC38 (2.5×10^5)	MC38 (1×10^6)	0/6
	MB49 (5×10^5)	6/6

The numbers in parentheses represent cells used for immunization or tumor challenge.

From Gong J. et al. (1997) J. N*at. Med.* **3**(5):558-561, 559

Furthermore, $CD8^+$ lymph node cells from FC/MUC1 vaccinated mice were capable of lysing MC-38 cells, MC-38/MUC1 cells and FC/MUC1 cells, but not the MUC1-negative syngeneic tumor line MB49 (Figure 2). Lymph node cells from naive mice were unable to lyse MC-38, MC-38/MUC1, or MB49 cells. Taken together, these data imply that the tumor protection afforded by the fusion cells is mediated by the education of immune effector cells and that these effector cells can lyse the parental tumor cells. It is of interest to note that vaccination with the parental tumor cells does not result in a potent $CD8^+$ anti-tumor response, but when the immune response is provoked with the fusion cells, the MC-38 cells are efficient targets and are rejected.

These studies demonstrate the feasibility of the present invention. That is, DC fusions can educate immune effector cells by presenting the antigens expressed by the tumor cells in the context of a professional APC environment. It is inferred from this data that: (1) the general methods of fusing murine DCs to murine tumor cells will apply to the fusion of human DCs to human tumor cells, and (2) human DC fusion cells will be potent agents at eliciting anti-tumor immune effector cells *in vitro*, the products of which can be used directly as therapeutics (*e.g.*, adoptive T cell transfer) or to further characterize the nature of the tumor rejection antigens.

The preceding discussion and examples are intended merely to illustrate the art. As it is apparent to one of skill in the art, various modifications can be made to the above without departing from the spirit and scope of this invention.

CLAIMS

What is claimed is:

1. A substantially pure population of educated, antigen-specific immune effector cells expanded in culture at the expense of hybrid cells, wherein the hybrid cells comprise antigen presenting cells (APCs) fused to cells that express one or more antigens.

2. The population according to claim 1, wherein the antigen presenting cells are dendritic cells (DCs).

3. The population according to claim 1, wherein the cells expressing the antigen(s) are tumor-specific.

4. The population according to claim 1, wherein the antigen-specific immune effector cells are cytotoxic T lymphocytes (CTLs).

5. The population according to claim 1, wherein the antigen-specific immune effector cells are genetically modified cells.

6. The population according to claim 1, wherein the hybrid cells are genetically modified cells.

7. The population according to claim 5, wherein the genetic modification comprises introduction of a polynucleotide.

8. The population according to claim 7, wherein the polynucleotide encodes a peptide, a ribozyme or an antisense sequence.

9. The population according to claim 1, wherein the APCs and the cells that express one or more antigens are autologous.

10. The population according to claim 1, wherein the APCs and the cells that express one or more antigens are allogeneic.

11. A substantially pure population of educated, antigen-specific immune effector cells produced by culturing immune effector cells with hybrid cells, wherein the hybrid cells are antigen presenting cells (APCs) fused to cells that express one or more antigens and wherein the educated, antigen-specific immune effector cells are expanded at the expense of the hybrid cells.

12. The population according to claim 11, wherein the antigen presenting cells are dendritic cells (DCs).

13. The population according to claim 11, wherein the cells recognizing the antigen(s) are tumor-specific.

14. The population according to claim 11, wherein the antigen-specific immune effector cells are cytotoxic T lymphocytes (CTLs).

15. The population according to claim 11, wherein the antigen-specific immune effector cells are genetically modified cells.

16. The population according to claim 11, wherein the hybrid cells are genetically modified cells.

17. The population according to claim 15, wherein the genetic modification comprises introduction of a polynucleotide.

18. The population according to claim 17, wherein the polynucleotide encodes a peptide, a ribozyme or an antisense sequence.

19. The population according to claim 11, wherein the APCs and the to cells that express one or more antigens are autologous.

20. The population according to claim 11, wherein the APCs and the cells that express one or more antigens are allogeneic.

21. The population according to claim 11, wherein the immune effector cells are naïve.

22. The population according to claim 11, wherein the immune effector cells are educated.

23. The population according to claim 11, wherein the immune effector cells are produced by culturing immune effector cells with hybrid cells in the presence of a cytokine.

24. The population of claim 23, wherein the cytokine is IL-2.

25. A method for producing antigen-specific immune effector cells comprising culturing immune effector cells in an effective amount of hybrid cells, wherein the hybrid cells comprise antigen presenting cells (APCs) fused to cells expressing one or more antigens and wherein the antigen-specific immune effector cells are produced at the expense of the hybrid cells.

