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(54) Title: METHODS AND COMPOSITIONS FOR DIAGNOSING AIDS AND OTHER DISEASES AND CONDITIONS INVOLVING IMMUNE SYSTEM ACTIVATION

(57) Abstract: This invention relates to methods and compositions suitable for monitoring the progression of AIDS, and other diseases whose progression involves immune system activation. In particular, the invention relates to the use of the TNF-Related Apoptosis-Inducing Ligand (TRAIL) and TRAIL Compounds to monitor the progression of AIDS, and other immune system diseases.

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Title of the Invention:

Methods and Compositions For Diagnosing AIDS And Other Diseases And Conditions Involving Immune System Activation

5 Field of the Invention:

This invention relates to methods and compositions suitable for monitoring the progression of AIDS, and other diseases whose progression involves immune system activation. In particular, the invention relates to the use of the TNF-Related Apoptosis-Inducing Ligand (TRAIL) and TRAIL Compounds to monitor the
10 progression of AIDS, and such other diseases.

Cross-Reference to Related Applications:

This application claims priority to United States Patent Application Serial No. 60/564,588, filed April 23, 2004 and United States Patent Application Serial No. 60/634,255, filed December 9, 2004, which applications are herein
15 incorporated by reference in their entirety.

Statement of Governmental Interest:

This invention was funded by the National Cancer Institute at the National Institutes of Health. The United States Government has certain rights to this invention.

20 Background of the Invention:**Diseases and Conditions Involving Immune System Activation**

A number of diseases and conditions are characterized by a progression that involves immune system activation. These diseases include Acquired Immunodeficiency Syndrome (AIDS), allergic asthma, Alzheimer's disease,
25 autoimmune disorders, Crohn's disease, Grave's disease, leukemia, atherosclerosis, lupus, multiple sclerosis, Parkinson's disease, transplant rejection,

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- etc. (Ringheim, G.E. *et al.* (2004) "NEURODEGENERATIVE DISEASE AND THE NEUROIMMUNE AXIS (ALZHEIMER'S AND PARKINSON'S DISEASE, AND VIRAL INFECTIONS)," J. Neuroimmunol. 147(1-2):43-49; Corry, D.B. *et al.* (2002) "BIOLOGY AND THERAPEUTIC POTENTIAL OF THE INTERLEUKIN-4/INTERLEUKIN-13 SIGNALING PATHWAY IN ASTHMA," Am. J. Respir. Med. 1(3):185-193; Fischer, A. (2004) "HUMAN PRIMARY IMMUNODEFICIENCY DISEASES: A PERSPECTIVE," Nat Immunol. 5(1):23-30; Franchini, G. *et al.* (2003) "T-CELL CONTROL BY HUMAN T-CELL LEUKEMIA/LYMPHOMA VIRUS TYPE 1," Int J Hematol. 78(4):280-296; Sandborn, W.J. *et al.* (2003) "NOVEL APPROACHES TO TREATING INFLAMMATORY BOWEL DISEASE: TARGETING ALPHA-4 INTEGRIN," Am J Gastroenterol. 98(11):2372-2382; Chitnis, T. *et al.* (2003) "ROLE OF COSTIMULATORY PATHWAYS IN THE PATHOGENESIS OF MULTIPLE SCLEROSIS AND EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS," J. Allergy Clin. Immunol. 112(5):837-850; Aw, M.M. (2003) "TRANSPLANT IMMUNOLOGY," J. Pediatr. Surg. 38(9):1275-80; Zhou, X. (2003) "CD4+ T CELLS IN ATHEROSCLEROSIS," Biomed Pharmacother. 57(7):287-291).

- The causal agent of AIDS has been found to be the retrovirus: Human Immunodeficiency Virus (HIV) (Wood, R.W. *et al.* (1987) "ACQUIRED IMMUNODEFICIENCY SYNDROME," Infect Dis Clin North Am. (1): 145-63; Gallo, R.C. *et al.* (2003) "THE DISCOVERY OF HIV AS THE CAUSE OF AIDS," N Engl J Med. 349(24): 2283-5; Holtgrave, D.R. (2004) "ESTIMATION OF ANNUAL HIV TRANSMISSION RATES IN THE UNITED STATES, 1978-2000," J Acquir Immune Defic Syndr. 35(1):89-92; Ebbesen, P.(1986) "THE GLOBAL EPIDEMIC OF AIDS," AIDS Res. (2 Suppl) 1: S23-8; Gallo, R.C.(1987) "THE AIDS VIRUS," Sci Am. 256(1):46-56; Morrow, C.D. *et al.* (1994) "VIRAL GENE PRODUCTS AND REPLICATION OF THE HUMAN IMMUNODEFICIENCY TYPE 1 VIRUS," Am J Physiol. 266(5 Pt 1): C1135-56).

- Infection with HIV is followed by a latency period, during which a progressive and slow destruction of the immune system occurs (Hessol, N.A. *et al.* (1990) "RELATIONSHIP BETWEEN AIDS LATENCY PERIOD AND AIDS SURVIVAL

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IMMUNODEFICIENCY VIRUS-INFECTED ASYMPTOMATIC INDIVIDUALS” J Exp Med 175, 331-40; Ameisen, J. C. *et al.* (1991) “CELL DYSFUNCTION AND DEPLETION IN AIDS: THE PROGRAMMED CELL DEATH HYPOTHESIS,” Immunol Today 12, 102-5). However, both CD4⁺ and CD8⁺ T cells are activated by HIV-1 infection, and none
5 of the current models adequately accounts for the preferential loss of CD4⁺ T cells (Groux, H. *et al.* (1992) “ACTIVATION-INDUCED DEATH BY APOPTOSIS IN CD4⁺ T CELLS FROM HUMAN IMMUNODEFICIENCY VIRUS-INFECTED ASYMPTOMATIC INDIVIDUALS,” J Exp Med 175, 331-40; Grossman, Z. *et al.* (2002) “CD4⁺ T-CELL DEPLETION IN HIV INFECTION: ARE WE CLOSER TO UNDERSTANDING THE CAUSE?”
10 Nat Med 8, 319-23).

AICD initiated by a variety of stimuli has been proposed as a potential mechanism for “bystander” cell killing. Among these stimuli, noninfectious viruses, which represent more than 95% of HIV-1 particles in plasma of patients, were shown to induce apoptosis of uninfected CD4⁺ and CD8⁺ T cells (Dimitrov,
15 D. S. *et al.* (1993) “QUANTITATION OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 INFECTION KINETICS,” J Virol 67, 2182-90; Piatak, M., Jr. *et al.* (1993) “HIGH LEVELS OF HIV-1 IN PLASMA DURING ALL STAGES OF INFECTION DETERMINED BY COMPETITIVE PCR,” Science 259, 1749-54; Esser, M.T. *et al.* (2001) “PARTIAL ACTIVATION AND INDUCTION OF APOPTOSIS IN CD4(+) AND CD8(+) T
20 LYMPHOCYTES BY CONFORMATIONALLY AUTHENTIC NONINFECTIOUS HUMAN IMMUNODEFICIENCY VIRUS TYPE 1,” J Virol 75, 1152-64; Esser, M.T. *et al.* (2001) “DIFFERENTIAL INCORPORATION OF CD45, CD80 (B7-1), CD86 (B7-2), AND MAJOR HISTOCOMPATIBILITY COMPLEX CLASS I AND II MOLECULES INTO HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 VIRIONS AND MICROVESICLES: IMPLICATIONS
25 FOR VIRAL PATHOGENESIS AND IMMUNE REGULATION,” J. VIROL. 75(13):6173-6182).

Unfortunately, the complexity of AIDS has encumbered efforts to monitor the progression of the disease. The timing of the onset of clinical AIDS is difficult to predict because the clinical manifestations of HIV infection are variable (Stein,
30 H. *et al.* (1991) “LYMPHOID TISSUES AND AIDS: ROLE OF LYMPHOCYTES AND

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TIME IN HOMOSEXUAL AND BISEXUAL MEN," J Acquir Immune Defic Syndr. 3(11):1078-85; Berkelman, R.L. *et al.* (1989) "EPIDEMIOLOGY OF HUMAN IMMUNODEFICIENCY VIRUS INFECTION AND ACQUIRED IMMUNODEFICIENCY SYNDROME," Am J Med. 86(6 Pt 2):761-70). The latency period is characterized
5 by a decrease in the number of helper T cells, by qualitative defects in T cell function and by a relative decrease in the rate of virus replication.

The most characteristic feature of AIDS is the selective depletion of CD4⁺ cells. These immune system cells are the targets of HIV infection, and are destroyed by the HIV virus (Lang, W.H. *et al.* (1989) "PATTERNS OF T-
10 LYMPHOCYTES CHANGES WITH HUMAN IMMUNODEFICIENCY VIRUS INFECTION FROM SEROCONVERSION TO THE DEVELOPMENT OF AIDS," J. AIDS 2:63-69; Janeway CA, *et al.* (2001) "IMMUNOBIOLOGY," Ch.11, 5th ed. New York and London: Garland Publishing; Ghani, A.C. *et al.* (2001) "SURROGATE MARKERS FOR DISEASE PROGRESSION IN TREATED HIV INFECTION," J AIDS 28(2):226-231).

15 Significantly, the pathogenic mechanisms responsible for the extensive depletion of CD4⁺ T cells, that is a characteristic of AIDS, are not well understood. Although HIV-1-infected cells die, the frequency of infected cells is too low to account for the depletion of CD4⁺ T cells (Schnittman, S. M. *et al.* (1990) "INCREASING VIRAL BURDEN IN CD4⁺ T CELLS FROM PATIENTS WITH HUMAN
20 IMMUNODEFICIENCY VIRUS (HIV) INFECTION REFLECTS RAPIDLY PROGRESSIVE IMMUNOSUPPRESSION AND CLINICAL DISEASE," Ann Intern Med 113, 438-43; Grivel, J. C. *et al.* (2000) "HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 INDUCES APOPTOSIS IN CD4(+) BUT NOT IN CD8(+) T CELLS IN EX VIVO-INFECTED HUMAN LYMPHOID TISSUE," J Virol 74, 8077-84). In order to address this disparity,
25 indirect mechanisms involving the death of uninfected CD4⁺ T cells have been proposed (Badley, A. D. *et al.* (2000) "MECHANISMS OF HIV-ASSOCIATED LYMPHOCYTE APOPTOSIS," Blood 96, 2951-64). Several reports have suggested that the Activation-Induced Cell Death ("AICD") of T lymphocytes may be a major contributor to CD4⁺ T cell depletion (Groux, H. *et al.* (1992) "ACTIVATION-
30 INDUCED DEATH BY APOPTOSIS IN CD4⁺ T CELLS FROM HUMAN

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FOLLICULAR DENDRITIC CELLS (FDC)” *Verh Dtsch Ges Pathol.* 75:4-19; Caetano, J.A. (1991) “IMMUNOLOGIC ASPECTS OF HIV INFECTION,” *Acta Med Port.* 4 Suppl 1:52S-58S; Reyes-Teran, G. *et al.* (1994) “PATHOGENESIS OF HUMAN IMMUNODEFICIENCY VIRUS INFECTION,” *Rev Invest Clin.* 1994 46(2):113-47).

5 Additionally, since there are no clinically detectable characteristics of the disease (i.e., disease “markers”) that can be universally employed to monitor the progression of the infection, disease progression must be monitored using “surrogate” markers that rely on characteristics that only generally correlate with the disease (see, Tsoukas, C.M. *et al.* (1994) “MARKERS PREDICTING PROGRESSION
10 OF HUMAN IMMUNODEFICIENCY VIRUS-RELATED DISEASE,” *Clin Microbiol Rev* 7(1):14-28). A surrogate marker should, ideally, identify patients at highest risk of disease progression, provide information on how an individual was infected, help clinicians accurately determine the stage of the HIV infection, predict development of opportunistic infections associated with the infection, and monitor the efficacy
15 of antiviral treatments. In addition, surrogate markers should be easily quantifiable, reliable, chemically available, and affordable. With respect to HIV, surrogate markers are measurable traits that correlate with disease progression from symptomless HIV infection to full blown clinical AIDS (Tsoukas, C.M. *et al.* (1994) “MARKERS PREDICTING PROGRESSION OF HUMAN IMMUNODEFICIENCY
20 VIRUS-RELATED DISEASE,” *Clin Microbiol Rev* 7(1):14-28).

 Prognostic or surrogate markers of HIV-1 disease progression that have been described include viral load, CD4⁺ T cell number, soluble immune system activation markers and T helper cell functional profiles (Ho, D. D. (1996) “VIRAL COUNTS COUNT IN HIV INFECTION,” *Science* 272:1124-1125; Mellors, J.W. *et al.*
25 (1996) “PROGNOSIS IN HIV-1 INFECTION PREDICTED BY THE QUANTITY OF VIRUS IN PLASMA,” *Science* 272 1167-1170). The number of HIV-1 particles in infected patient blood has been reported to be the best available surrogate marker of HIV-1 disease progression (Ho, D. D. (1996) “VIRAL COUNTS COUNT IN HIV INFECTION,” *Science* 272:1124-1125; Mellors, J.W. *et al.* (1996) “PROGNOSIS IN HIV-1
30 INFECTION PREDICTED BY THE QUANTITY OF VIRUS IN PLASMA,” *Science* 272:

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1167-1170). Additional prognostic surrogate markers of HIV-1 disease progression would be valuable, particularly those that investigate pathogenic mechanisms which result in CD4⁺ T cell depletion.

At present, only two surrogate markers are recognized by the United States Food & Drug Administration as clinically relevant to HIV progression: (1) the amount of viral RNA (i.e., the HIV-1 viral load) and (2) the absolute number of peripheral CD4⁺ T cells (i.e., the CD4⁺ T cell count) (see, Tsoukas, C.M. *et al.* (1994) "MARKERS PREDICTING PROGRESSION OF HUMAN IMMUNODEFICIENCY VIRUS-RELATED DISEASE," Clin Microbiol Rev 7(1):14-28; De Milito, A. *et al.* (2003) "SURROGATE MARKERS AS A GUIDE TO EVALUATE RESPONSE TO ANTIRETROVIRAL THERAPY," Curr. Med. Chem. 10(5):349-365; Scondras, D. (1997) "IT'S TIME TO APPROVE MORE SURROGATE MARKERS," AIDS Treatment News: Issue # 267; Ghani, A.C. *et al.* (2001) "SURROGATE MARKERS FOR DISEASE PROGRESSION IN TREATED HIV INFECTION," JAIDS 28(2):226-231; Mellors, J.W. *et al.* (1997) "PLASMA VIRAL LOAD AND CD4⁺ LYMPHOCYTES AS PROGNOSTIC MARKERS OF HIV-1 INFECTION," Ann Intern Med. 126(12):946-54).

The total amount of HIV virus present in a patient at a given time, i.e., the "viral load," is a particularly widely employed HIV surrogate marker (Mellors, J. *et al.* (1996) "PROGNOSIS IN HIV-1 INFECTION PREDICTED BY THE QUANTITY OF VIRUS IN PLASMA," Science 272: 1167-1170; Baker, R. (1996) "HIV VIRAL LOAD SUPERCEDES CD4 COUNT AS BEST MARKER FOR PREDICTING RISK OF AIDS AND DEATH," Bulletin of Experimental Treatments for AIDS; Pereira, R.S. (1997) "VIRAL LOAD TESTING: WHICH TEST FOR WHAT? BHIVA GUIDELINES COORDINATING COMMITTEE. BRITISH HIV ASSOCIATION GUIDELINES FOR ANTI-RETROVIRAL TREATMENT OF HIV-SEROPOSITIVE INDIVIDUALS." Lancet 349: 1086-1092). Thus, to confirm an acute HIV infection in symptomatic individuals with potential HIV risk factors, current United States guidelines recommend use of HIV RNA (viral load) tests (U.S. Public Health Service, (August 13, 2001) "GUIDELINES FOR THE USE OF ANTIRETROVIRAL AGENTS IN HIV-INFECTED ADULTS AND ADOLESCENTS").

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Unfortunately, however, present methods for assessing viral load, typically involve the use of the polymerase chain reaction (PCR) to amplify HIV-specific gene sequences, and are in some ways unsatisfactory (Craddock, M. (1995) "HIV: SCIENCE BY PRESS CONFERENCE," *Reappraising AIDS* 3(5): 1-4; Peter, J.B. *et al.* (2004) "MOLECULAR-BASED METHODS FOR QUANTIFYING HIV VIRAL LOAD," *AIDS Patient Care STDS*. 18(2):75-9; Johnson, C. "VIRAL LOAD AND THE PCR- WHY THEY CAN'T BE USED TO PROVE HIV INFECTION," *Continuum* vol. 6 (3), <http://www.virusmyth.net/aids/continuum/article8.htm>). In particular, PCR amplification is highly susceptible to cross-contamination; a drawback that has limited the clinical use of PCR in monitoring HIV infection (Schnittman, S.M. *et al* (1991) "FREQUENT DETECTION OF HIV-1-SPECIFIC MRNAS IN INFECTED INDIVIDUALS SUGGESTS ONGOING ACTIVE VIRAL EXPRESSION IN ALL STAGES OF DISEASE," *AIDS Res Hum Retroviruses* 7(4):361-7; Blattner, W.A. (1989) "Retroviruses," pp 545-592. In *Viral Infections in Humans*, third edition, edited by A Evans. Plenum Medical Book Company, New York). Additionally, viral culture methods are complicated and susceptible to contamination (Bootman, J.S. *et al.* (1992) "AN INTERNATIONAL COLLABORATIVE STUDY TO ASSESS A SET OF REFERENCE REAGENTS FOR HIV-1 PCR," *J. Vir. Meth.* 37:23; Teo, I.A. *et al.* (1995) "PCR IN SITU: ASPECTS WHICH REDUCE AMPLIFICATION AND GENERATE FALSE-POSITIVE RESULTS," *Histochem. J.* 27:660). While such methods quantitate overall viral production in a patient, they do not enumerate the number of patient cells infected (Eleopulos, E. *et al.* (1994) "IS HIV REALLY HIDING IN THE LYMPH NODES?" *Letter to Nature*; Sloand, E. *et al.* (1991) "HIV TESTING: STATE OF THE ART," *JAMA* 266:2861).

Viral load testing has helped to define the natural history and prognosis of cohorts of people with HIV infection (Rhame, F.S. *et al.* (1989) "THE CASE FOR WIDER USE OF TESTING FOR HIV INFECTION," *N Engl J Med*; 320:1248-1254; Carpenter, C.C. *et al.* (1997) "ANTIRETROVIRAL THERAPY FOR HIV INFECTION IN 1997: UPDATED RECOMMENDATION OF THE INTERNATIONAL AIDS SOCIETY - USA PANEL," *JAMA*; 278:1962-1969; Panel on Clinical Practices for Treatment of HIV Infection. (November 5, 1997) "GUIDELINES FOR THE USE OF ANTIRETROVIRAL

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AGENTS IN HIV-INFECTED ADULTS AND ADOLESCENTS,” DHHS and Henry J. Kaiser Family Foundation; Hammer, S.M. *et al.* (1997) “A CONTROLLED TRIAL OF TWO NUCLEOSIDE ANALOGUES PLUS INDINAVIR IN PERSONS WITH HUMAN IMMUNODEFICIENCY VIRUS INFECTION AND CD4 CELL COUNTS OF 200 PER CUBIC
5 MILLILITER OR LESS,” *N Engl J Med* 337:724-733). If viral load testing is not available, current treatment guidelines recommend testing for p24 antigen, a viral protein. In either case, the diagnosis is to be confirmed by antibody testing.

Testing serum for antibodies to HIV with a standard ELISA is currently the most common, cost-effective, and accurate method of screening for HIV infection
10 (Centers for Disease Control (1988) “UPDATE: SEROLOGIC TESTING FOR ANTIBODY TO HUMAN IMMUNODEFICIENCY VIRUS,” *MMWR* 36:833-840; Schwartz, J.S. *et al.* (1988) “HUMAN IMMUNODEFICIENCY VIRUS TEST EVALUATION, PERFORMANCE AND USE,” *JAMA* 259:2574-2579; Burke, D.S. *et al.* (1988) “MEASUREMENT OF THE FALSE POSITIVE RATE IN A SCREENING PROGRAM FOR HUMAN
15 IMMUNODEFICIENCY VIRUS INFECTIONS,” *N Engl J Med* 319:961-964). Rapid serum HIV antibody tests, saliva- and urine- based antibody tests, and home HIV antibody testing kits have been approved by the Food and Drug Administration (FDA) and are being marketed (Bayer, R. *et al.* (1995) “SOUNDING BOARD: TESTING FOR HIV INFECTION AT HOME,” *N Engl J Med* 332:1296-1299; Phillips,
20 K.A. *et al.* (1995) “OCCASIONAL NOTES: POTENTIAL USE OF HOME HIV TESTING,” *N Engl J Med* 332:1308-1310; Sax, P.E. *et al.* “NOVEL APPROACHES TO HIV ANTIBODY TESTING,” *AIDS Clinical Care* 9:1-6). A variation of the standard antibody test, presently approved in the U.S. only for research, is the sensitive/less sensitive or “detuned” ELISA (Mirken, B. (2001) “HIV TESTING 101,” *AIDS
25 Treatment News Issue #375 (Part 2 of 2)*). The detuned test takes advantage of the fact that antibody levels rise in a predictable pattern during roughly the first four to six months after infection, eventually reaching a plateau that often stays roughly constant for many years.

In light of the problems attending to the use of viral load and CD4⁺ T cell
30 count to diagnose AIDS, other HIV surrogate markers have been proposed. For

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example, cellular markers like HLA-DR⁺, IL-2R⁺, and T cells; as well as soluble markers like beta-2 microglobulin (β_2 -M), neopterin, soluble Interleukin receptor (sIL-2R), soluble CD4 (sCD4), and soluble CD8 (sCD8) have been proposed (Tsoukas, C.M. *et al.* (1994) "MARKERS PREDICTING PROGRESSION OF HUMAN IMMUNODEFICIENCY VIRUS-RELATED DISEASE," Clin Microbiol Rev. 7(1):14-28).
5 In addition, some biological markers like beta-2 microglobulin, neopterin, interferon, other cytokines and their receptors are being used as alternative markers (Anderson, R.E. *et al.* (1990) "LONGITUDINAL ANALYSIS OF LABORATORY MARKERS DURING HIV INFECTION," VI Intl. Conf. AIDS, San Francisco;
10 Anderson, R.E. *et al.* (1990) "USE OF β_2 -MICROGLOBULIN LEVEL AND CD4 LYMPHOCYTE COUNT TO PREDICT DEVELOPMENT OF ACQUIRED IMMUNODEFICIENCY SYNDROME IN PERSONS WITH HUMAN IMMUNODEFICIENCY VIRUS INFECTIONS," Arch. Intern. Med 150:73-77).

Unfortunately, the use of known surrogate markers to evaluate HIV
15 progression has a number of limitations. No known surrogate marker consistently reflects AIDS progression in all individuals. Additionally, there is a concern that by monitoring one surrogate marker (such as CD4⁺ titers), it is possible to overlook treatments that for some reason fail to affect the chosen marker but which would nevertheless be effective in treating the disease. For these reasons, the use of
20 combinations of surrogate markers has been proposed. For example, a study performed to test the ability of cellular markers (i.e., the number of CD4⁺ T cells, the number of CD8⁺ T cells and the ratio of CD4⁺ T cells to CD8⁺ T cells) and serologic markers (i.e., serum levels of neopterin, beta 2-microglobulin, soluble interleukin-2 receptors, IgA, and HIV p24 antigen) to predict the progression of
25 clinically acquired immunodeficiency syndrome (AIDS) revealed that the level of CD4⁺ T cells in combination with serum level of either neopterin or beta 2-microglobulin were most powerful predictors of progression to AIDS (Fahey, J.L. *et al.* (1990) "THE PROGNOSTIC VALUE OF CELLULAR AND SEROLOGIC MARKERS IN INFECTION WITH HUMAN IMMUNODEFICIENCY VIRUS TYPE 1," N Eng J Med
30 322(3):166-72). While the use of combinations of markers may address

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deficiencies associated with individual markers, the use of multiple assays is associated with a corresponding increase in assay complexity and potential error.

Although a marker must have a biologically relevant relationship to disease progression, the relationship cannot effectively be utilized in evaluating a treatment regimen unless the marker also responds quickly to effective treatment. For example, while the level of plasma HIV RNA and the absolute numbers of peripheral CD4⁺ T cells have become *de facto* reference markers, additional parameters are still needed in clinical practice in order to refine antiretroviral treatments and to discriminate adequately between apparently equivalent stages of disease (see, De Mito, A. *et al.* (2003) "SURROGATE MARKERS AS A GUIDE TO EVALUATE RESPONSE TO ANTIRETROVIRAL THERAPY," *Curr. Med. Chem.* 10 (5):349-365). To address these needs, prognostic markers of immune system activation that can be used to assess disease progression and which might be suitable for the monitoring and prognosis of antiretroviral therapies are desired (De Mito, A. *et al.* (2003) "SURROGATE MARKERS AS A GUIDE TO EVALUATE RESPONSE TO ANTIRETROVIRAL THERAPY," *Curr. Med. Chem.* 10 (5):349-365; Fahey, J.L. *et al.* (1990) "THE PROGNOSTIC VALUE OF CELLULAR AND SEROLOGIC MARKERS IN INFECTION WITH HUMAN IMMUNODEFICIENCY VIRUS TYPE 1," *N Eng J Med* 322(3):166-72; Mellors, J.W. *et al.* (1998) "PLASMA VIRAL LOAD AND CD4⁺ LYMPHOCYTES AS PROGNOSTIC MARKERS OF HIV-1 INFECTION," *Ann Intern Med.* 126(12):946-54; Cozzi Lepri, A. *et al.* (1998) "THE RELATIVE PROGNOSTIC VALUE OF PLASMA HIV RNA LEVELS AND CD4 LYMPHOCYTE COUNTS IN ADVANCED HIV INFECTION," *AIDS* 12(13):1639-43).

TNF-Related Apoptosis-Inducing Ligand (TRAIL)

TNF-Related Apoptosis-Inducing Ligand (TRAIL) is a member of the TNF superfamily. It induces apoptosis in human tumor cell lines and in virus-infected cells, but does not induce apoptosis in normal cells (Wiley, S. R. *et al.* (1995) "IDENTIFICATION AND CHARACTERIZATION OF A NEW MEMBER OF THE TNF FAMILY THAT INDUCES APOPTOSIS," *Immunity* 3, 673-682; Griffith, T. S., *et al.*

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(1998) "INTRACELLULAR REGULATION OF TRAIL-INDUCED APOPTOSIS IN HUMAN MELANOMA CELLS," J Immunol 161, 2833-28340; Herbeuval, J. P. *et al.* (2003) "MACROPHAGES FROM CANCER PATIENTS: ANALYSIS OF TRAIL, TRAIL RECEPTORS, AND COLON TUMOR CELL APOPTOSIS," J Natl Cancer Inst 95, 611-621; Jeremias, I. *et al.* (1998) "TRAIL/APO-2-LIGAND-INDUCED APOPTOSIS IN HUMAN T CELLS," Eur J Immunol 28, 143-152; Clarke, P. *et al.* (2003) "TWO DISTINCT PHASES OF VIRUS-INDUCED NF-KAPPA-B- REGULATION ENHANCE TRAIL-MEDIATED APOPTOSIS IN VIRUS-INFECTED CELLS," J Biol Chem; Gura, T. (1997) "HOW TRAIL KILLS CANCER CELLS, BUT NOT NORMAL CELLS," Science 277:768). TRAIL has been implicated in apoptosis of multiple cell types in HIV-infected patients, including CD4⁺ and CD8⁺ T cells (Lum, J. J. *et al.* (2001) "INDUCTION OF CELL DEATH IN HUMAN IMMUNODEFICIENCY VIRUS-INFECTED MACROPHAGES AND RESTING MEMORY CD4 T CELLS BY TRAIL/APO21," J Virol 75, 11128-11136; Katsikis, P. D. *et al.* (1997) "INTERLEUKIN-1 BETA CONVERTING ENZYME-LIKE PROTEASE INVOLVEMENT IN FAS-INDUCED AND ACTIVATION-INDUCED PERIPHERAL BLOOD T CELL APOPTOSIS IN HIV INFECTION. TNF-RELATED APOPTOSIS-INDUCING LIGAND CAN MEDIATE ACTIVATION-INDUCED T CELL DEATH IN HIV INFECTION," J Exp Med 186, 1365-1372; Jeremias, I. *et al.* (1998) "TRAIL/APO-2-LIGAND-INDUCED APOPTOSIS IN HUMAN T CELLS," Eur J Immunol 28, 143-152).

The two biologically active forms of TRAIL, membrane-bound (mTRAIL) and soluble TRAIL (sTRAIL), are regulated by type I interferon (interferon-alpha and beta: IFN- α and IFN- β) (Sato, K. *et al.* (2001) "ANTIVIRAL RESPONSE BY NATURAL KILLER CELLS THROUGH TRAIL GENE INDUCTION BY IFN-ALPHA/BETA," Eur J Immunol. 31:3138-3146). TRAIL can be secreted by leukocytes, including T lymphocytes, natural killer cells (Smyth, M.J. *et al.* (2001) "TUMOR NECROSIS FACTOR-RELATED APOPTOSIS-INDUCING LIGAND (TRAIL) CONTRIBUTES TO INTERFERON GAMMA-DEPENDENT NATURAL KILLER CELL PROTECTION FROM TUMOR METASTASIS," J Exp Med. 2001;193:661-670), dendritic cells (Vidalain, P.O. *et al.* (2000) "MEASLES VIRUS INDUCES FUNCTIONAL

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TRAIL PRODUCTION BY HUMAN DENDRITIC CELLS," J Virol. 74:556-559;
Vidalain, P.O. *et al.* "MEASLE VIRUS-INFECTED DENDRITIC CELLS DEVELOP
IMMUNOSUPPRESSIVE AND CYTOTOXIC ACTIVITIES," Immunobiology 204:629-
638), monocytes and macrophages (Herbeuval, J.P. *et al.* (2003) "MACROPHAGES
5 FROM CANCER PATIENTS: ANALYSIS OF TRAIL, TRAIL RECEPTORS, AND COLON
TUMOR CELL APOPTOSIS," J Natl Cancer Inst. 95:611-621). However, the role of
TRAIL in CD4+ T cell depletion in vivo during progression to AIDS remains to be
established. Several reports have shown that macrophages and activated monocytes
can play a role in CD4+ T cell depletion (Sperber, K. *et al.* (2003) "INDUCTION OF
10 APOPTOSIS BY HIV-1-INFECTED MONOCYTIC CELLS," J Immunol. 70:1566-1578;
Badley, A.D. *et al.* (1997) "MACROPHAGE-DEPENDENT APOPTOSIS OF CD4+ T
LYMPHOCYTES FROM HIV-INFECTED INDIVIDUALS IS MEDIATED BY FASL AND
TUMOR NECROSIS FACTOR. J Exp Med. 185:55-64; Miura, Y. *et al.* (2003) "TNF-
RELATED APOPTOSIS-INDUCING LIGAND (TRAIL) INDUCES NEURONAL APOPTOSIS
15 IN HIV-ENCEPHALOPATHY," J Med Dent Sci. 50:17-25). HIV-1 infection activates
circulating monocytes by inducing IFN- α (Zagury, D. *et al.* (1998) "Interferon
alpha and Tat involvement in the immunosuppression of uninfected T cells and C-
C chemokine decline in AIDS," Proc Natl Acad Sci U S A. 95:3851-3856) and
secretion of apoptotic cytokines such as tumor necrosis factor alpha (TNF- α),
20 CD30L, and FasL (Oyaizu, N. *et al.* (1997) "MONOCYTES EXPRESS FAS LIGAND
UPON CD4 CROSS-LINKING AND INDUCE CD4+ T CELLS APOPTOSIS: A POSSIBLE
MECHANISM OF BYSTANDER CELL DEATH IN HIV INFECTION," J Immunol.
1997;158:2456-2463). Furthermore, antigen-presenting cells such as monocytes
and dendritic cells have been implicated in AIDS pathogenesis (Badley, A.D. *et al.*
25 (2000) "Mechanisms of HIV-associated lymphocyte apoptosis," Blood 96:2951-
29643; Badley, A.D. *et al.* (1997) "MACROPHAGE-DEPENDENT APOPTOSIS OF
CD4+ T LYMPHOCYTES FROM HIV-INFECTED INDIVIDUALS IS MEDIATED BY FASL
AND TUMOR NECROSIS FACTOR. J Exp Med. 185:55-64).

Soluble TRAIL ("sTRAIL") is upregulated in HIV-1-infected patients
30 (Liabakk, N.B. *et al.* (2002) "DEVELOPMENT, CHARACTERIZATION AND USE OF
MONOCLONAL ANTIBODIES AGAINST STRAIL: MEASUREMENT OF STRAIL BY

ELISA,” *J Immunol Methods*. 259(1-2):119-28) and in vitro studies have linked TRAIL to the depletion of T cells from HIV-1-infected patients (Lum, J. J. *et al.* (2001) “INDUCTION OF CELL DEATH IN HUMAN IMMUNODEFICIENCY VIRUS-INFECTED MACROPHAGES AND RESTING MEMORY CD4 T CELLS BY TRAIL/APO21,” *J Virol* 75, 11128-36; Yang, Y. (2003) “MONOCYTES TREATED WITH HUMAN IMMUNODEFICIENCY VIRUS TAT KILL UNINFECTED CD4+ CELLS BY A TUMOR NECROSIS FACTOR-RELATED APOPTOSIS-INDUCED LIGAND-MEDIATED MECHANISM,” *J. Virol* 77: 6700-6708). Nonetheless, the identification of TRAIL-producing cells in vivo and the mechanism responsible for TRAIL-mediated T cell death remain to be established.

