(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
3 January 2003 (03.01.2003)

PCT

(10) International Publication Number
WO 03/000199 A2

(51) International Patent Classification[7]: A61K

(21) International Application Number: PCT/US02/20128

(22) International Filing Date: 25 June 2002 (25.06.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

60/300,731	25 June 2001 (25.06.2001)	US
10/056,922	24 January 2002 (24.01.2002)	US

(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier applications:

US	60/300,731 (CIP)
Filed on	25 June 2001 (25.06.2001)
US	10/056,922 (CIP)
Filed on	24 January 2002 (24.01.2002)

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/000199 A2

(54) Title: ILT3 AND ILT4-RELATED COMPOSITIONS AND METHODS

(57) Abstract: This invention provides compositions which comprise at least two of a CD4+CD25+ cell, IL-l0, a CD8+CD28- cell and a vitamin D_3 analog. This invention also provides methods for generating a tolerogenic antigen-presenting cell, and increasing the expression of ILT3 and/or ILT4 by an antigen-presenting cell. This invention further provides methods for inhibiting the onset of or treating the rejection of an antigenic substance and inhibiting the onset of or treating an autoimmune disease in a subject. This invention further provides methods for treating and preventing AIDS, cancer, and Hepatitis C-related disorders, and for identifying agents useful for such purposes. Finally, this invention provides related compositions and kits.

ILT3 AND ILT4-RELATED COMPOSITIONS AND METHODS

This application claims priority of U.S. Serial No. 60/300,731, filed June 25, 2001, and of U.S. Serial No. 10/056,922, filed January 24, 2002, the contents of which are hereby incorporated by reference into the present application.

This invention was made with support under Grant No. AI25210-15 from the National Institutes of Health. Accordingly, the United States Government has certain rights in the invention.

Throughout this application, various references are referred to within parentheses. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citation for these references may be found at the end of this application, preceding the claims.

Background of the Invention

The inhibitory activity shown by regulatory T (T_R) lymphocytes is believed to be central to the prevention of autoimmune diseases, allergies, transplant rejection and immune-deficiency disorders. Recent evidence indicates that multiple types of T_R cells may exist. Different subsets of $CD4^+$ and $CD8^+$ T lymphocytes show regulatory activities that are mediated by immunosuppressive cytokines or by contact-dependent mechanisms (1-4). In both humans and rodents one of the best-characterized populations of T_R cells are the $CD4^+CD25^+$ lymphocytes. After T cell receptor (TCR)-triggering, $CD4^+CD25^+$ T_R cells inhibit immune responses *in vivo* and *in vitro via* an antigen-presenting

cell (APC)-independent mechanism. This occurs in an antigen-nonspecific and major histocompatibility complex (MHC)-nonrestricted manner (1-6). Human as well as murine $CD4^+CD25^+$ T_R cells are anergic and express intracellular cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), a costimulatory receptor which delivers a negative or "off" signal to T cells (6, 7). CTLA-4 may account for the ability of $CD4^+CD25^+$ T cells to suppress immune responses *in vivo* (8). There is evidence that $CD4^+CD25^+$ T cell-mediated suppression of conventional $CD4^+CD25^-$ T cell activation in response to alloantigen, immobilized anti-CD3 and phytohemagglutinin (PHA) stimulation is based on contact-dependent, cytokine-independent, T cell-to-T cell interaction (5, 9). One hypothesis suggests that after TCR-mediated activation, $CD4^+CD25^+$ T cells express cell surface molecule(s) that mediate suppression by binding to a counter-receptor on $CD4^+CD25^-$ T cells. This counter-receptor may also require induction by TCR ligation (3).

A distinct subset of $CD4^+$ T_R cells, isolated by expanding human T cells primed with alloantigens in the presence of interleukin 10 (IL-10) was termed type 1 T_R (T_R1) cells (10). These cells inhibit both naïve and memory T cells in an antigen-specific manner *via* a mechanism that is partially dependent on the production of the immunoregulatory cytokines IL-10 and transforming growth factor-β (TGF-β) (10). Similarly, within the human $CD8^+$ subset, there exist antigen-specific T_R cells that suppress $CD4^+$ T helper (T_H) cell reactivity by producing IL-10 (11).

It has been previously shown that there is a distinct population of human T_R cells which are characterized by their $CD8^+CD28^-$ phenotype (12-17) and are referred to as T suppressor (T_S) cells (12-18). Like the $CD4^+$ T_R cells, $CD8^+CD28^-$ T_S cells can be generated *in vitro* after multiple rounds of stimulation of human peripheral blood mononuclear cells (PBMCs) with either allogeneic-(12) or xenogeneic-donor APCs(13). Similarly,

$CD8^+CD28^-$ T_S can be generated *in vitro* by priming PBMCs with self-APCs pulsed with nominal antigens such as MHC antigens or tetanus toxin (14). $CD8^+CD28^-$ T_S cells are MHC class I-restricted and suppress antigen-specific $CD4^+$ T_H cell responses, inhibiting their capacity to produce IL-2 and preventing up-regulation of CD40 ligand (CD40L)(12-15). Inhibition of $CD4^+$ T_H cell proliferation is not caused by killing either APCs or $CD4^+$ T_H cells. Neither is the suppressor effect mediated by the production of cytokines; instead it requires direct interactions between $CD8^+CD28^-$ T_S cells and the APCs used for priming (12, 13). In this system, the APCs act as a bridge between $CD8^+CD28^-$ T_S cells—which recognize peptide-MHC class I complexes on their cell surfaces—and $CD4^+$ T_H cells—which recognize peptide-MHC class II complexes on their cell surfaces(12). $CD8^+CD28^-$ T_S cells inhibit CD40-mediated up-regulation of costimulatory molecules such as CD80 and CD86 on APCs that present the peptide-MHC class I complexes to which the $CD8^+CD28^-$ T_S cells have been previously primed (12, 13, 16). The suppressed APCs are rendered unable to induce and sustain the full program of $CD4^+$ T_H cell activation due, at least in part, to the inhibition of NF-κB activation and transcription of costimulatory molecules in APCs (17).

Summary of the Invention

This invention provides a first composition which comprises at least two of a CD4+CD25+ cell, IL-10, a CD8+CD28- cell, and/or a vitamin D3 analog, in prophylactically or therapeutic amounts.

This invention further provides a composition which comprises the first instant composition and a pharmaceutically acceptable carrier.

This invention further provides method for generating a tolerogenic antigen-presenting cell which comprises contacting the cell with an effective amount of IL-10, a CD4+CD25+ and/or a vitamin D3 analog.

This invention further provides a method for increasing the expression of ILT3 and/or ILT4 by an antigen-presenting cell which comprises contacting the cell with an effective amount of IL-10, a CD4+CD25+ cell and/or a vitamin D3 analog.

This invention further provides a method for inhibiting the onset of rejection of an antigenic substance in a subject, which comprises administering to the subject a prophylactically effective amount of IL-10, a CD4+CD25+ cell, and/or a vitamin D3 analog.

This invention further provides a method for treating the rejection of an antigenic substance in a subject, which comprises administering to the subject a therapeutically effective amount of IL-10, a CD4+CD25+ cell, and/or a vitamin D3 analog.