26. The method according to claim 25, wherein the APCs are dendritic cells (DCs).

27. The method according to claim 25, wherein the DCs are derived from blood, bone marrow or skin and the immune effector cells are derived from tumor tissue.

28. The method according to claim 25, wherein the cells expressing the antigen(s) and the immune effector cells have been enriched from a tumor.

29. The method according to claim 25, wherein the APCs and the cells that express one or more antigens are autologous.

30. The method according to claim 25, wherein the APCs and the cells that express one or more antigens are allogeneic.

31. The method according to claim 25, wherein the immune effector cells are naïve.

32. The method according to claim 25, wherein the immune effector cells are eductated.

33. The method according to claim 25, further comprising culturing the immune effector cells in the presence of an effective amount of cytokine.

34. The method according to claim 33, wherein the cytokine is IL2.

35. A method of adoptive immunotherapy, comprising administering to a subject a substantially pure population of educated, antigen-specific immune effector cells expanded in culture at the expense of hybrid cells, wherein the hybrid cells comprise antigen presenting cells (APCs) fused to cells that express one or more antigens.

36. A method of adoptive immunotherapy comprising administering to a subject a substantially pure population of educated, antigen-specific immune effector cells made by culturing naïve immune effector cells with hybrid cells, wherein the hybrid cells are antigen presenting cells (APCs) fused to cells that express one or more antigens and wherein the educated, antigen-specific immune effector cells are expanded at the expense of the hybrid cells.

37. The method according to claim 35 or 36, wherein the APCs are dendritic cells (DCs).

38. The method according to claim 35 or 36, wherein the DCs are derived from blood, bone marrow or skin and the immune effector cells are derived from a tumor.

39. The method according to claim 35 or 36, wherein the cells that express one or more antigens and the immune effector cells have been enriched from a tumor.

40. The method according to claim 35 or 36, wherein the immune effector cells are cytotoxic T cells (CTLs).

41. The method according to claim 35 or 36, wherein the antigen-specific immune effector cells administered to the subject are allogeneic.

42. The method according to claim 35 or 36, wherein the antigen-specific immune effector cells administered to the subject are autologous.

43. The method according to claim 35 or 36, further comprising culturing the immune effector cells in the presence of an effective amount of a cytokine.

44. The method according to claim 43, wherein the cytokine is IL2.

45. A method of identifying a fragment of a gene encoding an antigen recognized by the population of antigen-specific immune effector cells according to claim 1 or 11, the method comprising:

(a) providing a population of first cells of claim 1 or 11, wherein the cells have an identified major histocompatibility complex (MHC) restriction and one or more second cells having a compatible major histocompatibility complex (MHC) to the first cell but which does not express antigen;

(b) identifying polynucleotides encoding a peptide sequence motif in the antigen displayed by the population of first cells of claim 1 or 11;

(c) identifying polynucleotides which are aberrantly expressed by the first cells as compared to one or more second cells; and

(d) comparing the polynuceotides identified in step (c) with the polynucleotides motifs identified in step (b) to identify the fragment of the gene encoding the antigen recognized by the immune effector cell.

46. The method of claim 45, wherein step (c) is performed prior to step (b).

47. A method of identifying a polypeptide encoding a sequence motif present in an antigen recognized by the population of antigen-specific immune effector cells according to claim 1 or 11, comprising:

(a) providing a cell population of antigen-specific immune effector cells of claim 1 or 13 and having an identified major histocompatibility complex (MHC) restriction; and

(b) identifying a polypeptide encoding a sequence motif in the antigen recognized by the immune effector cells.

48. A vaccine comprising the population of antigen-specific immune effector cells of claim 1 or 11 and a pharmaceutically acceptablel carrier.

49. A vaccine comprising antigen-specific immune effector cells according to the method of claim 11.

50. A vaccine comprising an antigen identified according to the method of claim 45.

51. A vaccine comprising an antigen identified according to the method of claim 47.

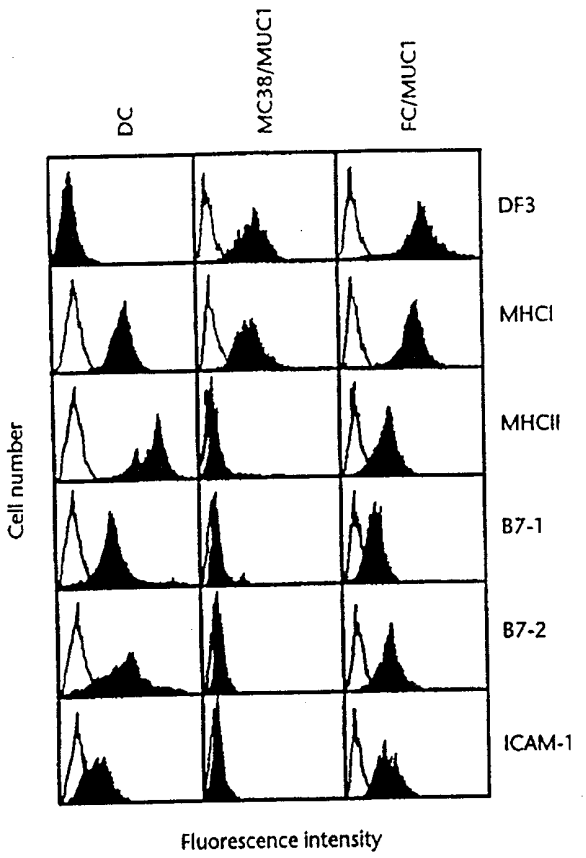
DC
MC38/MUC1
FC/MUC1
DF3
MHCI
MHCII
B7-1
B7-2
ICAM-1
Cell number
Fluorescence intensity