Among the five TRAIL receptors described, only TRAIL-R1 death receptor 4 (DR4) and TRAIL-R2 (DR5) induce cell death upon ligation (Golstein, P. (1997) “CELL DEATH: TRAIL AND ITS RECEPTORS,” *Curr Biol* 7, R750-3; Pan, G. *et al.* (1997) “THE RECEPTOR FOR THE CYTOTOXIC LIGAND TRAIL,” *Science* 276, 111-3). DR5 gene is regulated by the transcription factor p53 which can be upregulated by type I interferons (Wu, G. S. *et al.* (1997) “KILLER/DR5 IS A DNA DAMAGE-INDUCIBLE P53-REGULATED DEATH RECEPTOR GENE,” *Nat Genet* 17, 141-3; Takaoka, A. *et al.* (2003) “INTEGRATION OF INTERFERON-ALPHA/BETA SIGNALING TO P53 RESPONSES IN TUMOUR SUPPRESSION AND ANTIVIRAL DEFENSE,” *Nature* 424, 516-23). The other receptors, TRAIL-R3 decoy receptor 1 (DcR1), TRAIL-R4 (DcR2), and osteoprotegerin (OPG) lack a death domain and do not trigger apoptosis (Degli-Esposti, M. A. *et al.* (1997) “THE NOVEL RECEPTOR TRAIL-R4 INDUCES NF-KAPPAB AND PROTECTS AGAINST TRAIL-MEDIATED APOPTOSIS, YET RETAINS AN INCOMPLETE DEATH DOMAIN,” *Immunity* 7, 813-20). The amino acid sequence of TRAIL is known, as is the N-terminal cytoplasmic domain (residues TRAIL residues 38-281), which comprises sTRAIL. sTRAIL production is presently thought to involve a shedding mechanism that involves cleavage by proteases (see, Liabakk, N.B. *et al.* (2002) “DEVELOPMENT, CHARACTERIZATION AND USE OF MONOCLONAL ANTIBODIES AGAINST sTRAIL: MEASUREMENT OF sTRAIL BY ELISA,” *J Immunol Methods*. 259(1-2):119-28; Martinez-Lorenzo, M.J. *et al.* (1999) “ACTIVATED T CELLS RELEASE BIOACTIVE FAS LIGAND AND

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APO2 LIGAND IN MICROVESICLES," J. Immunol. 163:1274-1281; Mariani, S.M. *et al.* (1998) "SURFACE EXPRESSION OF TRAIL/APO-2 LIGAND IN ACTIVATED MOUSE T AND B CELLS," Eur. J. Immunol. 28(5):1492-1498; Mariani, S.M. *et al.* (1998) "DIFFERENTIAL REGULATION OF TRAIL AND CD95 LIGAND IN TRANSFORMED
5 CELLS OF THE T AND B LYMPHOCYTE LINEAGE," Eur J Immunol. 28(3):973-982).

In sum, despite progress in the use of surrogate markers, such as viral load and CD4⁺ T cell count, to evaluate the progression of AIDS and other diseases whose progression involves immune system activation, a need remains for a method for more effectively monitoring the progression of such diseases. The
10 present invention is directed to such need.

Summary of the Invention:

This invention relates to methods and markers for monitoring the progression of AIDS, and other diseases whose progression involves immune system activation. In particular, the invention relates to the use of the TNF-Related
15 Apoptosis-Inducing Ligand (TRAIL) and TRAIL Compounds to monitor the progression of HIV infection, and to diagnose AIDS and other immune system diseases. Preferably, this is accomplished by assessing the presence or concentration of TRAIL and especially mTRAIL, sTRAIL, the TRAIL DR5 receptor molecule, and biological molecules that activate TRAIL or its receptor or
20 that are expressed by cells that express activated TRAIL or TRAIL DR5 receptor molecules in an individual's blood or other bodily fluids. These biological molecules include p53, α -interferon and β -interferon (IFN α/β). The invention further contemplates assessing the presence or concentration of molecules that are not TRAIL Compounds (as that term is defined herein) in an individual's blood or
25 other bodily fluids in concert with an assessment of the presence or concentration of one or more TRAIL Compound(s) in order to determine the presence or severity of a disease involving immune system activation. Such additional compounds include CD69 and HLA-DR.

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The present invention thus provides a new surrogate marker that has mechanistic implications for HIV-1 pathogenesis, and which directly correlates with viral load but not necessarily inversely with CD4⁺ T cell count. Soluble TRAIL produced by monocytes can interact with TRAIL death receptors that can be induced by CD 4⁺ T cells, and contribute to apoptosis-induced loss of CD4⁺ T cells in progression to AIDS. The present invention thus permits, by measuring levels of the TNF-Related Apoptosis-Inducing Ligand, one to distinguish among HIV-1 infected patients with high viral load, HIV-1 infected patients with low viral loads and uninfected healthy individuals. The invention provides an advantage over other surrogate marker (e.g., viral load measurement and CD4⁺ T cell count) in monitoring the progression of retroviral infection in that TRAIL is a death molecule involved in CD4⁺ T cell depletion in HIV/AIDS. Furthermore, TRAIL is a marker of immune function that directly correlates with HIV viral load.

In detail, the invention provides a method for determining whether a mammal suffers from a disease or condition of involving immune system activation, wherein the method comprises assaying for the presence or concentration of a TRAIL Compound in a biological fluid of the mammal.

The invention particularly concerns the embodiment of such method wherein the mammal is selected from the group consisting of a human, simian, feline, bovine, equine, canine, ovine or porcine mammal.

The invention particularly concerns the embodiments of such methods wherein the method comprises an immunoassay that determines the presence or concentration of the TRAIL Compound, the immunoassay comprising the steps of:

- (a) contacting a sample of the biological fluid with an antibody specific for the TRAIL Compound, the contacting being under conditions sufficient to permit the TRAIL Compound if present in the sample to bind to the antibody and form a TRAIL Compound – antibody complex;

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- (b) contacting the formed TRAIL Compound – antibody complex with a molecule capable of specific binding to the complex, the contacting being under conditions sufficient to permit the molecule to bind to the complex and form an extended complex; and
- 5 (c) determining the presence or concentration of the TRAIL Compound in the biological fluid by determining the presence or concentration of the formed extended complex in the sample.

The invention particularly concerns the embodiments of such methods wherein the TRAIL Compound is selected from the group consisting of sTRAIL,
10 mTRAIL, the TRAIL DR5 receptor molecule, a biological molecule that activates TRAIL, and a biological molecule that activates the TRAIL DR5 receptor (especially, wherein the biological molecule that activates the TRAIL DR5 receptor is p53.

The invention particularly concerns the embodiments of such methods
15 wherein the method reveals the presence of the TRAIL Compound, or wherein the method reveals the concentration of the TRAIL Compound.

The invention particularly concerns the embodiments of such methods wherein the disease or condition involving immune system activation is selected from the group consisting of HIV infection, AIDS, cancer, atherosclerosis,
20 Alzheimer's disease, inflammation, autoimmune disorder, allergic asthma, Crohn's disease, Grave's disease, lupus, multiple sclerosis, Parkinson's disease, allograft transplant rejection, and graft vs host disease. The invention further concerns the embodiments of such methods wherein the disease or condition involving immune system activation is cancer, and particularly colon cancer, lung cancer, breast
25 cancer, pancreatic cancer, leukemia, myeloma, skin cancer, brain cancer, cervical cancer, or testicular cancer.

The invention additionally concerns an immunoassay that determines the presence or concentration of a TRAIL Compound in a biological fluid of a mammal, wherein the immunoassay comprises the steps of:

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- 5 (a) contacting a sample of the biological fluid with an antibody specific for the TRAIL Compound, the contacting being under conditions sufficient to permit the TRAIL Compound if present in the sample to bind to the antibody and form a TRAIL Compound – antibody complex;
- (b) contacting the formed TRAIL Compound – antibody complex with a molecule capable of specific binding to the complex, the contacting being under conditions sufficient to permit the molecule to bind to the complex and form an extended complex; and
- 10 (c) determining the presence or concentration of the TRAIL Compound in the biological fluid by determining the presence or concentration of the formed extended complex in the sample.

The invention additionally concerns the embodiment of such immunoassay wherein the TRAIL Compound is selected from the group consisting of sTRAIL, 15 mTRAIL, the TRAIL DR5 receptor molecule, a biological molecule that activates TRAIL, and a biological molecule that activates the TRAIL DR5 receptor (especially, wherein the biological molecule that activates the TRAIL DR5 receptor is p53

The invention additionally concerns the embodiment of such immunoassay 20 wherein the mammal is selected from the group consisting of a human, simian, feline, bovine, equine, canine, ovine or porcine mammal.

The invention additionally concerns the embodiments of such immunoassays wherein the immunoassay is an immunochromatographic immunoassay, wherein:

25 in the step (a), the biological sample is placed in contact with a first porous carrier, the first porous carrier containing a non-immobilized, labeled antibody specific for the TRAIL Compound;

in the step (b), the formed TRAIL Compound– antibody complex is placed in contact with a second porous carrier, the second porous carrier being in

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communication with the first porous carrier, and containing an immobilized molecule capable of specific binding to the complex; and

in the step (c), the presence or concentration of the TRAIL Compound in the biological fluid is determined by detecting the presence of the labeled antibody
5 specific for the TRAIL Compound in the second porous carrier.

The invention additionally concerns the embodiments of such immunoassays wherein the immunoassay reveals the presence of the TRAIL Compound or wherein the immunoassay reveals the concentration of the TRAIL Compound.

10 The invention additionally concerns the embodiments of such immunoassays wherein the immobilized molecule capable of specific binding to the complex is an rsTRAIL molecule.

The invention additionally concerns the embodiments of such immunoassays wherein the TRAIL Compound is sTRAIL, and the immobilized
15 molecule capable of specific binding to the complex is an anti-sTRAIL antibody.

The invention additionally concerns the embodiments of such immunoassays wherein the immunoassay determines whether a mammal suffers from a disease or condition involving immune system activation selected from the group consisting of HIV infection, AIDS, cancer, atherosclerosis, Alzheimer's
20 disease, inflammation, autoimmune disorder, allergic asthma, Crohn's disease, Grave's disease, lupus, multiple sclerosis, Parkinson's disease, allograft transplant rejection, and graft vs host disease. The invention further concerns the embodiments of such methods wherein the disease or condition involving immune system activation is cancer, and particularly colon cancer, lung cancer, breast
25 cancer, pancreatic cancer, leukemia, myeloma, skin cancer, brain cancer, cervical cancer, or testicular cancer.f

The invention additionally concerns a kit for measuring the presence or concentration of a TRAIL Compound in a biological fluid of a mammal, wherein

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the kit comprises a hollow casing comprising a multilayer filter system, and first and second porous carriers, wherein the second porous carrier is in communication with the first porous carrier, and the first porous carrier is in communication with the multilayer filter system, a portion of which is accessible from the casing;

5 wherein:

the first porous carrier contains a non-immobilized, labeled antibody that specifically binds the TRAIL Compound; and

the second porous carrier contains an immobilized, unlabeled molecule that binds to a TRAIL Compound – antibody complex.

10 The invention additionally concerns the embodiments of such kits wherein the kit permits the presence of the TRAIL Compound or the concentration of the TRAIL Compound to be determined.

The invention additionally concerns the embodiments of such kits wherein the immobilized molecule capable of specific binding to the complex is an

15 rsTRAIL molecule.

The invention additionally concerns the embodiments of such kits wherein the TRAIL Compound is selected from the group consisting of sTRAIL, mTRAIL, the TRAIL DR5 receptor molecule, a biological molecule that activates TRAIL, and a biological molecule that activates the TRAIL DR5 receptor (especially,
20 wherein the biological molecule that activates the TRAIL DR5 receptor is p53) , and the immobilized molecule capable of specific binding to the complex is an anti-sTRAIL antibody.

The invention additionally concerns the embodiments of such kits wherein the kit is suitable for determining whether a mammal suffers from a disease or
25 condition of involving immune system activation selected from the group consisting of HIV infection, AIDS, cancer, atherosclerosis, Alzheimer's disease, inflammation, autoimmune disorder, allergic asthma, Crohn's disease, Grave's disease, lupus, multiple sclerosis, Parkinson's disease, allograft transplant rejection, and graft vs host disease. The invention further concerns the

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embodiments of such kits wherein the disease or condition involving immune system activation is cancer, and particularly colon cancer, lung cancer, breast cancer, pancreatic cancer, leukemia, myeloma, skin cancer, brain cancer, cervical cancer, or testicular cancer.

5 In light of the findings of the present invention that antibodies against IFN- α/β reduced CD4⁺ T cell death, and blocked the effect of each of the molecules required for inducing TRAIL-mediated apoptosis, one aspect of the present invention concerns the recognition that anti-type I interferon antibodies can be used as an anti-HIV-1 therapeutic agent.

10 **Brief Description of the Figures:**

Figure 1 shows increased plasma levels of TRAIL in AIDS patients.

Figure 1, Panel A shows plasma samples from 107 HIV-1-infected patients and 53 uninfected controls which are tested for their soluble TRAIL content by ELISA.

Two groups of HIV-1-infected patients were defined depending on their viral load.

15 The mean values of plasma TRAIL were 852 $\pm$ 52 pg/ml for 55 control donors, 1339 $\pm$ 79 pg/ml for 49 HIV-1-infected patients with undetectable viral load (<50 RNA copies/ml of blood) and 2242 $\pm$ 131 pg/ml for 58 HIV-1-infected patients with higher viral load (>50 RNA copies/ml of blood). P = p values from unpaired *t* test.

Figure 1, Panel B shows correlation between TRAIL levels observed in patients

20 and TRAIL levels predicted by the invention. **Figure 1, Panel C** presents longitudinal data of four HIV-1-infected patients who, at time = 0, begin antiretroviral therapy. Patients were followed for 40 weeks. TRAIL level was measure by ELISA. Data are representative of 4 different group of the 8 patients tested.

25 **Figure 2** shows effects of AT-2 HIV-1 particles on TRAIL secretion.

Figure 2, Panel A shows PBMC from HIV-1 uninfected or HIV-1 infected individuals cultured for 3 days in the presence or absence of AT-2 HIV-1_{MN} or HIV-1_{Ada}. Levels of TRAIL are detected using ELISA. **Figure 2, Panel B** shows CD4⁺, CD8⁺ T cells, DC, macrophages and monocytes from infected and

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noninfected individuals cultured for 3 days in presence of AT-2 HIV-1_{MN}. Mean values (+/- standard errors) are shown for six independent experiments for each condition tested. **Figure 2, Panel C** shows monocytes from HIV-1 uninfected donors cultured for 1 day in the presence of microvesicles (controls) or AT-2 HIV-1_{MN}. Cells are analyzed for mTRAIL by FACS. Monocytes exposed to AT-2 HIV-1_{MN} exhibit increased expression of mTRAIL compared to controls (p<0.0001). Data are representative of 6 independent experiments performed. **Figure 2, Panel D** shows monocytes from HIV-1 uninfected donors cultured for 1 day in the presence of microvesicles or AT-2 HIV-1_{MN}. TRAIL mRNA level quantified by real time PCR. Mean values with standard errors are shown for four independent experiments. **Figure 2, Panel E** shows monocytes from HIV-1 uninfected donors cultured in the presence of different concentration of AT-2 HIV-1_{MN} and sTRAIL quantified by ELISA. Mean values with standard errors are shown for six independent experiments.

Figure 3 shows effect of IFN type I antibodies on TRAIL production by monocytes from HIV-1 uninfected donors. **Figure 3, Panel A** shows monocytes cultured for 3 days in the presence of AT-2 HIV-1_{MN} with blocking antibodies against IFN- α , IFN- β or both. The level of sTRAIL is quantified by ELISA. **Figure 3, Panel B** shows monocytes cultured for 1 day in the presence of AT-2 HIV-1_{MN}, with or without blocking antibodies against IFN- α and IFN- β . The level of TRAIL mRNA is quantified by real time PCR analysis. **Figure 3, Panel C** shows monocytes cultured for 3 days in the presence of recombinant IFN- α or IFN- β . The level of sTRAIL is quantified by ELISA. Mean values with standard errors presented in **Figure 3, Panels A, B, and C** are representative of four independent experiments for each condition are tested. **Figure 3, Panel D** shows IFN- α levels by monocytes cultured for 1 (open bars) or 3 (closed bars) days in presence of microvesicles (control), AT-2 HIV-1_{MN} or AT-2 HIV-1_{Ada}. **Figure 3, Panel E** shows STAT1 and STAT2 expression. Monocytes were cultured 24h in the presence of AT-2 HIV-1_{MN}, AT-2 HIV-1_{Ada} or recombinant IFN- α/β (10 ng/mL) and production of STAT1 and STAT2 was analyzed by western-

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blot. β -actin was used as a loading control. **Figure 3, Panel F** shows the results of a blocking assay: monocytes were cultured for 3 days with AT-2 HIV-1_{MN} and in presence of: (1) isotype control antibody; (2) the CD4 binding inhibitor soluble CD4 (2 μ g/ml); (3) the CXCR4 inhibitor AMD-3100 (2 μ g/ml); (4) the fusion inhibitor T20 (2 μ g/ml). Monocytes cultured without AT-2 HIV-1_{MN} were used as control. TRAIL level was quantified by ELISA. Mean values with standard errors are shown for 3 independent experiments for each condition tested.

Figure 4 shows effect of infectious HIV-1 on TRAIL production by monocytes. **Figure 4, Panel A** shows monocytes cultured for 3 days in the presence of AT-2 HIV-1_{MN}, HIV-1_{MN} or infectious HIV-1_{LAV}. sTRAIL level is determined by ELISA. Mean values with standard errors are shown for six independent experiments for each condition tested. **Figure 4, Panel B** shows monocytes from HIV-1- donors cultured 3 days in the presence of live HIV-1_{MN} with blocking antibodies against IFN α , IFN β or both. The level of sTRAIL is quantified by ELISA. Mean values with standard errors are shown for 6 independent experiments for each condition tested. **Figure 4, Panel C** shows monocytes from HIV-1⁻ donors cultured for 1 day in the presence of microvesicles (controls) or live HIV-1_{MN}. Cells are analyzed for mTRAIL by FACS. Monocytes exposed to HIV-1_{MN} overexpressed mTRAIL compared to controls ($p < 0.0001$). Data are representative of 6 independent experiments performed.

Figure 5 shows effect of infectious HIV-1 on sTRAIL production in tonsil culture. Ex vivo human lymphoid tissue were culture for 12 days in presence or absence of HIV-1_{LAV}. sTRAIL was measured at day 3, 6, 9 and 12 by ELISA. Culture medium was changed at 3-day intervals.

Figure 6 shows an analysis of TRAIL and DR5 in HIV-1-infected patients and HIV-1-uninfected controls: **Panel A:** DR5 mRNA analysis of PBMC from 9 HIV-1-uninfected controls and 16 HIV-1-infected patients. Horizontal bars indicates mean values that were significantly different, $p = 0.03$. **Panel B:** Plasma TRAIL levels and flow cytometric analysis of Annexin V⁺ DR5⁺ CD4⁺ T cells

from 16 uninfected control donors and 22 HIV-1-infected patients. *p* values were calculated using a two-tailed Student's *t* test. **Panel C:** Linear regression analysis of DR5 mediated cell death (DR5⁺ AnnexinV⁺) among apoptotic cells (AnnexinV⁺) was performed in the same cohort of patients (n=22) and controls (n=16).

Figure 7 shows annexin V and STAT expression in T cells: **Figure 7, Panel A:** Flow cytometric analysis of positively-selected CD4⁺ and CD8⁺ T cells from healthy, HIV-1-uninfected blood donors cultured for 1 (left) and 6 (right) days alone or in the presence of microvesicles (controls) or with: HIV-1_{MN}; HIV-1_{Ada}; influenza A virus (Flu); HIV-1_{MN} plus Flu; or HIV-1_{Ada} plus Flu. Infectious HIV-1 was used in the example illustrated. Very similar results were obtained when AT-2 HIV-1 was used. * represents *p* values <0.05 between treated T cells (HIV-1, and HIV-1⁺Flu) compared to control T cells, using a two-tailed Student's *t* test. **Figure 7, Panel B:** Annexin V/Propidium double-positive cells resulting from culture of CD4⁺ or CD8⁺ T cells with AT-2 HIV-1_{MN} plus Flu or AT-2 HIV-1_{Ada} plus Flu. **Figure 7, Panel C:** Effect of IFN- α/β -specific antibodies on inhibition of apoptosis in CD4⁺ T cells cultured with infectious (HIV-1) or noninfectious (AT-2 HIV-1) plus Flu. Effect of culturing CD4⁺ T cells with Flu, AT-2 HIV-1_{MN} or AT-2 HIV-1_{MN} plus Flu on IFN- α production (**Figure 7, Panel D**) and phosphorylated STAT1 activation (**Figure 7, Panel E**). **Figure 7, Panel F:** Western blot analysis of STAT1 and STAT2 protein after exposure of CD4⁺ T cells to AT-2 HIV-1_{MN}, Flu, AT-2 HIV-1_{MN} plus Flu, and effect of anti-IFN- α/β antibodies on STAT expression. Data of **Figure 7, Panel A** are mean values with standard errors of 6 independent experiments for each condition tested. Data of **Figure 7, Panels B, C, D, E and F** are representative of 4 independent experiments.

Figure 8 shows an analysis of TRAIL expression and production: **Figure 8, Panel A:** Flow cytometric analysis of CD4⁺ and CD8⁺ T cells cultured with Flu, infectious HIV-1_{MN}, or HIV-1_{MN} plus Flu. **Figure 8, Panel B:** TRAIL gene expression after culture of CD4⁺ T cells with AT-2 HIV-1_{MN} plus Flu, and effect of

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anti-IFN- α/β antibodies. **Figure 8, Panel C:** Effect of anti-IFN- α/β antibodies on mTRAIL expression induced by culture with AT-2 HIV-1_{MN} plus Flu. **Figure 8, Panel D:** Effect of culturing CD4⁺ T cells with IFN- α , IFN- β or IFN- α/β on the percentage of CD4⁺ T cells expressing mTRAIL. **Figure 8, Panel E:** Western blot analysis of TRAIL production after culture of CD4⁺ T cells with AT-2 HIV-1_{MN}, Flu, AT-2 HIV-1_{MN} plus Flu, AT-2 HIV-1_{MN} plus Flu plus anti-IFN- α/β antibodies, or rIFN- α/β . **Figure 8, Panel F:** Effect of anti-IFN- α/β antibodies, monoclonal RIK-2 anti-TRAIL antibody, anti-DR5 antibody, and soluble CD4-IgG (sCD4) on the percentage of Annexin V⁺ cells resulting from culture of CD4⁺ T cells with AT-2 HIV-1_{MN} plus Flu. Data of panel **Figure 8, Panels B and F** are mean values with standard errors of 6 independent experiments for each condition tested. Data of panels **Figure 8, Panels A, C, D and E** are representative of 4 independent experiments.

Figure 9 shows DR5 expression and p53 transcription and synthesis:

Figure 9, Panel A: Flow cytometric analysis of DR5 expression on CD4⁺ and CD8⁺ T cells after culture with Flu, infectious HIV-1_{MN}, or Flu plus infectious HIV-1_{MN} (Identical results were obtained using AT-2 HIV-1_{MN}). **Figure 9, Panel B:** Two-color flow cytometric analysis of Annexin V/DR5 double positive CD4⁺ T cells cultured alone (control) or with AT-2 HIV-1_{MN} plus Flu. **Figure 9, Panel C:** DR5 gene expression in CD4⁺ T cells cultured with AT-2 HIV-1_{MN} plus Flu, and inhibition by anti-IFN- α/β antibodies. **Figure 9, Panel D:** Flow cytometric analysis of: AT-2 HIV-1_{MN} plus Flu-induced DR5 expression by CD4⁺ T cells and inhibition by anti-IFN- α/β antibodies (upper) and lack of effect of recombinant IFN- α/β plus HIV-1_{MN} on DR5 expression (middle) and apoptosis (lower) of CD4⁺ T cells. **Figure 9, Panel E:** Western blot analysis of DR5 protein production by CD4⁺ T cells cultured with AT-2 HIV-1_{MN}, Flu, AT-2 HIV-1_{MN} plus Flu or rIFN- α/β , and inhibition of DR5 by anti-IFN- α/β antibodies. **Figure 9, Panel F:** Effect of culture of CD4⁺ T cells with AT-2 HIV-1_{MN} plus Flu on p53 gene expression and inhibition by anti-IFN- α/β antibodies. **Figure 9, Panel G:** Effect of culture of CD4⁺ T cells with AT-2 HIV-1_{MN}, Flu, or AT-2 HIV-1_{MN} plus Flu on p53 protein

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production. **Figure 9, Panel H:** Western blot analysis of p53 production by CD4⁺ T cells cultured with AT-2 HIV-1_{MN}, Flu, AT-2 HIV-1_{MN} plus Flu or rIFN- α/β , and inhibition by anti-IFN- α/β antibodies. Data of **Figure 9, Panels C, F and G** are mean values with standard errors of 6 independent experiments for each condition tested. Data of **Figure 9, Panels A, B, D, E and H** are representative of 4 independent experiments.

Figure 10 illustrates a “Two-Hit” model of type I interferon-dependent TRAIL~DR5-mediated selective apoptosis of CD4⁺ T cells in AIDS progression. **Top:** Exposure of CD4⁺ T cells to either infectious or noninfectious HIV-1 or to other viruses induces expression of mTRAIL and activation of STAT1 but not apoptosis. **Bottom:** Activation of STAT2 and expression of p53 and DR5 requires two hits that involve either infectious or noninfectious HIV-1 (first hit) plus a second event or virus (second hit) (our examples: influenza or measles viruses). Exposure of CD4⁺ T cells to both hits is required to activate STAT2 (also increased STAT1) and p53 that results in DR5 expression. Each of these virus-induced events is blocked by antibodies against IFN- α/β . None of the above virus-induced changes were observed in CD8⁺ T cells. Because rIFN- α/β did not replace the second virus for inducing p53, DR5 and apoptosis, “?” indicates a signal or event of the two-hit model (that triggers p53 and DR5). pTRAIL = plasma TRAIL, mTRAIL = membrane TRAIL.

Description of the Preferred Embodiments:

As discussed above, only two surrogate markers (i.e., HIV-1 viral load and CD4⁺ T cell count) have previously been recognized as sufficiently correlative of AIDS progression to be employed clinically. The present invention relates to methods and compositions suitable for monitoring the progression of AIDS, and other diseases whose progression involves immune system activation.

One aspect of the present invention derives from the recognition that the “TNF-Related Apoptosis-Inducing Ligand” (“TRAIL”) Compounds can be employed as a surrogate marker for the progression of AIDS and other diseases

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whose progression involves immune system activation. The positive correlation between sTRAIL and HIV progression (i.e. the marker directly correlates with viral load but not with CD4 count) has mechanistic implications for HIV-1 pathogenesis. Because of the selectivity of TRAIL and its receptor for CD4⁺ T cells, the use of TRAIL Compounds as surrogate marker(s) for evaluating the progression of AIDS and other diseases of immune system activation provides an advantage over viral load and CD4 determinations.

The depletion of CD4⁺ T cells that results from HIV-1 infection remains unsolved despite more than 20 years of investigation of AIDS pathogenesis. The percentage of CD4⁺ T cells that is HIV-1 infected prior to and during the early decrease in CD4⁺ T cell numbers cannot account for the severe loss of this cell population (Grivel, J.C. *et al.* (2000) "HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 INDUCES APOPTOSIS IN CD4(+) BUT NOT IN CD8(+) T CELLS IN EX VIVO-INFECTED HUMAN LYMPHOID TISSUE," J Virol. 74:8077-8084; Schnittman, S.M. *et al.* (1990) "INCREASING VIRAL BURDEN IN CD4⁺ T CELLS FROM PATIENTS WITH HUMAN IMMUNODEFICIENCY VIRUS (HIV) INFECTION REFLECTS RAPIDLY PROGRESSIVE IMMUNOSUPPRESSION AND CLINICAL DISEASE," Ann Intern Med. 113:438-443). Other studies have implicated "bystander" apoptotic death of activated, uninfected T cells during progression to AIDS (Badley, A.D. *et al.* (2000) "Mechanisms of HIV-associated lymphocyte apoptosis," Blood 96:2951-2964). TNF-related apoptosis-inducing ligand (TRAIL), a member of the TNF superfamily (Wiley, S.R. *et al.* (1995) "IDENTIFICATION AND CHARACTERIZATION OF A NEW MEMBER OF THE TNF FAMILY THAT INDUCES APOPTOSIS," Immunity 3:673-682), may be involved in CD4⁺ T cell depletion, since TRAIL, produced by monocytes exposed to the HIV-1 Tat protein, killed uninfected CD4⁺ T cells (Yang, Y. *et al.* (2003) "MONOCYTES TREATED WITH HUMAN IMMUNODEFICIENCY VIRUS TAT KILL UNINFECTED CD4(+) CELLS BY A TUMOR NECROSIS FACTOR-RELATED APOPTOSIS-INDUCED LIGAND-MEDIATED MECHANISM," J Virol. 77:6700-6708). Moreover, soluble TRAIL was found in HIV-1-infected patients (Liabakk, N.B. *et al.* (2002) "DEVELOPMENT, CHARACTERIZATION AND USE OF MONOCLONAL ANTIBODIES AGAINST STRAIL: MEASUREMENT OF STRAIL BY ELISA," J Immunol Methods.

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259:119-128; Stylianou, E. *et al.* "RAISED SERUM LEVELS OF INTERLEUKIN-18 IS ASSOCIATED WITH DISEASE PROGRESSION AND MAY CONTRIBUTE TO VIROLOGICAL TREATMENT FAILURE IN HIV-1-INFECTED PATIENTS," Clin Exp Immunol. 132:462-466), and may be responsible for the death of neurons in AIDS patients leading to dementia (Ryan, L.A. *et al.* (2004) "TNF-RELATED APOPTOSIS-INDUCING LIGAND MEDIATES HUMAN NEURONAL APOPTOSIS: LINKS TO HIV-1-ASSOCIATED DEMENTIA," J Neuroimmunol. 148:127-139). TRAIL induces apoptosis in human tumor cell lines (Griffith, T.S. *et al.* (1998) "INTRACELLULAR REGULATION OF TRAIL-INDUCED APOPTOSIS IN HUMAN MELANOMA CELLS," J Immunol. 161:2833-2840) and in infected cells (Jeremias, I. *et al.* (1998) "TRAIL/Apo-2-ligand-induced apoptosis in human T cells," Eur J Immunol. 28:143-152; Clarke, P. *et al.* (2003) "TWO DISTINCT PHASES OF VIRUS-INDUCED NF-KAPPAB- REGULATION ENHANCE TRAIL-MEDIATED APOPTOSIS IN VIRUS-INFECTED CELLS," J Biol Chem. 278(20):18092-18100 [Epub Mar 13. 2003]) but not in normal cells (Gura, T. (1997) "HOW TRAIL KILLS CANCER CELLS, BUT NOT NORMAL CELLS," Science 277:768).

As used herein, the term "TRAIL Compound" is intended to include not only the naturally occurring or recombinant fragments of TRAIL (such as mTRAIL and sTRAIL,), but also the TRAIL DR5 receptor molecule, and biological molecules that activate TRAIL (such as IFN α/β) or its receptor or that are expressed by cells that express activated TRAIL or TRAIL DR5 receptor molecules or fragments of such molecules.

Preferred TRAIL Compounds

TRAIL (sTRAIL / mTRAIL / rsTRAIL)

TRAIL, its naturally occurring fragments (such as m-TRAIL or sTRAIL) or polynucleotide molecules that encode such molecules, or fragments of any such molecules are preferred TRAIL Compounds of the present invention. TRAIL is a membrane-associated protein composed of multiple domains: an N-terminal cytoplasmic domain, an internal transmembrane domain, and a C-terminal

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extracellular domain (amino acid residues 38-281 of **SEQ ID NO:1**); the C-terminal extracellular domain being responsible for inducing apoptosis (Wiley, S. R. *et al.* (1995) "IDENTIFICATION AND CHARACTERIZATION OF A NEW MEMBER OF THE TNF FAMILY THAT INDUCES APOPTOSIS," *Immunity* 3, 673-682). Although

5 TRAIL is thus generally membrane-bound, under certain circumstances bioactive TRAIL is released from cells (Liabakk, N.B. *et al.* (2002) "DEVELOPMENT, CHARACTERIZATION AND USE OF MONOCLONAL ANTIBODIES AGAINST sTRAIL: MEASUREMENT OF sTRAIL BY ELISA," *J Immunol Methods*. 259(1-2):119-28; Martinez-Lorenzo, M.J. *et al.* (1999) "ACTIVATED T CELLS RELEASE BIOACTIVE

10 FAS LIGAND AND APO2 LIGAND IN MICROVESICLES," *J. Immunol.* 163:1274-1281.

The amino acid sequence of TRAIL (**SEQ ID NO:1**) is presented below, with the N-terminal cytoplasmic domain (residues 38-281) underlined:

SEQ ID NO:1

15	MAMMEVQGGP	SLGQTCVLIV	IFTVLLQSLC	VAVTYVYFTN	<u>ELKQMQDKYS</u>
	1 10	20	30	40	50
	<u>KSGIACFLKE</u>	<u>DDSYWDPNDE</u>	<u>ESMNSPCWQV</u>	<u>KWQLRQLVRK</u>	<u>MILRTSEETI</u>
	51 60	70	80	90	100
	<u>STVQEKQQNI</u>	<u>SPLVRERGPQ</u>	<u>RVAAHITGTR</u>	<u>GRSNTLSSPN</u>	<u>SKNEKALGRK</u>
	101 110	120	130	140	150
20	<u>INSWESSRSG</u>	<u>HSFLSNLHLR</u>	<u>NGELVIHEKG</u>	<u>FYYIYSQTYF</u>	<u>RFQEEIKENT</u>
	151 160	170	180	190	200
	<u>KNDKQMVQYI</u>	<u>YKYTSYPDPI</u>	<u>LLMKSARNSC</u>	<u>WSKDAEYGLY</u>	<u>SIYQGGIFEL</u>
	201 210	220	230	240	250
25	<u>KENDRIFVSV</u>	<u>TNEHLIDMDH</u>	<u>EASFFGAFLV</u>	<u>G</u>	
	251 260	270	280	281	

A nucleic acid molecule encoding TRAIL is presented below as **SEQ ID NO:2**. TRAIL is encoded by residues 88-930 of **SEQ ID NO:2** (the start and stop codons of TRAIL are underlined). The sequence encoding sTRAIL (residues 38-281 of **SEQ ID NO:1**) is encoded by residues 199-930 of **SEQ ID NO:2**.

