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(54) Title: DETECTION OF HIV-INFECTED CELL SUBSETS

(57) Abstract: The present invention provides methods of detecting HIV-infected cells of a specific cell subset. The methods of detecting HIV in a specific cell subset are carried out using one or more cell subset-defining antibody to identify cells of the desired subset and one or more HIV detection reagents consisting of HIV-specific antibodies or CD4-based HIV gp120 detection probes to detect intracellular HIV proteins within cells of the desired subset.



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DETECTION OF HIV-INFECTED CELL SUBSETS

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims priority to U.S. application Serial No. 60/783,317, filed March 17, 2006, which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

The present invention relates, in general, to the analysis of human immunodeficiency virus (HIV, or "an AIDS virus") infection and, more particularly, to the areas of methods of detecting HIV-infected cell subsets, and to assessing coreceptor expression on HIV-infected cell subsets. The present invention may be applicable in the treatment of human immunodeficiency virus (HIV, or "an AIDS virus") infection, in the selection of antiretroviral therapy, and to improve predictions of disease prognosis.

BACKGROUND OF THE INVENTION

The detection of cells infected with human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS), remains challenging because of the high level of genetic variability present in the viral population. Based on sequence variability, strains or isolates of HIV-1 are classified into three groups: group M (the "major" group); group O (the "outlier" group) O; and group N, the "new" group. Within group M there are known to be at least nine genetically distinct subtypes (or clades) of HIV-1, designated subtypes A, B, C, D, F, G, H, J and K, and the strains within each subtype exhibit significant variability. The inter-strain variability of HIV proteins has limited the range of HIV types detectable using antibody-based assays because of inter-strain variability in the non-conserved epitopes. There remains a need for practical methods capable of detecting HIV-infected cells despite the high degree of viral sequence variability.

HIV leads to disease as a result of the depletion of CD4⁺ T helper cells and the consequent inability to fight opportunistic infections. HIV primarily infects activated CD4⁺ T cells. Infected CD4⁺ T helper cells become targets for killing by HIV-specific cytotoxic CD8⁺ T cells, but also die from a variety of other causes. Because CD4⁺ T cells activated in response

to HIV antigen are themselves targets for HIV infection, HIV infection induces a constant supply of target cells, thus leading to further rounds of replication and immune destruction. The result is a reduction of HIV-specific CD4⁺ T cells, thereby depleting the arm of the immune system that controls replication of the virus.

Douek et al., 2002, Nature 417:95-98, studied the progressive loss of CD4⁺ T cells following infection and showed that HIV-specific CD4⁺ T cells are preferentially infected by HIV. They suggest that this provides a potential mechanism to explain the loss of HIV-specific CD4⁺ T-cell responses, and consequently the loss of immunological control of HIV replication.

In addition, HIV infected T cells can become non-proliferating memory cells, and during the chronic stage of HIV infection, most infected cells are CD4⁺ T memory cells. These non-proliferating memory cells form a latent reservoir of infected CD4⁺ T cells that can survive for many years, even in the presence of the current anti-HIV drugs (HAART - highly active anti-retroviral therapy) that appear to suppress HIV replication completely. This is because an infected non-proliferating CD4⁺ T memory cell does not actively replicate virus (i.e., does not make the viral proteins and genomic RNA), although the cell still harbors a DNA copy of HIV (the provirus) integrated into its chromosomes. On reactivation of the memory cells by antigen, viral replication resumes.

HIV infection of CD4⁺ T cells begins with the fusion of the viral envelope with the outer membrane of a target cell. Fusion is initiated when the viral envelope glycoprotein gp120 binds first to CD4 and then to a coreceptor, primarily CC chemokine receptor 5 (CCR5) or CXCR4 chemokine receptor 4 (CXCR4). The coreceptor tropism of the virus has been mapped to the V3 region of Gp120.

Regardless of the route of transmission, the viruses that first appear in the acute phase of infection are CCR5-tropic (R5 virus). Throughout the early stages of disease, R5 viruses predominate. Years after chronic infection is established, the CXCR4-tropic virus (X4 virus) phenotype appears in about 40%–50% of infected individuals. The emergence of X4 virus is predictive of rapid depletion of CD4⁺ cells and acceleration of HIV-1 disease progression, which may be related to the ability of CXCR4 viruses to infect an expanded spectrum of crucial target cells as compared to CCR5 strains.

US Patent No. 6,727,060, issued April 27, 2004, describes methods of analyzing HIV-1 coreceptor use, along with applications in the clinical care of HIV-1-infected patients. The methods for determining whether CXCR4 or CCR5 strains are present in a patient comprise isolating HIV from an infected patient and transforming cells with an HIV envelope gene from the isolated HIV strains such that the cells now express HIV envelope protein. The transformed cells are selectively fused with an indicator cell line that expresses an HIV envelope-compatible coreceptor (e.g., CD4 and either CCR5 or CXCR4). As fusion only occurs when an envelope protein interacts with a compatible coreceptor present on the surface of indicator cells, coreceptor use of the HIV isolated from the patient is indicated by whether the transformed cells fuse with either CCR5 or CXCR4 indicator cells.

Detection of HIV nucleic acids can be carried out using various nucleic acid amplification-based methods. Alternatively, a suitable method for determining whether infection of the indicator cell line occurred is contacting an epitope of HIV and identifying whether binding occurs, without binding to a control. In particular, the specificity assay of the invention can be carried out by employing antibodies directed against the HIV p24 antigen, as described by Kusunoki et al. (1999) *Nucleosides Nucleo.* 18:1705, or by using commercial ELISA assay kits, available, for example, from NEN Life Science Products, Boston.

Flow cytometric methods of analyzing intracellular cytokine expression in highly purified blood cell lineages has been described. See, for example, Picker et al., *Blood* 86(4):1408-1419 (1995); Waldrop et al., *J. Clin. Invest.* 99:1739-1750 (1997); Ghanekar et al., *J. Immunol.* 157:4028-4036 (1996); de Saint-Vis et al., *J. Immunol.* 160:1666-1676 (1998), all incorporated herein by reference). Suni et al., *J. Immunol.* 212:89-98 (1998), and US Patent publication 2001/0006789, both incorporated herein by reference, described an assay for concurrent expression of intracellular cytokines and cell surface proteins in antigen-stimulated T lymphocytes without prior T cell purification.

SUMMARY OF THE INVENTION

The present invention relates to methods of detecting HIV-infected cells of a particular cell type. The methods involves labeling cell-surface markers that identify the particular cell type of interest using labeled, target-specific detection reagents, and intracellular HIV proteins

using at least one detection reagent that binds to HIV proteins. Cells in which both cell-surface markers and intracellular HIV proteins have been labeled are detected, counted, or otherwise analyzed by cytometry, preferably, flow cytometry. The present invention provides improved methods of detecting, identifying, or enumerating cells infected with HIV.

One aspect of the present invention relates to a method of detecting HIV-infected cells of a particular cell subset, comprising:

contacting a sample containing said cells with at least one cell subset-defining antibody and at least one HIV detection reagent that binds to HIV proteins; and

cytometrically detecting the intracellular binding of said HIV detection reagent by cells in the defined cell subset.

In one embodiment, the HIV detection reagent is a broadly reactive HIV-specific antibody. The variability of HIV proteins, in particular, the variability of the gp120 coat protein, typically limits the range of HIV types detectable using antibodies. Recently, a number of broadly reactive HIV-specific antibodies have been described (see below). One novel aspect of the invention is the use of these broadly reactive HIV-specific antibodies, preferably in combinations of two or more, for the detection of intracellular HIV proteins in infected cells. In one embodiment, the methods of the present invention are carried out using at least one species, preferably multiple species, of these broadly reactive HIV-specific antibodies. Preferred broadly reactive HIV-specific antibodies include F105, 2F5, 296-13H11, 4E10 and M14.

In another embodiment, the HIV detection reagent is a CD4-based HIV gp120 probe that binds to the CD4-binding domain of HIV gp120 envelope glycoprotein. Despite the high overall variability of gp120, the gp120 CD4-binding region is relatively conserved, as binding of gp120 to CD4 is necessary for entry into a host cell. The gp120 glycoprotein binds the most amino-terminal of the four immunoglobulin-like domains within the extracellular portion of CD4. A CD4-based HIV gp120 detection probe useful in the present invention comprises the extracellular portion of CD4, or a fragment thereof that binds to HIV gp120.

In a preferred embodiment, the CD4-based detection probe is a chimeric molecule comprising the extracellular portion of CD4, or a fragment thereof that binds to HIV gp120

(typically at least the V1 domain), and an immunoglobulin or immunoglobulin-like chain, or fragment thereof. Preferably, the CD4-based detection probe is a fusion protein expressed from a recombinant nucleic acid sequence encoding (from 5' to 3') the extracellular portion of CD4, or a fragment thereof that binds to HIV gp120, and an immunoglobulin chain have an N-terminal deletion encompassing the variable region. Typically, the immunoglobulin portion will contain at least the Fc region.