This invention further provides a method for inhibiting the

onset of an autoimmune disease in a subject, which comprises administering to the subject a prophylactically effective amount of IL-10, a CD4+CD25+ cell, and/or a vitamin D3 analog.

This invention further provides a method for treating autoimmune disease in a subject, which comprises administering to the subject a therapeutically effective amount of IL-10, CD4+CD25+ cell, and/or vitamin D3 analog.

This invention further provides a second composition of matter comprising an agent that specifically binds to ILT3 and/or ILT4.

This invention further provides a composition which comprises the second instant composition and a pharmaceutically acceptable carrier.

This invention further provides a method for decreasing the expression of ILT3 and/or ILT4 by an antigen-presenting cell which comprises contacting the cell with the second instant composition.

This invention further provides a method for inhibiting the onset of AIDS or cancer in a subject, which comprises administering to the subject a prophylactically effective amount of the second instant composition and a pharmaceutically acceptable carrier.

This invention further provides a method for treating AIDS or cancer in an afflicted subject, which comprises administering to the subject a therapeutically effective amount of the second instant composition and a pharmaceutically acceptable carrier.

This invention further provides a method for inhibiting the

onset of a Hepatitis C-related disorder in a subject infected with the Hepatitis C virus, which comprises administering to the subject a prophylactically effective amount of the second instant composition and a pharmaceutically acceptable carrier.

This invention further provides a method for treating a Hepatitis C-related disorder in a subject infected with the Hepatitis C virus, which comprises administering to the subject a prophylactically effective amount of the second instant composition and a pharmaceutically acceptable carrier.

This invention further provides a method for determining the degree to which a subject is immunocompromised, which comprises determining the expression level of ILT3 and/or ILT4 in antigen-presenting cells of the subject and comparing the expression level so determined to the ILT3 and/or ILT4 expression level in antigen-presenting cells of a subject whose immune system is normal or compromised to a known degree.

This invention further provides a method for determining the likelihood that a subject's immune system will reject an antigenic substance if introduced into the subject, which comprises determining the expression level of ILT3 and/or ILT4 in the antigen-presenting cells of the subject, and comparing the expression level so determined to the expression level of ILT3 and/or ILT4 determined in antigen-presenting cells of a subject whose immune system has a known likelihood for rejecting the antigenic substance.

This invention further provides a method for determining whether an agent is an immunosuppressant or an immunostimulant which comprises (a) contacting the agent with an antigen-presenting cell and (b) determining the resulting expression level of ILT3 and/or ILT4 in the cell, an increase of ILT3

and/or ILT4 expression resulting from step (a) indicating that the agent is an immunosuppressant, and a decrease of ILT3 and/or ILT4 expression resulting from step (a) indicating that the agent is an immunostimulant.

This invention further provides a method for determining whether an agent is an immunosuppressant or an immunostimulant which comprises (a) administering the agent to a subject and (b) determining the resulting expression level of ILT3 and/or ILT4 in the subject's antigen-presenting cells, an increase of ILT3 and/or ILT4 expression resulting from step (a) indicating that the agent is an immunosuppressant, and a decrease of ILT3 and/or ILT4 expression resulting from step (a) indicating that the agent is an immunostimulant.

Finally, this invention also provides for a kit practicing any of the above-identified methods, comprising (a) an agent useful for quantitating ILT3 and/or ILT4 or nucleic acid encoding same, and (b) instructions for use.

Brief Description of the Figures

Figures 1A and B

CD8+CD28- T_S inhibit CD4+ T_H proliferation and render APC tolerogenic. (a) The addition of monoclonal anti-ILT3 or a cocktail of monoclonal anti-ILT4 plus anti-HLA class I to cultures containing CD4+ T_H, CD8+CD28- T_S and stimulating APC partially abrogates the T_S effect on T_H proliferation; (b) rIL2 restores T_H proliferation in response to APC tolerized by exposure to T_S.

Figures 2A-C

CD8+CD28- T_S upregulate the expression of both ILT3 and ILT4 on APC. (a) ILT3 and ILT4 mRNA are increased in APC co-cultured with CD8+CD28- T_S (b) Time course of ILT3 and ILT4 mRNA induction in APC co-cultured with CD8+CD28- T_S. (c) Expression of ILT3, ILT4 and CD86 on CD14+ monocytes and $CD11c^+$ HLA DR^+ DC before and after exposure to CD8+CD28- T_S.

Figures 3A-D

ILT3 and ILT4 transduction of KG1 APC. (a) Map of MIG retroviral expression vectors encoding ILT3 and ILT4. (b) Fluorescence histogram of ILT3 and ILT4 expression on the surface of ILT3-MIG-KG1, ILT4-MIG-KG1 or MIG-KG1 control. (c) CD80 expression on the cell surface of MIG-KG1, ILT3-MIG-KG-1 and ILT4-MIG-KG1 in cultures with or without KG1-primed CD4+ T_H. (d) Proliferative responses of naïve and memory CD4+ T_H to ILT3-MIG-KG1 and ILT4-MIG-KG1 in cultures with or without anti-ILT3, or rIL2.

Figures 4A and 4B

Molecular and functional changes accompany ILT3 expression in

KG1 APCs. (a) NF-kB activation induced by CD4+ T_H in KG1 APC is suppressed by CD8+CD28- T_S as determined by EMSA using Sp1 specific probe as control. 1mg of nuclear extract from KG1 APC was used. (b) Inhibition of NF-kB activation in ILT3-MIG-KG1 clone A and clone B.

Figures 5A-C

Expression of ILT3 and ILT4 in APC from the spleen of transplant donors after preincubation with recipient's CD8+CD28- T cells. (a) ILT3 and ILT4 mRNA in CD14+ donor splenocytes treated and untreated with CD8+CD28- T_S cells from the corresponding heart transplant recipient. (b) ILT3 and ILT4 expression on the cell surface of CD14+ splenocytes from the same donors and from HLA mismatched controls, before and after incubation with recipients' CD8+CD28- T cells. (c) CD86 expression on donor CD14+ splenocytes incubated with CD40-L (D1.1) transfected cells in the presence or absence of CD8+CD28- T cells from the corresponding recipient.

Figure 6

Cytotoxic activity of CD8+ T cells from recipient with acute rejection. Annexin V and PI staining of CD20+ and CD14+ splenocytes from the transplant donor incubated with or without CD8+CD28- T cells or unfractionated CD8+ T cells from the corresponding recipient.

Figures 7A-C

The relationship between CD8+CD28- T cells and ILT3/ILT4 expression on monocytes from immunologically deficient, HIV-infected patients. (a) Phenotypic characterization of peripheral blood T lymphocytes in HIV-infected vs. Non-infected. (b) Percentage of monocytes expressing ILT4. (c) ILTa mRNA levels measured by semiquantitative RT-PCR in CD14+ cells of HIV-infected and healthy individuals.

Figure 8

Direct correlation between the frequency of $CD8^+$ $CD28^-$ T cells and the percentage of $ILT4^+$ monocytes in the population of HIV infected patients.

Detailed Description of the Invention

This invention provides a first composition which comprises at least two of a CD4+CD25+ cell, IL-10, a CD8+CD28- cell and/or a vitamin D_3 analog, in prophylactically or therapeutic amounts. In the preferred embodiment, the CD4+CD25+ cell is a CD4+CD25+Ro+ cell, and the CD8+CD28- cell is a CD8+CD28-CD27+ cell. In one embodiment, the composition further comprises a pharmaceutically acceptable carrier. In another embodiment, the CD8+CD28- and CD4+CD25+ cells and IL-10 are human.