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SEQ ID NO:2

```

      1 cctcactgac tataaaagaa tagagaagga agggcttcag tgaccggctg cctggctgac
    61 ttacagcagt cagactctga caggatcATG gctatgatgg aggtccaggg gggacccagc
    121 ctgggacaga cctgcgtgct gatcgtgac ttcacagtgc tcctgcagtc tctctgtgtg
  5   181 gctgtaactt acgtgtactt taccaacgag ctgaagcaga tgcaggacaa gtactccaaa
    241 agtggcattg cttgtttctt aaaagaagat gacagttatt gggaccccaa tgacgaagag
    301 agtatgaaca gcccctgctg gcaagtcagg tggcaactcc gtcagctcgt tagaaagatg
    361 attttgagaa cctctgagga aaccatttct acagttcaag aaaagcaaca aaatatttct
    421 cccctagtga gagaaagagg tcctcagaga gtagcagctc acataactgg gaccagagga
  10   481 agaagcaaca cattgtcttc tccaaactcc aagaatgaaa aggcctctgg ccgcaaaata
    541 aactcctggg aatcatcaag gagtgggcat tcattcctga gcaacttgca cttgaggaat
    601 ggtgaactgg tcatccatga aaaagggttt tactacatct attcccaaac atactttcga
    661 tttcaggagg aaataaaaga aaacacaaag aacgacaaac aaatgggtcca atatattttac
    721 aaatacacaa gttatcctga ccctatatgt ttgatgaaaa gtgctagaaa tagttgttgg
  15   781 tctaaagatg cagaatatgg actctattcc atctatcaag ggggaatatt tgagcttaag
    841 gaaaatgaca gaatttttgt ttctgtaaca aatgagcact tgatagacat ggaccatgaa
    901 gccagttttt tcggggcctt tttagttggc TAActgacct ggaaagaaaa agcaataacc
    961 tcaaagtgac tattcagttt tcaggatgat acactatgaa gatgtttcaa aaaatctgac
  20  1021 caaaacaaac aaacagaaaa cagaaaacaa aaaaacctct atgcaatctg agtagagcag
    1081 ccacaaccaa aaaattctac aacacacact gttctgaaag tgactcactt atcccaagaa
    1141 aatgaaattg ctgaaagatc tttcaggact ctacctcata tcagtttgct agcagaaatc
    1201 tagaagactg tcagcttcca aacattaatg caatggttaa catctctgt ctttataatc
    1261 tactccttgt aaagactgta gaagaaagcg caacaatcca tctctcaagt agtgtatcac
  25  1321 agtagtagcc tccaggtttc cttaagggac aacatcctta agtcaaaaga gagaagaggc
    1381 accactaaaa gatcgagttt tgccctgggtc agtggtcac acctgtaatc ccaacatttt
    1441 gggaacccaa ggtgggtaga tcacgagatc aagagatcaa gaccatagtg accaacatag
    1501 tgaaacccca tctctactga aagtgcacaa attagctggg tgtgttgga catgcctgta
    1561 gtccagctta cttgagaggc tgaggcagga gaatcgtttg aacccgggag gcagagggtg
  30  1621 cagtgtggtg agatcatgcc actacactcc agcctggcga cagagcgaga cttgggtttca
    1681 aaaaaaaaaa aaaaaaaaaa cttcagtaag tacgtgttat ttttttcaat aaaattctat
    1741 tacagtatgt caaaaaaaaaa aaaaaaaaaa

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In one embodiment, invention contemplates methods and compositions for monitoring the presence of biologically active forms of TRAIL, and in particular, for monitoring biologically active forms of TRAIL that are membrane associated or naturally secreted from cells. Such molecules include membrane bound TRAIL (“mTRAIL”) and soluble (i.e., non-membrane bound) TRAIL (“sTRAIL”). As used herein, the term “TRAIL Compound” is intended to refer to naturally occurring or recombinant fragments of TRAIL. sTRAIL is the release form of membrane TRAIL, and corresponds to the extracellular domain of TRAIL (amino acid residues 38-281 of **SEQ ID NO:1**); it is encoded by residues 199-930 of **SEQ ID NO:2**. Without intending to be bound thereby, the production of sTRAIL may involve a shedding mechanism that involves cleavage by proteases (see, Liabakk, N.B. *et al.* (2002) “DEVELOPMENT, CHARACTERIZATION AND USE OF

MONOCLONAL ANTIBODIES AGAINST STRAIL: MEASUREMENT OF STRAIL BY ELISA," J Immunol Methods. 259(1-2):119-28; Martinez-Lorenzo, M.J. *et al.* (1999) "ACTIVATED T CELLS RELEASE BIOACTIVE FAS LIGAND AND APO2 LIGAND IN MICROVESICLES," J. Immunol. 163:1274-1281; Mariani, S.M. *et al.* (1998)

5 "SURFACE EXPRESSION OF TRAIL/APO-2 LIGAND IN ACTIVATED MOUSE T AND B CELLS," Eur. J. Immunol. 28(5):1492-1498; Mariani, S.M. *et al.* (1998) "DIFFERENTIAL REGULATION OF TRAIL AND CD95 LIGAND IN TRANSFORMED CELLS OF THE T AND B LYMPHOCYTE LINEAGE," Eur J Immunol. 28(3):973-982). sTRAIL has been used as a surrogate marker for assessing hepatitis B viral

10 infection (Han, L.H. *et al.* (2002) "DETECTION OF SOLUBLE TRAIL IN HBV INFECTED PATIENTS AND ITS CLINICAL IMPLICATIONS," World J Gastroenterol. 8(6):1077-1080), and multiple sclerosis (Wandinger, K.P. *et al.* (2003) "TNF-RELATED APOPTOSIS INDUCING LIGAND (TRAIL) AS A POTENTIAL RESPONSE MARKER FOR INTERFERON-BETA TREATMENT IN MULTIPLE SCLEROSIS," Lancet

15 361(9374):2036-2043). Recombinant forms of sTRAIL (denoted herein as "rsTRAIL") may be produced by expressing a TRAIL fragment comprising the C-terminal extracellular domain. For example, Wiley, S. R. *et al.* expressed a recombinant sTRAIL comprising amino acid residues 95-281 of **SEQ ID NO:1** (U.S Patent No. 5,763,223; Wiley, S. R. *et al.* (1995) "IDENTIFICATION AND

20 CHARACTERIZATION OF A NEW MEMBER OF THE TNF FAMILY THAT INDUCES APOPTOSIS," Immunity 3, 673-682). Other rsTRAIL molecules comprising the C-terminal extracellular domain can be employed (for example, rsTRAIL molecules comprising amino acid residues 101-281, or 114-281, of **SEQ ID NO:1**) to induce apoptosis. See also, Wang, L.H. *et al.* (2002) "DENSITY CULTIVATION FOR

25 PREPARATION OF RECOMBINANT SOLUBLE HUMAN TRAIL. Acad. J. Sec. Mil. Med. Univ. 23(2):132-135; Wang, X.J. *et al.* (2000) "THE CLONING, EXPRESSION AND REFOLDING OF TNF-RELATED APOPTOSIS INDUCING LIGAND," J. Beijing Med. Univ. 32(5):387-390. sTRAIL has been expressed in *Escherichia coli* (Xia, X.X. *et al.* (2004) "Purification and Characterization of Recombinant sTRAIL

30 Expressed in *Escherichia coli*," Acta Biochimica Biophysica Sinica 36(2):118-122). sTRAIL ELISA detection kits are available commercially (Diacclone).

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Antibodies to sTRAIL and to TRAIL can be readily produced, or obtained commercially (Diacclone; Alexis Corporation; Trinoca Biochem).

TRAIL DR5 Receptor

The TRAIL DR5 receptor and polynucleotides that encode the TRAIL DR5
 5 receptor, or fragments of such molecules, are TRAIL Compounds of the present invention. The amino acid sequence of the TRAIL DR5 receptor is provided below as **SEQ ID NO:11**. A nucleic acid sequence that encodes the TRAIL DR5 receptor is provided below as **SEQ ID NO:12** (see, GenBank Accession Nos. AAB71412 and AF020501, herein incorporated by reference). The start and stop
 10 codons for the TRAIL DR5 receptor are underlined.

SEQ ID NO:11

	MEQRGQNAPA	ASGARKRHGP	GPREARGARP	GLRVPKTLVL	VVAAVLLLVLS
	10	20	30	40	50
15	AESALITQQD	LAPQQRVAPQ	QKRSSPSEGL	CPPGHHISED	GRDCISCKYG
	60	70	80	90	100
	QDYSTHWNDL	LFCLRCTRCD	SGEVELSPCT	TTRNTVCQCE	EGTFREEDSP
	110	120	130	140	150
	EMCRKCRITGC	PRGMVKVGDC	TPWSDIECVH	KESGIIIGVT	VAAVVLIVAV
	160	170	180	190	200
20	FVCKSLWKK	VLPYLKGICS	GGGGDPERVD	RSSQRPGAED	NVLNEIVSIL
	210	220	230	240	250
	QPTQVPEQEM	EVQEPAEPTG	VNMLSPGESE	HLLEPAEAER	SQRRRLVPA
	260	270	280	290	300
25	NEGDPTETLR	QCFDDFADLV	PFDSWEPLMR	KLGLMDNEIK	VAKAEAAGHR
	310	320	330	340	350
	DTLYTMLIKW	VNKTGRDASV	HTLLDALETL	GERLAKQKIE	DHLLSSGKFM
	360	370	380	390	400
	YLEGNADSAM	S			
	410	411			

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SEQ ID NO:12

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      1 ATGgaacaac ggggacagaa cgccccggcc gcttcggggg cccgaaaag gcacggccca
    61 ggacccaggg aggcgcgggg agccaggcct gggctccggg tccccaagac ccttggtgctc
   121 gttgtcgccg cggtcctgct gttggtctca gctgagtctg ctctgatcac ccaacaagac
    5 181 ctagctcccc agcagagagt ggccccacaa caaaagaggt ccagcccctc agagggattg
   241 tgtccacctg gacaccatat ctcagaagac ggtagagatt gcatctcctg caaatatgga
   301 caggactata gcactcactg gaatgacctc cttttctgct tgcgctgcac caggtgtgat
   361 tcaggtgaag tggagctaag tccctgcacc acgaccagaa acacagtgtg tcagtgcgaa
   421 gaaggcacct tccgggaaga agattctcct gagatgtgcc ggaagtgccg cacagggtgt
  10 481 cccagaggga tggtaagggt cggtgattgt acaccctgga gtgacatcga atgtgtccac
   541 aaagaatcag gcatcatcat aggagtcaca gttgcagccg tagtcttgat tgtggctgtg
   601 tttgtttgca agtctttact gtggaagaaa gtccttcctt acctgaaagg catctgctca
   661 ggtggtggtg gggacctga gcgtgtggac agaagctcac aacgacctgg ggctgaggac
   721 aatgtcctca atgagatcgt gagtatcttg cagcccaccc aggtccctga gcaggaaatg
  15 781 gaagtccagg agccagcaga gccaacaggt gtcaacatgt tgtcccccg ggagtcagag
   841 catctgctgg aaccggcaga agctgaaagg tctcagagga ggaggctgct ggttccagca
   901 aatgaagggt atcccactga gactctgaga cagtgtctcg atgactttgc agacttggtg
   961 ccctttgact cctgggagcc gctcatgagg aagttgggcc tcatggacaa tgagataaag
  1021 gtggctaaag ctgaggcagc gggccacagg gacacctgtg acacgatgct gataaagtgg
  20 1081 gtcaacaaaa ccgggcgaga tgcctctgtc cacacctgc tggatgcctt ggagacgctg
  1141 ggagagagac ttgccaagca gaagattgag gaccacttgt tgagctctgg aaagttcatg
  1201 tatctagaag gtaatgcaga ctctgccatg tccTAA

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The TRAIL DR5 receptor is known to be involved in apoptosis, and to be affected by p53 (Golstein, P. (1997) "CELL DEATH: TRAIL AND ITS RECEPTORS,"

25 Curr Biol 7, R750-3; Pan, G. *et al.* (1997) "THE RECEPTOR FOR THE CYTOTOXIC LIGAND TRAIL," Science 276, 111-113; Wu, G. S. *et al.* (1997) "KILLER/DR5 IS A DNA DAMAGE-INDUCIBLE P53-REGULATED DEATH RECEPTOR GENE," Nat Genet 17, 141-3; Takaoka, A. *et al.* (2003) "INTEGRATION OF INTERFERON-ALPHA/BETA SIGNALING TO P53 RESPONSES IN TUMOUR SUPPRESSION AND

30 ANTIVIRAL DEFENSE," Nature 424, 516-23); Miranda-Carus, M.E. *et al.* (2004) "RHEUMATOID ARTHRITIS SYNOVIAL FLUID FIBROBLASTS EXPRESS TRAIL-R2 (DR5) THAT IS FUNCTIONALLY ACTIVE," Arthritis Rheum. 50(9):2786-2793; Ren, Y.G. *et al.* (2004) "DIFFERENTIAL REGULATION OF THE TRAIL DEATH RECEPTORS DR4 AND DR5 BY THE SIGNAL RECOGNITION PARTICLE," Mol Biol Cell.

35 15(11):5064-74. Epub 2004 Sep 08; Dicker, F. *et al.* (2004) "FAS-LIGAND (CD178) AND (TRAIL) SYNERGISTICALLY INDUCE APOPTOSIS OF CD40-ACTIVATED CHRONIC LYMPHOCYTIC LEUKEMIA B CELLS," Blood. 2004 Aug 31 [Epub ahead of print]; Liu, X. *et al.* (2004) "p53 UPREGULATES DEATH RECEPTOR 4 EXPRESSION THROUGH AN INTRONIC P53 BINDING SITE," Cancer Res. 64(15):5078-5083;

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Sheikh, M.S. (2004) "MYC TAGGING ALONG THE TRAIL TO DEATH RECEPTOR 5," Cell Cycle. 2004 Jul 8;3(7) [Epub ahead of print]; Aggarwal, B.B. *et al.* (2004) "REGULATION OF TRAIL-INDUCED APOPTOSIS BY ECTOPIC EXPRESSION OF ANTI-APOPTOTIC FACTORS," Vitam Horm. 67:453-483.

- 5 It has been proposed that TRAIL DR5 may be a potential anti-cancer therapy (Yagita, H. *et al.* (2004) "TRAIL AND ITS RECEPTORS AS TARGETS FOR CANCER THERAPY," Cancer Sci. 95(10):777-783; Delmas, D. *et al.* (2004) "REDISTRIBUTION OF CD95, DR4 AND DR5 IN RAFTS ACCOUNTS FOR THE SYNERGISTIC TOXICITY OF RESVERATROL AND DEATH RECEPTOR LIGANDS IN
- 10 COLON CARCINOMA CELLS," Oncogene. 2004 Oct 11 [Epub ahead of print]; Crowder, C. *et al.* (2004) "PML¹ MEDIATES IFN{ALPHA} INDUCED APOPTOSIS IN MYELOMA BY REGULATING TRAIL INDUCTION," Blood 2004 Sep 30 [Epub ahead of print].

P53

- 15 The p53 protein and polynucleotides that encode the p53 protein, or fragments of such molecules, are TRAIL Compounds of the present invention. The p53 protein is encoded by the p53 gene, which is a very well described cancer gene (see, e.g., Vogelstein, B. *et al.* (2004) "CANCER GENES AND THE PATHWAYS THEY CONTROL," Nat Med. 10(8):789-799; Okada, H. *et al.* (2004) "PATHWAYS OF
- 20 APOPTOTIC AND NON-APOPTOTIC DEATH IN TUMOUR CELLS," Nat Rev Cancer. 4(8):592-603). The amino acid sequence of the human p53 protein is provided below as **SEQ ID NO:13**. A nucleotide sequence that encodes this protein is provided below as **SEQ ID NO:14** (see, GenBank Accession Nos. K03199 and AAA59989, herein incorporated by reference). The start and stop codons for p53
- 25 are underlined.

SEQ ID NO:13:

MEEPQSDPSV	EPPLSQETFS	DLWKLLPENN	VLSPPLPSQAM	DDLMLSPDDI
10	20	30	40	50
EQWFTEDPGP	DEAPRMPEAA	PPVAPAPAAP	TPAAPAPAPS	WPLSSSVPSQ
30	60	80	90	100

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	KTYQGSYGFR	LGFLHSGTAK	SVTCTYSPAL	NKMFCQLAKT	CPVQLWVDST
	110	120	130	140	150
	PPPGTRVRAM	AIYKQSQHMT	EVVRRCPHHE	RCSDSDGLAP	PQHLIRVEGN
	160	170	180	190	200
5	LRVEYLDDRN	TFRHSVVVPY	EPPEVGSDCT	TIHYNMCNS	SCMGMNRRP
	210	220	230	240	250
	ILTIITLED	SGNLLGRNSF	EVHVCACPGR	DRRTEENLR	KKGEPHHELP
	260	270	280	290	300
10	PGSTKRALPN	NTSSSPQPKK	KPLDGEYFTL	QIRGRERFEM	FRELNEALEL
	310	320	330	340	350
	KDAQAGKEPG	GSRAHSSHLK	SKKGQSTSRH	KKLMFKTEGP	DSD
	360	370	380	390	393

SEQ ID NO:14:

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15      1 gtcgaccctt tccacccctg gaagatggaa ataaacctgc gtgtgggtgg agtgtagga
      61 caaaaaaaaa aaaaaaaaaa tctagagcca ccgtccaggg agcaggtagc tgctgggctc
      121 cggggacact ttgcgttcgg gctgggagcg tgctttccac gacggtgaca cgcttcctg
      181 gattggcagc cagactgcct tccgggtcac tgccATGgag gagccgcagt cagatcctag
      241 cgctcgagccc cctctgagtc aggaacatt ttcagaccta tggaaactac ttcctgaaaa
      301 caacgtttctg tcccccttgc cgtcccaagc aatggatgat ttgatgctgt ccccgagcga
20      361 tattgaacaa tggttcactg aagaccagg tccagatgaa gctcccagaa tgccagaggc
      421 tgctcccccc gtggcccctg caccagcagc tcctacaccg gcggcccctg caccagcccc
      481 ctccctggccc ctgtcatctt ctgtcccttc ccagaaaacc taccagggca gctacggttt
      541 ccgtctgggc ttcttgcatc ctgggacagc caagtctgtg acttgacagt actcccctgc
      601 cctcaacaag atgttttgcc aactggccaa gacctgcctt gtgcagctgt gggttgattc
25      661 cacacccccg cccggcaccc gcgtccgcgc catggccatc tacaagcagt cacagcacat
      721 gacggagggt gtgaggcgct gccccacca tgagcgctgc tcagatagcg atgggtctggc
      781 ccctcctcag catcttatcc gagtggaagg aaatttgctg gtggagtatt tggatgacag
      841 aaacactttt cgacatagtg tgggtgtgcc ctatgagccg cctgagggtt gctctgactg
30      901 taccaccatc cactacaact acatgtgtaa cagttcctgc atgggcggca tgaaccggag
      961 gcccatcctc accatcatca cactggaaga ctccagtggg aatctactgg gacggaacag
     1021 ctttgagggt catgtttgtg cctgtcctgg gagagaccgg cgacagaggg aagagaatct
     1081 ccgcaagaaa ggggagcctc accacgagct gccccaggg agcactaagc gagcactgcc
     1141 caacaacacc agctcctctc cccagccaaa gaagaaacca ctggatggag aatatttcac
     1201 ccttcagatc cgtgggcgtg agcgcttcga gatgttcga gagctgaatg aggccttggg
35     1261 actcaaggat gccaggctg ggaaggagcc aggggggagc agggctcact ccagccacct
     1321 gaagtccaaa aagggtcagt ctacctccg ccataaaaaa ctcatgttca agacagaagg
     1381 gcctgactca gacTGAcatt ctccattct tgttccccac tgacagctc ccacccccat
     1441 ctctccctcc cctgccattt tgggttttgg gtctttgaac ccttgcttgc aataggtgtg
     1501 cgtcagaagc acccaggact tccatttgct ttgtccggg gctccactga acaagttggc
40     1561 ctgcactggg gttttgttgt ggggaggagg atggggagta ggacatacca gcttagattt
     1621 taagggtttt actgtgaggg atgtttggga gatgtaagaa atgttcttgc agttaagggt
     1681 tagtttataa tcagccacat tctaggtagg gaccacttc accgtactaa ccagggaagc
     1741 tgtccctcac tgttgaattc

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The expression of the p53 protein is affected by HIV-1 (Bakhanashvili, M.
45 *et al.* (2004) "P53 IN CYTOPLASM MAY ENHANCE THE ACCURACY OF DNA

- 35 -

- SYNTHESIS BY HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 REVERSE
TRANSCRIPTASE,” *Oncogene*. 23(41):6890-6899; Makino, E. *et al.* (2004)
“INTRODUCTION OF AN N-TERMINAL PEPTIDE OF S100C/A11 INTO HUMAN CELLS
INDUCES APOPTOTIC CELL DEATH,” *J Mol Med*. 82(9):612-620. Epub 2004 Jul 06;
- 5 Garden, G.A. *et al.* (2004) “HIV ASSOCIATED NEURODEGENERATION REQUIRES
P53 IN NEURONS AND MICROGLIA,” *FASEB J*. 18(10):1141-1143. Epub 2004 May
20; Oberst, A. *et al.* (2003) “A PLACE TO DIE FOR: APOPTOSIS IN CANCER AND
INFECTION, CAPRI 2002,” *Cell Death Differ*. 10(3):393-395; Little, R.F. *et al.*
(2003) “HIGHLY EFFECTIVE TREATMENT OF ACQUIRED IMMUNODEFICIENCY
10 SYNDROME-RELATED LYMPHOMA WITH DOSE-ADJUSTED EPOCH: IMPACT OF
ANTIRETROVIRAL THERAPY SUSPENSION AND TUMOR BIOLOGY,” *Blood*.
101(12):4653-4659. Epub 2003 Feb 27; Castedo, M. *et al.* (2002) “Cyclin-
dependent kinase-1: linking apoptosis to cell cycle and mitotic catastrophe,” *Cell*
Death Differ. 2002 Dec;9(12):1287-1293; Mahomed, A. *et al.* (2002) “HUMAN
15 IMMUNODEFICIENCY VIRUS INFECTION, BCL-2, P53 PROTEIN, AND KI-67 ANALYSIS
IN OCULAR SURFACE SQUAMOUS NEOPLASIA,” *Arch Ophthalmol*. 2002
May;120(5):554-558; Selliah, N. *et al.* (2001) “BIOCHEMICAL MECHANISMS OF
HIV INDUCED T CELL APOPTOSIS,” *Cell Death Differ*. 8(2):127-136; Wattre, P. *et*
al. (1996) “APOPTOSIS AND HUMAN VIRAL INFECTIONS,” *Ann Biol Clin (Paris)*
20 54(5):189-197; Gualberto, A. *et al.* (1995) “P53 AND SP1 INTERACT AND
COOPERATE IN THE TUMOR NECROSIS FACTOR-INDUCED TRANSCRIPTIONAL
ACTIVATION OF THE HIV-1 LONG TERMINAL REPEAT,” *J Biol Chem*.
270(34):19680-19683; Duan, L. *et al.* (1994) “THE TUMOR SUPPRESSOR PROTEIN
P53 STRONGLY ALTERS HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 REPLICATION,”
25 *J Virol*. 68(7):4302-4313). Indeed, HIV-1 infected patients with ocular surface
squamous neoplasia (OSSN) were commonly found to express p53 (Mahomed, A.
et al. (2002) (“HUMAN IMMUNODEFICIENCY VIRUS INFECTION, BCL-2, P53
PROTEIN, AND KI-67 ANALYSIS IN OCULAR SURFACE SQUAMOUS NEOPLASIA,”
Arch Ophthalmol. 2002 May;120(5):554-558). Gualberto, A. *et al.* (1995) (“P53
30 AND SP1 INTERACT AND COOPERATE IN THE TUMOR NECROSIS FACTOR-INDUCED
TRANSCRIPTIONAL ACTIVATION OF THE HIV-1 LONG TERMINAL REPEAT,” *J Biol*

Chem. 270(34):19680-19683) discloses a synergistic role for p53 and Sp1 in the mechanism of TNF induction of HIV-1 LTR-mediated transcription and suggest that Sp1 may play an important role in modulating certain functions of p53.

5 **Additional Compounds Whose Assay May Be Conducted In Concert
With The Assessment Of The Concentration Or Presence Of TRAIL
Compounds**

CD69

CD69 is an early activation marker of peripheral lymphocyte cells (Fogli,
M. *et al.* (2004) "SIGNIFICANT NK CELL ACTIVATION ASSOCIATED WITH
10 DECREASED CYTOLYTIC FUNCTION IN PERIPHERAL BLOOD OF HIV-1-INFECTED
PATIENTS,"*Eur J Immunol.* 34(8):2313-2321). CD69 is discussed in U.S. Patent
6,593,124.

Assays for CD69, and polynucleotides that encode CD69, or fragments of such molecules, may be used in concert with assays of TRAIL Compounds in order to monitor the progression of AIDS, and other diseases whose progression involves immune system activation. The amino acid sequence of CD69 is provided by **SEQ ID NO:15**. The sequence of a polynucleotide that encodes CD69 is provided by **SEQ ID NO:16** (see, GenBank Accession Nos. NM 001781 and NP 001772, herein incorporated by reference). The start and stop codons for CD69 are underlined.

SEQ ID NO:15:

25	MSSENCFAVE	NSSLHPESGQ	ENDATSPHFS	TRHEGSFQVP	VLCAMNVVF
	10	20	30	40	50
	ITILIIALIA	LSVGQYNCPG	QYTFSMPSDS	HVSSCEDWV	GYQRKCYFIS
	60	70	80	90	100
	TVKRSWTSAQ	NACSEHGATL	AVIDSEKDMN	FLKRYAGREE	HWVGLKKEPG
	110	120	130	140	150
	HPWKWSNGKE	FNNWFNVTGS	DKCVFLKNT	VSSMECEKNL	YWICNKPYP
	160	170	180	190	199

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SEQ ID NO:16:

```

1 agactcaaca agagctccag caaagacttt cactgtagct tgacttgacc tgagattaac
61 tagggaatct tgagaataaa gATGagctct gaaaattggt tcgtagcaga gaacagctct
121 ttgcatccgg agagtggaca agaaaatgat gccaccagtc cccatttctc aacacgtcat
5 181 gaagggtcct tccaagttcc tgtcctgtgt gctgtaatga atgtggctct catcaccatt
241 ttaatcatag ctctcattgc cttatcagtg ggccaatata attgtccagg ccaatacaca
301 ttctcaatgc catcagacag ccatgtttct tcatgctctg aggactgggt tggctaccag
361 aggaaatgct actttatttc tactgtgaag aggagctgga cttcagccca aaatgcttgt
421 tctgaacatg gtgctactct tgctgtcatt gattctgaaa aggacatgaa ctttctaaaa
10 481 cgatacgtag gtagagagga acactgggtt ggactgaaaa aggaacctgg tcacccatgg
541 aagtgggtcaa atggcaaaga atttaacaac tgggtcaacg ttacaggggc tgacaagtgt
601 gtttttctga aaaacacaga ggtcagcagc atggaatgtg agaagaattt atactggata
661 tgtaacaaac cttacaaaTA Ataaggaac atgttcactt attgactatt atagaatgga
721 actcaaggaa atctgtgtca gtggatgctg ctctgtgggc cgaagtcttc catagagact
15 781 ttgtgaaaaa aaattttata gtgtcttggg aattttcttc caaacagaac tatggaaaaa
841 aaggaagaaa ttccaggaaa atctgcactg tgggctttta ttgcatgag ctagaagcat
901 cacaggttga ccaataacca tgcccaagaa tgagaagaat gactatgcaa cctttggatg
961 cactttatat tattttgaat ccagaaataa tgaaataact aggcgtggac ttactattta
1021 ttgctgaatg actaccaaca gtgagagccc ttcattgcatt tgcactactg gaaggagtta
20 1081 gatgttggta ctagatactg aatgtaaaca aaggaattat ggctggtaac ataggttttt
1141 agtctaattg aatcccttaa actcagggag catttataaa tggacaaatg cttatgaaac
1201 taagatttgt aatattttctc tctttttaga gaaatttgcc aatttacttt gttatttttc
1261 cccaaaaaga atgggatgat cgtgtattta ttttttact tcctcagctg tagacaggtc
1321 cttttcgaat gtacatatat ctttgccttt ataactcttt atacagtgtc ttacagagaa
25 1381 aagacataag caaagactat gaggaatatt tgcaagacat agaatagtgt tggaaaatgt
1441 gcaatatgtg atgtggcaaa tctctattag gaaatattct gtaactttca gacctagaat
1501 aatactagtc ttataatagg tttgtgactt tcctaaatca attctattac gtgcaatact
1561 tcaatacttc atttaaaata tttttatgtg caataaaatg tatttgtttg tattttgtgt
1621 tcagtacaat tataagctgt ttttatatat gtgaaataaa agtagaataa acacaaaaaa
30 1681 aaaaaaaaaa aaaaaaaaaa aa

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Stevceva, L. *et al.* (2004) ("FUNCTIONAL SIMIAN IMMUNODEFICIENCY VIRUS GAG-SPECIFIC CD8+ INTRAEPITHELIAL LYMPHOCYTES IN THE MUCOSAE OF SIMMAC251- OR SIMIAN-HUMAN IMMUNODEFICIENCY VIRUS KU2-INFECTED MACAQUES," *Virology* 319(2):190-200) disclose that SIV-infected intraepithelial lymphocytes (IELs) expressed CD69 and produced IFN- γ . A relationship between immune activation antigens and HIV pathogenesis has recently been described (Dunn, S.J. *et al.* (2004) "IDENTIFICATION OF CELL SURFACE TARGETS FOR HIV-1 THERAPEUTICS USING GENETIC SCREENS," *Virology* 321(2):260-273); Sousa, A.E. *et al.* (2002) "CD4 T CELL DEPLETION IS LINKED DIRECTLY TO IMMUNE ACTIVATION IN THE PATHOGENESIS OF HIV-1 AND HIV-2 BUT ONLY INDIRECTLY TO THE VIRAL LOAD," *J Immunol.* 169(6):3400-3406; Palmer, B.E. *et al.* (2002) "DISCORDANCE BETWEEN FREQUENCY OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1)-SPECIFIC GAMMA INTERFERON-PRODUCING CD4(+) T CELLS AND

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HIV-1-SPECIFIC LYMPHOPROLIFERATION IN HIV-1-INFECTED SUBJECTS WITH ACTIVE VIRAL REPLICATION,” J Virol. 76(12):5925-36; Trimble, L.A. *et al.* (2000) “HUMAN IMMUNODEFICIENCY VIRUS-SPECIFIC CIRCULATING CD8 T LYMPHOCYTES HAVE DOWN-MODULATED CD3ZETA AND CD28, KEY SIGNALING MOLECULES FOR T-CELL ACTIVATION,” J Virol. 74(16):7320-7330; Rosok, B.I. *et al.* (1998) “CORRELATES OF APOPTOSIS OF CD4+ AND CD8+ T CELLS IN TONSILLAR TISSUE IN HIV TYPE 1 INFECTION,” Aids Res Hum Retroviruses 14(18):1635-1643; Voiculescu, C. *et al.* (1998) “EXPRESSION OF ACTIVATION SURFACE MARKERS, INTERLEUKIN-2 SYNTHESIS AND APOPTOSIS RATE IN FRESH OR CULTURED LYMPHOCYTES FROM HIV-INFECTED CHILDREN,” Roum Arch Microbiol Immunol. 57(1):33-44).

CD69 expression may be used in the diagnosis of HIV infection (Nielsen, S.D. *et al.* (1998) “EXPRESSION OF THE ACTIVATION ANTIGEN CD69 PREDICTS FUNCTIONALITY OF IN VITRO EXPANDED PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC) FROM HEALTHY DONORS AND HIV-INFECTED PATIENTS,” Clin Exp Immunol. 114(1):66-72; Prince, H.E. *et al.* (1997) “CD69 EXPRESSION RELIABLY PREDICTS THE ANTI-CD3-INDUCED PROLIFERATIVE RESPONSE OF LYMPHOCYTES FROM HUMAN IMMUNODEFICIENCY VIRUS TYPE 1-INFECTED PATIENTS,” Clin Diagn Lab Immunol. 4(2):217-222; Perfetto, S.P. *et al.* (1997) “MEASUREMENT OF CD69 INDUCTION IN THE ASSESSMENT OF IMMUNE FUNCTION IN ASYMPTOMATIC HIV-INFECTED INDIVIDUALS,” Cytometry. 30(1):1-9; Krowka, J.F. *et al.* (1996) “EXPRESSION OF CD69 AFTER IN VITRO STIMULATION: A RAPID METHOD FOR QUANTITATING IMPAIRED LYMPHOCYTE RESPONSES IN HIV-INFECTED INDIVIDUALS,” J Acquir Immune Defic Syndr Hum Retrovirol. 11(1):95-104). In accord with the principles of the present invention, assays of CD69 may be used in concert with the TRAIL Compounds of the present invention to monitor the progression of AIDS, and other diseases whose progression involves immune system activation.