HIV detection reagent suitable for use in the present methods may be used in combination to increase the detection sensitivity. Thus, depending on the application, it may be desirable to use a combination of one or more HIV-specific antibodies, preferably two or more, and more preferably three or more, together with a CD4-based gp120 probe. These HIV detection reagents can be labeled with the same detectable label such that a cumulative signal from all the labeled detection reagents bound to HIV proteins.

Cell subsets of particular interest are subsets of T cells, in particular, the CD4⁺ T cells, as HIV primarily infects CD4⁺ T cells. T cell subsets may be identified using multiple T cell subset-defining antibodies. In preferred embodiments, the present invention provides methods of detecting HIV-infected CD4⁺ T cells, comprising:

- contacting a sample containing peripheral blood mononuclear cells with at least one T cell subset-defining antibody and at least one HIV detection reagent that binds to HIV proteins; and

- cytometrically detecting the intracellular binding of said HIV detection reagent by cells in the defined T cell subset.

The sample of cells may be contacted with the labeled detection reagents either simultaneously, or in any order. The preferred order of staining will depend on the particular reagents used. To enable intracellular staining of the HIV proteins, cells are permeabilized prior to contacting with the labeled HIV-specific antibodies using well known reagents and methods, as described in references cited below.

During the chronic stage of HIV infection, most infected CD4⁺ T cells are non-proliferating CD4⁺ T memory cells that do not actively replicate virus (in particular, do not make viral proteins). On reactivation of the memory cells by antigen, viral replication resumes. Thus, to increase the amount of viral proteins present in the cell and improve the sensitivity of

detection in memory T cells, the cells preferably are activated. Activation can be carried out using any of the compounds known to activate T cells, including both specific and non-specific activators.

In one embodiment, activation of HIV protein production in T cells may be induced by contacting a sample containing peripheral blood mononuclear cells with an HIV protein, for example, gp160, gp140, or gp120, or fragment thereof. Embodiments of the invention in which activation of HIV protein production is induced by an HIV protein may benefit from the incorporation of a wash step following activation and prior to detection to minimize binding of the HIV detection reagents to residual activating protein.

In order to further increase the sensitivity of detection using the present methods, HIV proteins produced in the cell preferably are allowed to accumulate by blocking transport of the proteins out of the cell. Compounds that have the effect of blocking the export of proteins and allowing proteins to accumulate are known in the art and have been used in assays for the intracellular detection of cytokines (see below). Such compounds may be useful in the present invention to allow HIV proteins to accumulate intracellularly. Preferred compounds that can be used to block transport of newly synthesized proteins are Brefeldin A and monensin. Additionally, the export of synthesized HIV proteins may be inhibited by contacting the sample with an inhibitor of HIV protein export, such as inhibitors selected from the group consisting of inhibitors of glycosylation, HIV protease inhibitors, and inhibitors of myristoylation.

Thus, the present invention provides a method of detecting HIV-infected CD4⁺ T cells, comprising:

- contacting a sample containing peripheral blood mononuclear cells with an activator of protein synthesis;

- optionally adding to said sample an inhibitor of HIV protein export, selected from the group consisting of an inhibitor of glycosylation, an HIV protease inhibitor, an inhibitor of myristoylation, and an inhibitor of protein export;

- permeabilizing said cells;

- adding to said sample at least one T cell subset-defining antibody and at least one HIV detection reagent that binds to HIV proteins; and then

cytometrically detecting the intracellular binding of said HIV detection reagent by cells in the defined T cell subset.

The present invention further provides methods of determining the frequency of HIV-infected cells of a particular cell subset that express a cell-surface protein of interest, such as CXCR4 and/or CCR5. The expression of CXCR4 and/or CCR5 in HIV-infected cells is carried out using labeled CXCR4-specific and/or CCR5-specific detection reagents in addition to the cell subset-defining and HIV-specific detection reagents. The use of measurably distinct labels on each of the detection reagents enables the independent measurement of the expression of each protein.

Thus, in another embodiment, the present invention provides a method of determining the frequency of HIV-infected cells of a particular cell subset that express a cell-surface protein of interest, comprising:

contacting a sample containing said cells with at least one cell subset-defining antibody, at least one HIV detection reagent that binds to HIV proteins, and an antibody specific for said cell-surface protein; and

cytometrically detecting binding of said antibody specific for said cell-surface protein and intracellular binding of said HIV detection reagent by cells in the defined cell subset.

The present invention may be used to monitor changes in the relative frequencies of CXCR4⁺ and CCR5⁺ HIV-infected cell subsets. Changes in the relative frequencies may provide information regarding the tropism of the viral strains present in the patient and may be used as a diagnostic method. For example, increase in the frequency of CXCR4⁺, CCR5⁻ subsets may indicate a shift towards CXCR4-tropic virus, with a concomitant acceleration of HIV disease progression. A comparison of the frequencies of CXCR4⁺ and/or CCR5⁺ HIV-infected cell subsets to the frequencies of CXCR4⁺ and/or CCR5⁺ uninfected cells of the same subset from the patient also may provide information regarding the tropism of the viral strains present in the patient.

The methods of the present invention may be used in a number of applications, including:

1. to monitor shifts in coreceptor use associated with changes in HIV disease progression,

2. to determine the relative prevalence of CXCR4- or CCR5-tropic strains in patients infected with HIV undergoing antiretroviral therapy,
3. to monitor the suppression of CCR5 or CXCR4-tropic strains in patients infected with HIV undergoing antiretroviral therapy, or
4. to detect and/or monitor shifts in coreceptor use is useful for assessing the effectiveness of antiretroviral therapies.

These and other objects and embodiments are described in or are obvious from and within the scope of the invention, from the following Detailed Description.

DETAILED DESCRIPTION

In order that the invention herein described may be fully understood, a number of terms are explicitly defined, below. Terms not explicitly defined are intended to have their usual meaning in the fields of HIV biology and cytometry. Flow cytometry is described at length in the extensive literature in this field, including, for example, Landy et al. (eds.), *Clinical Flow Cytometry*, Annals of the New York Academy of Sciences Volume 677 (1993); Bauer et al. (eds.), *Clinical Flow Cytometry: Principles and Applications*, Williams & Wilkins (1993); Ormerod (ed.), *Flow Cytometry: A Practical Approach*, Oxford Univ. Press (1997); Jaroszeski et al. (eds.), *Flow Cytometry Protocols*, Methods in Molecular Biology No. 91, Humana Press (1997); and Practical Shapiro, *Flow Cytometry*, 4th ed., Wiley-Liss (2003); all incorporated herein by reference. Fluorescence imaging microscopy is described in, for example, Pawley (ed), *Handbook of Biological Confocal Microscopy*, 2nd Edition, Plenum Press (1989), incorporated herein by reference.

By "whole blood" is intended a fluid blood sample as drawn in the presence of an anticoagulant from a mammal and substantially unfractionated thereafter.

"Antibody" includes all products, derived or derivable from antibodies or from antibody genes, that are useful as markers in the flow cytometric methods described herein.

"Antibody" thus includes, inter alia, natural antibodies, antibody fragments, antibody derivatives, and genetically-engineered antibodies, antibody fragments, and antibody derivatives.

"Cell subset-defining antibody" refers to any antibody that may be used, alone or in combination with other antibodies, to facilitate identification of a particular subset of cells, and thus includes antibodies that are specific for epitopes displayed by cells of the subset. Typically, cell subsets may be identified by the presence of particular markers expressed by the cell and/or the absence of particular markers. By "absence" is intended a level of expression, as measured in an immunoassay, such as a flow cytometric assay, that is not significantly different from background.

"T-cell subset-defining antibody" refers to cell subset-defining antibody for the identification of a particular subset of T cells (also known as T lymphocytes).

A "cell activator", as used herein, refers to any substance that is capable of inducing or upregulating protein expression of a cell, including expression of cytokines, chemokines, cell surface proteins, or viral proteins from endogenous viral sequences. Suitable cell activators for use in the present invention are described below.

Antibodies are delineated by the standard CD nomenclature for the target molecule (see, e.g., Knapp W, Dorken K, Gilks WR, et al., eds. *Leukocyte typing IV: white cell differentiation antigens*. Oxford: Oxford University Press, 1989). For convenience, CD designations (e.g., CD4) are used herein to refer to not only the antibody cluster of differentiation, but also the corresponding antigen. This usage is common in the scientific literature, and one of skill in the art will understand the usage from the context.

The term "broadly reactive", with reference to HIV-specific antibodies, refers herein to an HIV-specific antibody that recognizes (i.e., binds to) conserved epitopes on HIV proteins from a multiplicity of strains within a plurality of HIV subtypes (clades).

The term "neutralizing antibody" refers to an antibody that reacts with an infectious agent, usually a virus, and destroys or inhibits its infectiveness and virulence.