This invention further provides a method for generating a tolerogenic antigen-presenting cell which comprises contacting the cell with an effective amount of IL-10, a CD4+CD25+ cell and/or a vitamin D_3 analog. In an embodiment, the antigen-presenting cell is a human antigen-presenting cell. The contacting can be performed, for example, *in vivo*, *ex vivo*, or *in vitro*. In another embodiment, the method further comprises contacting the antigen-presenting cell with a CD8+CD28- cell. In another embodiment, the antigen-presenting cell is a dendritic cell or a monocyte.

This invention further provides a method for increasing the expression of ILT3 and/or ILT4 by an antigen-presenting cell which comprises contacting the cell with an effective amount of IL-10, a CD4+CD25+ cell and/or a vitamin D_3 analog. In one embodiment, the antigen-presenting cell is a human antigen-presenting cell. In another embodiment, the contacting is performed *in vivo*, *ex vivo*, or *in vitro*. In a further embodiment, the method further comprises contacting the antigen-presenting cell with a CD8+CD28- cell. In a further embodiment, the antigen-presenting cell is a dendritic cell or a monocyte.

This invention further provides a method for inhibiting the onset of rejection of an antigenic substance in a subject, which comprises administering to the subject a prophylactically effective amount of IL-10, a CD4+CD25+ cell, and/or a vitamin D_3 analog. In one embodiment, the antigenic substance is a transplanted cell, tissue or organ. In another embodiment, the antigenic substance is xenogeneic, allogeneic, and/or a prosthetic device. In the preferred embodiment, the subject is human.

This invention further provides a method for treating the rejection of an antigenic substance in a subject, which comprises administering to the subject a therapeutically effective amount of IL-10, a CD4+CD25+ cell, and/or a vitamin D_3 analog. In one embodiment, the antigenic substance is a transplanted cell, tissue or organ. In another embodiment, the antigenic substance is xenogeneic, allogeneic, and/or a prosthetic device. In the preferred embodiment, the subject is human.

This invention further provides a method for inhibiting the onset of an autoimmune disease in a subject, which comprises administering to the subject a prophylactically effective amount of IL-10, a CD4+CD25+ cell, and/or a vitamin D_3 analog. In one embodiment, the disease is selected from the group consisting of autoimmune encephalomyelitis, lupus, rheumatoid arthritis, multiple sclerosis, myasthenia gravis, psoriasis and Crohn's disease. In the preferred embodiment, the subject is human.

This invention further provides a method for treating autoimmune disease in a subject, which comprises administering to the subject a therapeutically effective amount of IL-10, CD4+CD25+ cell, and/or vitamin D3 analog. In one embodiment,

the disease is selected from the group consisting of autoimmune encephalomyelitis, lupus, rheumatoid arthritis, multiple sclerosis, myasthenia gravis, psoriasis and Crohn's disease. In another embodiment, the subject is human.

This invention further provides a second composition of matter comprising an agent that specifically binds to ILT3 and/or ILT4. In one embodiment, the agent is an anti-ILT3 or ILT4 antibody, or antigen-binding portion thereof.

This invention further provides a composition which comprises the second composition and a pharmaceutically acceptable carrier.

This invention further provides a method for decreasing the expression of ILT3 and/or ILT4 by an antigen-presenting cell which comprises contacting the cell with the second composition. In one embodiment, the antigen-presenting cell is a human antigen-presenting cell. In another embodiment, the contacting is performed *in vivo*, *ex vivo*, or *in vitro*. In another embodiment, the antigen-presenting cell is a dendritic cell or a monocyte.

This invention further provides a method for inhibiting the onset of AIDS or cancer in a subject, which comprises administering to the subject a prophylactically effective amount of the second composition. In the preferred embodiment, the subject is human.

This invention further provides a method for treating AIDS or cancer in an afflicted subject, which comprises administering to the subject a therapeutically effective amount of the second composition. In the preferred embodiment, the subject is human.

This invention further provides a method for inhibiting the onset of a Hepatitis C-related disorder in a subject infected with the Hepatitis C virus, which comprises administering to the subject a prophylactically effective amount of the second composition. In the preferred embodiment, the subject is human. Hepatitis C-related disorders include by example cirrhosis and liver cancer.

This invention further provides a method for treating a Hepatitis C-related disorder in a subject infected with the Hepatitis C virus, which comprises administering to the subject a prophylactically effective amount of the second composition. In the preferred embodiment, the subject is human.

This invention further provides a method for determining the degree to which a subject is immunocompromised, which comprises determining the expression level of ILT3 and/or ILT4 in antigen-presenting cells of the subject and comparing the expression level so determined to the ILT3 and/or ILT4 expression level in antigen-presenting cells of a subject whose immune system is normal or compromised to a known degree. In one embodiment, the antigen-presenting cell is a dendritic cell or a monocyte. In the preferred embodiment, the subject is human. In one embodiment, determining the expression level of ILT3 and/or ILT4 comprises determining the level of mRNA encoding same. In another embodiment, determining the expression level of ILT3 and/or ILT4 comprises determining the level of ILT3 and/or ILT4 protein. In the methods of this invention, determining the amount of ILT3 and ILT4 expression can be performed, for example, using whole blood, isolated APCs, or isolated monocytes.

This invention further provides a method for determining the likelihood that a subject's immune system will reject an antigenic substance if introduced into the subject, which comprises determining the expression level of ILT3 and/or ILT4 in the antigen-presenting cells of the subject, and comparing the expression level so determined to the expression level of ILT3 and/or ILT4 determined in antigen-presenting cells of a subject whose immune system has a known likelihood for rejecting the antigenic substance. In one embodiment, the antigen-presenting cell is a dendritic cell or a monocyte. In the preferred embodiment, the subject is human. In one embodiment, determining the expression level of ILT3 and/or ILT4 comprises determining the level of mRNA encoding same. In another embodiment, determining the expression level of ILT3 and/or ILT4 comprises determining the level of ILT3 and/or ILT4 protein. In an embodiment, the antigenic substance is a transplanted cell, tissue or organ. The antigenic substance can be, for example, xenogeneic, allogeneic, or a prosthetic device.

This invention further provides a method for determining whether an agent is an immunosuppressant or an immunostimulant which comprises (a) contacting the agent with an antigen-presenting cell and (b) determining the resulting expression level of ILT3 and/or ILT4 in the cell, an increase of ILT3 and/or ILT4 expression resulting from step (a) indicating that the agent is an immunosuppressant, and a decrease of ILT3 and/or ILT4 expression resulting from step (a) indicating that the agent is an immunostimulant. In the preferred embodiment, the antigen-presenting cell is human. In another embodiment, the antigen-presenting cell is a dendritic cell or a monocyte. In one embodiment, determining the expression level of ILT3 and/or ILT4 comprises determining the level of mRNA encoding same. In another embodiment, determining the expression level

of ILT3 and/or ILT4 comprises determining the level of ILT3 and/or ILT4 protein.