HLA-DR

The HLA-DR antigen is part of the major histocompatibility complex (MHC) that identifies self from non-self and is pivotal in immune recognition of foreign tissue and infective agents and presents a variety of cellular proteins necessary for immunity (Andersson, G. (1998) "EVOLUTION OF THE HUMAN HLA-DR REGION," *Front Biosci.* 27;3:d739-745; Auger, I. *et al.* (1997) "HLA-DR AND THE DEVELOPMENT OF RHEUMATOID ARTHRITIS," *Autoimmunity* 26(2):123-128; Cabrera, T. *et al.* (1995) "BIOLOGICAL IMPLICATIONS OF HLA-DR EXPRESSION IN TUMOURS," *Scand J Immunol.* 41(4):398-406; Klajman, A. *et al.* (1990) "THE IMPORTANCE OF HLA-DR IN AUTOIMMUNE DISEASES," *Isr J Med Sci.* 26(12):691-692; Redman, C.W. (1983) "HLA-DR ANTIGEN ON HUMAN TROPHOBLAST: A REVIEW," *Am J Reprod Immunol.* 3(4):175-177; Charron, D.J. (1982) "HLA-DR ANTIGENS: CELLULAR EXPRESSION AND MOLECULAR STRUCTURE," *Ann Immunol (Paris)*. 133D(2):155-169; Tiwari, J.L. *et al.* (1981) "HLA-DR AND DISEASE ASSOCIATIONS," *Prog Clin Biol Res.* 58:151-163).

Assays for HLA-DR (especially HLA-DR alpha), and polynucleotides that encode HLA-DR or fragments of such molecules, may be used in concert with assays of TRAIL Compounds in order to monitor the progression of AIDS, and other diseases whose progression involves immune system activation. The amino acid sequence of HLA-DR alpha is provided by **SEQ ID NO:17**. The sequence of a polynucleotide that encodes HLA-DR alpha is provided by **SEQ ID NO:18** (see, GenBank Accession Nos. NM_019111 and NP_061984, herein incorporated by reference). The start and stop codons for HLA-DR are underlined.

SEQ ID NO:17:

25	MAISGVPVLG	FFIIAVLMSA	QESWAIKEEH	VIIQAEFYLN	PDQSGEFMFD
	10	20	30	40	50
	FDGDEIFHVD	MAKKETVWRL	EEFGRFASFE	AQGALANIAV	DKANLEIMTK
	60	70	80	90	100
30	RSNYTPITNV	PPEVTVLTSN	PVELREPNVL	ICFIDKF TPP	VVNVTWLRNG
	110	120	130	140	150

- 40 -

KPVTTGVSET VFLPREDHLF RKFHYLPFLP STEDVYDCRV EHWGLDEPLL
 160 170 180 190 200
 KHWEFDAPSP LPETTENVVC ALGLTVGLVG IIIGTIFIHK GLRKSNAER
 210 220 230 240 250
 5 RGPL
 254

SEQ ID NO:18:

1 acattctctt ttcttttatt cttgtctgtt ctgcctcact cccgagctct actgactccc
 61 aacagagcgc ccaagaagaa aATGgccata agtggagtcc ctgtgctagg atttttcatc
 10 121 atagctgtgc tgatgagcgc tcaggaatca tgggctatca aagaagaaca tgtgatcatc
 181 caggccgagt tctatctgaa tcctgaccaa tcaggcgagt ttatgtttga ctttgatggt
 241 gatgagattt tccatgtgga tatggcaaag aaggagacgg tctggcggct tgaagaattt
 301 ggacgatttg ccagctttga ggctcaagggt gcattggcca acatagctgt ggacaaagcc
 361 aacctggaaa tcatgacaaa gcgctccaac tatactccga tcaccaatgt acctccagag
 15 421 gtaactgtgc tcacgaacag ccctgtggaa ctgagagagc ccaacgtcct catctgtttc
 481 atagacaagt tcaccccacc agtgggtcaat gtcacgtggc ttcgaaatgg aaaacctgtc
 541 accacaggag tgtcagagac agtcttctctg cccagggaag accacctttt ccgcaagttc
 601 cactatctcc ccttcctgcc ctcaactgag gacgtttacg actgcagggt ggagcactgg
 661 ggcttgatg agcctcttct caagcactgg gagtttgatg ctccaagccc tctcccagag
 20 721 actacagaga acgtgggtgtg tgccctgggc ctgactgtgg gtctgggtgg catcattatt
 781 gggaccatct tcatcatcaa gggattgcgc aaaagcaatg cagcagaacg cagggggcct
 841 ctgTAAGgca catggagggtg atgggtgttc ttagagagaa gatcactgaa gaaacttctg
 901 ctttaattggc tttaacaaagc tggcaatatt acaatccttg acctcagtga aagcagtcat
 961 cttcagcatt ttccagccct atagccaccc caagtgtgga tatgcctctt cgattgctcc
 25 1021 gtactctaac atctagctgg cttccctgtc tattgccttt tcctgtatct attttccctct
 1081 attttccatc attttattat caccatgcaa tgcctctgga ataaaacata caggagtctg
 1141 tctctgctat ggaatgcccc atggggcatc tcttgtgtac ttattgttta aggtttcctc
 1201 aaactgtgat ttttctgaac acaataaact attttgatga tcttgggtgg aaaaaaaaaa
 1261 aaaaaaa

30 Higher levels of HLA-DR have been found in HIV-1 infected individuals,
 relative to uninfected individuals (Khudiakova, N.E. (2004) ("SOLUBLE HLA
 CLASS I AND HLA CLASS II ANTIGENS IN THE BLOOD OF HIV INFECTED
 PATIENTS," Zh Mikrobiol Epidemiol Immunobiol. (1):42-45). Additionally,
 Resino, S. *et al.* (2003) reported that children having low viral loads exhibited
 35 enhanced HLA-DR expression relative to children having higher viral loads
 (Resino, S. *et al.* (2003) "CHARACTERIZING THE IMMUNE SYSTEM AFTER LONG-
 TERM UNDETECTABLE VIRAL LOAD IN HIV-1-INFECTED CHILDREN," J Clin
 Immunol. 23(4):279-289). This finding of an inverse correlation between viral
 load and HLA-DR expression appears to be found in multiple reports (see, for
 40 example, Sindhu, S.T. *et al.* (2003) "VIRUS LOAD CORRELATES INVERSELY WITH

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THE EXPRESSION OF CYTOTOXIC T LYMPHOCYTE ACTIVATION MARKERS IN HIV-1-
 INFECTED/AIDS PATIENTS SHOWING MHC-UNRESTRICTED CTL-MEDIATED
 LYSIS," Clin Exp Immunol. 132(1):120-127). However, it has also been reported
 that untreated AIDS patients had higher levels of HLA-DR than HAART-treated
 5 patients (see, Lange, C.G. *et al.* (2002) "IMPACT OF SUPPRESSION OF VIRAL
 REPLICATION BY HIGHLY ACTIVE ANTIRETROVIRAL THERAPY ON IMMUNE
 FUNCTION AND PHENOTYPE IN CHRONIC HIV-1 INFECTION," J Acquir Immune
 Defic Syndr. 30(1):33-40; Resino, S. (2001) "RELATIONSHIP BETWEEN T-CELLS
 SUBSETS AND PROGNOSTIC MARKERS IN HIV-1-INFECTED CHILDREN," Med Clin
 10 (Barc). 2001 Jul 14;117(6):201-206).

It has been reported that HLA-DR may be used to predict HIV-progression
 (Gascon, R.L. *et al.* (2002) "INCREASED HLA-DR EXPRESSION ON PERIPHERAL
 BLOOD MONOCYTES IN SUBSETS OF SUBJECTS WITH PRIMARY HIV INFECTION IS
 ASSOCIATED WITH ELEVATED CD4 T-CELL APOPTOSIS AND CD4 T-CELL
 15 DEPLETION," J Acquir Immune Defic Syndr. 30(2):146-153; Choi, B.S. *et al.*
 (2002) "THE CD28/HLA-DR EXPRESSIONS ON CD4+T BUT NOT CD8+T CELLS
 ARE SIGNIFICANT PREDICTORS FOR PROGRESSION TO AIDS," Clin Exp Immunol.
 2002 Jan;127(1):137-144; Leng, Q. *et al.* (2001) "IMMUNE ACTIVATION
 CORRELATES BETTER THAN HIV PLASMA VIRAL LOAD WITH CD4 T-CELL
 20 DECLINE DURING HIV INFECTION," J Acquir Immune Defic Syndr. 27(4):389-397;
 [Authors Not Listed] (1999) "T-CELL ACTIVATION PREDICTIVE OF POOR SURVIVAL
 IN ADVANCED DISEASE," AIDS Treat News. 317:3; Aldhous, M.C. *et al.* (1996)
 "CD4 AND CD8 LYMPHOCYTES IN DIAGNOSIS AND DISEASE PROGRESSION OF
 PEDIATRIC HIV INFECTION," Pediatr AIDS HIV Infect. 7(1):20-30).

25 **α -Interferon / β -interferon (IFN α/β)**

Type I interferon (interferon-alpha and beta: IFN- α and IFN- β) regulate
 membrane-bound (mTRAIL) and soluble TRAIL (sTRAIL) (Sato, K. *et al.* (2001)
 "ANTIVIRAL RESPONSE BY NATURAL KILLER CELLS THROUGH TRAIL GENE
 INDUCTION BY IFN-ALPHA/BETA," Eur J Immunol. 31:3138-3146), and are TRAIL

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Compounds of the present invention. The amino acid sequence of interferon alpha is provided by **SEQ ID NO:19**. The sequence of a polynucleotide that encodes interferon alpha is provided by **SEQ ID NO:20** (see, GenBank Accession No. AL390882, herein incorporated by reference). The gene encoding IFN α is located at positions 1806-2829 of **SEQ ID NO:20**. Position numbering is from AL390882. The start and stop codons for interferon alpha are underlined.

SEQ ID NO:19:

	MALSFSLLMA	VLVLSYKSIC	SLGCDLPQTH	SLGNRRALIL	LAQMGRISPF
	10	20	30	40	50
10	SCLKDRHDFG	FPQEEFDGNQ	FQKAQAISVL	HEMIQQTFLN	FSTKDSSATW
	60	70	80	90	100
	EQSLLEKFST	ELNQQLNDLE	ACVIEVGVVE	ETPLMNVDSI	LAVKKYFQRI
	110	120	130	140	150
15	TLYLTEKKYS	PCAWEVVRAE	IMRSFSLSKI	FQERLRRKE	
	160	170	180	189	

SEQ ID NO:20:

	1801	caaggttcaa	ggttacccat	ctcaagtagc	ctagcaatat	tggcaacatc	cca <u>ATG</u> gcc
	1861	tgtccttttc	tttactgatg	gccgtgctgg	tgctcagcta	caaatccatc	tgttctctgg
	1921	gctgtgatct	gcctcagacc	cacagcctgg	gtaataggag	ggccttgata	ctcctggcac
20	1981	aaatgggaag	aatctctcct	ttctcctgcc	tgaaggacag	acatgacttt	ggattccccc
	2041	aggaggagtt	tgatggcaac	cagttccaga	aggctcaagc	catctctgtc	ctccatgaga
	2101	tgatccagca	gacottcaat	ctcttcagca	caaaggactc	atctgctact	tgggaacaga
	2161	gcctcctaga	aaaatttttc	actgaactta	accagcagct	gaatgacctg	gaagcctgcg
	2221	tgatacagga	ggttgggggtg	gaagagactc	ccctgatgaa	tgtggactcc	atcctggctg
25	2281	tgaagaaata	cttccaaaaga	atcactcttt	atctgacaga	gaagaaatac	agcccttgtg
	2341	cctgggaggt	tgtcagagca	gaaatcatga	gatccttctc	tttatcaaaa	atttttcaag
	2401	aaagattaag	gaggaaggaa	<u>TGA</u> aacctgt	ttcaacatgg	aaatgatctg	tattgactaa
	2461	tacaccagtc	cacacttcta	tgacttctgc	catttcaaa	actcatttct	cctataacca
	2521	ccgcatgagt	tgaatcaaaa	ttttcagatc	ttttcaggag	tgtaaggaaa	catcatgttt
30	2581	acctgtgcag	gcactagtcc	tttacagatg	accatgctga	tagatcta	tatctatcta
	2641	ttgaaatatt	tatttattta	ttagatttaa	attatttttg	tccatgta	attatgtgta
	2701	cttttacatt	gtgttatatc	aaaatatgtt	atttatattt	agtcaatata	ttattttctt
	2761	tttattaatt	tttactatta	aaacttctta	tattatttgt	ttattcttta	ataaagaaat
	2821	accaagccca					

35 The amino acid sequence of interferon beta is provided by **SEQ ID NO:21**. The sequence of a polynucleotide that encodes interferon beta is provided by **SEQ ID NO:22** (see, GenBank Accession No. AL390882, herein incorporated by reference). The gene encoding IFN β is located at positions 90503-91361 of **SEQ**

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ID NO:22. Position numbering is from AL390882. The start and stop codons for interferon beta are underlined.

SEQ ID NO:21:

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5  MTNKCLLQIA   LLLCFSTTAL   SMSYNLLGFL   QRSSNFQCQK   LLWQLNGRLE
    10          20          30          40          50

   YCLKDRMNFD   IPEEIKQLQQ   FQKEDAALTI   YEMLQNIFAI   FRQDSSSTGW
    60          70          80          90         100

  NETIVENLLA   NVYHQINHLK   TVLEEKLEKE   DFTRGKLMSS   LHLKRYYGRI
    110         120         130         140         150

10  LHYLKAKEYS   HCAWTIVRVE   ILRNFYFINR   LTGYLRN
    160         170         180         187

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SEQ ID NO:22:

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90541 tcgaagcctt tgctctggca caacaggtag taggcgacac tgttcgtgtt gtcaacATGa
15  90601 ccaacaagtg tctcctccaa attgctctcc tgttgtgctt ctccactaca gctctttcca
    90661 tgagctacaa cttgcttgga ttctacaaaa gaagcagcaa ttttcagtgt cagaagctcc
    90721 tgtggcaatt gaatgggagg cttgaatact gcctcaagga caggatgaac tttgacatcc
    90781 ctgaggagat taagcagctg cagcagttcc agaaggagga cgccgcattg accatctatg
    90841 agatgctcca gaacatcttt gctattttca gacaagattc atctagcact ggctggaatg
20  90901 agactattgt tgagaacctc ctggctaata tctatcatca gataaaccat ctgaagacag
    90961 tcctggaaga aaaactggag aaagaagatt tcaccagggg aaaactcatg agcagctctgc
    91021 acctgaaaag atattatggg aggattctgc attacctgaa ggccaaggag tacagtcact
    91081 gtgcctggac catagtcaga gtggaaatcc taaggaactt ttacttcatt aacagactta
    91141 caggttacct ccgaaacTGA agatctccta gcctgtgcct ctgggactgg acaattgctt
25  91201 caagcattct tcaaccagca gatgctgttt aagtgactga tggctaatagt actgcatatg
    91261 aaaggacact agaagatttt gaaattttta ttaaattatg agttattttt atttatttaa
    91321 attttatttt ggaaaataaa ttatttttgg tgcaaaagtc aacatggcag ttttaatttc

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Preferred Methods for the Use of TRAIL Compounds

The present invention provides a method for determining whether a

30 mammal (especially a human, simian, feline, bovine, equine, canine, ovine or porcine mammal) suffers from a disease or condition of involving immune system activation, wherein the method comprises assaying for the presence or concentration of one or more TRAIL Compound(s) in a biological fluid of said mammal. As used herein, examples of such diseases and conditions whose

35 progression involves "immune system activation" include HIV (FIV, SIV, etc.) infection, AIDS, certain cancers (e.g., colon cancer, lung cancer, breast cancer,

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pancreatic cancer, leukemia, myeloma, skin cancer, brain cancer, cervical cancer, testicular cancer, etc.), atherosclerosis, Alzheimer's disease, inflammatory diseases and autoimmune disorders (especially, allergic asthma, Crohn's disease, Grave's disease, lupus, multiple sclerosis, Parkinson's disease, allograft transplant
5 rejection, graft vs. host disease, etc.

In one embodiment, the methods and compositions of the present invention may be used to diagnose whether such a mammal is healthy, or suffers from such diseases and conditions. In an alternative embodiment, the methods and compositions of the present invention may be used to monitor the progression,
10 stage, or severity of a disease or condition in humans and other mammals known to suffer from such a disease or condition.

The present invention particularly provides a method for diagnosing AIDS and other diseases and conditions involving immune system activation. In a preferred embodiment, the present invention permits one to distinguish among
15 HIV-1-infected patients with high viral loads, patients with low viral loads and uninfected healthy control blood donors versus by measuring plasma levels of one or more TRAIL Compounds, and to relate the quantity of viral particles in an individual to the level(s) of TRAIL Compounds expressed or secreted by cells (e.g., monocytes) and to recognize that the quantity of HIV-1-mediated TRAIL
20 Compound production by such cells is regulated by type I interferon.

In accord with a second aspect of the present invention, since TRAIL Compound expression/production is a marker of immune system activation (e.g., TRAIL is produced after HIV-1 infection), the TRAIL Compounds can be used as markers for evaluating the progression of all diseases and conditions involving
25 immune system activation.

In one embodiment, the invention measures the presence or concentration of a TRAIL Compound in a fluid of a patient. In another preferred embodiment the invention measures the presence or concentration of sTRAIL or mTRAIL in a fluid of a patient. Suitable fluid can comprise blood, serum, plasma, urine, saliva,

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semen, cerebrospinal fluid, lymph, etc. Preferably, such fluid will be blood, serum, or plasma. The determination of the presence or concentration of sTRAIL may be accomplished using any molecule capable of specific binding with sTRAIL (e.g., a receptor or ligand of sTRAIL). In preferred embodiments, the invention will

5 employ an immunoassay to determine the concentration or presence of sTRAIL in such a fluid. Such assays use the immunospecific binding of an antibody and an antigen to facilitate the detection of the sTRAIL. As used herein the term “antibody” is intended to encompass both naturally produced immunoglobulins (e.g., IgG, etc.), as well as fragments of such molecules (e.g., Fab, F(ab)₂, etc.) that

10 are capable of specifically binding an antigen, as well as recombinant antigen-binding molecules (e.g., humanized antibodies, single chain antibodies, etc.). In a preferred embodiment, the invention will employ a PE-conjugated mouse IgG1 anti-human TRAIL monoclonal antibody.

Any of a wide variety of assay formats may be used in accordance with the

15 methods of the present invention. Such formats may be heterogeneous or homogeneous, sequential or simultaneous, competitive or noncompetitive. U.S. Patent Nos. 5,563,036; 5,627,080; 5,633,141; 5,679,525; 5,691,147; 5,698,411; 5,747,352; 5,811,526; 5,851,778; and 5,976,822 illustrate several different assay formats and applications. Such assays can be formatted to be quantitative, so as to

20 measure the concentration or amount of the selected TRAIL Compound, or they may be formatted to be qualitative, so as to measure the presence or absence of the selected TRAIL Compound. Enzymatic-binding assay formats (e.g., Enzyme-Linked Immunosorbent Assay (ELISA)), are particularly preferred.

Heterogeneous immunoassay techniques typically involve the use of a solid

25 phase material to which the reaction product becomes bound, but may be adapted to involve the binding of non-immobilized antigens and antibodies (i.e., a solution-phase immunoassay). The reaction product is separated from excess sample, assay reagents, and other substances by removing the solid phase from the reaction mixture (e.g., by washing). One type of solid phase immunoassay that may be

30 used in accordance with the present invention is a sandwich immunoassay. In a

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typical sandwich assay, the more TRAIL Compound analyte (e.g., sTRAIL, mTRAIL, etc.) present in the sample, the greater the amount of label present on the solid phase. This type of assay format is generally preferred, especially for the visualization of low analyte concentrations, because the appearance of label on the solid phase is more readily detected.

In accordance with a preferred embodiment of such a heterogeneous assay, a “first” antibody bound to a solid support (i.e., immobilized) is incubated in the presence of a biological sample being tested for the presence of sTRAIL or mTRAIL. A “second” antibody, which has been detectably labeled and which is capable of specifically binding with the TRAIL Compound is provided. In one sub-embodiment, the “first” antibody” will be an anti-TRAIL antibody; alternatively, it may be an anti-“first” antibody. In either case, the presence of a TRAIL Compound in the sample being tested results in the immobilization of the detectably labeled “second” antibody. The extent of such immobilization is proportional to the amount of TRAIL Compound present in the sample.

As will be appreciated, the “first” antibody may be incubated with the biological sample in an unbound state and then subsequently bound to the solid support (i.e., immobilizable). The supports are then preferably extensively treated (e.g., by washing, etc.) to substantially remove unbound detectably labeled antibody that may be present. Sandwich assay formats are described by Schuurs *et al.* U.S. Patent Nos. 3,791,932 and 4,016,043, and by Pankratz, *et al.*, U.S. Patent No. 5,876,935. The “second” antibody may be a natural immunoglobulin isolated from nonhuman primates (e.g., anti-human IgG murine antibody, anti-human IgG goat antibody, etc.), or can be produced recombinantly or synthetically. It may be an intact immunoglobulin, or an immunoglobulin fragment (e.g., FAb, F(Ab)₂, etc.). As desired, other binding molecules (capable of binding to the TRAIL Compound employed) may be employed in concert with or in lieu of such second antibodies.

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To eliminate the bound-free separation step and reduce the time and equipment needed for a chemical binding assay, a homogeneous assay format may alternatively be employed. In such assays, one component of the binding pair may still be immobilized; however, the presence of the second component of the binding pair is detected without a bound-free separation. Examples of homogeneous optical methods are the EMIT method of Syva, Inc. (Sunnyvale, CA), which operates through detection of fluorescence quenching; the laser nephelometry latex particle agglutination method of Behringwerke (Marburg, Germany), which operates by detecting changes in light scatter; the LPIA latex particle agglutination method of Mitsubishi Chemical Industries (Tokyo, Japan); the TDX fluorescence depolarization method of Abbott Laboratories (Abbott Park, IL); and the fluorescence energy transfer method of Cis Bio International (Paris, France). Any of such assays may be adapted for use in accordance with the objectives of the present invention.

The binding assay of the present invention may be configured as a competitive assay. In a competitive assay, the more sTRAIL or mTRAIL present in the test sample, the lower the amount of label present on the solid phase.

In a manner similar to the sandwich assay, a competitive assay can be conducted by providing a defined amount of a labeled selected TRAIL Compound (such as sTRAIL, mTRAIL or rsTRAIL) and determining whether the fluid being tested contains a TRAIL Compound that would compete with the labeled antibody for binding to the support. In such a competitive assay, the amount of captured label is inversely proportional to the amount of analyte present in the test sample. Smith (U.S. Patent No. 4,401,764) describes a competitive assay format using a mixed binding complex that can bind analyte or labeled analyte but in which the analyte and labeled analyte cannot simultaneously bind the complex. Clagett (U.S. Patent No. 4,746,631) describes an immunoassay method using a reaction chamber in which an analyte/ligand/marker conjugate is displaced from the reaction surface in the presence of test sample analyte and in which the displaced analyte/ligand/marker conjugate is immobilized at a second reaction site. The

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conjugate includes biotin, bovine serum albumin, and synthetic peptides as the ligand component of the conjugate, and enzymes, chemiluminescent materials, enzyme inhibitors, and radionucleotides as the marker component of the conjugate. Li (U.S. Patent No. 4,661,444) describes a competitive immunoassay using a
5 conjugate of an anti-idiotypic antibody and a second antibody, specific for a detectable label, in which the detectable response is inversely related to the presence of analyte in the sample. Allen (European Patent Appln. No. 177,191) describes a binding assay involving a conjugate of a ligand analog and a second reagent, such as fluorescein, in which the conjugate competes with the analyte
10 (ligand) in binding to a labeled binding partner specific for the ligand, and in which the resultant labeled conjugate is then separated from the reaction mixture by means of solid phase carrying a binding partner for the second reagent. This binding assay format combines the use of a competitive binding technique and a reverse sandwich assay configuration; i.e., the binding of conjugate to the labeled
15 binding member prior to separating conjugate from the mixture by the binding of the conjugate to the solid phase. The assay result, however, is determined as in a conventional competitive assay in which the amount of label bound to the solid phase is inversely proportional to the amount of analyte in the test sample. Chieriegatt *et al.* (GB Patent No. 2,084,317) describe a similar assay format using
20 an indirectly labeled binding partner specific for the analyte. Mochida *et al.* (U.S. Patent No. 4,185,084) also describe the use of a double-antigen conjugate that competes with an antigen analyte for binding to an immobilized antibody and that is then labeled. This method also results in the detection of label on a solid phase in which the amount of label is inversely proportional to the amount of analyte in
25 the test sample. Sadeh *et al.* (U.S. Patent No. 4,243,749) describe a similar enzyme immunoassay in which a hapten conjugate competes with analyte for binding to an antibody immobilized on a solid phase. Any of such variant assays may be used in accordance with the present invention.

In all such assay formats, at least one component of the assay reagents (e.g.,
30 a TRAIL Compound, an antibody, etc.) will preferably be labeled or otherwise rendered detectable by the evolution or quenching of light. Any of a variety of

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detectable moieties (e.g., a fluorescent moiety, a radioactive moiety, an enzyme, a magnetic or paramagnetic particle or bead, a chemiluminescent moiety, etc.) may be employed. Radioisotopic-binding assay formats (e.g., a radioimmunoassay, etc.) employ a radioisotope as such label; the signal is detectable by the evolution
5 of light in the presence of a fluorescent or fluorogenic moiety (see Lucas *et al.* (U.S. Patent No. 5,698,411) and Landrum *et al.* (U.S. Patent No. 5,976,822)). Enzymatic-binding assay formats (e.g., an ELISA, etc.) employ an enzyme as a label; the signal is detectable by the evolution of color or light in the presence of a chromogenic or fluorogenic moiety. Other labels, such as paramagnetic labels,
10 materials used as colored particles, latex particles, colloidal metals such as selenium and gold, and dye particles (see U.S. Patent Nos. 4,313,734; 4,373,932, and 5,501,985) may also be employed. The use of enzymes (especially alkaline phosphatase, β -galactosidase, horse radish peroxidase, or urease) as the detectable label is preferred.

15 The presence of enzymatic labels may be detected through the use of chromogenic substrates (including those that evolve or adsorb fluorescent, UV, visible light, etc.) in response to catalysis by the enzyme label. Detection of label can be accomplished using multiple detectors, multipass filters, gratings, or spectrally distinct fluors (see e.g., U.S. Patent No. 5,759,781), etc. It is particularly
20 preferred to employ peroxidase as an enzyme label, especially in concert with the chromogenic substrate 3, 3', 5, 5'-tetramethylbenzidine (TMB). In the case of labeling of the antibodies with peroxidase as enzyme, it is possible to use the periodate technique (Nakane, P.K. *et al.* (1974) "PEROXIDASE-LABELED ANTIBODY. A NEW METHOD OF CONJUGATION," J Histochem Cytochem. 22:1084-
25 90) or a method reported in which the partners are linked with a heterobifunctional reagent (Ishikawa, E. *et al.* (1983) "ENZYME-LABELING OF ANTIBODIES AND THEIR FRAGMENTS FOR ENZYME IMMUNOASSAY AND IMMUNOHISTOCHEMICAL STAINING," J Immunoassay. 4(3):209-327).

Any of a wide variety of solid supports may be employed in the
30 immunoassays of the present invention. Suitable materials for the solid support are

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synthetics such as polystyrene, polyvinyl chloride, polyamide, or other synthetic polymers, natural polymers such as cellulose, as well as derivatized natural polymers such as cellulose acetate or nitrocellulose, and glass, especially glass fibers. The support can take the form of spheres, rods, tubes, and microassay or
5 microtiter plates. Sheet-like structures such as paper strips, small plates, and membranes are likewise suitable. The surface of the carriers can be permeable and impermeable for aqueous solutions. For the method of the present invention, the substrate is coated on to a solid matrix. Any standard immunoassay solid matrix such as a microtitre plate, beads and the like can be used.

10 Although the foregoing description pertains to assaying for the presence of a TRAIL Compound (e.g., sTRAIL) in a biological sample that is a fluid, it will be appreciated that any biological sample that may be rendered fluidic (e.g., tissue or biopsy extracts, cellular extracts, extracts of feces, sputum, etc.) may likewise be employed in the assays of the present invention.

15 Materials for use in the assays of the invention are ideally suited for the preparation of a kit. Such a kit may comprise a carrier means being compartmentalized to receive in close confinement; one or more containers means vials, tubes and the like; each of the containers means comprising one of the separate elements to be used in the method. For example, in one embodiment, one
20 of the containers means may comprise as a first element a TRAIL Compound (especially, sTRAIL, mTRAIL or rsTRAIL) bound to a solid support. A second container may comprise as a second element a detectably labeled antibody, preferably in lyophilized form, or in solution, that specifically binds to the employed TRAIL Compound. In using the kit, a user adds to a container a
25 premeasured amount of a sample suspected of containing a measurable yet unknown amount of a TRAIL Compound, and a premeasured amount of support-bound TRAIL Compound and the detectably labeled antibody. To the extent that a TRAIL Compound (e.g., sTRAIL, etc.) is present in the sample being tested, it will compete for antibody binding to the immobilized TRAIL Compound. After an
30 appropriate time for incubation, an immune complex is formed and is separated

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from the supernatant fluid, and the immune complex or the supernatant fluid are detected, as by radioactive counting, addition of an enzyme substrate, and color development, or by inclusion of a chemical label (e.g., colloidal gold, latex beads, etc.). Thus, the amount of labeled antibody bound to the support will be inversely
5 proportional to the amount of the selected TRAIL Compound in the sample.

In an alternative embodiment, one of the containers means may comprise as a first element an anti-TRAIL Compound antibody bound to a solid support. A second container may comprise as a second element a detectably labeled antibody, preferably in lyophilized form, or in solution, that specifically binds to a TRAIL
10 Compound. In using the kit, a user adds to a container a premeasured amount of a sample suspected of containing a measurable yet unknown amount of the TRAIL Compound (e.g., sTRAIL), and a premeasured amount of support-bound antibody and the detectably labeled antibody. To the extent that the TRAIL Compound is present in the sample being tested, it will bind to the immobilized antibody, and
15 thus become itself immobilized. After an appropriate time for incubation, an immune complex is formed and is separated from the supernatant fluid, and the immune complex or the supernatant fluid are detected, as by radioactive counting, addition of an enzyme substrate, and color development, or by inclusion of a chemical label (e.g., colloidal gold, latex beads, etc.). Thus, the amount of labeled
20 antibody bound to the support will be directly proportional to the amount of the TRAIL Compound in the sample.

In addition, the kit may also contain one or more containers, each of which comprises a (different) predetermined amount of a TRAIL Compound (e.g., sTRAIL) or anti-TRAIL Compound antibody. These latter containers can be used
25 to prepare a standard curve into which can be interpolated the results obtained from the sample containing the unknown amount of the selected TRAIL Compound.

The present invention particularly relates to the use of immuno-chromatographic assay formats to detect a TRAIL Compound (especially sTRAIL), and particularly in the diagnosis or monitoring of HIV infection and

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AIDS progression. In a preferred immunochromatographic assay format, two contacting, but spatially distinct, porous carriers are employed. By way of illustration with respect to sTRAIL, the first such carrier may preferably contain a non-immobilized, labeled anti-sTRAIL antibody and the second such carrier will preferably contain an immobilized, but unlabeled antibody. The unlabeled antibody may be an anti-sTRAIL antibody (especially one which binds to a different epitope from that recognized by the labeled antibody) or an antibody that specifically binds to the species of unlabeled antibody employed (e.g., where labeled murine anti-sTRAIL IgG antibodies are employed, the unlabeled antibody may be an anti-murine IgG antibody, etc.). Other TRAIL Compounds may be employed in lieu of sTRAIL.

Preferably, the device will comprise a hollow casing constructed of, for example, a plastic material, etc., in which the first carrier will communicate indirectly with the interior of the casing via a multilayer filter system that is accessible from the device (e.g., by protruding therefrom or by being incompletely covered by the device), such that a serum, plasma, or whole blood test sample can be applied directly to the filter system and will permeate therefrom into the first porous carrier. In such a device, the permeation of fluid containing the TRAIL Compound will cause the non-immobilized labeled antibody of the first carrier to become bound to the migrating TRAIL Compound, and will then permeate into the second carrier. Because the second carrier contains immobilized antibody that binds the migrating complex, immune complexes entering the second carrier will be trapped therein.

Detection of labeled antibody in the carrier containing the immobilized unlabeled antibody thus indicates that a TRAIL Compound is present in the sample being evaluated. The assay can be made quantitative by measuring the quantity of labeled molecules that become bound within the second carrier.

Having now generally described the invention, the same will be more readily understood through reference to the following examples, which are

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provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

Example 1

5 **TNF-related Apoptosis-inducing Ligand (TRAIL) in HIV-1-infected Patients and its in vitro Production by Antigen-presenting Cells**

Since sTRAIL has been detected in HIV-1-infected patients, the levels of plasma sTRAIL from HIV-1-infected patients to those of uninfected control donors were compared in order to determine whether sTRAIL levels in AIDS patients were associated with viral load.