The term "inhibitor of HIV protein export", as used herein, refers to any compound which inhibits the loss of HIV proteins synthesized within the cell, regardless of mechanism, and thus includes both inhibitors of protein export through cellular processes and inhibitors of viral maturation, budding, or shedding. Inhibitors of HIV protein export include, for example

inhibitors of protein transport, inhibitors of glycosylation, inhibitors of HIV protease, inhibitors of myristoylation. Classes of inhibitors of glycosylation are described, below.

The term “CD4-based HIV gp120 detection probe”, or simply “CD4-based probe”, as used herein, refers to any protein or chimeric molecule that includes sequences from the extracellular portion of CD4 protein and is capable of binding to HIV gp120 envelope glycoprotein.

The term “chimeric” or “hybrid” molecule, as used herein, refers to a molecule that contains functional components derived from two or more independent molecule species. The independent molecule species can be used in whole or in part to produce the chimeric molecule.

In the description that follows, it is to be understood that the various particular combinations of reagents described below are considered to be embodiments of the invention, and that the invention is not limited to the particular combinations exemplified.

Methods of Detecting HIV in a specific cell-subset

The methods of detecting HIV in a specific cell-subset are carried out using one or more cell subset-defining antibody to identify cells of the desired subset and one or more HIV detection reagents, such as HIV-specific antibodies or CD4-based probes that bind to HIV gp120 envelope glycoprotein, to detect intracellular (or both intracellular and cell surface-expressed) HIV proteins within cells of the desired subset. Protocols useful for carrying out various steps of the methods of the present invention can be adapted from the published flow-cytometric methods for the detection of intracellular cytokines within specific cell subsets. For example, sample preparation, T cell activation, inhibition of protein secretion, staining of both intracellular and cell-surface proteins using fluorescently labeled antibodies, and analysis of cells using flow cytometry are carried out using protocols adapted from published methods. One of skill in the art will understand that the particular protocols described in the art are to be modified or optimized for use in the present invention, and that such modifications or optimizations can be carried out in a routine manner.

Flow-cytometric methods for the detection of intracellular cytokines within specific T cell subsets are described in

Picker et al., Blood 86(4):1408-1419 (1995);

Ghanekar et al., J. Immunol. 157:4028-4036 (1996);

Waldrop et al., J. Clin. Invest. 99:1739-1750 (1997);

de Saint-Vis et al., J. Immunol. 160:1666-1676 (1998);

Suni et al., 1998, Journal of Immunological Methods 212:89-98;

Nomura et al., 2000, Cytometry 40:60-68;

Maecker et al., 2001, Journal of Immunological Methods 255:27-40;

US Patent publication 2001/0006789; and

BD Fastimmune CFC Handbook: Performance Characteristics of Antigen-Specific Cytokine Flow Cytometry (CFC) Assays, (BD Biosciences, San Jose, CA);

each incorporated herein by reference. Betts et al., 2003, Journal of Immunological Methods 281:65-78, incorporated herein by reference, describe flow-cytometric methods for the detection of antigen-specific CD8⁺ cells using activation-induced cell-surface markers. Flow-cytometric methods for the intracellular detection of cytokines within dendritic cell subsets are described in US Patent No. 6,495,333, incorporated herein by reference.

The steps of the methods of the present invention are described more fully below.

Analogous methods and reagents for preparing samples and carrying out intracellular detection of proteins I

Samples

The methods of the present invention for detecting HIV in a specific cell-subset are carried out using any sample containing cells of the desired cell subset. Suitable sample preparation methods are selected from known methods according to the nature of the sample and the selected method of analysis. For example, cell subsets of interest may be cell suspended in a fluid, such as peripheral blood mononuclear cells (PBMCs), in which case flow cytometry is a preferred method of analysis, or may be adherent cells, in which case fluorescence microscopy is a preferred method of analysis.

In preferred embodiment, a sample containing PMBCs, such as either whole blood or purified PBMCs, is used. Whole blood provides the closest possible environment to in vivo. In

addition, the use of whole blood is convenient because it requires the least sample preparation when working with fresh cells. Suitable sample preparation methods are described in the references cited above.

Cell subset-defining antibodies

In preferred embodiments, HIV is detected with T cell subsets. T cells, and subsets of T cells, can be identified using a number of combinations of markers that are well-known in the art. For example, methods of counting CD4⁺ or CD8⁺ T cells are well known, and assays for carrying out these methods are commercially available. Antibody combinations used to identify T cell subsets for the purpose of counting may be used to identify T cell subsets in the methods of the present invention. For example, T cell subset-defining antibodies may be selected from those described in US Patent Nos., 4,895,796; 4,987,086; 5,156,951; 5,583,033; and 5,622,853; each incorporated herein by reference.

Preferred T cell subset-defining antibodies are commercially available from, for example, BD Biosciences (San Jose, CA). A preferred combination for use in the present invention is the combination of a CD3-specific antibody (to identify T cells) and a CD4-specific antibody, which together identify CD4⁺ T cells. Other T cell subset-defining antibodies may be used. Examples of T cell subsetting antibody combinations commercially available from BD Biosciences (San Jose, CA) are listed in the table below along with the dyes used to label the antibodies. Instructions for their use are provided in the literature accompanying each product.

	Antibody target proteins	Dyes
BD Tritest™	CD3/CD19/CD45	FITC, PE, PerCP
BD Tritest™	CD3/CD4/CD45	FITC, PE, PerCP
BD Tritest™	CD3/CD8/CD45	FITC, PE, PerCP
BD Multitest™	CD3/CD8/CD45/CD4	FITC, PE, PerCP, APC

In addition to T cells, HIV also infects a number of other cells, such as dendritic cells, macrophages and cells of the nervous system, including oligodendrocytes, astrocytes, neurones, glial cells and brain macrophages. Cell-surface markers (i.e., proteins) that identify these other cells are known in the art. One of skill in the art will be able to select suitable cell

subset-defining antibodies specific for cell-surface markers that identify other cell types in a manner analogous to that described herein for T cell subset-defining antibodies.

HIV Detection Reagents

I. HIV-specific antibodies

The methods of the present invention can be carried out using HIV-specific antibodies for the detection of HIV proteins expressed within the cell or on the surface of the cell. HIV exhibits a high level of genetic variability, both among strains within each subtype and between the various subtypes. The inter-strain variability of HIV proteins, e.g., the gp120 surface protein, typically limits the range of HIV types detectable using specific antibodies because of inter-strain variability in the non-conserved epitopes. For the present invention, it is beneficial to use antibodies exhibiting a specificity across HIV strains as broad as possible. Preferably, one or more, more preferably, two or more, broadly reactive HIV-specific antibodies, which bind to multiple strains within a plurality of subtypes, are used. Alternatively, or in combination, a multiplicity of HIV-specific antibodies are used, wherein the multiplicity includes antibodies specific to different HIV strains, such that the combination of antibodies exhibits reactivity with a multiplicity of HIV strains. In this manner, a broad range of HIV subtypes can be detected, despite the variable and unpredictable nature of the viral protein sequences present in the cells.

Preferred broadly reactive HIV-specific antibodies are the broadly reactive antibodies, including the broadly neutralizing antibodies, that have been described recently in the literature. Typically, these antibodies react with gag or gp41 proteins. For most, the specificities of these antibodies are described, and one of skill will be able to select combinations of antibodies such that combined specificity is of the desired breadth. The utility of these antibodies for the detection of intracellular HIV proteins in infected cells has not been investigated extensively. One novel aspect of the invention is the use of these broadly reactive HIV-specific antibodies, that were originally selected as broadly neutralizing antibodies, for the detection of intracellular HIV proteins in infected cells. Examples of broadly reactive HIV-specific antibodies suitable for use in the present invention include the neutralizing antibodies described in the following references, each incorporated herein by reference:

- Zhang M-Y, et. al. 2003. Broadly cross-reactive HIV neutralizing human monoclonal antibody Fab selected by sequential antigen panning of a phage display library. *J. Immunol. Methods* 283:17-25;
- Zhang M-Y, et. al. 2004. Identification and characterization of a new cross-reactive human immunodeficiency virus type 1-neutralizing human monoclonal antibody. *J. Virol.* 78:9233-9242
- Haynes BF, et. al. 2005. Cardiolipin polyspecific autoreactivity in two broadly neutralizing HIV-1 antibodies. *Science* 308:1906-1908;
- Brunel FM, et. al. 2006. Structure-function analysis of the epitope for 4E10, a broadly neutralizing human immunodeficiency virus type 1 antibody. *J. Virol.* 80: 1680-1687;
- Zwick MB, et. al. 2005. Anti-human immunodeficiency virus type 1 (HIV-1) antibodies 2F5 and 4E10 require surprisingly few crucial residues in the membrane-proximal external region of glycoprotein gp41 to neutralize HIV-1. *J. Virol.* 79:1252-1261;
- Riscitelli RA, et. al. 2005. Structure of the Fab fragment of F105, a broadly reactive anti-human immunodeficiency virus (HIV) antibody that recognizes the CD4 binding site of HIV type 1 gp120. *J. Virol.* 79:1360-1369;
- He et al., 2002, Efficient Isolation of Novel Human Monoclonal Antibodies with Neutralizing Activity Against HIV-1 from Transgenic Mice Expressing Human Ig Loci, *Journal of Immunology*, 2002, 169: 595–605;
- U.S. Patent No. 5,831,034; and
- U.S. Patent No. 6,228,361.