This invention further provides a method for determining whether an agent is an immunosuppressant or an immunostimulant which comprises (a) administering the agent to a subject and (b) determining the resulting expression level of ILT3 and/or ILT4 in the subject's antigen-presenting cells, an increase of ILT3 and/or ILT4 expression resulting from step (a) indicating that the agent is an immunosuppressant, and a decrease of ILT3 and/or ILT4 expression resulting from step (a) indicating that the agent is an immunostimulant. In one embodiment, determining the expression level of ILT3 and/or ILT4 comprises determining the level of mRNA encoding same. In another embodiment, determining the expression level of ILT3 and/or ILT4 comprises determining the level of ILT3 and/or ILT4 protein.

Finally, this invention provides a kit for practicing the above-identified methods, comprising (a) an agent useful for quantitating ILT3 and/or ILT4 or nucleic acid encoding same, and (b) instructions for use. In one embodiment, the agent is an antibody specific for ILT3 and/or ILT4. In another embodiment, the agent is a nucleic acid that specifically hybridizes to a nucleic acid encoding ILT3 and/or ILT4.

This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

The abbreviations used herein are: TCL - T cell line; Th - T helper cell; Ts - T suppressor cell; PBMC - peripheral blood mononuclear cell; APC - antigen-presenting cell; DCs -

dendritic cells; APCs - antigen-presenting cells; CD40L=CD40 Ligand; Mean fluorescence intensity - MFI; TNF - tumor necrosis factor; PE - Phycoerythrin; PI - Propidium Iodide; ILT3 - immunoglobulin (Ig)-like transcript 3; ILT4 - immunoglobulin (Ig)-like transcript 4; KIR - killer cell inhibitory receptor; TCR - T cell receptor.

Experimental Details

First Series of Experiments

A. Experimental Synopsis

General

The immunoglobulin like transcripts ILT3 and ILT4 belong to a family of inhibitory receptors expressed by human monocytes and dendritic cells. We now demonstrate that $CD8^+CD28^-$ alloantigen specific T-suppressor cells induce the upregulation of ILT3 and ILT4 on monocytes and dendritic cells rendering these antigen presenting cells (APC) tolerogenic. Tolerogenic APC show reduced expression of costimulatory molecules and induce antigen specific unresponsiveness in $CD4^+$ T helper cells. Study of human heart transplant recipients showed that rejection-free patients have circulating T-suppressor cells, which induce the upregulation of ILT3 and ILT4 in donor APC. These findings demonstrate an important mechanism of immune regulation.

Detailed

To gain a better insight into the precise molecular basis that underlies the anergizing capacity acquired by APCs exposed to $CD8^+CD28^-$ T_S cells, allospecific human T cell lines (TCLs) were generated and the $CD8^+CD28^-$ T_S cells from these lines were used to modulate the function of monocytes and immature dendritic cells (DCs). As an allostimulator, the myelomonocytic cell line KG1 were used; KG1 cells show many of the phenotypic characteristics of immature DCs (19). After exposure to $CD8^+CD28^-$ T_S cells, these APCs show increased expression of the genes encoding immunoglobulin (Ig)-like transcript 3 (ILT3) and

ILT4. The inhibitory receptors ILT3 and ILT4, which are expressed by monocytes and DCs, belong to a family of Ig-like inhibitory receptors that are structurally and functionally related to killer cell inhibitory receptors (KIRs) (20-24). The subset of ILT receptors, which includes ILT3 and ILT4, displays a long cytoplasmic tail containing immunoreceptor tyrosine-based inhibitory motifs (ITIMs). These receptors mediate inhibition of cell activation by recruiting tyrosine phosphatase SHP-1 (20-24). Coligation of ILTs in monocytes inhibits Ca^{2+} mobilization and tyrosine phosphorylation triggered by antibody ligation of FcγRII (also known as CD32), HLA-DR and FcγRI (also known as CD64) (20). Although the ligand for ILT3 is unknown, ILT4 binds HLA-A, HLA-B, HLA-C and HLA-G (20, 22). The present study demonstrates that $CD8^+CD28^-$ T_S cells induce the up-regulation of ILT3 and ILT4 on monocytes and dendritic cells, rendering these APC capable of anergizing $CD4^+$ T_H cells.

B. Materials and Methods

Transplant patients

Citrate-anticoagulated whole blood was from the recipients of cadaver donor heart transplants treated at New York Presbyterian Medical Center. The average number of HLA mismatches between the organ donors and transplant recipients was 2.6±1.4 for HLA-A and HLA-B and 1.8±0.2 for HLA-DR. All patients were treated with standard immunosuppressive therapy. Endomyocardial biopsies were done to monitor rejection in heart allograft recipients according to a standard time schedule as described (43).

Spleens from cadaver donors were obtained and used for histocompatibility testing; splenocyte suspensions were cryopreserved at the time of transplantation. All experiments were done in compliance with the relevant laws and institutional Good Clinical Practice guidelines and were Institutional Review Board-approved.

Generation of alloreactive TCLs

PBMCs from healthy volunteers were separated from peripheral blood by Ficoll-Hypaque centrifugation. Responding PBMCs ($1X10^6$/ml) were stimulated in 24-well plates with irradiated (1600 rad) APCs ($0.5X10^6$/ml) obtained from allogeneic PBMCs *via* the depletion of $CD2^+$ T cells. Alternatively, responding PBMCs were stimulated with irradiated (3000 rad) cells ($0.5X10^6$/ml) from the KG1 myelomonocytic cell line. The KG1 cell line (ATCC, Manassas, VA) expresses HLA-A30, HLA-B35, HLA-B51, HLA-BW4, HLA-BW6, HLA-DRB1*1101 and HLA-DRB1*1401.

Cells were cultured for 7 days in complete medium (RPMI 1640 supplemented with 10% human serum, 2 mM l-glutamine and 50 μg/ml of gentamycin) (Gibco-BRL, Grand Island, NY). After 7 days, responding cells were collected, washed and rechallenged with the original stimulating cells. Three days later, rIL-2 (Boehringer Mannheim, Indianapolis, IN) was added (10 U/ml) and the cultures were expanded for an additional 4 days. Proliferation assays were done on day 14.

Three-day proliferation assay

Before testing, responding T cells were depleted of natural killer cells with the use of goat anti-mouse IgG magnetic beads (Dynal, Lake Success, NY) coupled with mAbs to CD16 and CD56 (Becton Dickinson, San Jose, CA). $CD4^+$ and $CD8^+$ T cells were

obtained from natural killer and $CD14^+$-depleted cell suspensions by negative selection with the use of $CD8^+$ and $CD4^+$ magnetic beads, respectively (Dynal). $CD8^+$ T cell suspensions were then twice depleted of $CD28^+$ T cells with the use of goat anti-mouse IgG beads (Dynal) coupled with monoclonal anti-CD28 (Becton Dickinson). The purity of the $CD4^+$ and $CD8^+CD28^-$ T cell subsets was monitored by cytofluorographic analysis. All $CD4^+$ and $CD8^+CD28^-$ T cell suspensions that were used in functional assays contained <2% $CD16^+CD56^+$ cells. $CD4^+$ T cells were >98% positive for the CD4 and CD45RO markers. The population of $CD8^+CD28^-$ T cells contained >98% cells that were positive for CD8 and <2% $CD28^{hi}$ cells.