10 Exposure of peripheral blood T cells from HIV-1 uninfected donors to infectious HIV-1 or to HIV-1 that had been rendered noninfectious by chemical treatment (Adrithiol-2) (AT-2 HIV-1 particles) results in the expression of membrane TRAIL on CD4⁺ but not CD8⁺ T cells. Therefore, the question of whether mTRAIL and/or sTRAIL would be produced by monocytes, macrophages, 15 and/or dendritic cells from HIV-1-infected and uninfected blood donors upon exposure to AT-2 HIV-1, as well as to infectious HIV-1 was investigated. Both HIV-1_{MN} (CXCR4-tropic) and HIV-1_{Ada} (CCR5-tropic) were used to determine if TRAIL production was HIV-1 coreceptor dependent. Since type I interferons (IFN- α and IFN- β) are required for TRAIL expression and production, the 20 possibilities that inhibition of this pathway would interfere with TRAIL; and that agents that inhibit HIV-1 infection would block HIV-1-induced production of sTRAIL were also investigated. An ex vivo tonsil lymphoid tissue histoculture model was used to determine whether HIV-1 would induce TRAIL in primary human lymphoid tissue.

25 **Methods**

Patients. Peripheral blood was collected from 107 HIV-1-infected patients. Longitudinal study of 8 HIV-1-infected patients who, at time = 0, begin antiretroviral therapy. Patients were followed for 40 weeks. One patient (1) was not on antiretroviral therapy (ART) prior to the commencement of the study, and 30 was then started on Tenofovir, D4T, and Kaletra. The patient went off ART and did

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not start another regimen prior to the end of the study. A second patient (II) was naïve prior to starting the study and was enrolled into a study that included Trizivir, Combivir, and Sustiva. Therapy continued through week 40. A third patient (III) was naïve prior to starting the study and was enrolled in a study that
5 included Trizivir, Combivir, and Sustiva. The patient was switched to DDI, Combivir, and Sustiva, which continued through week 40. A fourth patient (IV) was on ART prior to the commencement of the study and was switched to Kaletra and Sustiva at the start of TRAIL study.

Preparation of AT-2-inactivated virions. HIV-1_{MN} (X4-tropic) and HIV-1_{Ada} (R5-tropic) are propagated as described by Rossio, J. L. *et al.* (1998)
10 (“INACTIVATION OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 INFECTIVITY WITH PRESERVATION OF CONFORMATIONAL AND FUNCTIONAL INTEGRITY OF VIRION SURFACE PROTEINS,” J Virol 72, 7992-8001). Viral supernatants are inactivated with 1 mM AT-2 for 18 h at 4°C before purification. Virus preparations are
15 produced by sucrose gradient banding in a continuous-flow centrifuge (Rossio, J. L. *et al.* (1998) “INACTIVATION OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 INFECTIVITY WITH PRESERVATION OF CONFORMATIONAL AND FUNCTIONAL INTEGRITY OF VIRION SURFACE PROTEINS,” J Virol 72, 7992-8001; Arthur, L. O. *et al.* (1998) “CHEMICAL INACTIVATION OF RETROVIRAL INFECTIVITY BY TARGETING
20 NUCLEOCAPSID PROTEIN ZINC FINGERS: A CANDIDATE SIV VACCINE,” AIDS Res Hum Retroviruses 14 Suppl 3, S311-9). Microvesicles, used as a control reagent, are isolated from supernatants of uninfected cell cultures in a manner identical to that used for virus preparation from infected cells (Bess, J.W. *et al.* (1997) “MICROVESICLES ARE A SOURCE OF CONTAMINATING CELLULAR PROTEINS FOUND
25 IN PURIFIED HIV-1 PREPARATIONS,” Virology 230:134-144).

Isolation and culture of PBMC and T cells. PBMC are isolated by density centrifugation (Ficoll-Hypaque: Uppsala, Sweden) from citrate-anti-coagulated peripheral blood obtained from healthy, HIV-1-seronegative donors by the Department of Blood Transfusion, NIH, Bethesda. Cells are cultured in RPMI
30 medium (Invitrogen, Gaithersburg, MD) with 10% fetal bovine serum (Sigma, St.

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Louis, MO). CD4⁺ and CD8⁺ cells were purified from PBMC by positive selection with anti-CD4⁺ or anti-CD8⁺ beads (Miltenyi Biotech, Auburn, CA).

Heparinized venous blood was collected from HIV-1-infected patients. CD4⁺ and CD8⁺ cells are purified from PBMC by positive selection with anti-CD4
5 or anti-CD8 beads (Miltenyi Biotech, Auburn, CA). Macrophages and dendritic cells are generated using monocytes as described by Chomarat, P. (2000) ("IL-6 SWITCHES THE DIFFERENTIATION OF MONOCYTES FROM DENDRITIC CELLS TO MACROPHAGES," Nat Immunol. 1:510-514). Cells were incubated with AT-2 HIV-1 at a final concentration of 50 ng/mL p24 equivalent. Infectious HIV-1MN and
10 HIV-1LAV were used at the same concentration. For all the experiments, T cells isolated by both negative and positive selection are used interchangeably, without noticeable differences in the experimental outcome.

Ex vivo human lymphoid tissue preparation and infection. Human tonsils were obtained from patients undergoing routine tonsillectomy within 6 h from surgery.
15 Tissues were kept in PBS and washed in complete culture medium composed of RPMI 1640 (Invitrogen) containing 15% heat-inactivated FCS (Sigma), non-essential amino acids (1mM), sodium pyruvate 1mM, L-glutamine (292 µg/ml), amphotericin B (2,5 µg/ml) (Gibco BRL, Grand Island, NJ), timentin (310 µg/ml; SmithKline Bechman, Philadelphia, PA), and gentamicin (50 µg/ml; Quality
20 Control, Inc., MD). Tissues were then sectioned into 2 mm³ blocks and placed on top of medium-hydrated collagen sponge gel (Gelfoam, Pharmacia & Upjohn) in complete medium at the air-liquid interface in 6 well plate (Costar, Cambridge, MA) with 9 blocks per sponge in each well in 4 ml of medium. 24 h later, the culture supernatant was replaced with fresh medium containing 1% Pen/Strep
25 (Gibco BRL), and each tissue block was infected with 1 ng p24_{gag} of an HIV-1 LAV.04 viral stock (3µl, 120 TCID₅₀). Culture medium was sampled and changed every 3 days.

Incubations of PBMC and isolated T cell subsets with viruses. Cells are cultured at 1 x 10⁶ cells/ml in RPMI 10% FCS in 12-well plates. For incubation

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with AT-2 HIV-1, the inactivated virus preparations are added at the initiation of culture to a final concentration of 50 ng/mL p24 equivalent. Infectious HIV-1_{MN} and HIV-1_{LAV} are used at the same concentration.

Plasma quantification of HIV-1 RNA. Plasma HIV-1 RNA is measured by
5 quantitative RT-PCR and all data are expressed in copies/ml (Amplicor Monitor; Roche Diagnostic Systems, Branchburg, NY, USA; detection limit 50 copies/ml).

Example 2

Flow Cytometry Investigation

Membrane TRAIL detection. Monocytes are cultured for 24 h in the absence or
10 presence of AT-2 HIV-1_{MN}, AT-2 HIV-1_{Ada} and infectious HIV-1_{MN} viruses. The cells are washed twice in PBS. Membrane TRAIL expression is determined by incubating cells for 20 minutes at room temperature with PE-conjugated mouse IgG1 anti-human TRAIL monoclonal antibody, (clone B-S23 Diaclone, Besançon France) or with relevant control isotype-matched antibodies (at 5 µg/mL each); in
15 PBS containing 2% mouse serum (SIGMA, St. Louis, MO).

Data acquisition and analysis. Cells are acquired on FACSCalibur flow cytometer (Becton Dickinson), using CellQuest software (Becton Dickinson Immunocytometry System). Samples are gated on viable cells by forward and side light scatters, and at least 50,000 live cell events are acquired for each sample.
20 Acquired data is analyzed using Cellquest software (Becton Dickinson).

Soluble TRAIL measurement. Levels of soluble TRAIL are measured in serum of HIV⁺ or HIV⁻ individuals, in PBMC and monocytes supernatants with the commercial TRAIL ELISA kit (Diaclone, Besancon, France), according to the manufacturer's instructions.

25 **Blocking assay.** To block TRAIL production, monocytes are cultured 3 days with AT-2 HIV-1_{MN/Ada} in the presence of CD4 binding inhibitor soluble CD4 (2 µg/ml), or the fusion inhibitor T20 (2 µg/ml), or the CXCR4 inhibitor AMD-310 (2 µg/ml) (AIDS Research and Reagents Program, NIAID, Bethesda, MD), or in

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presence of both sheep polyclonal anti-human IFN- α (2000 IFN- α neutralizing U/ml) and sheep polyclonal anti-human IFN- β antibodies (500 IFN- β neutralizing U/ml) (BioSource International, Camarillo, CA). Recombinant IFN- α and IFN- β (Peprotech Inc, Rocky Hill, NJ) are used at 200 U/ml. TRAIL production is

5 quantified by ELISA.

Example 3 Real Time Quantitative RT-PCR

RNA extraction and reverse transcription. Total RNA is extracted from monocytes with the acid guanidium thiocyanate-phenol-chloroform method

10 (Chomczynski, P. *et al.* (1987) "SINGLE-STEP METHOD OF RNA ISOLATION BY ACID GUANIDINIUM THIOCYANATE-PHENOL-CHLOROFORM EXTRACTION," Anal Biochem 162:156-159), modified for TRIzol (Invitrogen, Carlsbad, CA, USA). The amount and the purity of the extracted RNAs are determined by spectrophotometric analysis at 260nm and 280nm. One μ g of total RNA is reverse

15 transcribed into first-strand cDNA in a 20 μ l final volume containing 1 μ M of random hexanucleotide primers, 1 μ M of oligo dT and 200 U of Molony murine leukemia virus reverse transcriptase. (Promega, Madison, WI, USA).

Real Time PCR. cDNA quantification for TRAIL and GAPDH is obtained with a real time PCR technique. Real time PCR is conducted with the ABI Prism

20 7900HT (Applied Biosystems, Foster City, CA, USA). All reactions are performed using a SYBR green PCR mix (QuantiTect SYBR Green PCR, Qiagen, Valencia, CA, USA), according to the following thermal profile: denaturation at 95°C for 15 sec, annealing at 60°C (61°C for TRAIL) for 15 sec, extension at 72°C for 15 sec (data collection is performed during the extension step). The primer

25 sequences are designed using the Primer Express Software v2.0 provided with the ABI Prism 7900HT

TRAIL	Forward	(SEQ ID NO:3)	GCTCTGGGCC	GCAAAT
	Reverse	(SEQ ID NO:4)	TGCAAGTTGC	TCAGGAATGA A
GAPDH	Forward	(SEQ ID NO:9)	CCACCCATGG	CAAATTCC
	Reverse	(SEQ ID NO:10)	TGGGATTTCC	ATTGATGACA AG

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All reactions are performed in triplicate (denaturation at 95°C for 15 sec, annealing at 60°C (61°C for TRAIL) for 15 sec, extension at 72°C for 15 sec).. Data analysis is performed with the SDS2.1 software, provided with the ABI Prism 7900HT. The threshold level is determined by the software according to the optimization of the standard curve. Standards are obtained by amplification of a control sample in a PCR reaction, using the same primers, reagents and conditions optimized for the real time analysis. Arbitrary quantity values are assigned to the resulting standard and 4-fold serial dilutions are made to obtain an 8-point standard curve. Results are presented as ratios between the target gene and the GAPDH mRNA.

Western blot. Monocytes were cultured with AT-2 HIV-1_{MN}, AT-2 HIV-1_{ADA}, recombinant IFN- α (10 ng/ml) or IFN- β (10 ng/ml), anti-IFN- α and anti-IFN- β (R & D Systems, Minneapolis MN). After 24 h, the cells were pelleted, washed in PBS and lysed (1% NP-40, 200mM NaCl, 50mM Tris pH 7.5, supplemented with protease inhibitor cocktail [Roche, Indianapolis, IN]). Total protein was quantified by BCA (Pierce, Rockford, IL) and 10 μ g of protein was mixed 1:1 with SDS Protein Gel Buffer (Quality Biologics, Gaithersburg, MD). Samples were run on a 10% or 15% SDS-PAGE gel (Bio-Rad, Hercules, CA). After transfer, the blot was blocked in 3% milk/TBST then incubated with anti-STAT1 or anti-STAT2 (Upstate Biotechnology, Inc., Lake Placid, NY) antibodies followed by anti-rabbit HRP secondary antibody (Jackson, West Grove, PA). ECL was performed and bands were visualized on Hyperfilm (Amersham, Piscataway, NJ). B-actin was visualized as a loading control using a mouse monoclonal antibody (Sigma).

Statistical Analysis. All experiments were repeated a minimum of three times. All comparisons of data were made with a two-sided Student's *t* test (data meet the requirements for a Student's *t* test). Differences in plasma TRAIL between HIV-1-infected patients and healthy donors were also analyzed using F-test to compare variances. All the statistic data were analyzed using Prism software (GraphPad, San Diego, CA).

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Example 4 TRAIL Levels In Plasma Of AIDS Patients And Uninfected Controls

To determine whether TRAIL is produced in AIDS patients as a result of infection with HIV-1, plasma from patients with AIDS, containing higher levels of soluble TRAIL, is tested and compared to healthy donors. As shown in **Figure 1, Panel A** the mean values of plasma sTRAIL are $852 \pm$ pg/ml for 53 control donors, 1339 ± 79 pg/ml for 49 HIV-1-infected patients with undetectable viral load (<50 mRNA copies/ml of blood) and 2240 ± 131 pg/ml for 58 HIV-1-infected patients with detectable viral load (>50 mRNA copies/ml of blood). These results suggest that in vivo TRAIL expression (and particularly sTRAIL expression) is increased in HIV-infected patients and is linked to the viral load. However, there appeared to be a saturation of the sTRAIL level in patients (12/58) with viral loads exceeding 40,000 RNA copies/mL, since higher viral loads were not associated with further increases in sTRAIL.

The correlation between viral loads and the CD4 counts of the 59 patients (with high viral load) and the TRAIL levels in plasma is determined. As shown in **Figure 1, Panel B** a positive correlation between viral load and TRAIL in plasma was found. The sTRAIL values obtained from the 58 HIV-1-infected patients with detectable viral load are plotted and compared with the predicted TRAIL values (solid diagonal line), assuming a perfect correlation between TRAIL and viral load.

The data of **Figure 1, Panel C** provide a longitudinal study of four HIV-1-infected patients who began HAART therapy and were subsequently followed for 40 weeks with measurement of plasma viral load and sTRAIL. Patient I exhibited an initial parallel drop in viral load and sTRAIL, followed by a concomitant rebound in both of these parameters. This rebound in viral load may reflect development of HIV-1 drug resistance. Patient II showed a continuous parallel drop in viral load and sTRAIL. Patient III showed three-of-four points in which changes in viral load and sTRAIL levels were similar. Patient IV, who had received ART prior to enrollment into this study, exhibited a parallel flat and low profile for both viral load and sTRAIL throughout the 40 weeks. This patient's

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initial low viral may have been due to successful ART prior to enrollment, which continued during this ART protocol.

Thus, both cross-sectional and longitudinal association between higher plasma viral loads and elevated sTRAIL levels were observed in two different cohorts of HIV-1-infected patients, and a parallel between ART-induced reduction in viral load and sTRAIL levels was observed.

Example 5 **Noninfectious HIV-1 Induces TRAIL Production By Monocytes**

To study the effects of noninfectious viruses, PBMC from healthy HIV-uninfected donors and AIDS patients are cultured in the presence of microvesicles (negative control), AT-2 HIV-1_{MN} (CXCR4-tropic) or AT-2 HIV-1_{Ada} (CCR5-tropic) for 3-to-6 days. sTRAIL levels in the culture supernatants are determined by ELISA. TRAIL production in untreated cultures or in cultures of PBMC from control donors treated with microvesicles (n=10) is not detected. In contrast unstimulated PBMC from AIDS patients produced 190 ± 10 pg/ml of sTRAIL (n=16). Exposure to AT-2 HIV-1_{MN} for 3 days induced sTRAIL production by PBMC from control donors (800 ± 160), as well as from AIDS patients (760 ± 140). Similarly, exposure to AT-2 HIV-1_{Ada} induced TRAIL production by both PBMC from controls (900 ± 300) and HIV-1-infected patients (640 ± 300) (**Figure 2, Panel A**).

To determine which cell types present in PBMC are producing sTRAIL in response to AT-2 HIV-1, isolated CD4⁺ and CD8⁺ T cells and monocyte-derived macrophages and dendritic cells are tested for TRAIL-production after culture with AT-2 HIV-1. Monocytes from HIV-1-infected patients, but not from healthy donors, produced sTRAIL (130 ± 40 pg/ml) when cultured without AT-2 HIV-1. Monocytes produced high levels of sTRAIL after exposure to AT-2 HIV-1 (MN or Ada) (**Figure 2 Panel B**). No significant differences were observed between monocytes from uninfected donors and AIDS patients. Exposure of monocyte-derived dendritic cells from HIV-1-infected but not from uninfected donors

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produced significant amounts (210 ± 20 pg/mL), although lower levels of sTRAIL than monocytes (1100 ± 210) (**Figure 2, Panel B**). Because membrane and soluble TRAIL are two forms of active TRAIL, the expression of mTRAIL was also studied. Increased expression of mTRAIL in AT-2 HIV-1-exposed monocytes
5 (p<0.0001), compared to microvesicle-treated or untreated monocytes (controls) is found. After AT-2 HIV-1_{MN} exposure for 3 days, 80 ± 8 % of monocytes expressed mTRAIL with a MFI = 19 ± 3 compared to 7% (MFI = 6 ± 1) of control monocytes (**Figure 2, Panel C**). Similar results are obtained using AT-2 HIV-1_{Ada}.

Monocytes from uninfected donors treated with AT-2 HIV-1_{MN} are also
10 tested for TRAIL mRNA expression using a real time quantitative PCR. TRAIL mRNA is barely detectable in untreated or microvesicles-treated monocytes, whereas exposure to AT-2 HIV-1_{MN} induced 20-fold increase in TRAIL mRNA expression (p=0.0045) (**Figure 2, Panel D**). These results indicated that monocytes expressed TRAIL mRNA and produced sTRAIL and mTRAIL upon
15 exposure to AT-2 HIV-1. In contrast, sTRAIL was not produced by CD4+ or CD8+ T cells nor by macrophages. However, CD4+ T cells express mTRAIL but not sTRAIL after exposure to AT-2 HIV-1.

To investigate whether low amount of virus induce sTRAIL production, a dose response experiment is performed in which monocytes were cultured for 3
20 days with dilutions of AT-1 HIV-1, spanning 4 logs of concentration. Very low concentrations of AT-2 HIV-1_{MN} induced measurable production of sTRAIL (**Figure 2, Panel E**). Optimal concentrations of AT-2 HIV-1_{MN} for sTRAIL production were in the 5-to-50 ng/ml (p24^{CA} equivalents) range, with no further increase in TRAIL at higher virus concentrations. Therefore, AT-2 HIV-1 was
25 used at 50 ng/ml for subsequent experiments.

Example 6

Noninfectious HIV-1 Induced TRAIL Production By The Type I IFN Pathway

To test whether type I IFN are required for TRAIL production by monocytes isolated from PBMC of control donors, an attempt was made to block

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TRAIL production using anti-IFN- α and/or anti-IFN- β antibodies. Anti-IFN- α Ab or anti-IFN- β Ab inhibited the sTRAIL production of AT-2 HIV-1-exposed monocytes by 25 ± 11 % and 40 ± 3 % inhibition of TRAIL production, respectively. When used together, these antibodies reduced TRAIL production by 76 ± 4 % (n = 6 experiments) in monocytes exposed to AT-2 HIV-1_{MN} (p<0.00001) (**Figure 3, Panel A**). Similar results were obtained using HIV-1_{Ada}. The effect of Ab against type I interferons on transcription of the TRAIL gene is also tested. Addition of anti-IFN- α and - β blocking mAb decreased TRAIL mRNA expression by 70 ± 30 % (p=0.046) (**Figure 3, Panel B**). To determine whether IFN- α and IFN- β were both involved in TRAIL production, monocytes were cultured in the presence of recombinant IFN- α or IFN- β . IFN- α induced 730 ± 200 pg/ml and IFN- β 1650 ± 300 pg/ml of sTRAIL (**Figure 3, Panel C**). Because AT-2 HIV-1 induction of TRAIL is IFN- α/β -dependent, it was verified that monocytes from uninfected donors cultured with AT-2 HIV-1_{MN} or AT-2 HIV-1_{Ada} produced IFN- α (**Figure 3, Panel D**). Moreover, it is demonstrated that AT-2 HIV-1_{MN} and AT-2 HIV-1_{Ada}, as well as recombinant IFN- α/β , increased monocyte expression of STAT1 and STAT2 (**Figure 3, Panel E**), the principal signaling molecules for type I IFN (Qureshi, S.A. *et al.* (1995) "TYROSINE-PHOSPHORYLATED STAT1 AND STAT2 PLUS A 48-KDA PROTEIN ALL CONTACT DNA IN FORMING INTERFERON-STIMULATED-GENE FACTOR 3," *Proc Natl Acad Sci U S A.* 92:3829-3833; Leung, S. *et al.* (1995) "ROLE OF STAT2 IN THE ALPHA INTERFERON SIGNALING PATHWAY," *Mol Cell Biol.* 15:1312-1317). Taken together, these findings indicate that AT-2 HIV-1-induced expression and production of TRAIL by monocytes is dependent on type I interferons.

Tests were conducted as to whether noninfectious HIV-1-induced production of sTRAIL would be inhibited by: 1) soluble CD4, that blocks the interaction between viral gp120 and CD4 expressed on CD4⁺ cells; 2) AMD-310, that blocks CXCR4 coreceptor binding; 3) T20, that prevents fusion of the virus with the CD4 cell membrane. The results (**Figure 3, Panel F**) demonstrate that only soluble CD4 inhibited HIV-1_{MN} from inducing sTRAIL production. These

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results are consistent with a triggering mechanism that is dependent on very early CD4/gp120 binding events, but which does not require early post-binding events such as co-receptor engagement or membrane fusion.

All of the results presented above were reproduced using the infectious counterparts of HIV-1_{MN} and HIV-1_{Ada}. Infectious HIV-1_{LAV}, which is commonly used in tonsil model (Grivel, J.C. *et al.* (1999) "CCR5- AND CXCR4-TROPIC HIV-1 ARE EQUALLY CYTOPATHIC FOR THEIR T-CELL TARGETS IN HUMAN LYMPHOID TISSUE," Nat Med. 1999;5:344-346), also induced TRAIL production in monocytes.

10 **Example 7**
Infectious HIV-1 Induces IFN Type I-Dependent TRAIL Production
By Monocytes

The effect of infectious HIV-1 on induction of TRAIL production by monocytes, similar to that obtained using noninfectious HIV-1, is tested. PBMC are cultured for 3 days in the presence of infectious HIV-1_{MN} at the same concentration used for noninfectious HIV-1. Infectious HIV-1_{LAV} is also tested for TRAIL production. As shown on **Figure 4, Panel A** HIV-1_{MN}-stimulated monocytes produced 1400±190 pg/ml of sTRAIL. This level of sTRAIL is not significantly different from the level detected after AT-2 HIV-1_{MN} exposure (p<0.5). HIV-1_{LAV} produces only a slightly lower level of sTRAIL. The combination of anti-IFN α and anti-IFN β mAb reduces production of sTRAIL by monocytes cultured with HIV-1_{MN} by 90±3 % (p<0.0001) (**Figure 4, Panel B**). Infectious HIV-1_{MN} also induces mTRAIL expression on monocytes at similar levels to those induced by noninfectious virus. After exposure to infectious HIV-1_{MN}, 75±8 % of monocytes expressed mTRAIL, with a MFI=15±3 (**Figure 4, Panel C**).

Example 8
Infection With HIV-1 Induces TRAIL Production In Tonsil Tissue Culture

To investigate whether TRAIL could be produced in primary lymphoid organs, human tonsils were infected with infectious HIV-1_{LAV}, the HIV-1 strain

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used previously in this model (Grivel, J.C. *et al.* (1999) "CCR5- AND CXCR4-TROPIC HIV-1 ARE EQUALLY CYTOPATHIC FOR THEIR T-CELL TARGETS IN HUMAN LYMPHOID TISSUE," Nat Med. 1999;5:344-346), and the TRAIL production was measured at different times ranging from 1-to-12 days. As shown in **Figure 5**,
5 **Panels A-C**, for each of three independent experiments using tissues from three different individuals, increased TRAIL secretion after infection with HIV-1_{Lav} compared to uninfected tonsils ($p < 0.001$) is observed. These results suggest that sTRAIL can be found in the primary lymphoid organs of HIV-1-infected patients, as well as in their blood (see **Figure 1**).

10 **Example 9**
TNF-Related Apoptosis-Inducing Ligand (TRAIL) In HIV-1-Infected Patients
And Its In Vitro Production By Antigen-Presenting Cells

As indicated above, elevated sTRAIL levels are found in the plasma of HIV-1-infected patients compared to healthy control donors. After in vitro
15 exposure to either infectious or noninfectious HIV-1, it is found that both sTRAIL and mTRAIL are produced by monocytes. Monocyte-derived dendritic cells from HIV-1-infected patients also produce low levels of sTRAIL. In contrast, sTRAIL is not produced by macrophages or by CD4⁺ or CD8⁺ T cells upon exposure to HIV-1. TRAIL production is associated with increased transcription of the TRAIL
20 gene in monocytes exposed to HIV-1 particles, and is mediated by the type I IFN pathway. Induction of sTRAIL by HIV-1 is likely to be dependent on CD4-gp120 interaction since it is blocked by soluble CD4. Finally, the ex vivo tonsil model demonstrates that TRAIL can be produced in primary lymphoid tissue after HIV-1 infection.

25 Soluble TRAIL was reported in HIV-1-infected patients (Liabakk, N.B. *et al.* (2002) "Development, Characterization And Use Of Monoclonal Antibodies Against Strail: Measurement Of Strail By ELISA," J Immunol Methods. 259:119-128) and in vitro studies linked TRAIL to the depletion of T cells from HIV-1-infected patients (Yang, Y. *et al.* (2003) "Monocytes Treated with Human
30 Immunodeficiency Virus Tat Kill Uninfected CD4(+) Cells by a Tumor Necrosis

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Factor-Related Apoptosis-Induced Ligand-Mediated Mechanism,” J Virol. 77:6700-6708; Miura, Y. *et al.* (2001) “Critical Contribution of Tumor Necrosis Factor-related Apoptosis-inducing Ligand (TRAIL) to Apoptosis of Human CD4(+) T Cells in HIV-1-infected hu-PBL-NOD-SCID Mice. J Exp Med. 5 2001;193:651-660)^{5,29}. However, the identification of TRAIL-producing cells in vivo, the association of viral load with the level of sTRAIL produced, and the mechanism responsible for TRAIL-mediated T cell death remain to be established. The present study demonstrates that sTRAIL can be detected in the plasma of HIV-1-infected patients. Furthermore, the amount of sTRAIL is higher in patients with
10 elevated viral load, than in patients with low or undetectable viral loads. However, plasma from all HIV-1 patients tested showed higher TRAIL content than plasma from healthy donors. Our 40-week longitudinal study of patients on ART showed striking parallel changes between viral load and TRAIL levels. Thus when ART decreased viral load, we observed a concomitant decrease in plasma TRAIL, which
15 may be one of the reasons for the efficacy of antiviral therapy.

More than 95% of HIV-1 virions detected in HIV-1-infected patients’ plasma do not possess culturable infectivity (Piatak, M, Jr. *et al.* (1993) “HIGH LEVELS OF HIV-1 IN PLASMA DURING ALL STAGES OF INFECTION DETERMINED BY COMPETITIVE PCR,” Science. 259:1749-1754), which raises the possibility that
20 noninfectious virus particles contribute to HIV-1 pathogenesis. The above Examples demonstrate that the major cellular source of TRAIL is monocytes, which produce TRAIL in response to both infectious and chemically-inactivated X4 (MN) and R5 (Ada) HIV-1. Interestingly, PBMC and DC from HIV-1-infected donors produced sTRAIL without further stimulation, suggesting that previous
25 contact with HIV-1 was sufficient to induce sTRAIL secretion. However, after 5 days of culture, sTRAIL production disappeared if the patients’ PBMC were not stimulated by HIV-1 particles, suggesting that continuous cell-virus interaction is required to maintain sTRAIL production.

Type I interferons are produced as a result of viral infection and can exert
30 anti-viral effects (Sato, K. *et al.* (2001) “ANTIVIRAL RESPONSE BY NATURAL

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KILLER CELLS THROUGH TRAIL GENE INDUCTION BY IFN-ALPHA/BETA,” *Eur J Immunol.* 31:3138-3146). Type I interferons also induce TRAIL expression through the interferon-stimulated response element (ISRE), which binds IFN-inducible genes (Kayagaki, N. *et al.* (1999) “TYPE I INTERFERONS (IFNS) REGULATE TUMOR NECROSIS FACTOR-RELATED APOPTOSIS-INDUCING LIGAND (TRAIL) EXPRESSION ON HUMAN T CELLS: A NOVEL MECHANISM FOR THE ANTITUMOR EFFECTS OF TYPE I IFNS,” *J Exp Med.* 1999;189:1451-1460. The above Examples demonstrate that in monocytes, type I interferons are induced by exposure to HIV-1 virions, thereby providing a mechanism for induction of TRAIL production in response to HIV-1. Recombinant IFN- α or IFN- β induced sTRAIL production, while antibodies against these interferons greatly reduced both TRAIL gene transcription and production in monocytes exposed to AT-2 HIV-1. The blockade of TRAIL production by soluble CD4, strongly suggests that the interaction between viral gp120 and the CD4 molecule on monocytes is required for HIV-1-induction of sTRAIL production. Conversely, the above-described findings that AMD-310 and T20 did not significantly inhibit sTRAIL production suggest that co-receptor binding and viral fusion with the target cell membrane are not required for HIV-1 activation of sTRAIL production by monocytes. Thus, only the earliest interaction between HIV-1 and CD4 on monocytes is important for initiation of TRAIL production.

HIV-1 induced TRAIL production was also tested in an ex vivo human tonsil model that resembles human lymphoid organ where critical events of HIV disease occur. Increased TRAIL secretion was seen in tonsil organ cultures from three different donors after HIV-1 infection. TRAIL production was maintained over an extended time period; higher TRAIL production was observed in infected rather than in uninfected tonsil cultures after 10 days. Detection of TRAIL in these HIV-1-infected cultures is consistent with increased apoptosis of CD4⁺ T cells after HIV-1 infection of tonsil cultures (Grivel, J.C. *et al.* (2000) “HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 INDUCES APOPTOSIS IN CD4(+) BUT NOT IN CD8(+) T CELLS IN EX VIVO-INFECTED HUMAN LYMPHOID TISSUE,” *J Virol.*

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74:8077-8084). Because CD4⁺ T cells express mTRAIL but do not produce sTRAIL upon exposure to HIV-1, the sTRAIL detected in plasma of HIV-1-infected patients is unlikely to have been secreted by CD4⁺ T cells, and may have been produced by monocytes and/or dendritic cells after exposure to HIV-1.

- 5 Furthermore, since B cells, present in large number in tonsil, did not produce TRAIL, it is possible that dendritic cells in the tonsil were the source of sTRAIL in the employed HIV-1-infected tonsil cultures.

The findings of the present study, in combination with the present observation that the membrane DR5 death receptors, required for induction of cell death via TRAIL, are expressed on CD4⁺ but not CD8⁺ T cells from HIV-1-infected patients, suggest a plausible mechanism for preferential loss of CD4⁺ T cells, in HIV-1 infection. In this model sTRAIL and/or mTRAIL produced by monocytes contribute to TRAIL-mediated death of CD4⁺ T cells in HIV-1-infected patients by directly interacting with the DR5 death receptor expressed on CD4⁺ T cells.

Establishment of the in vivo relevance of our in vitro findings to the loss of CD4⁺ T cells in HIV-1-infected patients may point toward novel therapeutic approaches for maintaining immune cell numbers and function. In particular, as indicated above, sCD4 was the most efficient inhibitor of TRAIL production, suggesting that this reagent might provide effective therapy that would inhibit the interaction of either infectious or noninfectious HIV-1 with the CD4 molecule that results in TRAIL production by CD4-expressing cells.

In sum, there is now considerable in vitro evidence that TNF-related apoptosis-inducing ligand (TRAIL) is involved in HIV-1 pathogenesis by inducing CD4⁺ T cell death characteristic of AIDS. Therefore, the levels of TRAIL in plasma samples from 107 HIV-1-infected and 53 uninfected controls, as well as in longitudinal plasma samples from patients who started antiretroviral therapy (ART) were tested. TRAIL was elevated in plasma of HIV-1-infected patients compared to uninfected individuals, and patients receiving ART showed decreased

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plasma TRAIL levels that correlated with reduction in viral load. In vitro exposure to infectious and noninfectious HIV-1 induced TRAIL in monocytes and marginally in DC, but not in macrophages, or T cells. Interestingly, the HIV-1 entry inhibitor, soluble CD4, blocked HIV-1-induced production of TRAIL.

- 5 Furthermore, production and gene expression of TRAIL by monocytes were regulated by type I interferon via STAT1/STAT2 signaling molecule. Ex vivo HIV-1 infection of human tonsil lymphoid tissue also resulted in increased TRAIL production. The above examples demonstrate that plasma TRAIL is elevated in HIV-1-infected patients, and is decreased by ART therapy. The high production of
- 10 TRAIL by antigen-presenting cells may contribute to the death of CD4⁺ T cells during progression to AIDS.