The HIV Immunology Database (Los Alamos National Laboratory) provides continuously updated information on monoclonal antibodies (mAbs) against HIV-1 produced by various techniques, including cellular methods utilizing Epstein-Barr transformation, phage display technology, antigen stimulation of lymphoid cells in vitro, and preparation of hybridomas from cells of transgenic mice. Gorny and Zolla-Pazner, "Human Monoclonal Antibodies that Neutralize HIV-1" In Bette T. M. Korber and et. al., editors, *HIV Immunology and HIV/SIV Vaccine Databases 2003*. pages 37--51. Los Alamos National Laboratory, incorporated herein by reference, provides a review of neutralizing mAbs, i.e., those that are able to inhibit the infectivity of HIV virions, which target target several clusters of neutralizing epitopes in the HIV-1 envelope proteins, including V1, V2, V3, CD4bs, CD4i in gp120, and cluster I, cluster II, and a region adjacent to cluster II in gp41. Antibodies that

can be classified as broadly and potently neutralizing for HIV-1 primary isolates include mAbs 2G12, IgG1b12, and 447-52D (specific for gp120) and 2F5 and 4E10 (specific for gp41). Certain other monoclonal reagents are capable of broad neutralization as Fab fragments but not as IgG molecules. The suitability of particular antibodies or antibody fragments that are identified in the HIV Immunology Database as broad neutralizing antibodies can be determined in a routine manner following the guidance provided herein.

Preferably, the methods of the present invention are carried out using at least one species, preferably multiple species, of these broadly reactive HIV-specific antibodies. Preferred broadly reactive HIV-specific antibodies include 2G12, IgG1b12, 447-52D, F105, 2F5, 296-13H11, 4E10 and M14.

In general, an antibody or combination of antibodies are selected to obtain a desired breadth of reactivity. In most applications it is desirable to detect HIV infection regardless of the subtype or within-subtype strain, and antibodies are selected as described above to be broadly reactive. However, in some applications, it may be adequate or desirable to detect only a limited number of strains or subtypes of HIV. For example, if infection with a specific HIV strain is to be measured, then antibodies that react specifically only with the strain of interest are used. Such strain-specific assays enable, for example, the measurement of relative infection frequencies in different cell types of a multiply infected patient in order to assess any differences in cell-type tropism. A large number of HIV-specific antibodies having more limited breadth of reactivity have been described in the literature, and the present invention can be carried out using one or more antibodies selected from these. One of skill in the art can select appropriate antibodies or combinations of antibodies to obtain the desired breadth of HIV reactivity.

II. CD4-based HIV gp120 Detection Probe

The methods of the present invention can be carried out using a CD4-based HIV gp120 detection probe that binds to the HIV gp120 envelope glycoprotein expressed within the cell or on the surface of the cell. The CD4 protein amino acid sequence and the gene sequence encoding the CD4 protein are both known and publicly available from, for example, GenBank.

CD4 consists of four immunoglobulin-like extracellular domains (designated V1-V4 or, equivalently, D1-D4), a transmembrane domain, and a cytoplasmic segment. The CD4 amino acid sequence and the gene sequence encoding the CD4 protein are both known and are publicly available from, for example, GenBank. The gp120 glycoprotein binds the most amino-terminal of the four immunoglobulin-like domains of CD4 (see, for example, Berger et al., 1988, Proc. Natl. Acad. Sci. 85:2357-2361; Wu et al., 1997 Nature 387(29): 527-530; and Kwong et al., 1998, Nature 393(18): 648-659, each incorporated herein by reference). Despite the high overall variability of gp120, the gp120 CD4-binding region is relatively conserved, as binding of gp120 to CD4 is necessary for entry into a host cell. The CD4 protein sequence that is recognized by gp120 is used herein as the basis of an HIV detection reagent, thus providing a detection reagent capable of binding to HIV regardless of subtype. A CD4-based HIV gp120 detection probe useful in the present invention comprises the extracellular portion of CD4 (e.g., sCD4), or a fragment thereof that binds to HIV gp120. For use in the present methods, the detection reagent is labeled, either directly or through any suitable linker, using a cytometrically detectable label. Fluorescent dyes and labeling methods suitable for use in the present invention are well known in the art.

In one embodiment, the CD4-based detection probe is a chimeric molecule comprising the extracellular portion of CD4, or a fragment thereof that binds to HIV gp120 (typically at least the V1 domain), and an immunoglobulin or immunoglobulin-like chain, or fragment thereof. Preferably, the CD4-based detection probe is a fusion protein expressed from a recombinant nucleic acid sequence encoding (from 5' to 3') the extracellular portion of CD4, or a fragment thereof that binds to HIV gp120, and an immunoglobulin chain have an N-terminal deletion encompassing the variable region. Typically, the immunoglobulin portion will contain at least the Fc region. The CD4 component of the probe provides a gp120-binding sequence, and the immunoglobulin component provides a multimerization domain and facilitates labeling of the probe using reagents and chemistries used for the routine labeling of antibodies.

Soluble forms of CD4, termed sCD4, comprising the extracellular portion of CD4 have been described as therapeutic inhibitor of HIV infection, based on the ability of sCD4 to bind to gp120 and thereby competitively inhibit viral attachment to CD4-expressing cells. Such sCD4 molecules, suitably labeled, can be used in the present invention as a CD4-based gp120 probe.

In addition, various sCD4-containing chimeric molecules, such as chimeric sCD4-immunoglobulin molecules, have been described in, for example, U.S. Patent No. 7,070,991 and U.S. Patent No. 6,117,656; both incorporated herein by reference. Chimeric sCD4-immunoglobulin molecules combine the immunoglobulin-like CD4 extracellular domains with (at least) the Fc portion of immunoglobulins. Such chimeric sCD4-immunoglobulin molecules and complexes, suitably labeled, can be used in the present invention as a CD4-based gp120 probe. The natural multimerization property of the immunoglobulin component of these chimeric molecules facilitates the formation of dimeric (IgG components) or multimeric (IgM or IgA components) complexes. Dimeric or multimeric complexes provide improved binding avidity as a result of their multiple CD4 sequences capable of binding to gp120, and are preferred for use in the present invention.

Chimeric CD4-based probes suitable for use in the present invention can be synthesized routinely following the methods described in the art cited above. Further teaching is provided by the teaching in the art of the synthesis of analogous chimeric proteins. For example, WO 93/10220, incorporated herein by reference, describes the synthesis of chimeric proteins consisting of the extracellular region of a major histocompatibility complex (MHC) glycoprotein linked to the constant region of an immunoglobulin molecule, i.e., having an N-terminal deletion encompassing the variable regions. In these molecules, the MHC component of the probe provides a T cell recognition site, and the immunoglobulin component provides a multimerization domain and facilitates labeling of the protein. The natural multimerization property of the immunoglobulin component of these chimeric molecules facilitates the formation of dimeric (IgG components) or multimeric (IgM or IgA components) complexes. Dimeric or multimeric complexes provide improved binding avidity as a result of their multiple binding regions, in this case the T cell recognition sites. U.S. Patent Nos. 6,015,884; 6,140,113; and 6,448,071, each incorporated herein by reference, describe similar divalent and multivalent complexes of MHC-immunoglobulin chimeric molecules. One of skill in the art will be able to produce dimeric and multimeric CD4-based probes of the present invention in a manner analogous to that used to produce dimeric and multimeric complexes of MHC-immunoglobulin chimeric molecules by substituting the CD4 immunoglobulin like extracellular domain for the MHC immunoglobulin-like domain.

CD4-based HIV gp120 detection probes consisting of chimeric sCD4-immunoglobulin molecules have a number of desirable properties. First, as such chimeric molecules form multimeric complexes by virtue of the natural multimerization property of the immunoglobulin portion, these probes should exhibit improved binding sensitivity. Because of the structural similarity between the extracellular portion of CD4 and an immunoglobulin, the chimeric protein is expected to fold properly and behave structurally like an immunoglobulin. The chimeric molecules may be labeled using the standard fluorescent dyes and well known chemistries developed for the labeling of an immunoglobulin (i.e., antibody labeling). Furthermore, the intracellular staining can be carried out using conditions (e.g., permeabilization conditions) optimized for antibody labeling of intracellular antigens (e.g., cytokines).

Detection Labels

A wide range of suitable detection labels are known in the art and described in, for example, the references cited herein. The choice of detection labels will depend on the particular combination of detection reagents selected and the capabilities of the instrument used for detection. One of skill in the art can select in a routine manner an appropriate set of labels that will enable the independent assessment of each of the targets. Methods of conjugating labels to antibodies are well-known in the art. A large number of dyes are commercially available from a variety of sources, such as, for example, Molecular Probes (Eugene, OR) and Exciton (Dayton, OH), that provide great flexibility in selecting a set of dyes having the desired spectral properties.