Proliferation assays were done after two or three cycles of stimulation of human T cells with allogeneic CD2-depleted APCs or KG1 cells. Responding $CD4^+$ T cells ($1X10^5$ cells/well) were stimulated in triplicate with irradiated DCs ($2.5X10^4$ cells/well), $CD14^+$ monocytes ($1X10^5$) or KG1 cells ($5X10^4$) in the absence or presence of human $CD8^+CD28^-$ T cells ($1X10^5$ cells/well). Cultures were set up in 96-well trays in a total volume of 0.2 ml. In some experiments, monoclonal anti-ILT3, a mixture of mAbs to ILT4 and HLA class I (W6/32, ATCC) or exogenous rIL-2 (10 U/ml) were added at the start of incubation. After 48 h of incubation, the cultures were pulsed with [^{3}H]thymidine and collected 18 h later. [^{3}H]thymidine incorporation was determined by scintillation spectrometry.

Monocytes and DCs

Monocytes were obtained from PBMCs with the use of a Monocyte Negative Selection Kit (Dynal). Immature DCs were generated by culturing monocytes in 6-well plates at a concentration of $2X10^6$ cells per well for 7 days; GM-CSF (1000 U/ml, R&D Systems, Minneapolis, MN) and IL-4 (1000 U/ml, R&D Systems)

were added on days 0, 2, 4, and 6 as described (44, 45). Immature DCs were $CD14^-CD11c^+HLA\text{-}DR^+$, as shown by flow cytometry analysis.

Flow cytometry

Flow cytometry studies were done with a FACScan (Becton Dickinson). CaliBRITE beads, from Becton Dickinson, were run under the FACSComp program to calibrate the instrument. Human $CD4^+$ and $CD8^+CD28^-$ T cell subsets were defined by staining with phycoerythrin (PE)-conjugated monoclonal antibodies (mAbs) to CD3, CD28 and CD45RO; fluorescein isothiocyanate (FITC)-conjugated mAbs to CD4 and CD8; and mixtures of mAbs to FITC-CD3, PE-CD4, peridinine chlorophyll protein (PerCP)-CD8 and allophycocyanin-CD45 or FITC-CD3, PE-CD16-CD56, PerCP-CD19 and allophycocyanin-CD45 (Becton Dickinson). Other mAbs we used to stain $CD8^+$ T cells included cychrome-CD38 and cychrome-HLA-DR (Becton Dickinson).

To study the expression of ILT3 or ILT4 and costimulatory molecules on normal APCs from peripheral blood or spleen cell suspensions, APCs were incubated with T_S cells for 18 h with or without CD40L-transfected D1.1 cells (see Results). The cells were then collected, washed and saturating amounts of mAbs to ILT3 or ILT4 were added (21, 22). After 30 min on ice, cells were washed twice, stained with PE-goat-anti-mouse or PE-goat-anti-rat (Caltech, Burlingame, CA), then washed twice again, incubated with mouse or rat IgG (as a blocking antibody, Vector Labs, Burlingame, CA), washed twice, then stained with mAbs to FITC-CD14 or cychrome-CD11c, FITC-HLA-DR and PerCP-CD3 (Pharmingen, San Diego, CA). In other samples, mAbs to PE-CD80 and PE-CD86 were added along with markers for monocytes and DCs. $CD3^+$ T cells were gated-out and $CD14^+$ monocytes or $CD14^-CD11c^+HLA\text{-}DR^+$ immature DCs were analyzed with CellQuest

software on a G4 Apple Macintosh Computer. Annexin V and PI staining of target APCs was done as described (12). Five parameter analyses (forward scatter, side scatter and three fluorescence channels) were used for list mode data analysis. The FL3 channel was used as a fluorescence trigger and FL1 and FL2 as analysis parameters.

cDNA microarray profiling

Total RNA (1 μg) extracted from $1X10^6$-$5X10^6$ KG1 cells or CD2-depleted normal APCs was radioactively labeled (with ^{33}P) by reverse transcriptase (Superscript, BRL, Rockville, MD) and hybridized to a human UniGene Filter (GF211, Research Genetics, Huntsville, AL) at 42°C for 16 h according to the manufacturer's instructions. After washing, the gene filter was exposed to a phosphorimaging screen and analyzed by Pathways 2 Software (Research Genetics, Huntsville, AL).

Semi-quantitative RT-PCR

First-strand cDNA was synthesized from 1 μg of total RNA with a cDNA synthesis kit (Roche Diagnostic, Indianapolis, IN). The following primers were used in PCR reactions. ILT4: 5' primer ACCCCCTGGACTCCTGATCAC; 3' primer TGGAGTCTCTGCGTACCCTCC (expected size, 834 bp). ILT3: 5' primer CAGACAGATGGACACTGAGG; 3' primer AGAATCAGGTGACTCCCAAC (expected size, 320 bp). Primers for GADPH were as described (46). ILT3 and ILT4 PCR reactions were done at 30 cycles and GADPH PCR reactions were done at 23 or 24 cycles. PCR products were analyzed on agarose gel stained with ethidium bromide. RT-PCR products were quantified by digital imaging of the ethidium bromide agarose gel with a Kodak System 120; the images were analyzed on a computer with Kodak 1D Software (Kodak, Rochester, NY). Values for ILT3 and ILT4 expression were normalized with the use of GAPDH

expression values measured in the same cDNA dilutions. The normalized signals for each gene in untreated APCs were given a value of 1. Data were expressed as the mean ± s.d. of all four different dilutions.

Construction of retroviral vectors containing ILT3 and ILT4

Full-length ILT3 and ILT4 cDNAs were cloned from KG1 cells by RT-PCR into the pcDNA4/TO/myc-His vector (Invitrogen, Carlsbad, CA) in-frame with a COOH-terminal c-Myc tag. The Myc-tagged ILT3 and ILT4 inserts were subcloned into the *Bgl*II site of a green fluorescence protein (GFP) - retroviral vector called MIG (for MSCV-IRES-GFP) (47). The ILT3-MIG and ILT4-MIG inserts were completely sequenced from both strands to confirm that the correct sequence had been inserted. ILT3-MIG, ILT4-MIG or MIG alone (50 μg), PCL-eco (20 μg) and VSV-G (5 μg) were used to transfect 293T cells with the calcium phosphate method. Viral supernatants were collected 48 h after transfection and filtered through 0.45-μm membranes before use.

Generation and characterization of KG1.ILT3 and KG1.ILT4 cells

Retroviral transduction was *via* the centrifugal enhancement method (48). Briefly, KG1 cells were resuspended in viral supernatant (1-2 ml/10^6 cells) with 8 μg/ml of polybrene (Sigma Chemical Co., St. Louis, MO), then centrifuged at 2500*g* for 2 h at 30 °C. Infected cells were resuspended in fresh Iscove's modified Eagle's medium and cultured overnight. After three consecutive spin-infections and overnight cultures, cells expressing high amounts of GFP were sorted with a FACStar Plus (Becton Dickinson). The sorted KG1.ILT3 and KG1.ILT4 cells,

which were typically >95% GFP^+, were used within 2-3 weeks. For each experiment, two or three independent transductants were tested.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared and EMSAs were done as described (49). Double-stranded NF-κB oligomers (AGCTTCAGAGGGACTTTCCTCTGA) and double-stranded Sp1 oligomers (CCCTTGGTGGGGGCGGGGCCTAAGCTGCG) were used. KG1 cells incubated with $CD4^+$ T_H cells were separated from the mixture with the use of $CD34^+$ Dynal beads. For supershift assays, nuclear extracts prepared from $CD4^+$ T_H cell-treated KG1 cells were incubated with antibodies to the NF-κB subunits p50 or p65 (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min at 4°C before the labeled NF-κB probe was added.