Example 10
A Model for Type I Interferon-dependent CD4⁺ T Cell Apoptosis in AIDS: Two Hits to Death

- 15 The pathogenic mechanisms responsible for the extensive depletion of CD4⁺ T cells characteristic of AIDS have not previously been well understood. As indicated above, although HIV-1-infected T cells die, the frequency of infected cells appears to be too low to account for the depletion of CD4⁺ T cells (Harper, M.E. *et al.* (1986) "DETECTION OF LYMPHOCYTES EXPRESSING HUMAN T-
- 20 LYMPHOTROPIC VIRUS TYPE III IN LYMPH NODES AND PERIPHERAL BLOOD FROM INFECTED INDIVIDUALS BY IN SITU HYBRIDIZATION," *Proc Natl Acad Sci U S A* 83:772-776; Grivel, J.C. *et al.* (2000) "HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 INDUCES APOPTOSIS IN CD4⁽⁺⁾ BUT NOT IN CD8⁽⁺⁾ T CELLS IN EX VIVO-INFECTED HUMAN LYMPHOID TISSUE," *J Virol* 74:8077-8084; Schnittman, S.M. *et al.* (1990)
- 25 "INCREASING VIRAL BURDEN IN CD4⁺ T CELLS FROM PATIENTS WITH HUMAN IMMUNODEFICIENCY VIRUS (HIV) INFECTION REFLECTS RAPIDLY PROGRESSIVE IMMUNOSUPPRESSION AND CLINICAL DISEASE," *Ann Intern Med* 113:438-443; Schnittman, S.M. *et al.* (1989) "THE RESERVOIR FOR HIV-1 IN HUMAN PERIPHERAL BLOOD IS A T CELL THAT MAINTAINS EXPRESSION OF CD4," *Science*
- 30 245:305-308). Therefore, indirect mechanisms involving the death of uninfected CD4⁺ T cells have been proposed (Badley, A.D. *et al.* (2000) "MECHANISMS OF

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HIV-ASSOCIATED LYMPHOCYTE APOPTOSIS,” *Blood* 96:2951-2964; Finkel, T.H. *et al.* (1995) “APOPTOSIS OCCURS PREDOMINANTLY IN BYSTANDER CELLS AND NOT IN PRODUCTIVELY INFECTED CELLS OF HIV- AND SIV-INFECTED LYMPH NODES,” *Nat Med* 1:129-134). Several reports suggested that activation-induced cell death (AICD) of T lymphocytes might be a major contributor to CD4⁺ T cell depletion (Groux, H. *et al.* (1992) “ACTIVATION-INDUCED DEATH BY APOPTOSIS IN CD4⁺ T CELLS FROM HUMAN IMMUNODEFICIENCY VIRUS-INFECTED ASYMPTOMATIC INDIVIDUALS,” *J Exp Med* 175:331-340; Ameisen, J.C. *et al.* (1991) “CELL DYSFUNCTION AND DEPLETION IN AIDS: THE PROGRAMMED CELL DEATH HYPOTHESIS,” *Immunol Today* 12:102-105). However, both CD4⁺ and CD8⁺ T cells are activated by HIV-1 infection (Groux, H. *et al.* (1992) “ACTIVATION-INDUCED DEATH BY APOPTOSIS IN CD4⁺ T CELLS FROM HUMAN IMMUNODEFICIENCY VIRUS-INFECTED ASYMPTOMATIC INDIVIDUALS,” *J Exp Med* 175:331-340; Grossman, Z. *et al.* (2002). “CD4⁺ T-CELL DEPLETION IN HIV INFECTION: ARE WE CLOSER TO UNDERSTANDING THE CAUSE”? *Nat Med* 8:319-323), and both subsets of T cells are susceptible to apoptosis (Meyaard, L. *et al.* (1992) “PROGRAMMED DEATH OF T CELLS IN HIV-1 INFECTION,” *Science* 257:217-219). Therefore, current models of AIDS pathogenesis do not adequately account for the preferential loss of CD4⁺ T cells during progression to AIDS.

Type I interferons (IFN α/β) have anti-viral activity, including against HIV-1 (Yamamoto, J.K. *et al.* (1986) “HUMAN ALPHA- AND BETA-INTERFERON BUT NOT GAMMA- SUPPRESS THE IN VITRO REPLICATION OF LAV, HTLV-III, AND ARV-2,” *J Interferon Res* 6:143-152). A recent study indicated that murine viral infections that induce IFN- α/β production, activate STAT1 and STAT2, resulting in expression and synthesis of p53 and apoptosis of virus-infected cells by an yet undefined (Takaoka, A. *et al.* (2003) “INTEGRATION OF INTERFERON-ALPHA/BETA SIGNALLING TO P53 RESPONSES IN TUMOUR SUPPRESSION AND ANTIVIRAL DEFENSE,” *Nature* 424:516-523) but important mechanism that can control viral infection (Vilcek, J. (2003) “BOOSTING P53 WITH INTERFERON AND VIRUSES,” *Nat Immunol* 4:825-826). However, if this cascade of type I interferon-dependent

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events leading to apoptosis of virally-infected cells also occurs when a virus interacts with target cells without productively infecting them, then these virus-exposed uninfected target cells might be signaled to die along with infected cells. In HIV-1 infection, where a large but variable percentage of viral particles are defective (Dimitrov, D.S. (1993) "QUANTITATION OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 INFECTION KINETICS," J Virol 67:2182-2190; Piatak, M., Jr. *et al.* (1993) "HIGH LEVELS OF HIV-1 IN PLASMA DURING ALL STAGES OF INFECTION DETERMINED BY COMPETITIVE PCR," Science 259:1749-1754), interaction of CD4⁺ T cells with viral particles might induce apoptosis of CD4⁺ T cells without evidence of extensive HIV infection.

As indicated above, TNF-related apoptosis-inducing ligand (TRAIL), a member of the TNF superfamily (Wiley, S.R. *et al.* (1995) "IDENTIFICATION AND CHARACTERIZATION OF A NEW MEMBER OF THE TNF FAMILY THAT INDUCES APOPTOSIS," Immunity 3:673-682), induces apoptosis in human tumor cell lines (Griffith, T.S. *et al.* (1998) "INTRACELLULAR REGULATION OF TRAIL-INDUCED APOPTOSIS IN HUMAN MELANOMA CELLS," J Immunol 161:2833-2840; Herbeval, J.P. *et al.* (2003) "MACROPHAGES FROM CANCER PATIENTS: ANALYSIS OF TRAIL, TRAIL RECEPTORS, AND COLON TUMOR CELL APOPTOSIS," J Natl Cancer Inst 95:611-621), and in virus-infected cells (Jeremias, I. *et al.* (1998) "TRAIL/APO-2-LIGAND-INDUCED APOPTOSIS IN HUMAN T CELLS," Eur J Immunol 28:143-152; Clarke, P. *et al.* (2003) "TWO DISTINCT PHASES OF VIRUS-INDUCED NF-KAPPAB-REGULATION ENHANCE TRAIL-MEDIATED APOPTOSIS IN VIRUS-INFECTED CELLS," J Biol Chem. 278(20):18092-100. Epub 2003 Mar 13), but not in normal cells (Gura, T. (1997) "How TRAIL KILLS CANCER CELLS, BUT NOT NORMAL CELLS," Science 277:768). TRAIL has five receptors, two (DR4 and DR5) that induce cell death, and three others that lack the death domain (Pan, G. *et al.* (1997) "THE RECEPTOR FOR THE CYTOTOXIC LIGAND TRAIL," Science 276:111-113). The TRAIL gene is regulated by type I interferons (Gong, B. *et al.* (2000) "GENOMIC ORGANIZATION AND TRANSCRIPTIONAL REGULATION OF HUMAN APO2/TRAIL GENE," Biochem Biophys Res Commun 278:747-752), and the DR5 gene is transcriptionally regulated by p53 (Wu, G.S. *et al.* (1997) "KILLER/DR5 IS A

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- DNA DAMAGE-INDUCIBLE P53-REGULATED DEATH RECEPTOR GENE,” *Nat Genet* 17:141-143). TRAIL may be involved in CD4⁺ T cell depletion, since TRAIL, produced by monocytes exposed to the HIV-1 Tat protein, killed uninfected CD4⁺ T cells (Yang, Y. *et al.* (2003) “MONOCYTES TREATED WITH HUMAN
- 5 IMMUNODEFICIENCY VIRUS TAT KILL UNINFECTED CD4⁽⁺⁾ CELLS BY A TUMOR NECROSIS FACTOR-RELATED APOPTOSIS-INDUCED LIGAND-MEDIATED MECHANISM,” *J Virol* 77:6700-6708). Moreover, soluble TRAIL was found in HIV-1-infected patients (Liabakk, N.B. *et al.* (2002) “DEVELOPMENT, CHARACTERIZATION AND USE OF MONOCLONAL ANTIBODIES AGAINST STRAIL:
- 10 MEASUREMENT OF STRAIL BY ELISA,” *J Immunol Methods* 259:119-128; Stylianou, E. *et al.* (2003) “RAISED SERUM LEVELS OF INTERLEUKIN-18 IS ASSOCIATED WITH DISEASE PROGRESSION AND MAY CONTRIBUTE TO VIROLOGICAL TREATMENT FAILURE IN HIV-1-INFECTED PATIENTS,” *Clin Exp Immunol* 132:462-466), and may be responsible for the death of neurons in AIDS
- 15 patients leading to dementia (Ryan, L.A. *et al.* (2004) “TNF-RELATED APOPTOSIS-INDUCING LIGAND MEDIATES HUMAN NEURONAL APOPTOSIS: LINKS TO HIV-1-ASSOCIATED DEMENTIA,” *J Neuroimmunol* 148:127-139). TRAIL has been implicated, in vitro, in the apoptosis of HIV-1-infected cells (Lum, J.J. *et al.* (2001) “INDUCTION OF CELL DEATH IN HUMAN IMMUNODEFICIENCY VIRUS-INFECTED
- 20 MACROPHAGES AND RESTING MEMORY CD4 T CELLS BY TRAIL/APO2L,” *J Virol* 75:11128-11136), including CD4⁺ and CD8⁺ T cells (Jeremias, I. *et al.* (1998) “TRAIL/APO-2-LIGAND-INDUCED APOPTOSIS IN HUMAN T CELLS,” *Eur J Immunol* 28:143-152; Katsikis, P.D. *et al.* (1997) “INTERLEUKIN-1 BETA CONVERTING ENZYME-LIKE PROTEASE INVOLVEMENT IN FAS-INDUCED AND ACTIVATION-
- 25 INDUCED PERIPHERAL BLOOD T CELL APOPTOSIS IN HIV INFECTION. TNF-RELATED APOPTOSIS-INDUCING LIGAND CAN MEDIATE ACTIVATION-INDUCED T CELL DEATH IN HIV INFECTION,” *J Exp Med* 186:1365-1372). However, TRAIL and DR5 expression on CD4⁺ T cells in HIV-1-infected patients remain to be determined.
- 30 The results provided in this Example demonstrate that HIV-1-infected patients exhibited: 1) elevated levels of TRAIL in their plasma, 2) increased

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percentage of CD4⁺ T cells that expressed DR5 and the apoptotic marker Annexin V. Because a higher percentage of apoptotic CD4⁺ T cells was found than is known to be infected (see above), the possibility that uninfected CD4⁺ T cells can undergo TRAIL-mediated apoptotic death as the result of a noninfectious interaction with HIV-1 was investigated. To accomplish this, purified CD4⁺ and CD8⁺ T cells from HIV-1-seronegative blood donors were exposed to HIV-1 that had been rendered noninfectious with aldrithiol-2 (AT-2), an HIV-1 inactivation strategy that maintains the integrity of the viral envelope (Rossio, J.L. *et al.* (1998) "INACTIVATION OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 INFECTIVITY WITH PRESERVATION OF CONFORMATIONAL AND FUNCTIONAL INTEGRITY OF VIRION SURFACE PROTEINS," J Virol 72:7992-8001; Arthur, L.O. *et al.* (1998) "CHEMICAL INACTIVATION OF RETROVIRAL INFECTIVITY BY TARGETING NUCLEOCAPSID PROTEIN ZINC FINGERS: A CANDIDATE SIV VACCINE," AIDS Res Hum Retroviruses 14 Suppl 3:S311-319). Therefore, the expression of TRAIL, STAT1, STAT2, p53 and DR5, as well as apoptosis in CD4⁺ and CD8⁺ T cells cultured with infectious or noninfectious HIV-1 was studied. The dependence of this T cell death cascade on type I interferons was investigated, and the dependence of apoptosis on HIV-1 binding to the CD4 molecule expressed by T cells was tested. A two-hit model is presented in which infectious or noninfectious HIV-1 (first hit) can activate type I interferons and TRAIL, and in synergy with a second infection or event (second hit), result in p53 and DR5-mediated apoptosis of CD4⁺ T cells.

Materials and Methods

HIV-1 positive patients. Venous blood was collected from 48 HIV-1-infected patients.

Preparation of AT-2-inactivated virions. HIV-1_{MN} (X4-tropic) and HIV-1_{Ada} (R5-tropic) were propagated as described above.

Isolation and culture of PBMC and T cells. PBMC and T cells were isolated as described above. CD4⁺ and CD8⁺ cells were purified from PBMC by either positive selection with anti-CD4 or anti-CD8 beads or by negative selection

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using cocktail of antibodies that bind all but CD4⁺ or CD8⁺ T cells (Miltenyi Biotech, Auburn, CA). For all the experiments, T cells isolated by both negative and positive selection were used interchangeably, without noticeable differences in the experimental outcome. The purity of CD4⁺ and CD8⁺ cells after purification
5 was >98% and >99%, respectively.

Incubation of PBMC and isolated T cell subsets with viruses. Cells were cultured at 1×10^6 cells/ml in RPMI 10% FCS in 12-well plates. For incubation with AT-2 HIV-1, the inactivated virus preparations were added at the initiation of culture at a final concentration of 500 ng/mL p24^{CA} equivalent. Infectious HIV-1_{MN}
10 was also used at 500ng/mL p24^{CA} equivalent. The ability of the infectious HIV-1 to actually infect cultures was confirmed by measuring p24 levels in the 6-day culture supernatants by ELISA (Beckman-Coulter, Miami, FL). Infectious influenza A (PR8) virus with a hemagglutinin A titer of 1:8192, was used at a 1/400 dilution in culture wells. Edmonston "wild-type" (EDW) measles virus
15 produced in Vero cell with a titer of 4.6×10^6 pfu/ml was used at a 1/500 dilution.

IFN- α detection. CD4⁺ and CD8⁺ T cells from HIV-1-seronegative donors were cultured for 24 h with or without influenza A and/or AT-2 HIV-1 viruses. Supernatants were collected and tested for IFN- α by ELISA (R & D Systems, Minneapolis, MN).

20 *Apoptosis study.* Cells were centrifuged, washed in Annexin buffer (1.4 M NaCl, 25 mM CaCl₂, and 100 mM MOPS, pH 7.14), and incubated for 20 minutes with medium only (negative control) or with FITC-conjugated Annexin V (Annexin V-FITC) (CALTAG, Burlingame, CA) in the dark at room temperature. After two washes, propidium iodide (100 μ g/mL) (CALTAG) was added and
25 analysis of apoptotic cells was confined to propidium iodide-negative cells to eliminate necrotic cells and membrane fragments that might bind Annexin V-FITC.

Detection of Membrane TRAIL. Isolated CD4⁺ and CD8⁺ T cells were cultured 24 h in absence or presence of influenza A virus and AT-2 HIV-1_{MN} or

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AT-2 HIV-1_{Ada}. The cells were washed twice in PBS. Membrane TRAIL expression was determined by incubating cells for 20 minutes at room temperature with PE-conjugated mouse IgG1 anti-human TRAIL monoclonal antibody (RIK-2), (Ebioscience, San Diego, CA) or with control isotype-matched antibodies (at 5
5 μ g/mL each) in PBS containing 2% mouse serum (SIGMA, St. Louis, MO). Cells were washed twice in ice-cold PBS and analyzed by flow cytometry. The same protocol was used for the staining of DR5 (Ebioscience), CD4, and CD8 (BD Bioscience, San Jose, CA). For STAT1 detection, an intracellular staining was performed using Fix and Perm kit (Caltag) and STAT1 phosphorylated monoclonal
10 antibody (BD Bioscience), according to the manufacturer's instructions.

Data acquisition and analysis. Cells were acquired on FACSCalibur flow cytometer (Becton Dickinson), using CellQuest software (Becton Dickinson Immunocytometry System). Samples were gated on viable cells by forward and side light scatters, and at least 50,000 live cell events were acquired for each
15 sample. Acquired data were analyzed using Cellquest software (Becton Dickinson).

RNA extraction and reverse transcription. Total RNA was extracted from CD4⁺ T cells with after 12 h of culture with HIV-1MN and influenza viruses, using the acid guanidium thiocyanate-phenol-chloroform method (Chomczynski, P. *et al* (1987) "SINGLE-STEP METHOD OF RNA ISOLATION BY ACID GUANIDINIUM
20 THIOCYANATE-PHENOL-CHLOROFORM EXTRACTION," Anal Biochem 162:156-159), modified for TRIzol (Invitrogen, Carlsbad, CA, USA).

Real Time PCR. cDNA quantification for TRAIL, DR-5, p53 and GAPDH was obtained with a real time PCR technique. Real time PCR was conducted as described above. Primers for TRAIL and GAPDH are as described above.
25 Primers for DR-5 are: Forward (SEQ ID NO:5) 5'-GGGCCACAGGGACACCTT-3'; Reverse (SEQ ID NO:6) 5'-GCATCTCGCCCGGTTTT-3'. Primers for p53 are: Forward (SEQ ID NO:7) 5'-CATGAGCGCTGCTCAGATAG-3'; Reverse (SEQ ID NO:8) 5'-ACACGCAAATTCCTTCCAC-3'.

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All reactions were performed in triplicate. Data analysis was performed with the SDS2.1 software. The threshold level was determined by the software according to the optimization of the standard curve. Standards were obtained by amplification of a control sample in a PCR reaction, using the same primers, reagents and conditions optimized for the real time analysis. Arbitrary quantity values were assigned to the resulting standard and 4-fold serial dilution were made to obtain a 8-point standard curve. Results are presented as ratios between the target gene and the GAPDH mRNA.

Blocking assays. Cells were cultured 24 h with AT-2 HIV-1 and influenza A virus in the presence of mouse blocking monoclonal anti-TRAIL (RIK-2) (Ebioscience) (Kayagaki, N. *et al.* (1999) "INVOLVEMENT OF TNF-RELATED APOPTOSIS-INDUCING LIGAND IN HUMAN CD4⁺ T CELL-MEDIATED CYTOTOXICITY," J Immunol 162:2639-2647), anti-DR5 (Imgenex, San Diego, CA) at 1, 5 and 10 µg/mL, soluble CD4 (AIDS Reagent Program, Bethesda, MD) at 2 µg/mL, or the combination of sheep polyclonal anti-human IFN-α (2000 IFN-α neutralizing U/mL) and sheep polyclonal anti-human IFN-β (500 IFN-β neutralizing U/mL) antibodies (BioSource International, Camarillo, CA). Mouse IgG isotype control antibody (BD Bioscience) was used at 5 µg/ml. Apoptosis was assessed by the Annexin V method described above. Results shown are for 5 µg/mL (anti-TRAIL) and 1 µg/mL (anti-DR5).

Nuclear p53 measurements. CD4⁺ T cells were culture for 24 h with or without influenza A and AT-2 HIV-1 viruses. Cells were lysed and nuclear p 53 was quantified by ELISA (Active Motif, Carlsbad, CA, USA) according to the according to the manufacturer's instructions.

Western Blot. PBMC's from uninfected donors were separated from whole blood by Ficoll centrifugation and CD4⁺ T cells were isolated by magnetic beads (Miltenyi, Auburn , CA). Cells were then resuspended in DMEM supplemented with 10% FBS (Hyclone, Logan, UT) and 1% Pen-Strep-Glut (Invitrogen, Carlsbad, CA) and stimulated with influenza virus, X4 AT-2 HIV-1, recombinant

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IFN- α (100 ng/ml) and IFN- β (100 ng/ml), anti-IFN- α and anti-IFN- β (R & D Systems) either singly or in combination. After 24 h, the cells were pelleted, washed in PBS and lysed (1% NP-40, 200mM NaCl, 50mM Tris pH 7.5, supplemented with protease inhibitor cocktail [Roche, Indianapolis, IN]). Total
5 protein in the lysate was quantified using BCA (Pierce, Rockford, IL) and 10 μ g of protein was mixed 1:1 with SDS Protein Gel Buffer (Quality Biologics, Gaithersburg, MD). The samples were run on a 10% or 15% SDS-PAGE gel (Bio-Rad, Hercules, CA). After transfer to a nitrocellulose membrane, the blot was blocked in 5% milk/TBST then incubated with either anti-STAT1, anti-STAT2
10 (Upstate Biotechnology, Inc., Lake Placid, NY), anti-TRAIL or anti-DR5 (Imgenix, San Diego, CA), anti-p53 (Calbiochem, La Jolla, CA), monoclonal antibodies followed by either anti-mouse or anti-rabbit HRP conjugated secondary antibody (Jackson, West Grove, PA). ECL was performed and bands were visualized on Hyperfilm (Amersham, Piscataway, NJ). β -actin was visualized as a
15 loading control using a monoclonal antibody (Sigma, St. Louis, MO).

Statistical analysis. All the experiments were repeated at least four times and the p values (p) were calculated using a two-tailed Student's *t* test. The linear regression analysis of DR5 mediated cell death (DR5⁺ AnnexinV⁺) among apoptotic cells (AnnexinV⁺) was performed with Microsoft Excel.

20 **Results**

Detection of TRAIL and DR5 in HIV-1-infected patients and uninfected controls. The levels of DR5 mRNA level in PBMC were compared from 16 HIV-1-infected patients and 9 HIV-1-uninfected controls. PBMC from HIV-1-infected patients expressed 2-fold more DR5 mRNA than PBMC from HIV-1-uninfected
25 controls (p=0.03) (**Figure 6, Panel A**). An additional 22 patients and 16 controls were tested for plasma TRAIL levels, and for the percentage of CD4⁺ T cells that were positive for Annexin V and DR5. Elevated levels of plasma TRAIL were detected in the HIV-1-infected patients (2617 $\pm$ 729 pg/ml) compared to controls, (730 $\pm$ 320 pg/ml) (p=0.0001). An increase in the percentage of CD4⁺ T cells that

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expressed the Annexin V ($p=3 \times 10^{-5}$) and DR5 ($p=2 \times 10^{-6}$) was observed (**Figure 6, Panel B**). Furthermore, a strong correlation is found between DR5 expression and apoptosis in $CD4^+$ T cells from HIV-1-infected patients ($R^2=0.67$, $p=3.10^{-6}$). In contrast, there was no correlation between these parameters in HIV⁻ healthy individuals ($R^2=0.17$, $p=0.11$) (**Figure 6, Panel C**). This result indicates that 67% of the death of $CD4^+$ T cells in these patients can be explained by DR5 expression, while no death of $CD4^+$ T cells can be attributed to DR5 expression in healthy individuals. These findings of increased plasma TRAIL levels combined with increased DR5 gene and protein expression in $CD4^+$ T cells from HIV-1-infected patients suggest the participation of TRAIL-mediated apoptosis in $CD4^+$ T cell depletion in AIDS.

Annexin V and STAT expression in virus-exposed T cells. To determine the role of HIV-1 in the expression of TRAIL and DR5 observed in HIV-1-infected patients, peripheral blood T cells were cultured from HIV-1-seronegative donors with infectious or noninfectious HIV-1. The effects of infectious HIV-1_{MN} (X4) and HIV-1_{Ada} (R5), as well as their noninfectious viral counterparts that had been inactivated by aldrithiol 2 (AT-2 HIV-1) (Arthur, L.O. *et al.* (1998) "CHEMICAL INACTIVATION OF RETROVIRAL INFECTIVITY BY TARGETING NUCLEOCAPSID PROTEIN ZINC FINGERS: A CANDIDATE SIV VACCINE," AIDS Res Hum Retroviruses 14 Suppl 3:S311-319) were tested, on T cell apoptosis. $CD4^+$ and $CD8^+$ T cells were positively-selected from PBMC and cultured for one-to-six days with these HIV-1 preparations, or with microvesicles (control), and the percentage of Annexin V-positive T cells was determined, using multicolor flow cytometry. Infectious HIV-1_{MN} or HIV-1_{Ada} did not induce significant apoptosis of either $CD4^+$ or $CD8^+$ T cells after one day. However, it was found that 32% and 31% of $CD4^+$ T cells were Annexin V positive when cultured 6 days with HIV-1_{MN} or HIV-1_{Ada}, respectively (**Figure 7, Panel A**). This apoptosis was approximately two fold above T cells cultured with microvesicles (16%). This difference was significant at $p=0.04$ for HIV-1_{MN} and $p=0.03$ HIV-1_{Ada}.

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T cells from HIV-1-seronegative donors were then co-cultured with HIV-1 plus a second virus to determine whether this viral contribution would induce apoptosis. Influenza A virus was chosen as the second virus because it was shown to co-localize with HIV-1 in mucosa-associated lymphoid tissue in vivo (Doherty, P.C. (1995) "ANATOMICAL ENVIRONMENT AS A DETERMINANT IN VIRAL IMMUNITY," J Immunol 155:1023-1027), and induced death of CD4⁺ T cells in PBMC from asymptomatic HIV-1-infected individuals (Clerici, M. *et al.* (1996) "ANTIGEN-STIMULATED APOPTOTIC T-CELL DEATH IN HIV INFECTION IS SELECTIVE FOR CD4⁺ T CELLS, MODULATED BY CYTOKINES AND EFFECTED BY LYMPHOTOXIN," Aids 10:603-611). T cells cultured with infectious Flu alone did not undergo apoptosis. However, co-culture of infectious HIV-1 plus Flu virus induced the death of 45-50% (day 1) and 90-95% (day 6) of CD4⁺ T cells, which are significantly less than CD4⁺ T cells cultured with microvesicles (controls) ($p < 0.001$). In contrast less than 15% and 30% of CD8⁺ T cells on the same days (**Figure 7, Panel A**). Identical results were obtained using noninfectious AT-2 HIV-1_{MN} and AT-2 HIV-1_{Ada}. Similar effects are observed between HIV-1 and measles virus, indicating that induction of CD4⁺ T cell death was not unique to Flu virus. It was verified that 28-30% of Annexin V⁺ CD4⁺, but only 3-6% of CD8⁺ T cells progressed to late stage apoptosis using propidium iodide (**Figure 7, Panel B**). Because virus infections induce type I interferons, which can be involved in apoptosis (Takaoka, A. *et al.* (2003) "INTEGRATION OF INTERFERON-ALPHA/BETA SIGNALLING TO P53 RESPONSES IN TUMOUR SUPPRESSION AND ANTIVIRAL DEFENSE," Nature 424:516-523), the role of type I interferons in apoptosis of HIV-1-exposed T cells was investigated. Antibodies against IFN- α/β dramatically reduced death of CD4⁺ T cells cultured with infectious HIV-1 and Flu virus, or with AT-2 HIV-1_{MN} and Flu virus (**Figure 7, Panel C**). **Figure 7, Panel C** also illustrates how similar the flow cytometric Annexin V curves are between infectious (upper) and noninfectious HIV-1 (lower) plus Flu.

The data of **Figure 7, Panel C** strongly suggest that type I interferons are important in the CD4⁺ T cell death that is induced by either infectious or noninfectious HIV-1 plus Flu. In fact, the comparison of the flow cytometry data

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of Annexin V⁺ CD4⁺ T cells was identical irrespective of whether infectious HIV-1 plus Flu or noninfectious AT-2 HIV-1 plus Flu were used to induce apoptosis. Furthermore, an equal inhibition of death was observed with anti-IFN α/β mAbs, indicating that type I interferons were required for inducing apoptosis by either
5 infectious or noninfectious HIV-1 plus Flu in the above-described system. If apoptosis of CD4⁺ T cells is inhibited by type I interferon-specific antibodies, then IFN- α or IFN- β might be detectable in our culture supernatants. Therefore, tests were conducted to determine whether enriched CD4⁺ or CD8⁺ T cells would produce IFN- α when culture with AT-2 HIV-1, Flu virus or the combination of
10 both. It was found that CD4⁺ but not CD8⁺ cells produced IFN- α after exposure to AT-2 HIV-1, Flu and the combination of these viruses (**Figure 7, Panel D**).

Because STAT1 and STAT2 are signaling molecules for type I interferons (Qureshi, S.A. *et al.* (1995) "TYROSINE-PHOSPHORYLATED STAT1 AND STAT2 PLUS A 48-KDA PROTEIN ALL CONTACT DNA IN FORMING INTERFERON-STIMULATED-
15 GENE FACTOR 3," Proc Natl Acad Sci U S A 92:3829-3833; Leung, S. *et al.* (1995) "ROLE OF STAT2 IN THE ALPHA INTERFERON SIGNALING PATHWAY," Mol Cell Biol 15:1312-1317), tests were conducted to determine whether STAT1 and STAT2 would be affected by co-culture of CD4⁺ T cells with HIV-1 and Flu. Flow cytometric and western blot analyses showed that STAT1 and STAT2 were
20 modulated by exposure of T cells to HIV-1 and Flu virus. AT-2 HIV-1_{MN}, Flu or AT-2 HIV-1_{MN} plus Flu induced similar levels of phosphorylated STAT1 expression on CD4⁺ T cells (**Figure 7, Panel E**), with a slightly higher percentage of STAT1⁺ CD4⁺ T cells resulting from culture with AT-2 HIV-1_{MN} plus Flu. Western blot analysis confirmed that Flu plus AT-2 HIV-1_{MN} was required to
25 increase STAT1, and to induce high levels of STAT2, and that both STAT1 and STAT 2 production were inhibited by anti IFN- α/β antibodies (**Figure 7, Panel F**). Thus, type I interferons are involved in the death of CD4⁺ T cells exposed to HIV-1 and Flu virus.

Analysis of TRAIL expression and production in virus-exposed T cells.

30 Since interferons regulate TRAIL expression (Gong, B. *et al.* (2000) "GENOMIC

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ORGANIZATION AND TRANSCRIPTIONAL REGULATION OF HUMAN APO2/TRAIL GENE," Biochem Biophys Res Commun 278:747-752), and because TRAIL was detected in the plasma of HIV-1-infected patients, TRAIL expression was measured on T cells cultured with HIV-1 and Flu virus alone or in combination.

- 5 Membrane TRAIL (mTRAIL) was detected on CD4⁺ (**Figure 8, Panel A**) but not CD8⁺ T cells cultured with Flu or HIV-1_{MN} alone, or with HIV-1_{MN} plus Flu. Noninfectious AT-2 HIV-1 also induced TRAIL expression on CD4⁺ T cells (data not shown). Exposure of CD4⁺ T cells to HIV-1_{MN} plus Flu induced TRAIL mRNA expression that was blocked by IFN- α/β antibodies (**Figure 8, Panel B**).
- 10 Anti-IFN- α/β antibodies also inhibited virus-induced expression of mTRAIL, suggesting that type I interferons are paramount for the induction of TRAIL in virus exposed-T cells (**Figure 8, Panel C**). IFN- α and/or IFN- β both induced mTRAIL expression on CD4⁺ T cells when added exogenously (**D**). Western blot analysis verified that TRAIL was produced upon culture of CD4⁺ T cells with AT-
- 15 2 HIV-1_{MN}, Flu, AT-2 HIV-1_{MN} plus Flu or IFN- α/β (**Figure 8, Panel E**). Antibodies against IFN- α/β greatly inhibited TRAIL production by AT-2 HIV-1_{MN} plus Flu (**Figure 8, Panel E**). The involvement of TRAIL in apoptosis of CD4⁺ T cells exposed to HIV-1_{MN} plus Flu was confirmed by experiments in which anti-IFN- α/β antibodies, the TRAIL-specific RIK-2 monoclonal antibody, and anti-DR5
- 20 antibody each inhibited CD4⁺ T cell apoptosis by 75% (p= 0.0001), 40% (p= 0.02) and 60 % (p= 0.03), respectively (**Figure 8, Panel F**). Soluble CD4 inhibited CD4⁺ T cell apoptosis by 90% (p= 0.0001) (**Figure 8, Panel F**), which demonstrates the involvement of the interaction between HIV-1 and CD4 for inducing apoptosis. Considered together, these results indicate that: (1) Flu- and/or HIV-1-induced
- 25 TRAIL expression and (2) the interaction between HIV-1 and CD4 is essential for inducing CD4⁺ T cell death via a type I interferon dependent mechanism.

DR5 expression and p53 transcription and synthesis in virus-exposed T cells. TRAIL-mediated apoptosis requires the expression of cellular TRAIL death receptors. Therefore, DR5 expression was measured on CD4⁺ and CD8⁺ T cells

30 after culture with HIV-1_{MN}, Flu or HIV-1_{MN} plus Flu. In contrast to TRAIL, which

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was induced by either HIV-1_{MN} or Flu alone, DR5 was selectively expressed only on CD4⁺ T cells cultured with both HIV-1_{MN} and Flu (**Figure 9, Panel A**). DR4 was not detected on CD4⁺ T cells, and CD8⁺ T cells did not express DR molecules. Involvement of DR5 was confirmed by its expression on the majority of cells
5 undergoing apoptosis after culture with AT-2 HIV-1_{MN} plus Flu (**Figure 9, Panel B**). In addition, culture of CD4⁺ T cells with AT-2 HIV-1_{MN} plus Flu induced DR5 mRNA and protein expression, both of which were blocked by anti-IFN- α/β antibodies (**Figure 9, Panels C and D (upper panel)**). Western blot and real time PCR analysis showed that both Flu and HIV-1_{MN} were required for DR5 expression
10 (**Figure 9, Panels C and E**), and that DR5 gene and protein expression were inhibited by anti-IFN- α/β antibodies (**Figure 9, Panels C and E**). However, addition of exogenous IFN- α/β did not induce DR5 (**Figure 9, Panel E**). Similarly, recombinant IFN- α/β in combination with HIV-1 did not induce DR5 or apoptosis (**Figure 9, Panel D (middle and lower panels)**) indicating that expression
15 of TRAIL (see **Figure 8, Panel D**) and DR5 have different requirements. Therefore, DR5 expression is the limiting component of TRAIL-mediated death in HIV-1-exposed cells.