Preferred fluorescent dyes for use with flow cytometric detection of the labeled cells are listed in the table below:

Dye	Dye Abbreviation
Fluorescein isothiocyanate	FITC
phycoerythrin	PE
Peridinin chlorophyll protein	PerCP
allophycocyanin	APC

Antibodies conjugated with fluorescent dyes suitable for identifying cell subsets are available commercially from a number of vendors. Preferred reagents are from BD Biosciences (San

Jose, CA). Preferred combinations of labeled antibodies for identifying CD4⁺ T cells include CD4 FITC and CD3 PerCP or CD3 PerCP/Cy5.5.

In general, to maximize detection sensitivity, the brightest label should be used to label the anti-HIV antibodies. PE or APC-labeled anti-HIV antibodies are preferred for the brightest staining.

Permeabilizing cells

In order to facilitate the staining of intracellular HIV proteins, the cells are permeabilized to allow the labeled detection reagents to pass through the cell membrane into the cell. Methods and reagents for permeabilizing cells to facilitate labeling intracellular proteins for detection using cytometric methods are well known in the art, are described in the references cited herein (see, e.g., the references cited above describing flow-cytometric methods for the detection of intracellular cytokines within specific T cell subsets), and are commercially available from a variety of companies. Preferred reagents are available from BD Biosciences (San Jose, CA).

Antibody Staining

Methods for staining both cell-surface proteins and intracellular proteins using the labeled detection reagents are well known in the art and are described in the references cited herein (see, e.g., the references cited above describing flow-cytometric methods for the detection of intracellular cytokines within specific T cell subsets. See, also, the product manuals and literature describing BD Fastimmune™ kits and reagents, available from BD Biosciences (San Jose, CA)).

Methods of Enhancing detection sensitivity

The detection of HIV-infected cells of a specific subsets can be enhanced using one or more of the following additional steps:

1. Adding an activator to the sample to induce protein synthesis;
2. Adding an inhibitor of HIV protein export to allow newly synthesized HIV proteins to accumulate in the cell;

It will be understood that the usefulness of each of the enhancement steps will depend on the cell subset of interest. For example, for the detection of HIV in resting CD4⁺ memory T cells, both the step of inducing HIV protein synthesis and inhibiting export of newly synthesized HIV protein may be advantageous. Each of these steps is described in more detail, below.

Cell Activation

Depending on the cell subset to be analyzed, it may be desirable to activate the cells to induce the synthesis of HIV proteins within the cell. In particular, activation may significantly enhance the ability to detect HIV proteins in CD4⁺ T memory cells, which do not produce HIV proteins in their resting state.

One method of inducing HIV protein synthesis in T cells is contact the T cells with a T cell-activating agent. Infectious virus can be detected in vitro, using nucleic acid detection assays, in HIV-infected CD4⁺ T memory cells following stimulation with T cell-activating agents (see Chun et al., 1997, Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. Proc. Natl. Acad. Sci. USA 94:13193; Finzi et al., 1997, Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. Science 278:1295; and Wong et al., 1997, Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. Science 278:1291; each incorporated herein by reference).

Activation of CD4⁺ T memory cells can be carried out using any of the compounds known to cause activation, including both specific and non-specific activators. In the present invention, it is desirable to use an activator that broadly activates CD4⁺ T memory cells in the sample regardless of the T cell specificity, although an activator of selected T cell subsets, including antigen-specific subsets, may be used.

Suitable activator compounds are well-known for use in, for example, measuring cytokine responses in activated T cells. Activators of T cells include, in addition to specific antigens, cytokines, and costimulatory molecules, polyclonal activators or mitogens that bypass the T cell receptor and activate large fractions of T cells regardless of the T cells' antigen specificity, including, for example, phytohaemagglutinin (PHA), phorbol 12-myristate 13 acetate (PMA), ionomycin (I), TNF α , and CD2/CD2R, which may be used in combination (e.g. PHA and IL-2; PHA and I; or PMA and I). Another class of activating agents, termed

superantigens, which activate specific subsets of T cells based on recognition of V_{β} sequences in the T cell receptor without regard to the T cell's antigen specificity (e.g. Staphylococcal enterotoxin B (SEB)), may be applicable in the present methods. The following references, each incorporated herein by reference, describe methods involving the detection of responding T cells activated in a non-specific manner by contact with a polyclonal activator or superantigen:

Maino et al., 1995, Cytometry 20:127-133;

Jung et al., 1993, J. Immunol. Methods 159:197-207

Elson et al., 1995, J. Immunol. 154(9):4294-4301

Prussin et al., 1995, J. Immunol. Methods 188:117-128

Picker et al., 1995, Blood 86:1408-1419

Application Note 1: Detection of Intracellular Cytokines in Activated Lymphocytes,
Becton Dickinson and Co.

In some applications, activation by specific antigens may be suitable. Antigen-specific activation of subsets of T cells has been achieved by contacting cells with a pool of overlapping peptides generated from a particular protein such that essentially all epitopes from the protein are presented to the cells (see, e.g., Maecker et al., 2001, J Immunol Methods 255: 27-40).

Alternatively, HIV protein synthesis can be induced in resting T cells that have not undergone classic T cell activation by contacting the cells with an isolated HIV protein or protein fragment. HIV encodes several proteins, including envelope and Nef, which trigger a variety of signaling pathways associated with cellular activation, thereby facilitating HIV replication in nondividing cells. The production of infectious virus from resting $CD4^{+}$ T cells isolated from HIV-infected individuals can be induced following exposure of these cells to, for example, gp140 envelope protein oligomers or gp120 (see Kinter et al., 2003, Journal of Immunology, 170: 2449-2455; and Cicala et al., 2002, Proc. Natl. Acad. Sci. USA 99(14): 9380-9385; both incorporated herein by reference). In a preferred embodiment of the invention, HIV protein synthesis is induced by contacting the cells with an HIV protein, or fragment thereof, such as a gp160, gp140, or gp120, or a fragment thereof.

Inhibiting HIV Protein Export

The sensitivity of detection of HIV proteins produced in the cell may be further increased by allowing newly synthesized proteins to accumulate by blocking transport of the proteins out of the cell. Compounds that have the effect of blocking the export of proteins and allowing proteins to accumulate are known in the art and have been used in assays for the intracellular detection of cytokines (see the references cited above relating to T cell activation). Such compounds may be useful in the present invention to allow HIV proteins to accumulate intracellularly, thereby increasing signal from bound labeled antibodies and improving detection. Preferred compounds that can be used to block transport of newly synthesized proteins are Brefeldin A (BFA) and monensin.

Alternatively, the export of viral proteins can be inhibited by the use of a compound that inhibits maturation, budding, or shedding of viral particles. Certain viral proteins undergo glycosylation, and inhibitors of glycosylation may also inhibit the maturation of HIV and release of viral particles. Assembly of infectious virions is dependent on the action of: (a) an aspartyl protease encoded by the viral pol gene and responsible for cleavage of the gag and gag-pol precursors into mature proteins and (b) cellular N-protein myristoyl transferase (NMT) which adds myristic acid to the N-terminus of gag, gag-pol and nef viral polypeptide precursors. Inhibitors of either protease cleavage or of myristoylation may also inhibit the inhibit the maturation and release of viral particles.

Inhibitors of glycosylation have been described. Brefeldin A, which inhibits processing of proteins through the golgi apparatus, may inhibit glycosylation of HIV proteins. Other inhibitors of glycosylation include castanospermine, a naturally occurring alkaloid and inhibitor of glucosidase-I served, a derivatives, such as N-Butyl deoxynojirimycin(butyl-DNJ) and 6-butyl-castanospermine. The following references, each incorporated herein by reference, describe glycosylation of HIV proteins and inhibitors of glycosylation.

Fenouillet and Jones, 1995, "The glycosylation of human immunodeficiency virus type 1 transmembrane glycoprotein (gp41) is important for the efficient intracellular transport of the envelope precursor gp160" J Gen Virol. 1995 Jun;76 (Pt 6):1509-14.

JOHNSON et al., "Synergistic inhibition of human immunodeficiency virus type 1 and type 2 replication in vitro by castanospermine and 3'-azido-3'-deoxythymidine" *ANTIMICROB AGENTS CHEMOTHER* 33:53-57 (1989).

RUPRECHT et al., "In vivo analysis of castanospermine, a candidate antiretroviral agent" *J AIDS* 2:149 (1989).

SUNKARA et al., "Anti-HIV activity of castanospermine analogs" *LANCET*, 1989;1:1206 (1989).

Both inhibitors of HIV protease and of myristoylation of HIV proteins have been described. See, for example, the following references, each incorporated herein by reference:

KOHL, N.R.; EMINI, E.A.; SCHLEIF, W.A.; ET AL. Active human immunodeficiency virus protease is required for viral infectivity. *PROC NATL ACAD SCI USA*, 85:4686-4690 (1988).