Statistical analysis

Analysis of the statistical differences between healthy controls and different groups of patients, with respect to the phenotype of $CD8^+$ T cells, were done with the use of one-way ANOVA tests followed by Scheffé criterion tests for multiple comparisons.

C. Results

$CD8^+CD28^-$ T_S cells inhibit APC allostimulatory capacity

It has been previously shown that $CD8^+CD28^-$ T_S cells from allospecific and xenospecific TCLs inhibit $CD4^+$ T_H cell proliferation in a dose-dependent manner (12, 13). Addition of

either exogenous IL-2 or monoclonal anti-CD28 restored $CD4^+$ T_H cell proliferation in the presence of $CD8^+CD28^-$ T_S cells, which indicates that the $CD4^+$ T_H cells were rendered anergic (12, 15). $CD8^+CD28^-$ T_S cells recognize MHC class I alloantigens on APCs and render the APC unable to stimulate $CD4^+$ T_H cell proliferation (12, 14).

To determine the effect of $CD8^+CD28^-$ T_S cells on $CD4^+$ T_H cell alloreactivity, 12 different TCLs were generated. For each TCL, T cells from a responder A were stimulated with CD2-depleted PBMCs from a stimulator B. After two rounds of allostimulation $CD4^+$ T_H and $CD8^+CD28^-$ T_S cells from each TCL were purified, and $CD4^+$ T_H cell alloreactivity was tested in 3-day proliferation assays. In these assays, $CD4^+$ T_H cells from responder A or mixtures of $CD4^+$ T_H and $CD8^+CD28^-$ T_S cells from responder A were stimulated with $CD14^+$ monocytes or $CD11c^+HLA\text{-}DR^+CD14^-$ immature DCs from stimulator B. Immature DCs were generated from monocytes cultured with granulocyte-monocyte colony-stimulating factor (GM-CSF) and IL-4. When KG1 cells were used as stimulators to generate TCLs, KG1 cells were also used in the proliferation assay. $CD8^+CD28^-$ T_S cells isolated from each of these TCLs inhibited the blastogenic response of $CD4^+$ T_H cells isolated from the same TCL to the specific stimulator by >80% (Figure 1a).

Whether APCs exposed to $CD8^+CD28^-$ T_S cells become tolerogenic was investigated. Monocytes or DCs from the donor used for TCL priming were preincubated with allospecific $CD8^+CD28^-$ T_S cells. Similarly, KG1 cells were preincubated with KG1-primed $CD8^+CD28^-$ T_S cells. After 18 h, these conditioned APCs were γ-irradiated and used for stimulating $CD4^+$ T_H cells in 3-day proliferation assays. $CD8^+CD28^-$ T_S cell-treated APCs induced little proliferation of allospecific $CD4^+$ T_H cells from the same TCL, whereas the proliferative responses of the same

allospecific $CD4^+$ T_H cells stimulated with untreated APCs were robust. Addition of recombinant IL-2 (rIL-2) to the allospecific $CD4^+$ T_H cells restored $CD4^+$ T_H responsiveness to $CD8^+CD28^-$ T_S cell-treated APCs (Figure 1b). Hence, APCs pretreated with $CD8^+CD28^-$ T_S cells are poor inducers of $CD4^+$ T_H cell activation; instead, these APCs induce $CD4^+$ T_H cell anergy.

Anergizing APCs express ILT3 and ILT4

To identify the molecular changes associated with the acquisition of a tolerogenic phenotype the following steps were taken. First the effects of $CD8^+CD28^-$ T_S cell exposure on the transcriptional profile of KG1 cells and normal APCs were analyzed using a cDNA microarray that contained probes for 4454 randomly selected human genes. Expression profiling showed changes in both KG1 cells and normal APCs after treatment with $CD8^+CD28^-$ T_S cells. Among the changes confirmed by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was the up-regulation of mRNA encoding ILT3 and ILT4 in T_S cell-treated APCs and KG1 cells (Figure 2a and 2b). The inhibitory receptors ILT3 and ILT4 are selectively expressed by monocytes and DCs and are thought to play a physiological role *in vivo* by negatively regulating the activation of APCs (20-24). Thus, ILT3 and ILT4 protein expression on the surfaces of monocytes and DCs pretreated with allospecific $CD8^+CD28^-$ T_S cells was examined. Flow cytometry analysis showed that $CD8^+CD28^-$ T_S cells induced the up-regulation of ILT3 and ILT4 cell surface expression on both monocytes and DCs, whereas the expression of costimulatory molecules, such as CD86, was down-regulated (Figure 2c).

These findings suggested that an inverse correlation exists between the up-regulation of ILT3 and ILT4 on APCs and the

expression of costimulatory molecules. Because CD40 ligation resulted in up-regulation of CD80 and CD86 on APCs, the effect of $CD8^+CD28^-$ T_S cells on CD40 signaling in APCs was tested. We incubated $CD8^+CD28^-$ T_S cells (from responder A) with monocytes or DCs from the specific stimulator (B) in the presence or absence of CD40L-transfected D1.1 cells (25, 26). The same allospecific $CD8^+CD28^-$ T_S cells that induced up-regulation of ILT expression by APCs also suppressed the CD40L-mediated up-regulation of CD80 and CD86 on the same APC.

Next it was determined whether ILT3 and ILT4 were responsible for the reduced capacity of APCs to stimulate $CD4^+$ T_H cell proliferation in the presence of $CD8^+CD28^-$ T_S cells. Monoclonal antibody (mAb) was added to ILT3 (24) or a mixture of mAbs to ILT4 (22) and HLA class I (the ligand for ILT4) to cultures containing allospecific $CD4^+$ T_H cells, $CD8^+CD28^-$ T_S cells and the APCs used for priming. Neither mAb to ILT3 nor the mixture of mAbs to ILT4 and HLA class I had any effect on T_S or T_H cell proliferation in response to the specific stimulator. However, mAb to ILT3 or a mixture of mAbs to ILT4 and HLA class I—but neither mAbs to ILT4 nor HLA class I alone—reversed by 49±4% the inhibitory effect of T_S cells on $CD4^+$ T_H cell proliferation in cultures containing mixtures of $CD4^+$ T_H cells, $CD8^+CD28^-$ T_S cells and monocytes or DCs (Figure 1a). These results indicated that the effect of $CD8^+CD28^-$ T_S cells on $CD4^+$ T_H cell proliferation is mediated by the inhibitory receptors ILT3 and ILT4 on APCs.