DR5 expression is regulated by the tumor suppressor p53 (24, 40). Therefore, we tested whether p53 gene expression and synthesis would be induced
20 by Flu, AT-2 HIV-1_{MN} or AT-2 HIV-1_{MN} plus Flu, and whether p53 induction would be inhibited by IFN- α/β antibodies. Both p53 gene expression (Fig. 4F) and p53 production (Fig. 4G,H) required CD4⁺ T cell stimulation with AT-2 HIV-1_{MN} plus Flu, and were blocked by anti-IFN- α/β . Thus, similar to the other components of this cascade to death, p53 activation and synthesis, which are necessary for DR5
25 expression (Wu, G.S. *et al.* (1997) "KILLER/DR5 IS A DNA DAMAGE-INDUCIBLE P53-REGULATED DEATH RECEPTOR GENE," *Nat Genet* 17:141-143; Sheikh, M.S. *et al.* (1998) "p53-DEPENDENT AND -INDEPENDENT REGULATION OF THE DEATH RECEPTOR KILLER/DR5 GENE EXPRESSION IN RESPONSE TO GENOTOXIC STRESS AND TUMOR NECROSIS FACTOR ALPHA," *Cancer Res* 58:1593-1598), are type I
30 interferon-dependent.

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In sum, as reported above, HIV-1-infected patients have more TRAIL in their plasma and more CD4⁺ T cells expressing DR5 and the apoptotic marker Annexin V than healthy donors. Furthermore, a strong correlation has been uncovered between DR5 expression and apoptosis in CD4 T cells from HIV-1-
5 infected patients ($r^2=0.67$, $p=3.10^{-6}$). In contrast, there was no correlation between these parameters in HIV⁻ healthy individuals ($r^2=0.17$, $p=0.11$). These results strongly suggest that TRAIL/DR5-mediated apoptosis is the major death mechanism that contribute to CD4⁺ T cell depletion in HIV-1-infected patients. The above-presented in vitro results demonstrate that CD4⁺ T cell death occurs
10 upon in vitro exposure of T cells to either infectious or noninfectious HIV-1 plus a second virus. While not intending to be limited thereby, the results suggest a model in which death is mediated by a cascade of molecular events, each of which is dependent on type I interferons. Thus, STAT1, STAT2, TRAIL, DR5, p53 and apoptosis were all inhibited by antibodies against IFN- α/β , and indicate a central
15 role for type I interferons in this HIV-1-associated death process. A recent study reported that the interaction of cell lines transfected with the gp120/41 HIV-1 envelope complex and CD4 results in expression of p53, leading to the expression of the proapoptotic protein Puma and apoptosis (Perfettini, J.L. *et al.* (2004) "NF- $\{KAPPA\}$ B AND P53 ARE THE DOMINANT APOPTOSIS-INDUCING TRANSCRIPTION
20 FACTORS ELICITED BY THE HIV-1 ENVELOPE," *J Exp Med* 199:629-640). The Puma model may be relevant here in that it revealed HIV-1-induced apoptosis of CD4 expressing cells in the absence of infection via a p53-dependent mechanism.

The present demonstration that apoptosis was partially inhibited by anti-TRAIL and anti-DR5 antibodies and almost completely inhibited by soluble CD4
25 indicate that: (1) TRAIL and its receptors are involved in this model of CD4⁺ T cell death; and (2) the interaction between HIV-1 and the CD4 molecule expressed on CD4⁺ T cells is essential for induction of apoptosis. The present findings that X4 and R5 co-receptor-using HIV-1 isolates were equally effective for inducing TRAIL and synergizing with Flu to induce DR5 and apoptosis indicates that
30 discriminatory co-receptor recognition is not important to the present invention. The finding that anti-TRAIL and anti-DR5 antibody did not completely block

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apoptosis raises the possibility that other death mechanisms may have contributed to the high levels of CD4⁺T cell death that we observed. However, antibodies against TNF, FASL and TWEAK had no effect in the proposed model. Furthermore, an earlier study reported that AT-2 HIV-1-induced T cell death that
5 was mediated by an unidentified non-Fas-FasL mechanism (Esser, M.T. *et al* (2001) "PARTIAL ACTIVATION AND INDUCTION OF APOPTOSIS IN CD4(+) AND CD8(+) T LYMPHOCYTES BY CONFORMATIONALLY AUTHENTIC NONINFECTIOUS HUMAN IMMUNODEFICIENCY VIRUS TYPE 1," J Virol 75:1152-1164).

Opportunistic infectious agents have been suggested to exacerbate HIV-1
10 disease by activating CD4⁺ T cells, rendering T helper cells susceptible to HIV-1 infection (Donovan, R.M. *et al.* (1996). "CHANGES IN VIRUS LOAD MARKERS DURING AIDS-ASSOCIATED OPPORTUNISTIC DISEASES IN HUMAN IMMUNODEFICIENCY VIRUS-INFECTED PERSONS," J Infect Dis 174:401-403), resulting in HIV-1 cytopathic death. Without intending to be bound thereby, the
15 present findings provide a new mechanism by which preferential CD4⁺ T cell apoptosis can occur. This mechanism involves the synergistic effects of either infectious or noninfectious HIV-1 and a second event (viral infection), both of which are essential for activating rate-limiting expression of DR5 death receptor molecule by a type I interferon-dependent mechanism. Although influenza A virus
20 was employed as the second virus (because it co-localizes with HIV-1 in primary lymphoid tissue (Doherty, P.C. (1995) "ANATOMICAL ENVIRONMENT AS A DETERMINANT IN VIRAL IMMUNITY," J Immunol 155:1023-1027)), the second event was not limited to influenza; measles virus, for example, also synergized with AT-2 HIV-1 to induce p53, DR5 and apoptosis of CD4⁺ T cells. Although
25 influenza and measles virus infections have not been associated with AIDS progression, both of these viruses fulfill the requirement of activating p53 and DR5 in presence of HIV-1. Thus, they provide an example of one way to selectively induce rapid and extensive CD4⁺ T cell death upon exposure to HIV-1. Because our findings show that p53 expression required two viruses to induce DR5, other
30 infection could provide the second event to induce DR5 and accelerate T cell depletion. Alternatively, it is possible that, in synergy with HIV-1, other non-viral

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events would induce p53 and DR5 resulting in apoptosis. These other events might include agents that would induce DNA damage, since p53 is induced by DNA damage (Wu, G.S. *et al.* (1997) "KILLER/DR5 IS A DNA DAMAGE-INDUCIBLE P53-REGULATED DEATH RECEPTOR GENE," *Nat Genet* 17:141-143).

5 The present finding that CD4⁺ T cells cultured with AT-2 HIV-1 plus recombinant IFN- α/β did not induce apoptosis suggests that type I interferons (induced by HIV-1) are required but not sufficient for inducing CD4⁺ T cell death. The central event for CD4⁺ T cell death in our findings was activation of p53 by the combined effect of HIV-1 and a second virus leading to the selective expression of DR5 on CD4⁺ T
10 cells.

The present demonstration that CD4⁺ T cells from HIV-1 patients were double-positive for Annexin V and DR5 and that TRAIL was detected in patients' plasma is consistent with the present in vitro data and suggests that the above-suggested in vitro model has clinical relevance. In contrast to the slow decline in
15 CD4 count during progression to AIDS, the accelerated in vitro model of TRAIL/DR5-mediated death suggested by such model occurred within six days. Part of this rapid CD4⁺ depletion could be due to the fact that T cell repopulation did not occur in the above-reported in vitro studies, in contrast to the in vivo setting in which CD4⁺ T cell repopulation was reported (Wei, X. *et al.* (1995)
20 "VIRAL DYNAMICS IN HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 INFECTION," *Nature* 373:117-122; Ho, D.D. *et al.* (1995) "RAPID TURNOVER OF PLASMA VIRIONS AND CD4 LYMPHOCYTES IN HIV-1 INFECTION," *Nature* 373:123-126). The fact that preferential apoptosis of CD4⁺ T cells was induced using non-infectious as well as infectious HIV-1 indicates that this synergistic effect is not
25 dependent on productive HIV-1-infection, and demonstrates that uninfected CD4⁺ T cells die in the suggested model (see report that uninfected CD4⁺ T cells can undergo bystander death during HIV-1 infection (Finkel, T.H. *et al.* (1995) "APOPTOSIS OCCURS PREDOMINANTLY IN BYSTANDER CELLS AND NOT IN PRODUCTIVELY INFECTED CELLS OF HIV- AND SIV-INFECTED LYMPH NODES," *Nat*
30 *Med* 1:129-134). The present findings also raise the possibility that non-infectious HIV-1 particles, which constitute a large portion of the plasma virus (Dimitrov,

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D.S. (1993) "QUANTITATION OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 INFECTION KINETICS," J Virol 67:2182-2190; Piatak, M., Jr. *et al.* (1993) "HIGH LEVELS OF HIV-1 IN PLASMA DURING ALL STAGES OF INFECTION DETERMINED BY COMPETITIVE PCR," Science 259:1749-1754), contribute to the decline in CD4
5 count by a similar mechanism. Nevertheless, it should be emphasized that the development of AIDS requires infectious HIV-1, which is the source of infectious and non-infectious virus particles that can induce apoptotic signals in CD4⁺ T cells.

The present invention demonstrates that cultures of T cells enriched for CD4⁺ by 98-99% produced IFN- α when cultured with HIV-1 and/or Flu.
10 However, type I IFN production could be due to CD4⁺ monocytes. IFN- α was reported to inhibit HIV-1 replication in vitro (Yamamoto, J.K. *et al.* (1986) "HUMAN ALPHA- AND BETA-INTERFERON BUT NOT GAMMA- SUPPRESS THE IN VITRO REPLICATION OF LAV, HTLV-III, AND ARV-2," J Interferon Res 6:143-152), and has been used for therapy in AIDS patients with Kaposi sarcoma
15 (Frissen, P.H. *et al.* (1997) "HIGH-DOSE INTERFERON-ALPHA2A EXERTS POTENT ACTIVITY AGAINST HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 NOT ASSOCIATED WITH ANTITUMOR ACTIVITY IN SUBJECTS WITH KAPOSIS SARCOMA," J Infect Dis 176:811-814). Type I interferons produced by plasmacytoid dendritic cells have been suggested to protect against AIDS progression (Soumelis, V. *et al.* (2001)
20 "DEPLETION OF CIRCULATING NATURAL TYPE 1 INTERFERON-PRODUCING CELLS IN HIV-INFECTED AIDS PATIENTS," Blood 98:906-912; Siegal, F.P. *et al.* (1999) "THE NATURE OF THE PRINCIPAL TYPE 1 INTERFERON-PRODUCING CELLS IN HUMAN BLOOD," Science 284:1835-1837). However, type I interferons induced in lymph nodes of macaques infected with SIV did not control viral replication (Abel,
25 K. *et al.* (2002) "THE RELATIONSHIP BETWEEN SIMIAN IMMUNODEFICIENCY VIRUS RNA LEVELS AND THE MRNA LEVELS OF ALPHA/BETA INTERFERONS (IFN-ALPHA/BETA) AND IFN-ALPHA/BETA-INDUCIBLE MX IN LYMPHOID TISSUES OF RHESUS MACAQUES DURING ACUTE AND CHRONIC INFECTION," J Virol 76:8433-8445). The present findings raise the possibility that type I interferons can be
30 harmful by setting the stage for the death of uninfected CD4⁺ T cells in patients whose CD4⁺ T cells have been activated to express TRAIL and DR5. In light of

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the present findings that antibodies against IFN- α/β reduced CD4⁺ T cell death, and blocked the effect of each of the molecules required for inducing TRAIL-mediated apoptosis, one aspect of the present invention concerns the recognition that anti-type I interferon antibodies can be used as an anti-HIV-1 therapeutic agent. See also reports that immune suppression is induced by IFN- α and can be blocked by anti-IFN- α antibodies (Zagury, D. *et al.* (1998) "INTERFERON ALPHA AND TAT INVOLVEMENT IN THE IMMUNOSUPPRESSION OF UNINFECTED T CELLS AND C-C CHEMOKINE DECLINE IN AIDS," Proc Natl Acad Sci U S A 95:3851-3856), and that immunization of AIDS patients against IFN- α reduced the rate of HIV-1 disease progression (Gringeri, A. *et al.* (1999) "ACTIVE ANTI-INTERFERON-ALPHA IMMUNIZATION: A EUROPEAN-ISRAELI, RANDOMIZED, DOUBLE-BLIND, PLACEBO-CONTROLLED CLINICAL TRIAL IN 242 HIV-1--INFECTED PATIENTS (THE EURIS STUDY)," J Acquir Immune Defic Syndr Hum Retrovirol 20:358-370.

The above-described two-hit model, based on the above-described data is illustrated in **Figure 10**. The model illustrates the requirement for STAT1 and STAT2 activation, as well as the induction of type I interferons, TRAIL, p53 and DR5 in CD4⁺ T cells. The top panel of **Figure 10** shows that only one hit is needed to induce TRAIL, but not p53 or DR5. The bottom panel of **Figure 10** indicates that two hits are required to induce the entire cascade of molecular events that are necessary for TRAIL-mediated death of CD4⁺ T cells. Both membrane TRAIL (mTRAIL), which is detected on CD4⁺ T cells after exposure to HIV-1 or influenza virus, and plasma TRAIL (pTRAIL) that is detected in patients can interact with DR5 that is expressed on CD4⁺ T cells, resulting TRAIL-mediated apoptosis. It is also noteworthy that exposure of monocytes from healthy blood donors to AT-2 HIV-1 results in soluble TRAIL production. Based on the above-presented findings, it is concluded that exposure to either infectious or noninfectious HIV-1 (first hit) in conjunction with a second virus (second hit) can induce the selective expression of TRAIL, p53 and DR5 on CD4⁺ T cells, resulting in apoptosis of uninfected CD4⁺ but not CD8⁺ T lymphocytes. The above-presented results indicate that HIV-1 and a second p53-activating event synergize to initiate a type I interferon-dependent cascade of molecular events that leads to

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TRAIL, p53 and DR5 expression, resulting in preferential apoptosis of uninfected CD4⁺ T cells. Thus, the present invention provide a mechanistic explanation for CD4⁺ T cell death that is independent of productive HIV-1 infection, but which may contribute to CD4⁺ T cell depletion in HIV-1-infected patients.

What Is Claimed Is:

- Claim 1. A method for determining whether a mammal suffers from a disease or condition involving immune system activation, wherein said method comprises assaying for the presence or concentration of a TRAIL Compound in a biological fluid of said mammal.
- 5
- Claim 2. The method of claim 1, wherein said mammal is selected from the group consisting of a human, simian, feline, bovine, equine, canine, ovine or porcine mammal.
- Claim 3. The method of claim 1, wherein said TRAIL compound is sTRAIL.
- 10 Claim 4. The method of claim 1, wherein said method comprises an immunoassay that determines the presence or concentration of said TRAIL Compound, said immunoassay comprising the steps of:
- (a) contacting a sample of said biological fluid with an antibody specific for said TRAIL Compound, said contacting being under conditions sufficient to permit said TRAIL Compound if present in said sample to bind to said antibody and form a TRAIL Compound – antibody complex;
- 15
- (b) contacting said formed TRAIL Compound – antibody complex with a molecule capable of specific binding to said complex, said contacting being under conditions sufficient to permit said molecule to bind to said complex and form an extended complex; and
- 20
- (c) determining the presence or concentration of said TRAIL Compound in said biological fluid by determining the presence or concentration of said formed extended complex in said sample.
- 25
- Claim 5. The method of claim 4, wherein said TRAIL Compound is selected from the group consisting of sTRAIL, mTRAIL, the TRAIL DR5

receptor molecule, a biological molecule that activates TRAIL, and a biological molecule that activates the TRAIL DR5 receptor.

- Claim 6. The method of claim 5, wherein said biological molecule that activates the TRAIL DR5 receptor is p53.
- 5 Claim 7. The method of claim 4, wherein said method reveals the presence of said TRAIL Compound.
- Claim 8. The method of claim 4, wherein said method reveals the concentration of said TRAIL Compound.
- 10 Claim 9. The method of claim 1, wherein said disease or condition involving immune system activation is selected from the group consisting of HIV infection, AIDS, cancer, atherosclerosis, Alzheimer's disease, inflammation, autoimmune disorder, allergic asthma, Crohn's disease, Grave's disease, lupus, multiple sclerosis, Parkinson's disease, allograft transplant rejection, and graft vs host disease.
- 15 Claim 10. The method of claim 9, wherein said disease or condition involving immune system activation is HIV infection or AIDS.
- Claim 11. An immunoassay that determines the presence or concentration of a TRAIL Compound in a biological fluid of a mammal, wherein said immunoassay comprises the steps of:
- 20 (a) contacting a sample of said biological fluid with an antibody specific for said TRAIL Compound, said contacting being under conditions sufficient to permit said TRAIL Compound if present in said sample to bind to said antibody and form a TRAIL Compound – antibody complex;
- 25 (b) contacting said formed TRAIL Compound – antibody complex with a molecule capable of specific binding to said complex, said contacting being under conditions sufficient

to permit said molecule to bind to said complex and form an extended complex; and

- (c) determining the presence or concentration of said TRAIL Compound in said biological fluid by determining the presence or concentration of said formed extended complex in said sample.

5

Claim 12. The immunoassay of claim 11, wherein said mammal is selected from the group consisting of a human, simian, feline, bovine, equine, canine, ovine or porcine mammal.

- 10 Claim 13. The immunoassay of claim 11, wherein said TRAIL Compound is selected from the group consisting of sTRAIL, mTRAIL, the TRAIL DR5 receptor molecule, a biological molecule that activates TRAIL, and a biological molecule that activates the TRAIL DR5 receptor.

- 15 Claim 14. The immunoassay of claim 13, wherein said biological molecule that activates the TRAIL DR5 receptor is p53.

Claim 15. The immunoassay of claim 11, wherein said immunoassay reveals the presence of said TRAIL Compound.

- 20 Claim 16. The immunoassay of claim 11, wherein said immunoassay reveals the concentration of said TRAIL Compound.

Claim 17. The immunoassay of claim 11, wherein said molecule capable of specific binding to said complex is an rsTRAIL molecule.

- 25 Claim 18. The immunoassay of claim 11, wherein said TRAIL Compound is sTRAIL, and said molecule capable of specific binding to said complex is an anti-sTRAIL antibody.

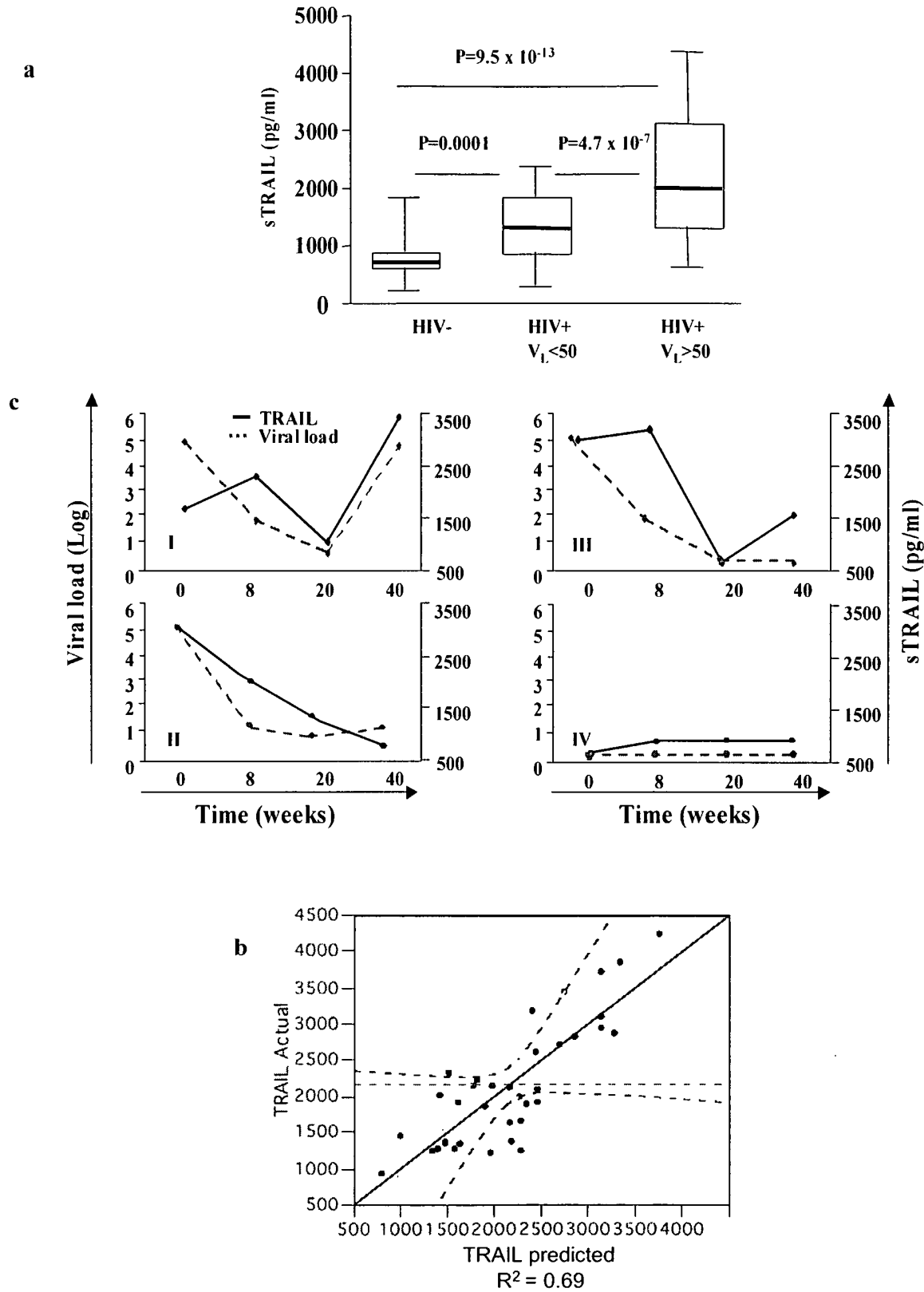
- Claim 19. The immunoassay of claim 11, wherein said immunoassay determines whether a mammal suffers from a disease or condition involving immune system activation selected from the group consisting of HIV infection, AIDS, cancer, atherosclerosis, Alzheimer's disease, inflammation, autoimmune disorder, allergic asthma, Crohn's disease, Grave's disease, lupus, multiple sclerosis, Parkinson's disease, allograft transplant rejection, and graft vs host disease.
- 5
- Claim 20. The immunoassay of claim 19, wherein said disease or condition involving immune system activation is HIV infection or AIDS.
- 10
- Claim 21. The immunoassay of claim 11, wherein said immunoassay is an immunochromatographic immunoassay, wherein:
- in said step (a), said biological sample is placed in contact with a first porous carrier, said first porous carrier containing a non-immobilized, labeled antibody specific for said TRAIL Compound;
- 15
- in said step (b), said formed TRAIL Compound– antibody complex is placed in contact with a second porous carrier, said second porous carrier being in communication with said first porous carrier, and containing an immobilized molecule capable of specific binding to said complex; and
- 20
- in said step (c), the presence or concentration of said TRAIL Compound in said biological fluid is determined by detecting the presence of said labeled antibody specific for said TRAIL Compound in said second porous carrier.
- 25
- Claim 22. The immunoassay of claim 21, wherein said immunoassay reveals the presence of said TRAIL Compound.
- Claim 23. The immunoassay of claim 21, wherein said immunoassay reveals the concentration of said TRAIL Compound.

- Claim 24. The immunoassay of claim 21, wherein said immobilized molecule capable of specific binding to said complex is an rsTRAIL molecule.
- 5 Claim 25. The immunoassay of claim 21, wherein said TRAIL Compound is sTRAIL, and said immobilized molecule capable of specific binding to said complex is an anti-sTRAIL antibody.
- 10 Claim 26. The immunoassay of claim 21, wherein said immunoassay determines whether a mammal suffers from a disease or condition involving immune system activation selected from the group consisting of HIV infection, AIDS, cancer, atherosclerosis, Alzheimer's disease, inflammation, autoimmune disorder, allergic asthma, Crohn's disease, Grave's disease, lupus, multiple sclerosis, Parkinson's disease, allograft transplant rejection, and graft vs host disease.
- 15 Claim 27. The immunoassay of claim 26, wherein said disease or condition involving immune system activation is HIV infection or AIDS.
- 20 Claim 28. A kit for measuring the presence or concentration of a TRAIL Compound in a biological fluid of a mammal, wherein said kit comprises a hollow casing comprising a multilayer filter system, and first and second porous carriers, wherein said second porous carrier is in communication with said first porous carrier, and said first porous carrier is in communication with said multilayer filter system, a portion of which is accessible from said casing; wherein: said first porous carrier contains a non-immobilized, labeled antibody that specifically binds said TRAIL Compound; and said second porous carrier contains an immobilized, unlabeled molecule that binds to a TRAIL Compound – antibody complex.
- 25

- Claim 29. The kit of claim 28, wherein said kit reveals the presence of said TRAIL Compound.
- Claim 30. The kit of claim 28, wherein said kit reveals the concentration of said TRAIL Compound.
- 5 Claim 31. The kit of claim 28, wherein said immobilized molecule capable of specific binding to said complex is an rsTRAIL molecule.
- Claim 32. The kit of claim 28, wherein said TRAIL Compound is sTRAIL, and said immobilized molecule capable of specific binding to said complex is an anti-sTRAIL antibody.
- 10 Claim 33. The kit of claim 28, wherein said kit is suitable for determining whether a mammal suffers from a disease or condition of involving immune system activation selected from the group consisting of HIV infection, AIDS, cancer, atherosclerosis, Alzheimer's disease, inflammation, autoimmune disorder, allergic asthma, Crohn's
- 15 disease, Grave's disease, lupus, multiple sclerosis, Parkinson's disease, allograft transplant rejection, and graft vs host disease.
- Claim 34. The immunoassay of claim 33, wherein said disease or condition of involving immune system activation is HIV infection or AIDS.

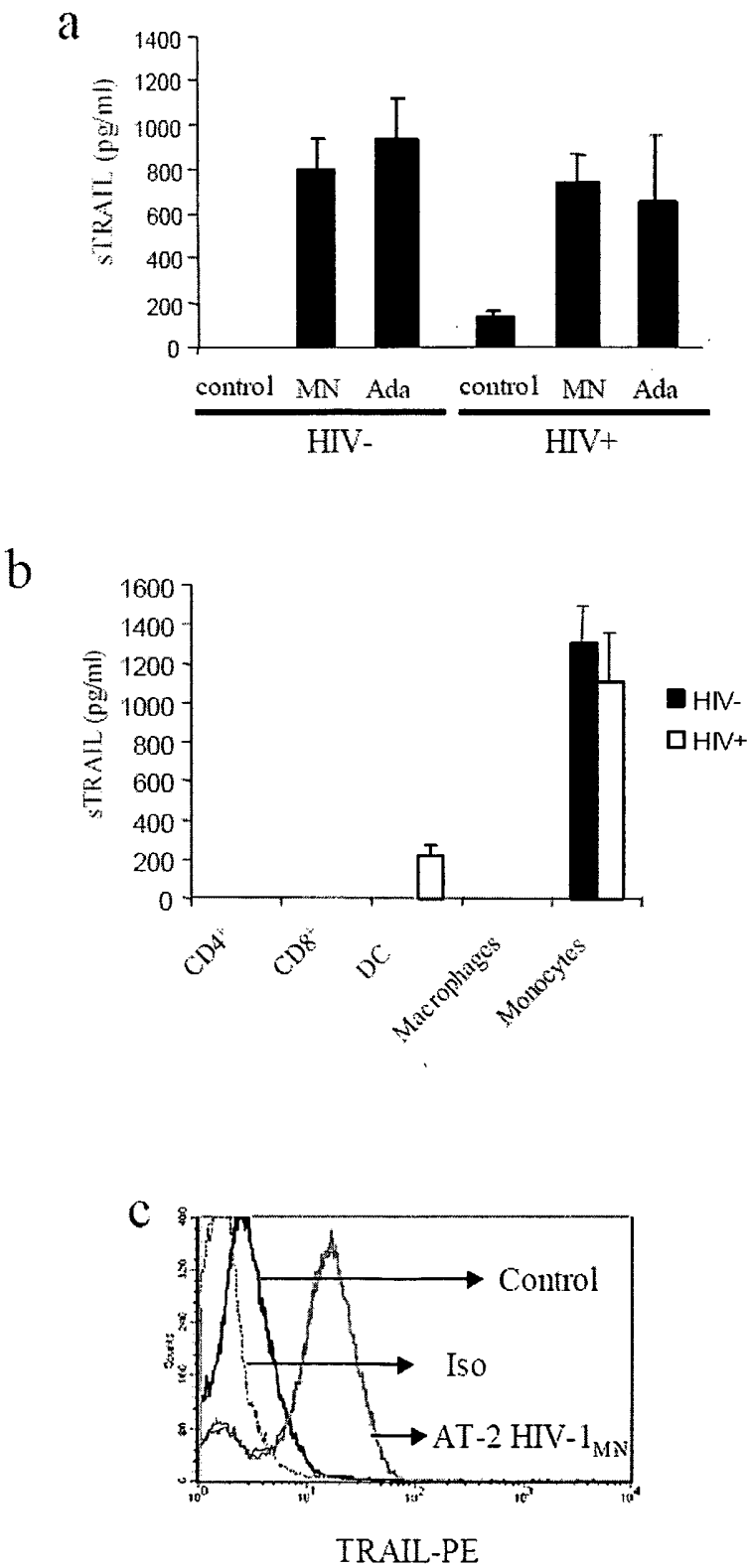
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Figure 1



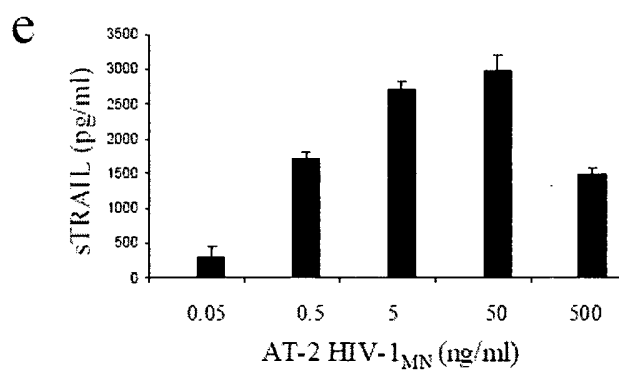
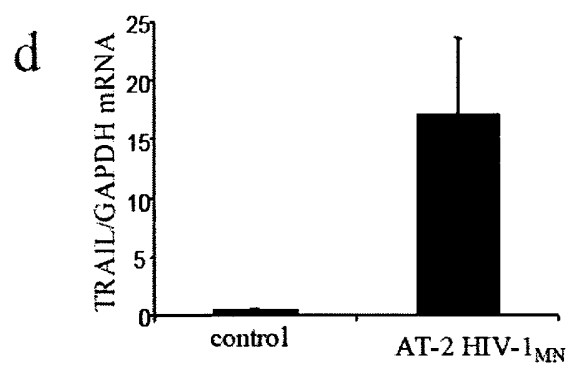
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Figure 2 (a-c)



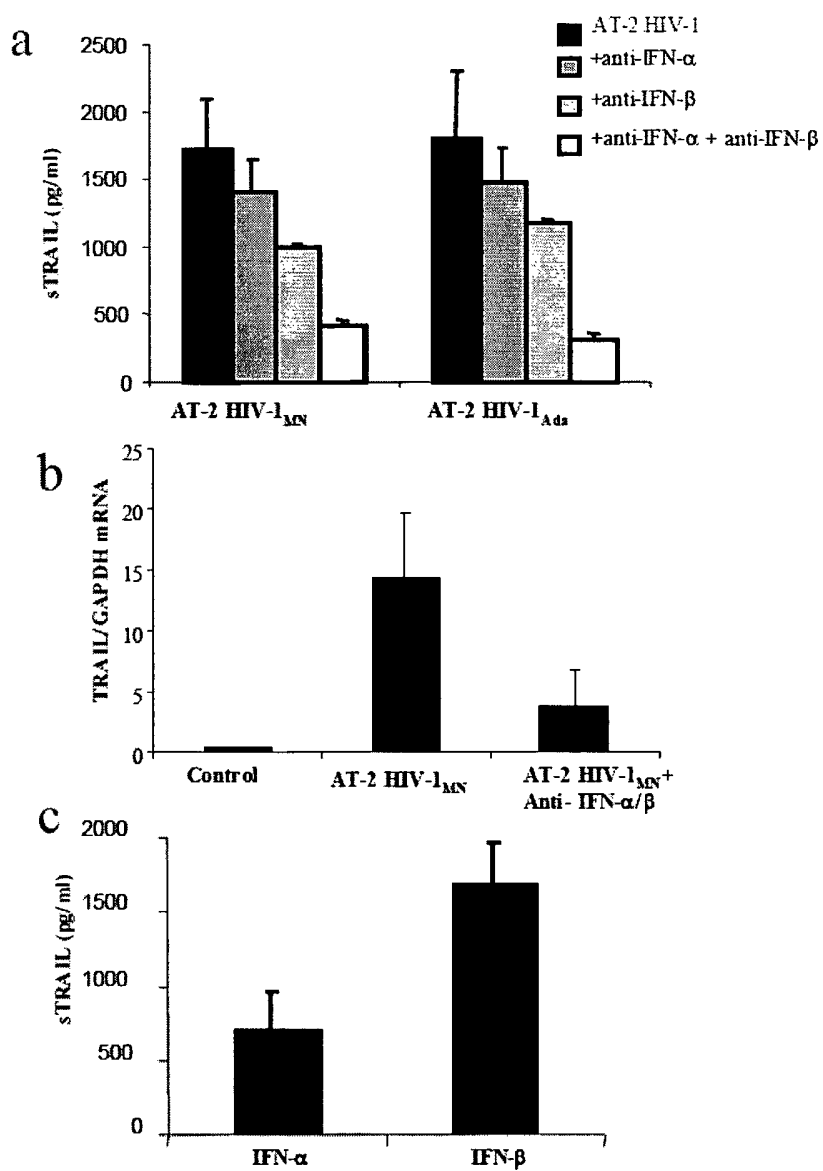
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Figure 2 (d-e)