PENG, C.; HO, B.K.; CHANG, T.W.; CHANG, N.T. Role of human immunodeficiency virus Type 1-specific protease in core protein maturation and viral infectivity. *J VIROL* 63:2550 (1989).

GOTTLINGER, H.G.; SODROSKI, J.G.; HASELTINE, W.A. Role of the capsid precursor processing and myristoylation in morphogenesis and infectivity of human immunodeficiency virus Type 1. *PROC NATL ACAD SCI USA* 86:5781-5789 (1989).

TOMASZEK, T.A.; MAGAARD, V.W.; BRYAN, H.G.; MOORE, M.L.; MEEK, T.D. Chromophoric peptide substrates for the spectrophotometric assay of hiv-1 protease. *BIOCHEM BIOPHYS RES COMMUN* 168:274-280 (1990).

HYLAND, L.J.; DAYTON, B.D.; MOORE, M.L.; SHU, A.Y.; HEYS, J.R.; MEEK, T.D. A radiometric assay for hiv-1 protease. *ANAL BIOCHEM* 188:408 (1990).

PHYLIP, L.H.; RICHARDS, A.D.; KAY, J.; ET AL. Hydrolysis of synthetic chromogenic substrates by hiv-1 and hiv-2 proteinases. *BIOCHEM BIOPHYS RES COMMUN* 171:439 (1990).

TAMBURINI, P.P.; DREYER, R.N.; HANSEN, J.; ET AL. A fluorometric assay for hiv-protease activity using high-performance liquid chromatography. *ANAL BIOCHEM* 186:363 (1990).

RICHARDS, A.D.; PHYLIP, L.H.; FARMERI, W.G.; ET AL. Sensitive, soluble chromogenic substrates for hiv-1 proteinase. *J BIOL CHEM* 265:7733-7736 (1990).

- MATAYOSHI, E.D.; WANG, G.T.; KRAFFT, G.A.; ERICKSON, J. Novel fluorogenic substrates for assaying retroviral proteases by resonance energy transfer. *SCIENCE* 247:99-958 (1990).
- BILLICH, A.; WINKLER, G. Colorimetric assay of hiv-1 proteinase suitable for high-capacity screening. *PEPTIDE RES* 3:274 (1990).
- ROBERTS, N.A.; MARTIN, J.A.; KINCHINGTON, D.; ET AL. Rational design of peptide-based hiv proteinase inhibitors. *SCIENCE* 248:358-361 (1990).
- MEEK, T.D.; LAMBERT, D.M.; DREYER, G.B.; ET AL. Inhibition of hiv-1 protease in infected t-lymphocytes by synthetic peptide analogues. *NATURE (LONDON)* 343:90-92 (1990).
- RICH, D.H.; GREEN, J.; TOTH, M.V.; MARSHALL, G.R.; KENT, S.B. Hydroxyethylamine analogues of the p17/p24 substrate cleavage site are tight-binding inhibitors of hiv protease. *J MED CHEM* 33:1285-1288 (1990).
- MCQUADE, T.J.; TOMASSELLI, A.G.; LIU, L.; ET AL. A synthetic hiv-1 protease inhibitor with antiviral activity arrests hiv-like particle maturation. *SCIENCE* 247:454-456 (1990).
- ASHORN, P.; MCQUADE, T.J.; THAISRIVONGS, S.; TOMASSELLI, A.G.; TARPLEY, W.G.; MOSS B. An inhibitor of the protease blocks maturation of human and simian immunodeficiency viruses and spread of infection. *PROC NATL ACAD SCI USA* 87:7472-7476 (1990).
- DREYER, G.B.; METCALF, B.W.; TOMASZEK, T.A.; ET AL. Inhibition of human immunodeficiency virus 1 protease in vitro: rational design of substrate analogue inhibitors. *PROC NATL ACAD SCI USA* 86:9752-9755 (1989).
- KEMPF, D.J.; NORBECK, D.W.; CODACOV, L.; ET AL. Structure-based, c2 symmetric inhibitors of hiv protease. *J MED CHEM* 33:2687-2689 (1990).
- ERICKSON, J.; NEIDHART, D.J.; VANDRIE, J.; ET AL. DESIGN, ACTIVITY AND 2.8 Å crystal structure of a c2 symmetric inhibitor complexed to hiv-1 protease. *SCIENCE* 249:527-533 (1990).
- BRYANT, M.L.; HEUCKEROTH, R.O.; KIMATA, J.T.; RATNER, L.; AND GORDON, J.I. Replication of human immunodeficiency virus 1 and moloney murine leukemia virus is inhibited by different heteroatom-containing analogs of myristic acid. *PROC NATL ACAD SCI USA*, 86:8655-8659 (1989).

Commercially available protease inhibitors that may be useful in the methods of the present invention are listed in the table, below.

Brand Name	Generic Name	Abbreviation	Experimental Code	Pharmaceutical Company
<u>Agenerase®</u>	amprenavir	APV	141W94 or VX-478	<u>GlaxoSmithKline</u>
<u>Aptivus®</u>	tipranavir	TPV	PNU-140690	<u>Boehringer Ingelheim</u>
<u>Crixivan®</u>	indinavir	IDV	MK-639	<u>Merck & Co.</u>
<u>Invirase®</u>	saquinavir	SQV	Ro-31-8959	<u>Hoffmann-La Roche</u>
<u>Kaletra®</u>	lopinavir + ritonavir	LPV	ABT-378/r	<u>Abbott Laboratories</u>
<u>Lexiva®</u>	fosamprenavir	FPV	GW-433908 or VX-175	<u>GlaxoSmithKline</u>
<u>Norvir®</u>	ritonavir	RTV	ABT-538	<u>Abbott Laboratories</u>
<u>Reyataz®</u>	atazanavir	ATZ	BMS-232632	<u>Bristol-Myers Squibb</u>
<u>Viracept®</u>	nelfinavir	NFV	AG-1343	<u>Pfizer</u>
	<u>brecanavir</u>		GW640385 or VX-385	<u>GlaxoSmithKline</u>
	<u>darunavir</u>		TMC-114	<u>Tibotec</u>

One of skill in the art will understand that the use of any of the above-mentioned compounds will involve routine experimentation in order to identify a suitable compound for any particular application and to determine a suitable concentration.

Analysis of Stained Cells

Following staining of cell-surface or intracellular proteins with labeled antibodies, the cells are analyzed cytometrically by, for example, flow cytometry or by imaging cytometry (e.g., scanning cytometry or fluorescent microscopy). The binding each a labeled antibody is detected by measuring a signal from the label at a level above a background level resulting from non-specific interactions. In general, HIV-infected cells in a defined cell subset are

recognized by the binding of both the subset-defining antibodies that identify the cell subset of interest, and HIV-specific antibodies that indicate the presence of intracellular HIV proteins.

Flow cytometric methods suitable for use in the present invention are analogous to the methods used to detect the synthesis of intracellular cytokines in defined T cell subsets. Such methods are well known in the art and described in, for example, Maino and Picker, 1998, *Cytometry (Communications in Clinical Cytometry)* 34: 207-215; and Nomura et al., *Cytometry* 40:60-68; both incorporated herein by reference. See also the references cited above relating to T cell activation, which describe the use of flow cytometric methods to detect simultaneously both intracellular and surface proteins. One of ordinary skill can carry out flow cytometric detection in the methods of the present invention in a routine manner following the guidance provided herein and in the references cited above and incorporated herein by reference.

Analysis of coreceptor expression

In some embodiment, the cell subsets of interest are defined in part by the expression of one or more chemokine receptors, such as CXCR4 or CCR5, that play a role in the initial stages of infection. The expression of CXCR4 and/or CCR5 in HIV-infected cells is carried out using labeled CXCR4-specific and/or CCR5-specific detection reagents in addition to other cell subset-defining and HIV-specific detection reagents. The use of measurably distinct labels on each of the detection reagents enables the independent measurement of the expression of each protein.

The present invention further provides methods to determine the frequency of HIV-infected cells of a particular cell subset that express a cell-surface protein of interest, such as CXCR4 and/or CCR5. Methods to determine the frequency of CXCR4- and/or CCR5-expressing HIV-infected cells are described more fully in the examples, below.

The present invention may be used to monitor changes in the relative frequencies of CXCR4⁺ and CCR5⁺ HIV-infected cell subsets. Changes in the relative frequencies may provide information regarding the tropism of the viral strains present in the patient and may be used as a diagnostic method. For example, increase in the frequency of CXCR4⁺, CCR5⁻ subsets may indicate a shift towards CXCR4-tropic virus, with a concomitant acceleration of HIV

disease progression. A comparison of the frequencies of CXCR4⁺ and/or CCR5⁺ HIV-infected cell subsets to the frequencies of CXCR4⁺ and/or CCR5⁺ uninfected cells of the same subset from the patient also may provide information regarding the tropism of the viral strains present in the patient.