Induction of T_H cell anergy

To further test the hypothesis that $CD8^+CD28^-$ T_S cell-induced up-regulation of ILT3 and ILT4 is responsible for the tolerogenic capacity acquired by APCs, ILT3 and ILT4 were overexpressed in KG1 cells as Myc fusion proteins *via* infection with recombinant retroviruses (Figure 3a). ILT3- or ILT4-transduced KG1 cells—referred to hereafter as KG1.ILT3 and KG1.ILT4 cells, respectively—expressed high amounts of ILT3 or ILT4, as shown by flow cytometry (Figure 3b) and confirmed by immunoblotting with anti-Myc. The basal expression of a variety of other markers—including HLA class I, HLA class II and costimulatory molecules—was similar in KG1 cells with empty vector alone (referred to hereafter as KG1.MIG cells) and in the KG1.ILT3 and KG1.ILT4 cells. In the presence of $CD4^+$ T_H cells, the percentage of $CD80^+$ cells increased from basal amounts to 34.1% in the KG1.MIG cells (Figure 3c). However, only 8.4% of the KG1.ILT3 cells and 10.2% of KG1.ILT4 cells expressed CD80 upon incubation with $CD4^+$ T_H cells (Figure 3c). Results obtained with three additional ILT3- and three ILT4-transduced KG1-independent clones—as well as four control KG1 clones transduced with vector alone—confirmed the finding that ILT3 and ILT4 overexpression interferes with the $CD4^+$ T_H cell-induced up-regulation of CD80.

In addition, KG1.ILT3 and KG1.ILT4 cells elicited much less proliferation of unprimed and KG1-primed $CD4^+$ T_H cells than KG1.MIG cells (Figure 3d). Addition of rIL-2 to the proliferation assays restored $CD4^+$ T_H cell proliferation, which supported the hypothesis that ILT3 or ILT4 overexpression renders KG1 cells tolerogenic (Figure 3d). Addition of anti-ILT3 to cultures that contained $CD4^+$ T_H and KG1.ILT3 cells restored the capacity of KG1.ILT3 cells to stimulate KG1-primed $CD4^+$ T_H cells. Similarly, the mixture of mAbs to ILT4 and HLA class I restored the capacity of KG1.ILT4 cells to stimulate T_H cell proliferation (Figure 3d). As overexpression of ILT3 and

ILT4 conferred KG1 cells with a tolerogenic capacity, whereas mAbs to ILT3 and ILT4 partially blocked this effect, it appears that ILT3 and ILT4 have an immunoregulatory effect upon APCs.

NF-κB activation is inhibited in ILT3-transduced APCs

Because T_S cells inhibit NF-κB-mediated transcription of co-stimulatory molecules in APCs (17), it was tested whether constitutive expression of ILT3 in KG1 cells mimics some of the known effects of $CD8^+CD28^-$ T_S cells on APCs. NF-κB activation was measured by electrophoresis mobility-shift assays (EMSAs) (17) with the use of nuclear extracts from KG1 cells incubated for 6 hours with $CD4^+$ T_H cells or mixtures of $CD4^+$ T_H and $CD8^+CD28^-$ T_S cells. $CD8^+CD28^-$ T_S cells inhibited T_H cell-induced NF-κB activation in KG1 cells, yet these cells had no effect on the DNA-binding activity of the transcription factor Sp1 used as a nuclear extract control of the treated APC (Figure 4a). Parallel studies done on KG1, KG1.MIG and KG1.ILT3 cells showed that ILT3 overexpression substantially reduced $CD4^+$ T_H cell-induced NF-κB activation after 12 h of incubation but did not change the DNA-binding activity of Sp1 (Figure 4b). Supershift experiments with antibodies specific for the p50 and p65 subunits of NF-κB showed that the observed bands represented p65-p50 complexes. Thus, ILT3-transduction led to the inhibition of T_H cell-induced NF-κB activation in KG1 cells.

ILT3 or ILT4 expression on donor APCs in vivo

To determine whether up-regulation of ILT3 and ILT4 on APCs plays a role *in vivo*, the effect of $CD8^+CD28^-$ T cells from 15 heart allograft recipients (transplanted within 6-8 months of the experiment) on APCs from their respective cadaver donors were examined.

$CD8^+CD28^-$ T cells isolated from recipient's fresh peripheral blood were incubated for 18 h with CD2-depleted spleen cells from the heart donor or from a control individual who shared no HLA class I antigens with the transplant donor. Sufficient numbers of monocytes isolated from cryopreserved cadaver spleen that could be stained for ILT3 and ILT4 were obtained in only 10 of the 15 cases. However, expression of the inhibitory receptors was measured by the more sensitive RT-PCR method in all 15 cases. Five of the fifteen recipients studied had no acute rejection episode within the first 6-8 months after transplantation. $CD8^+CD28^-$ T cells from each of these patients induced the up-regulation of either ILT3 (two patients) or ILT4 (three patients) mRNA levels (Figure 5a) and cell surface expression (Figure 5b) on APCs from their corresponding donor. This effect was specific to the donor's HLA class I antigens, as $CD8^+CD28^-$ T cells from the rejection-free patients did not induce up-regulation of either ILT3 or ILT4 on control APCs displaying HLA antigens to which the recipient had not been exposed *in vivo* (Figure 5b). $CD8^+CD28^-$ T cells from the remaining ten recipients had no effect on the level of ILT3 or ILT4 mRNA expressed by APCs. Nine of these ten heart transplant recipients experienced at least one episode of acute rejection (histological grade 2B or 3) within the first 6 months after transplantation. ILT3 and/or ILT4 was up-regulated in $CD8^+CD28^-$ T cells from all five patients without acute rejection induced, whereas $CD8^+CD28^-$ T cells from nine of ten patients with rejection had no such effect. This indicates that the capacity of T_S cells to induce up-regulation of ILT3 or ILT4 on donor monocytes is strongly associated with the absence of acute rejection ($P<0.002$).

The ability of recipient $CD8^+CD28^-$ T cells to inhibit CD40-triggered up-regulation of CD86 on donor APCs was also tested

to determine whether $CD8^+CD28^-$ T cells primed *in vivo* with allogeneic HLA antigens behaved in a similar manner to T_S cells generated *in vitro*. Thus, five patients who had remained rejection-free after transplantation and five patients who had experienced two or three episodes of acute rejection within the first 8 months after transplantation were examined. $CD8^+CD28^-$ T cells from the rejection-free patients inhibited CD40L-triggered up-regulation of CD86 on donor APCs. In contrast, $CD8^+CD28^-$ T cells from patients with acute rejection episodes did not inhibit CD40 signaling (Figure 5c). These findings suggested that similar to $CD8^+CD28^-$ T_S cells from an alloreactive TCL, $CD8^+CD28^-$ T cells from transplant recipients in quiescence induced the up-regulation of inhibitory receptors ILT3 and/or ILT4 and inhibited up-regulation of costimulatory molecules on APCs in an allospecific manner.

To establish whether T cells from transplant patients are cytotoxic to donor APCs, annexin V and propidium iodide (PI) were used to stain APCs incubated with $CD8^+CD28^-$ T cells and unfractionated $CD8^+$ T cells from the recipients. Similar to quiescent patients, $CD8^+CD28^-$ T cells from patients with rejection showed no cytotoxic T cell activity in response to donor APCs. However, the nonfractionated $CD8^+$ T cells (which contained both $CD28^-$ and $CD28^+$ T cells) from patients with rejection were capable of killing donor APCs. Unfractionated $CD8^+$ T cells from quiescent patients showed no cytotoxic activity. This suggests that $CD8^+CD28^+$ T cells from allosensitized recipients act as effectors of allograft rejection (Figure 6).