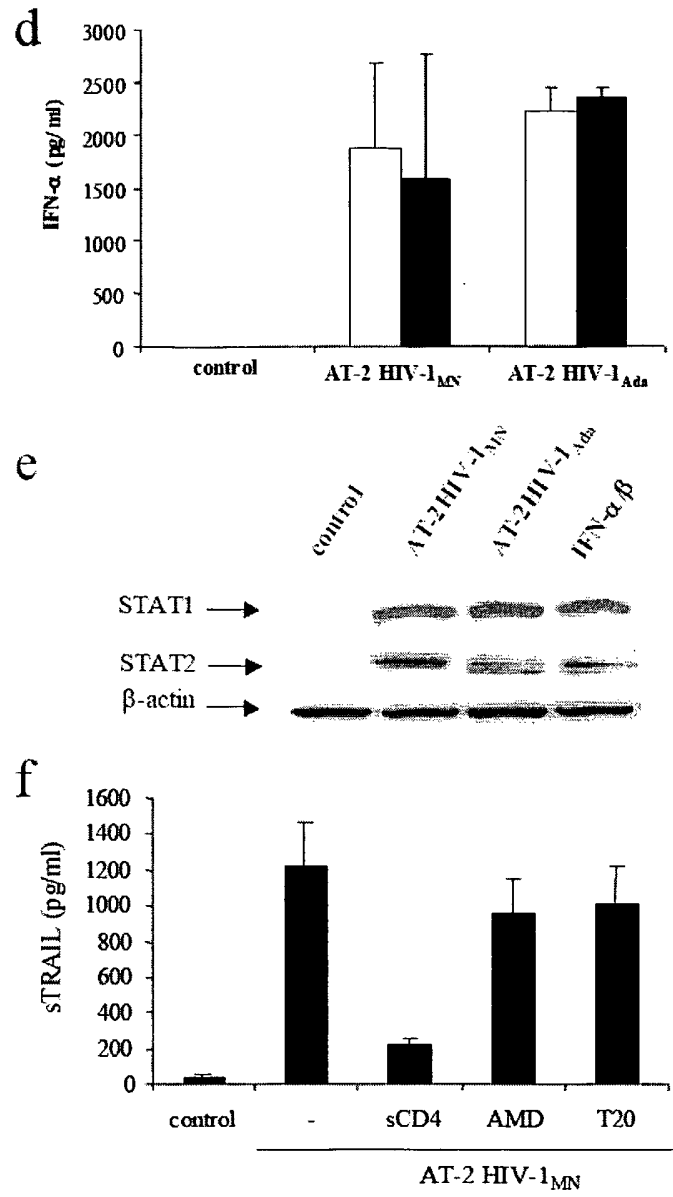
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Figure 3 (a-c)



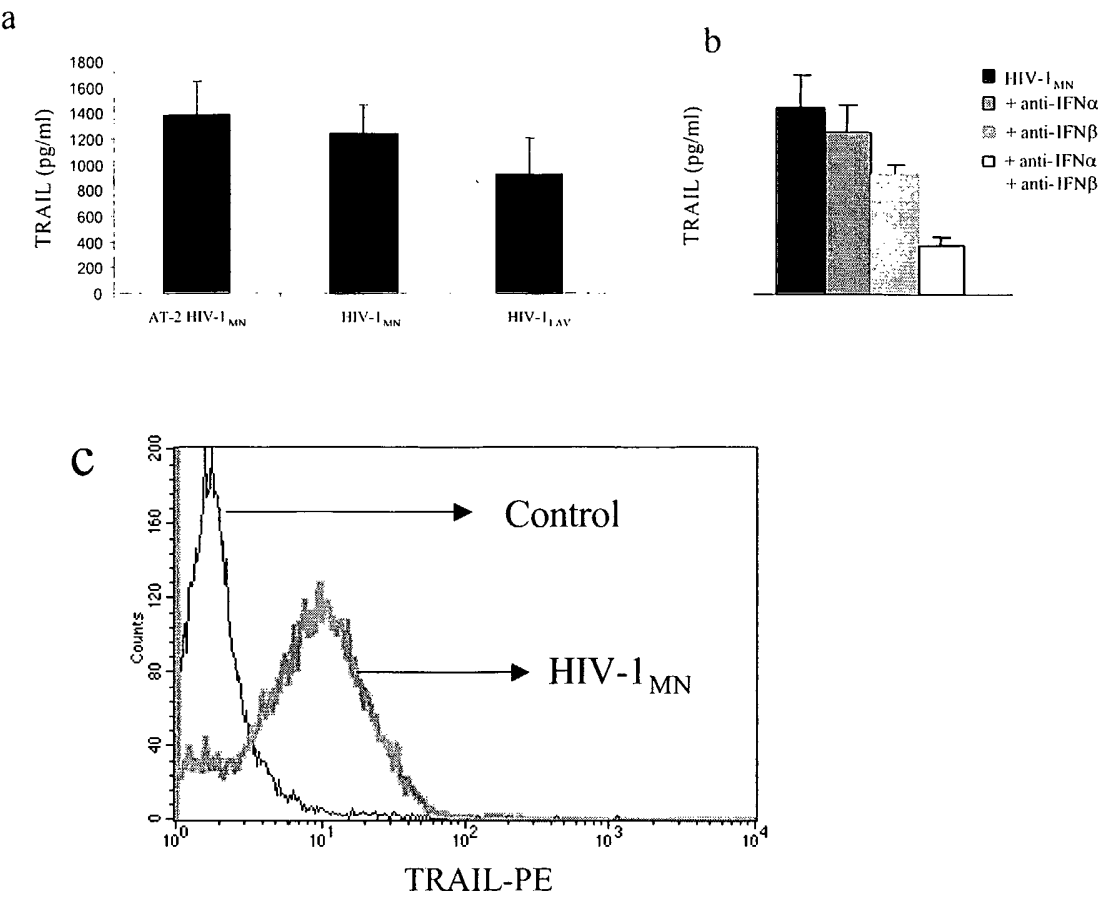
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Figure 3 (d-f)



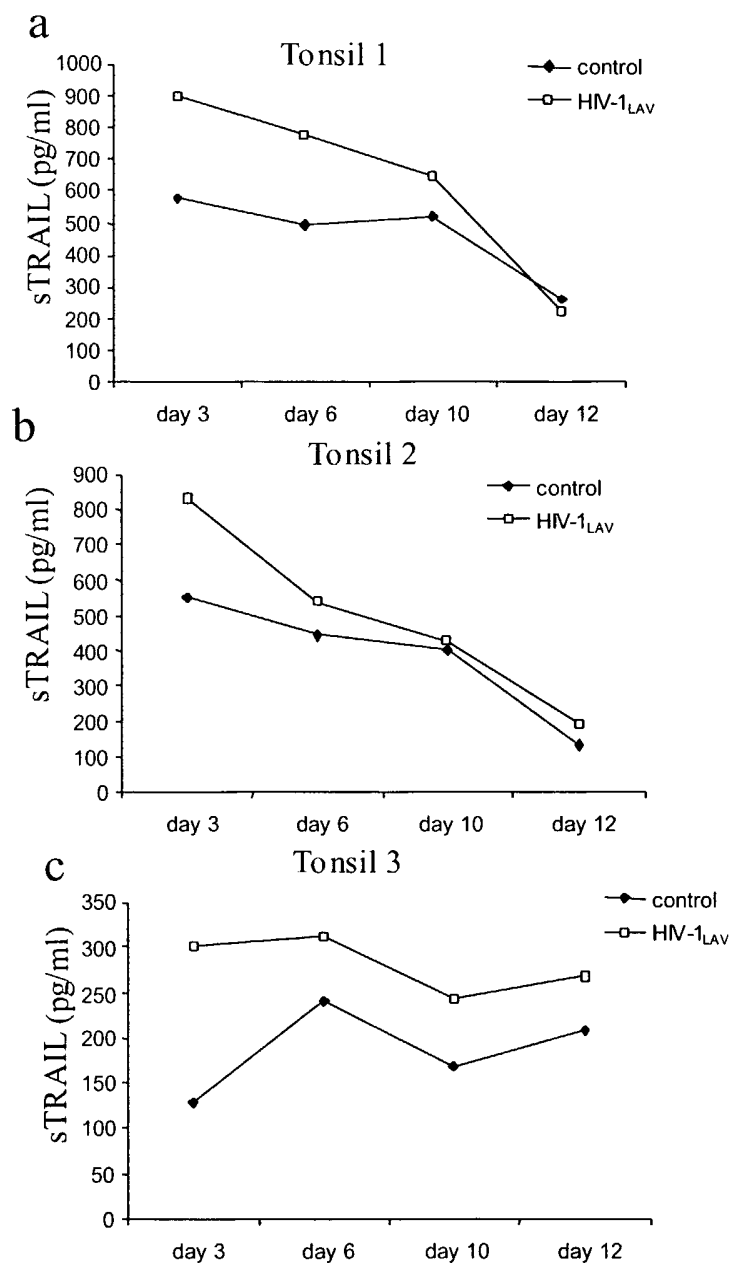
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Figure 4



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Figure 5



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Figure 6 (A)

A

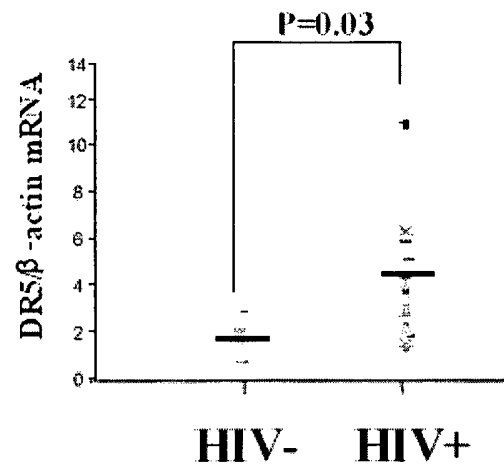
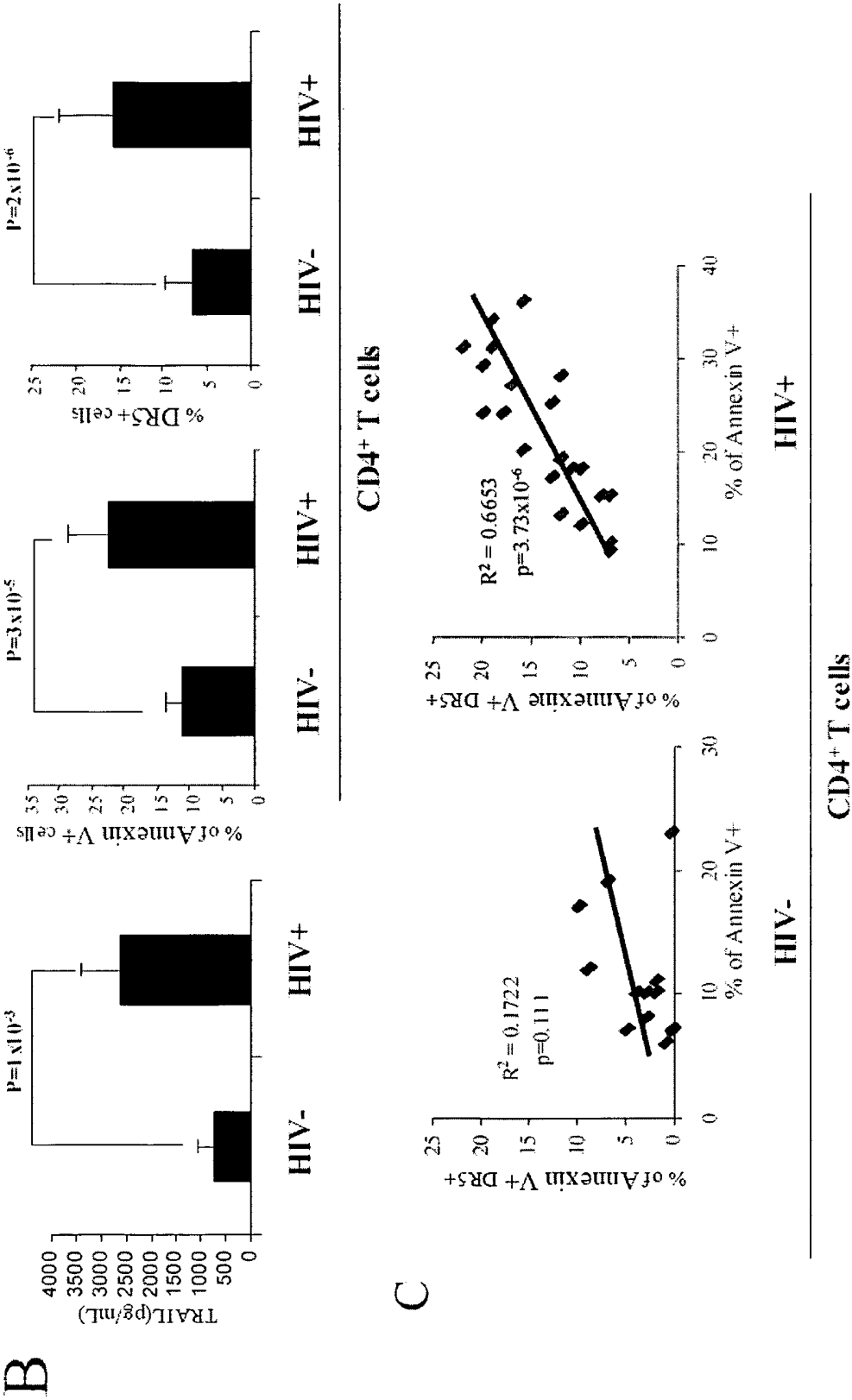
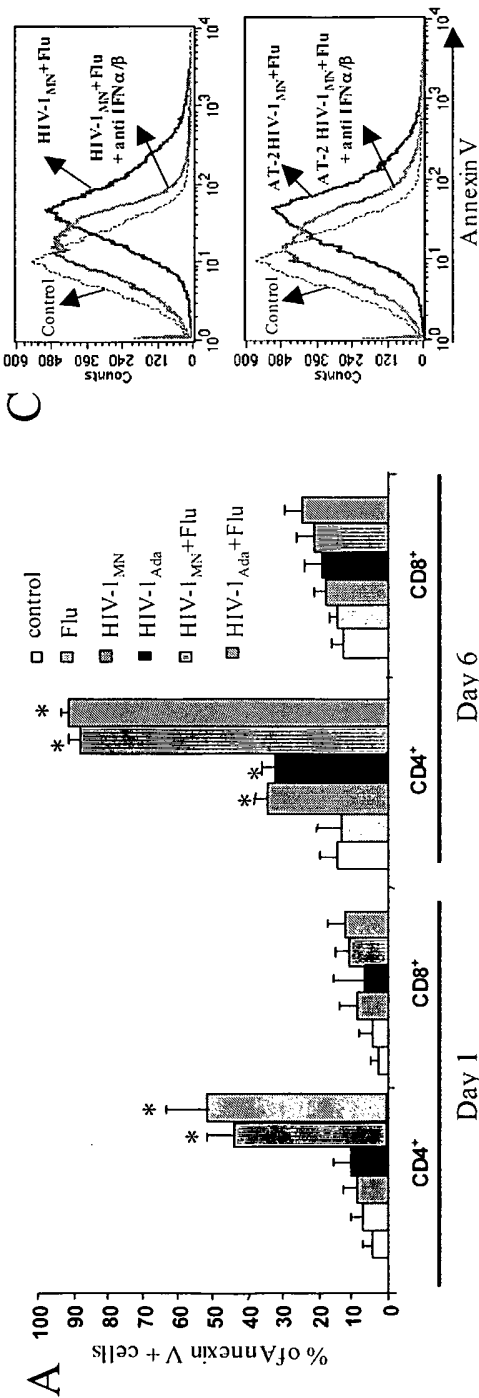


Figure 6, (B-C)



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Figure 7 (A, C)



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Figure 7 (B, D-F)

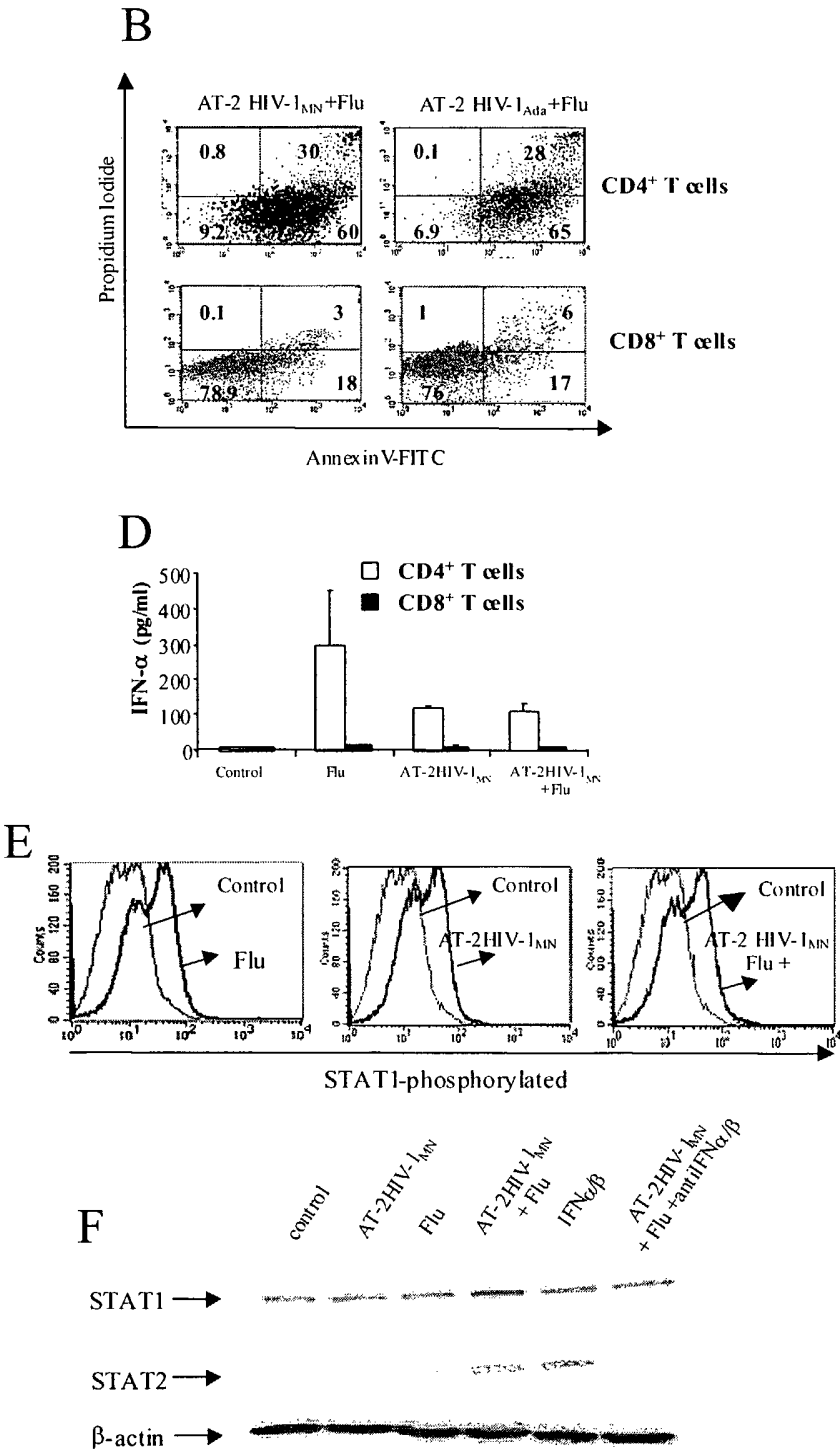
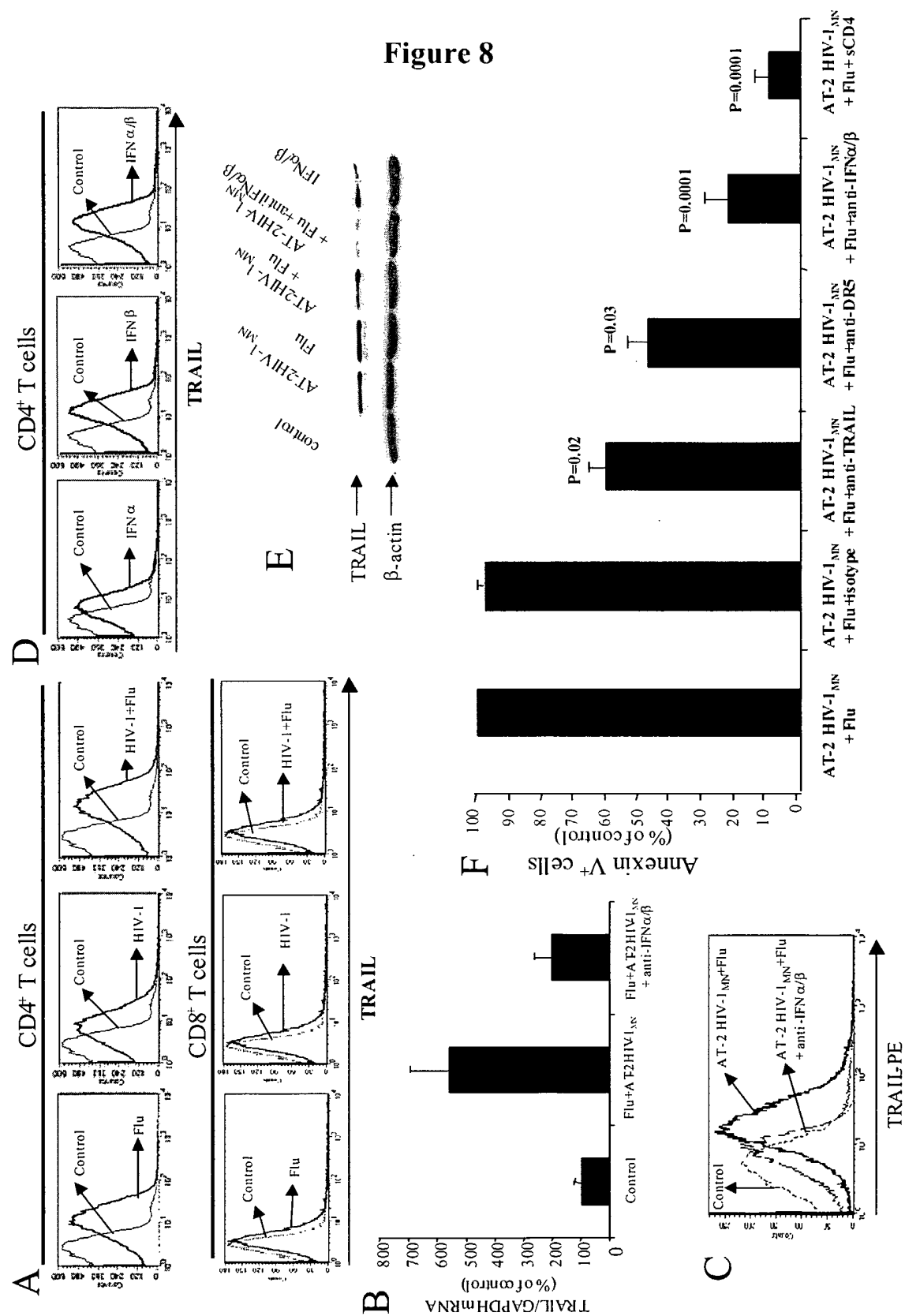
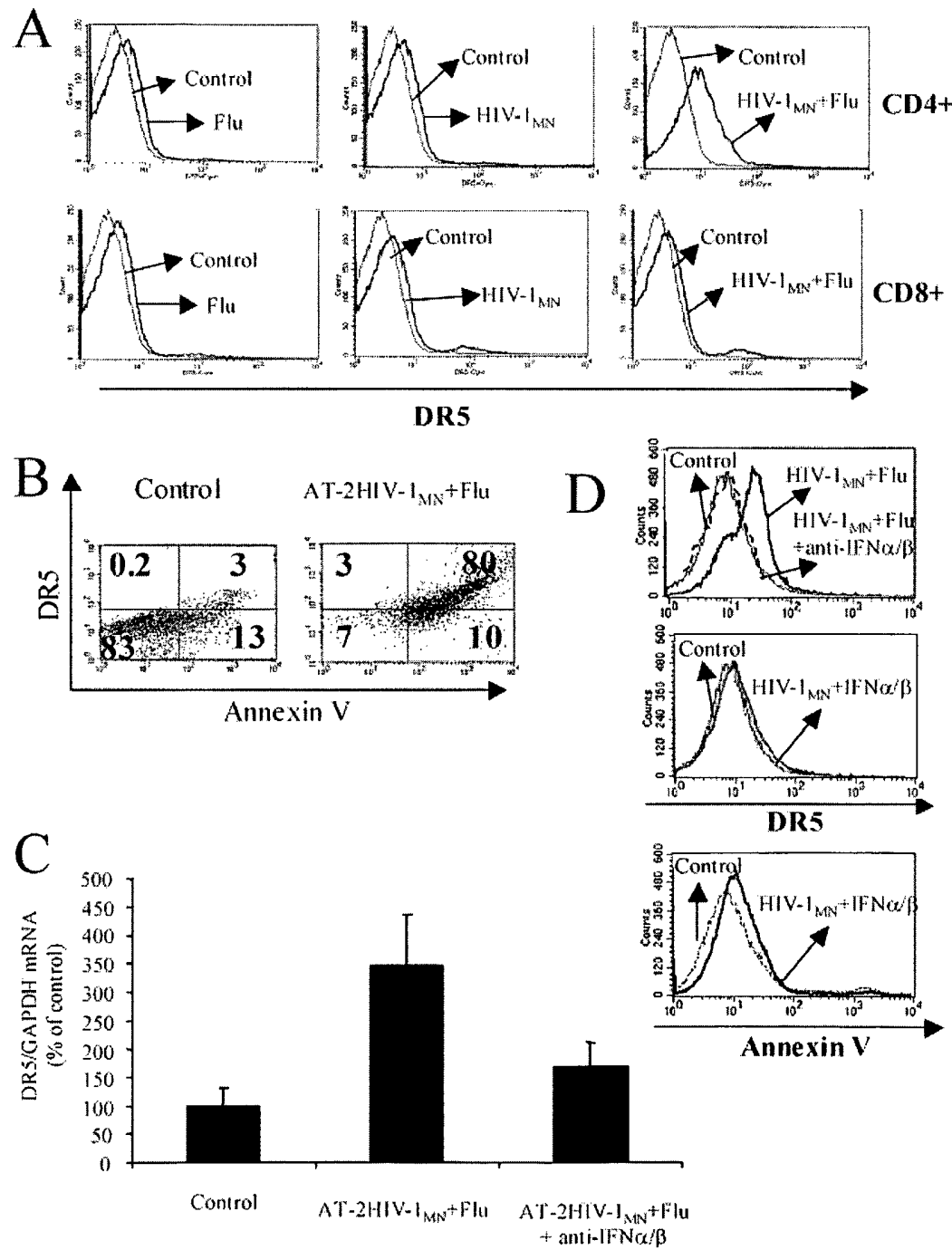


Figure 8



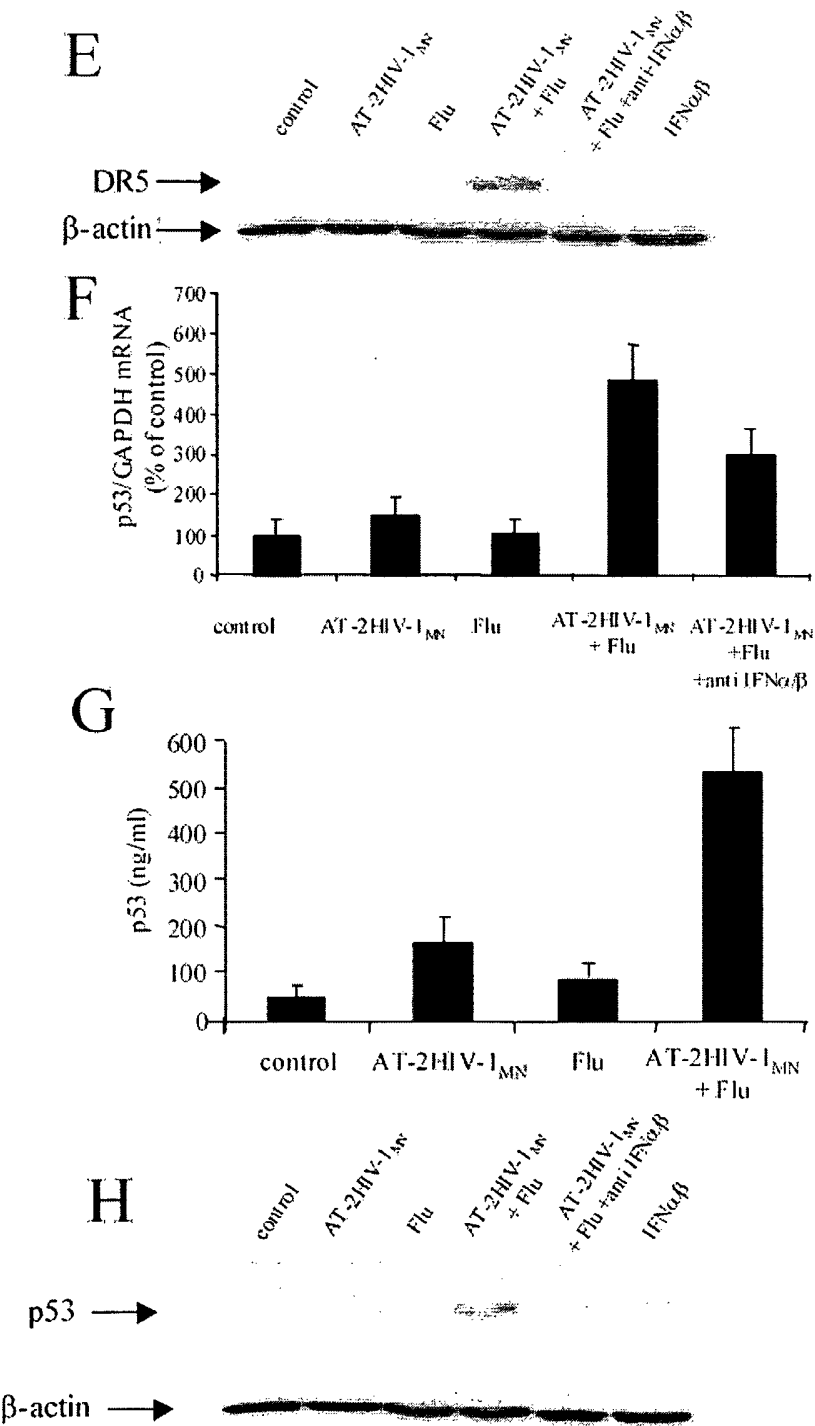
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Figure 9 (A-D)



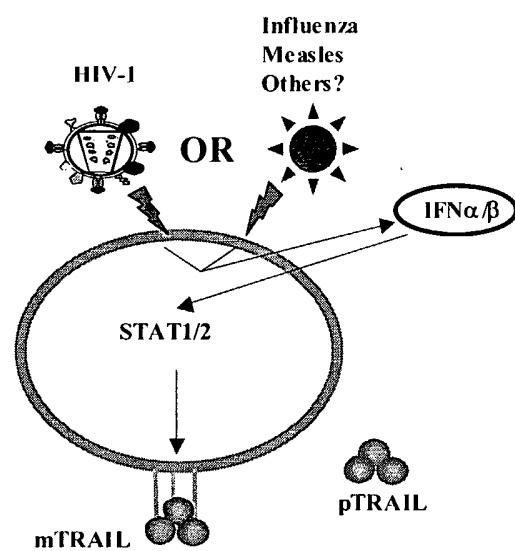
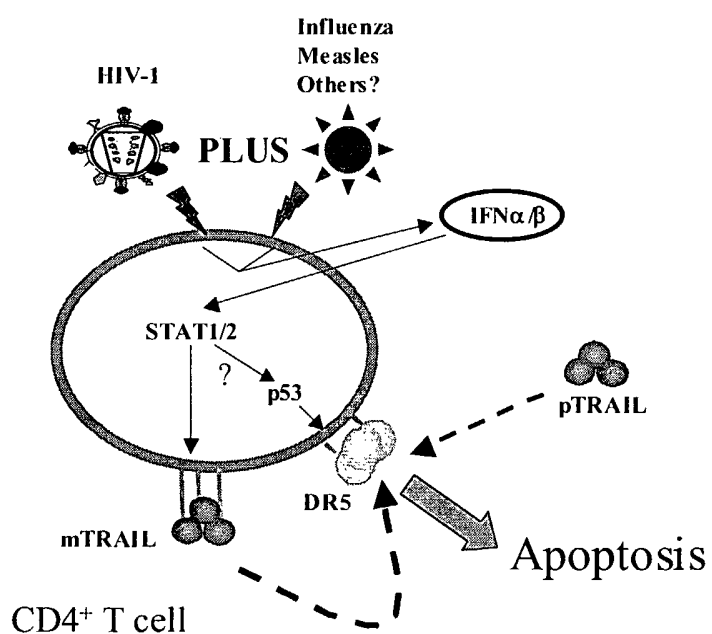
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Figure 9 (E-H)



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Figure 10

CD4⁺ T cellCD4⁺ T cell

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