Application of the diagnostic methods to detect and/or monitor shifts in coreceptor use may be useful for predicting disease progression over time, or for monitoring the effectiveness of antiretroviral therapy. Aspects of antiretroviral therapy that can be monitored, for example, are development of drug resistance and/or sensitivity. The diagnostic methods of the invention can be applied before initiating antiretroviral therapy to determine a suitable antiretroviral treatment regimen. The diagnostic methods of the claimed invention can also be applied after initiating antiretroviral therapy to monitor efficacy of a viral treatment regimen and where efficacy of the treatment is directly related to a shift in the coreceptor use.

Kits

The reagents of the present invention can be advantageously used as a kit for carrying out the method of the invention and could be employed in a variety of applications, e.g., as kits suitable for scientific, medical and/or diagnostic purposes. The manufacture of the kits follows preferably standard procedures that are known to the person skilled in the art. Kits can advantageously include instructions for use.

In the present invention, it is additionally understood that HIV is a lentivirus, and the skilled artisan can readily understand that from the teachings herein, and the knowledge in the art, within the ambit of the invention are herein embodiments wherein the virus is a lentivirus other than HIV, including SIV and FIV, as in U.S. Pat. Nos. 5,863,542 and 5,766,598, and wherein the coreceptors are analogous (e.g., homologous) to CCR5 and CXCR4.

EXAMPLES

The present invention is additionally described by way of the following illustrative, non-limiting Examples, that provide a better understanding of the present invention and of its many advantages.

Example 1

Methods for Intracellular HIV Detection

This example describes preferred methods for carrying out the invention in general. One of skill will recognize that assay performance depends on a number of application-specific factors, such as the cell type to be analyzed, the labeled antibodies selected, and the nature of the sample used. Routine optimization should be carried out for each application.

The methods of detecting HIV in a specific cell-subset are carried out using one or more cell subset-defining antibody to identify cells of the desired subset (i.e., subpopulation) and one or more HIV detection reagents consisting of HIV-specific antibodies or CD4-based HIV gp120 detection probes to detect intracellular HIV proteins within cells of the desired subset. Protocols useful for carrying out various steps of the methods of the present invention can be adapted from the published flow-cytometric methods for the detection of intracellular cytokines within specific cell subsets, cited above, in a routine manner.

Sample collection

The methods of the present invention can be carried out using samples of either whole blood or peripheral blood mononuclear cells (PBMCs), or lymphoid cells from other compartments, e.g., from lymph nodes. If the cells are to be activated using T cell activators, whole blood preferably is collected in sodium heparin tubes, such as a BD VACUTAINER® cell preparation tube (CPT) (BD, Franklin Lakes, NJ). Other anticoagulants, such as EDTA and ACD, bind calcium ions and severely compromise the functional capacity of lymphocytes. If PBMCs are to be used, the blood may be collected in either ACD- or heparin-containing tubes.

Reagents

Fluorescently labeled antibodies for identifying lymphocyte subsets are available commercially from a number of vendors. Preferred reagents are from BD Biosciences (San Jose, CA). A preferred combination of labeled antibodies for identifying CD4-positive and CD8-positive T-lymphocyte populations is a CD4-specific antibody labeled with FITC and a CD3-specific antibody labeled with PerCP.

One or more species of HIV-specific antibodies or CD4-based HIV gp120 detection probes are labeled with the brightest fluorochrome among those used in order to maximize detection sensitivity. If more than one species of detection reagents are used, all the species of HIV detection reagents are labeled using the same fluorochrome. PE or APC-labeled anti-HIV antibodies are preferred for use with FITC- and PerCP-labeled antibodies specific for cell-surface proteins, such as with the preferred combination of a CD4-specific antibody labeled with FITC and a CD3-specific antibody labeled with PerCP.

Activation and Inhibition of HIV Protein Export

Cells are activated as described above. Optionally, Brefeldin A is added in order to inhibit HIV protein export, as described above.

Lysis, Fixation, and Permeabilization

Red blood cells in the sample are lysed and the remaining mononuclear cells are fixed with 450 μ L of FACS® Lysing Solution (BD Biosciences) for 15 to 60 minutes at room temperature or 24 hours at 4°C prior to analysis.

Cells are resuspended in 0.5 ml of FACS™ Permeabilizing Solution (BD Biosciences) for 10 min at room temperature.

Immunofluorescent Staining

Staining of cell surface markers that identify lymphocyte subsets is carried out by adding the antibody conjugates to 50- μ L aliquots of samples and incubating the mixture for 15 minutes at room temperature.

Staining of both surface and intracellular epitopes is carried out simultaneously in a single staining step, following fixation and permeabilization of the cells. This is possible because the antibodies to human CD3- and CD4-specific antibodies available from BD Biosciences recognize determinants that are at least partially resistant to fixation and permeabilization. However, the staining procedure can be modified such that surface-staining is carried out

prior to fixation for use with antibodies to other determinants, or antibodies to the determinants obtained from other vendors.

It is expected that the optimal staining conditions can be optimized routinely. In general, staining of cells that have been fixed and permeabilized is carried out at room temperature for 30 minutes in a staining volume of 50–100 μ L. Cell staining is dependent on both antibody concentration and time. If the staining volume is increased, staining is carried out with an increased antibody concentration, or for a longer time, up to 60 minutes, or with intermittent mixing.

Flow Cytometric Analysis

Whole blood samples are analyzed using a flow cytometer. Suitable flow cytometers are commercially available from BD Biosciences (San Jose, CA). Preferred flow cytometers include the BD FACSCalibur™, BD FACSCanto™, BD FACSArray™ and LSRII flow cytometers.

An example of a flow cytometer suitable for use in the methods of the present invention is a BD FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA) with the four-color fluorescence detection option. This cytometer has two lasers, a blue laser (488 nm) and a red diode laser (~635 nm). Photomultiplier tubes (PMT) are used for measurement of dye fluorescence. The wavelength ranges detected for the measurement of forward scatter (FSC) and side scatter (SSC), and in each of the fluorescence detection channels (FL1-FL4), are shown in the table, below.

<u>Channel</u>	<u>Wavelength Range</u>
FSC	488/10 nm
SSC	488/10 nm
FL1	530/30 nm
FL2	585/42 nm
FL3	≥ 670 nm
FL4	661/16

Using these detector channels, the fluorescence of the preferred dyes is measured primarily in the channels as follows:

Detector	Dye	Dye Abbreviation
FL1	fluorescein isothiocyanate	FITC
FL2	Phycoerythrin	PE
FL3	peridinin chlorophyll protein	PerCP
FL4	Allophycocyanin	APC

Using the preferred reagent combinations, data are acquired with using fluorescence triggering in the FL3 channel (CD3 PerCP) to gate on the CD3-positive T cell population. Preferably, about 40,000–50,000 gated CD3⁺ events are collected, although the number needed will depend on the frequency of HIV⁺ cells within the CD3⁺ subset. Guidance on the number of events to collect (along with other aspects of flow-cytometric methods for the detection of intracellular cytokines within specific cell subsets) is provided in the BD Fastimmune CFC Handbook: Performance Characteristics of Antigen-Specific Cytokine Flow Cytometry (CFC) Assays, available from BD Biosciences, incorporated herein by reference.

Example 2

Assay for Coreceptor Expression

This example describes methods of detecting the expression of CCR5 and/or CXCR4 on HIV-infected CD4⁺ T cells, useful, for example, to determine the frequency of CCR5- and/or CXCR4- expressing HIV-infected CD4⁺ T cells. The methods are carried out essentially as described in Example 1, but with the addition of fluorescently labeled detection reagents specific to each of these cell-surface markers.

Both CCR5-specific antibodies and CXCR4-specific antibodies are commercially available from, for example, BD Biosciences (San Jose, CA). Both CCR5 and CXCR4-specific antibodies are commercially available conjugated to one of several dyes suitable for use in flow cytometry, or in unconjugated form. One of skill in the art will be able to select in a routine manner appropriate dyes for use in combination with the labeled T cell subset-defining reagents and the labeled HIV-specific antibodies, and to carry out conjugation of antibodies to the selected dyes using routine methods.

We claim:

1. A method of detecting HIV-infected cells of a particular cell subset, comprising:
contacting a sample containing said cells with at least one labeled T cell subset-defining antibody and at least one labeled HIV detection reagent that is a broadly reactive HIV-specific antibody or a CD4-based HIV gp120 probe; and
cytometrically detecting the intracellular binding of said HIV detection reagent by cells in the defined cell subset.
2. The method of claim 1, wherein said step of detecting is carried out using flow cytometry.
3. The method of claim 1, wherein said broadly reactive HIV-specific antibody is selected from the group consisting of 2G12, IgG1b12, 447-52D, F105, 2F5, 296-13H11, 4E10 and M14.
4. The method of claim 1, wherein said CD4-based HIV gp120 detection probe is a chimeric molecule comprising the extracellular domain of CD4, or a fragment thereof that binds to HIV gp120, and the Fc region of an immunoglobulin.
5. The method of claim 1, wherein said at least one T cell subset-defining antibody is an antibody that binds to CD4⁺ cells.
6. The method of claim 1, wherein said cells are permeabilized to facilitate intracellular staining of HIV proteins.
7. The method of claim 1, wherein said cells are activated by contacting said sample with an activating agent to induce synthesis of HIV proteins.
8. The method of claim 1, wherein said cells are activated by contacting said sample with a T cell activating agent to induce synthesis of HIV proteins.