Figure 1

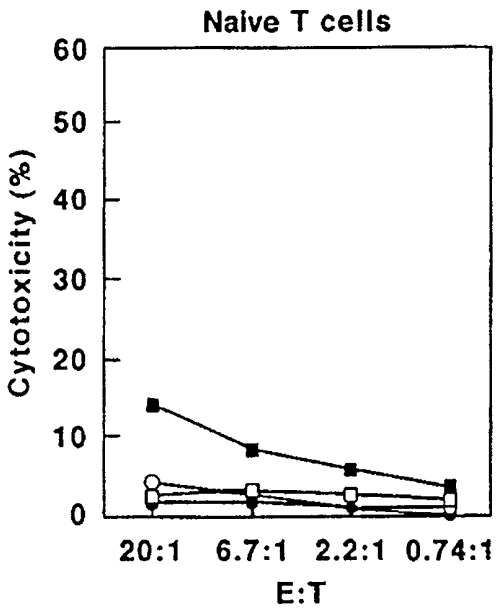

Figure 2A

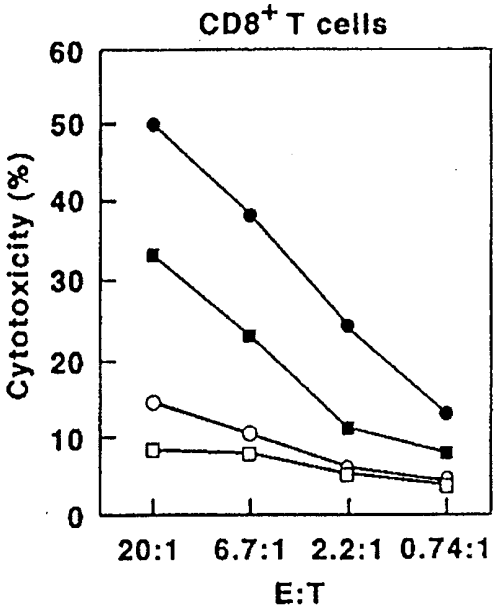

Figure 2B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/01464

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 35/00; C12N 15/85, 35/86; C12Q 1/68; G01N 33/53
US CL :435/6, 7.1, 373, 325; 424/93.1

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.1, 373, 325; 424/93.1

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GONG et al. Induction of antitumor activity by immunization with fusions of dendritic and carcinoma cells. Nature Medicine. May 1997, Vol. 3, No. 5, pages 558-561, see entire article.	1-51
X	CELLUZZI et al. Immunization with dendritic cells fused with tumor cells elicits potent tumor specific immunity. J. Invest. Dermatology. 23 April 1997, Vol. 108, No. 4, page 564, see entire abstract.	1-51
Y	WONG et al. Induction of primary human antigen-specific cytotoxic T lymphocytes in vitro using dendritic cells pulsed with peptides. J. Immunotherapy. 15 January 1998, Vol. 21, No. 1, pages 32-40, see entire document.	1-51

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

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 30 APRIL 1999	Date of mailing of the international search report 26 MAY 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MICHAEL C. WILSON Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/01464

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	GONG et al. Reversal of tolerance to human MUC1 antigen in MUC1 transgenic mice immunized with fusions of dendritic and carcinoma cells. Proc. Natl. Acad. Sci., USA. May 1998, Vol. 95, pages 6279-6283, see entire document.	1-51
A	WU et al. Treatment of hepatocellular carcinoma with the cellular tumor vaccines generated by in vitro modification of tumor cells with non gene transfer approaches. Advances in experimental medicine and biology. 1998, Vol. 451, pages 283-293, see entire document.	1-51

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, caplus, wpids, biosis, medline.
Search terms: chimeric cell, fused cell, dendritic, hybrid cell, cyotoxic T-lymphocyte, tumor infiltrating lymphocyte, tumor