9. The method of claim 8, wherein said T cell activating agent is selected from the group consisting of a CD3, CD28, phytohaemagglutinin (PHA), phorbol 12-myristate 13 acetate (PMA), ionomycin (I), Staphylococcal enterotoxin B (SEB), TNF- α , and CD2/CD2R.
10. The method of claim 7, wherein said activating agent is an HIV protein or a fragment thereof.
11. The method of claim 10, wherein said activating agent is gp160 or a fragment thereof.
12. The method of claim 10, wherein said activating agent is gp120 or a fragment thereof.
13. The method of claim 7, wherein export of synthesized proteins is inhibited by contacting said sample with an inhibitor of HIV protein export selected from the group consisting of an inhibitor of glycosylation, an HIV protease inhibitor, an inhibitor of myristoylation, and an inhibitor of protein export.
14. The method of claim 13, wherein said inhibitor of protein export is selected from the group consisting of Brefeldin A and monensin.
15. A method of detecting HIV-infected cells of a particular T cell subset, comprising:
contacting a sample containing peripheral blood mononuclear cells with at least one labeled T cell subset-defining antibody and at least one labeled HIV detection reagent that is a broadly reactive HIV-specific antibody or a CD4-based HIV gp120 probe; and
cytometrically detecting the intracellular binding of said HIV detection reagent by cells in the defined T cell subset.
16. The method of claim 15, wherein said step of detecting is carried out using flow cytometry.
17. The method of claim 15, wherein said broadly reactive HIV-specific antibody is selected from the group consisting of 2G12, IgG1b12, 447-52D, F105, 2F5, 296-13H11, 4E10 and M14.

18. The method of claim 15, wherein said CD4-based HIV gp120 detection probe is a chimeric molecule comprising the extracellular domain of CD4, or a fragment thereof that binds to HIV gp120, and the Fc region of an immunoglobulin.
19. The method of claim 15, wherein said at least one T cell subset-defining antibody is an antibody that binds to CD4⁺ cells.
20. The method of claim 15, wherein said cells are permeabilized to facilitate intracellular staining of HIV proteins.
21. The method of claim 15, wherein said cells are activated by contacting said sample with an activating agent to induce synthesis of HIV proteins.
22. The method of claim 15, wherein said cells are activated by contacting said sample with a T cell activating agent to induce synthesis of HIV proteins.
23. The method of claim 22, wherein said T cell activating agent is selected from the group consisting of a CD3, CD28, phytohaemagglutinin (PHA), phorbol 12-myristate 13 acetate (PMA), ionomycin (I), Staphylococcal enterotoxin B (SEB), TNF- α , and CD2/CD2R.
24. The method of claim 21, wherein said activating agent is an HIV protein or a fragment thereof.
25. The method of claim 24, wherein said activating agent is gp160 or a fragment thereof.
26. The method of claim 24, wherein said activating agent is gp120 or a fragment thereof.
27. The method of claim 21, wherein export of synthesized proteins is inhibited by contacting said sample with an inhibitor of HIV protein export selected from the group consisting of an inhibitor of glycosylation, an HIV protease inhibitor, an inhibitor of myristoylation, and an inhibitor of protein export.
28. The method of claim 27, wherein said inhibitor of protein export is selected from the group consisting of Brefeldin A and monensin.

29. A method of detecting HIV-infected cells of a T cell subset, comprising:
contacting a sample containing peripheral blood mononuclear cells with an activator of protein synthesis;
adding to said sample an inhibitor of HIV protein export selected from the group consisting of an inhibitor of glycosylation, an HIV protease inhibitor, an inhibitor of myristoylation, and an inhibitor of protein export;
permeabilizing said cells;
adding to said sample at least one labeled T cell subset-defining antibody and at least one labeled HIV detection reagent that is a broadly reactive HIV-specific antibody or a CD4-based HIV gp120 probe; and then
cytometrically detecting intracellular binding of said HIV detection reagent by cells in the defined T cell subset.
30. The method of claim 29, wherein said step of detecting is carried out using flow cytometry.
31. The method of claim 29, wherein said broadly reactive HIV-specific antibody is selected from the group consisting of 2G12, IgG1b12, 447-52D, F105, 2F5, 296-13H11, 4E10 and M14.
32. The method of claim 29, wherein said CD4-based HIV gp120 detection probe is a chimeric molecule comprising the extracellular domain of CD4, or a fragment thereof that binds to HIV gp120, and the Fc region of an immunoglobulin.
33. The method of claim 29, wherein said at least one T cell subset-defining antibody is an antibody that binds to CD4⁺ cells.
34. The method of claim 29, wherein said cells are permeabilized to facilitate intracellular staining of HIV proteins.
35. The method of claim 29, wherein said cells are activated by contacting said sample with an activating agent to induce synthesis of HIV proteins.

36. The method of claim 29, wherein said cells are activated by contacting said sample with a T cell activating agent to induce synthesis of HIV proteins.
37. The method of claim 36, wherein said T cell activating agent is selected from the group consisting of a CD3, CD28, phytohaemagglutinin (PHA), phorbol 12-myristate 13 acetate (PMA), ionomycin (I), Staphylococcal enterotoxin B (SEB), TNF- α , and CD2/CD2R.
38. The method of claim 35, wherein said activating agent is an HIV protein or a fragment thereof.
39. The method of claim 38, wherein said activating agent is gp160 or a fragment thereof.
40. The method of claim 38, wherein said activating agent is gp120 or a fragment thereof.
41. The method of claim 35, wherein export of synthesized proteins is inhibited by contacting said sample with an inhibitor of HIV protein export selected from the group consisting of an inhibitor of glycosylation, an HIV protease inhibitor, an inhibitor of myristoylation, and an inhibitor of protein export.
42. The method of claim 41, wherein said inhibitor of protein export is selected from the group consisting of Brefeldin A and monensin.
43. A method of determining the frequency of HIV-infected cells of a particular cell subset that express a cell-surface protein of interest, comprising:
 contacting a sample containing said cells with at least one labeled cell subset-defining antibody, at least one labeled HIV detection reagent that is a broadly reactive HIV-specific antibody or a CD4-based HIV gp120 probe, and an antibody specific for said cell-surface protein; and
 cytometrically detecting binding of said antibody specific for said cell-surface protein and intracellular binding of said HIV detection reagent by cells in the defined cell subset.
44. The method of claim 43, wherein said step of detecting is carried out using flow cytometry.

45. The method of claim 43, wherein said CD4-based HIV gp120 detection probe is a chimeric molecule comprising the extracellular domain of CD4, or a fragment thereof that binds to HIV gp120, and the Fc region of an immunoglobulin.
46. The method of claim 43, wherein said broadly reactive HIV-specific antibody is selected from the group consisting of 2G12, IgG1b12, 447-52D, F105, 2F5, 296-13H11, 4E10 and M14.
47. The method of claim 43, wherein said at least one T cell subset-defining antibody is an antibody that binds to CD4⁺ cells.
48. The method of claim , wherein said cells are permeabilized to facilitate intracellular staining of HIV proteins.
49. The method of claim 43, wherein said cells are activated by contacting said sample with an activating agent to induce synthesis of HIV proteins.
50. The method of claim 43, wherein said cells are activated by contacting said sample with a T cell activating agent to induce synthesis of HIV proteins.
51. The method of claim 50, wherein said T cell activating agent is selected from the group consisting of a CD3, CD28, phytohaemagglutinin (PHA), phorbol 12-myristate 13 acetate (PMA), ionomycin (I), Staphylococcal enterotoxin B (SEB), TNF- α , and CD2/CD2R.
52. The method of claim 49, wherein said activating agent is an HIV protein or a fragment thereof.
53. The method of claim 49, wherein said activating agent is gp160 or a fragment thereof.
54. The method of claim 49, wherein said activating agent is gp120 or a fragment thereof.
55. The method of claim 49, wherein export of synthesized proteins is inhibited by contacting said sample with an inhibitor of HIV protein export selected from the group

consisting of an inhibitor of glycosylation, an HIV protease inhibitor, an inhibitor of myristoylation, and an inhibitor of protein export.

56. The method of claim 55, wherein said inhibitor of protein export is selected from the group consisting of Brefeldin A and monensin.

57. The method of claim 43, wherein said cell-surface protein is a chemokine receptor.

58. The method of claim 57, wherein said cell-surface protein is selected from the group consisting of CXCR4 and CCR5.