Annexin V and PI staining of $CD20^+$ and $CD14^+$ splenocytes from the transplant donor incubated with or without $CD8^+CD28^-$ T cells or unfractionated $CD8^+$ T cells from the corresponding recipient.

Both $CD8^+CD28^-$ and the $CD8^+CD28^+$ T cells from transplant recipients and healthy controls were further characterized with respect to the frequency of $CD38^+$, $CD45RO^+$ and $HLA\text{-}DR^+$ cells within the $CD8^+$ subset. The percentage of $CD8^+CD28^-$ T cells was higher in heart recipients compared to healthy controls ($P<0.01$); this concurred with published data on liver transplant recipients (27). The frequency of $CD8^+CD28^-$ T cells that were expressing CD38, CD45RO and HLA-DR was also higher in transplant recipients compared to controls ($P<0.01$), yet there was no difference between patients with or without rejection. The frequency of CD38- and CD45RO-expressing T cells within the $CD8^+CD28^+$ subset was not significantly different between patients that had or had not undergone rejection or healthy controls. However, transplant recipients showed a higher frequency of $CD8^+CD28^+HLA\text{-}DR^+$ T cells compared to the controls ($P<0.01$) (Table 1). These results indicate that although compared to healthy controls, transplant patients show an expansion of T cells with memory and activation markers, the phenotype of $CD8^+CD28^-$ or $CD8^+CD28^+$ T cells from patients that had or had not undergone rejection does not differ with respect to these markers.

Table 1. Phenotypic Characterization of CD8+ T Cells From Transplant Patients and Healthy Controls

		Transplant patients	
Cell subsets	Controls (%)a	No rejection (%)b	Rejection (%)c
CD8+	20.70±4.95	20.17±1.94	20.89±4.04
CD8+CD28-	22.20±6.79	63.33±6.35	60.89±4.91
CD8+CD28+	77.30±6.91	38.33±5.13	39.22±4.94
CD8+CD28-CD38+	14.20±4.31	36.67±3.93	33.11±2.57
CD8+CD28+CD38+	4.05±1.39	6.80±1.94	5.67±2.12
CD8+CD28-CD45RO+	12.20±2.63	28.83±4.17	25.67±3.57
CD8+CD28+CD45RO+	37.20±4.73	39.67±5.57	35.44±4.07
CD8+CD28-HLA-DR+	7.19±3.48	36.24±3.99	38.56±5.05
CD8+CD28+HLA-DR+	10.51±5.44	20.17±5.00	23.78±3.60

a Data are mean ± S.D. from 20 individuals
b Data are mean ± S.D. from 6 individuals
c Data are mean ± S.D. from 9 individuals

D. Discussion

ILT3 and ILT4 are up-regulated in APCs after exposure to $CD8^+CD28^-$ T_S cells and are essential to the tolerogenic phenotype acquired by APCs. The $CD4^+$ T_H cell unresponsiveness induced by $CD8^+CD28^-$ T_S cell-treated APCs is characteristic of T cell anergy, as the loss of $CD4^+$ T_H cell proliferative capacity can be reversed by the addition of exogenous IL-2 (28). TCR-triggering (signal 1) in the absence of costimulation (signal 2) results in T cell anergy (29). Tolerogenic APCs showed decreased amounts of costimulatory molecules in conjunction with increased ILT3 and ILT4 expression.

Although it has been speculated that ITIM-bearing ILTs may control DC antigen-presenting functions, co-stimulation and cytokine production, their physiological significance was unknown. Now it has been demonstrated that up-regulation of

ILT3 and ILT4 renders monocytes and DCs tolerogenic. The finding that overexpression of ILT3 was associated with inhibition of NF-κB activation shows that, in the presence of $CD8^+CD28^-$ T_S cells, APCs have a reduced capacity to transcribe NF-κB-dependent costimulatory molecules (17). Although it is not clear how ILT3 and ILT4 overexpression interferes with CD40 signaling, it is possible that these receptors act through SHP phosphatases to modulate IκB phosphorylation and degradation, thus affecting NF-κB activation. This would inhibit the transcription of NF-κB-dependent genes that encode co-stimulatory molecules in DCs, thus promoting their capacity to induce $CD4^+$ T_H cell anergy (22-24).

Other strategies that inhibit the expression of co-stimulatory molecules, such as treatment with corticosteroids (30), vitamin D3 (31) and culture with a suboptimal dose of GM-CSF (32)can also successfully generate tolerogenic APCs (33). It remains to be seen, however, whether up-regulation of ILT3 and ILT4 also occurs in such APCs with tolerogenic activity.

In vivo evidence that ILT3 and ILT4 expression is associated with an anergizing APC phenotype was provided by a study of transplant patients. $CD8^+CD28^-$ T cells from quiescent patients induced up-regulation of ILT3 or ILT4 on donor monocytes and inhibited CD40-signaling (25).

$CD4^+$ T_H cells from transplant recipients recognize MHC alloantigens directly on donor APCs (direct pathway of allorecognition) or indirectly on self-APCs that have captured and processed antigens from dying graft cells (indirect pathway of allorecognition)(33). *In vitro* studies have shown that $CD8^+CD28^-$ T_S cells primed with allogeneic APCs inhibit the direct pathway (12), whereas $CD8^+CD28^-$ T_S cells primed with self-APCs pulsed with allopeptides inhibit the indirect

allorecognition pathway (14). The finding that $CD8^+CD28^-$ T cells from quiescent transplant recipients induced the up-regulation of ILT3 or ILT4 and inhibited CD40-signaling by donor APCs indicates the presence of a population of allospecific T_S cells that may inhibit the direct recognition pathway involved in allograft rejection. It is possible that suppression of the direct recognition pathway can be achieved by treating the organ with agents that induce the up-regulation of ILT3 and ILT4 on donor DCs before transplantation. After transplantation, donor DCs that are overexpressing ILT3 and ILT4 may induce T_H cell anergy *in situ* or in the draining lymph nodes. Apoptotic donor DCs will be captured and processed by recipient DCs in the lymph nodes. Recipient allopeptide-specific T cells may be cross-tolerized by these autologous DCs that present alloantigens in the absence of inflammatory cytokines. Alternatively, the indirect pathway could also be suppressed by inducing the overexpression of ILT3 and ILT4 on autologous (recipient) DCs that are generated and allopeptide-pulsed *ex vivo*.

Several other ITIM-bearing receptors, such as mouse PD-1, contribute to immune regulation, as mice that are homozygous for a disrupted PD-1 gene also develop autoimmune diseases (34). Other autoimmune disorders have been linked to single-point mutations in SHP-1 (35).

There is increasing evidence that DCs are central both to the activation and to the suppression of the immune response (35-39). The finding that $CD8^+CD28^-$ T_S cells induce up-regulation of ILT3 and ILT4 is critical to the tolerogenic properties acquired by APCs and supports the concept that the functional state of an APC dictates the outcome of an immune response (40, 41).

Although the focus in the description above has been focused on $CD8^+CD28^-$ T cell-mediated suppression, T_R cells with other phenotypes and/or their cytokines may act *via* a similar mechanism. This idea is supported by recent experiments that showed that within 20 h of incubation with IL-10, the expression of ILT3 and ILT4 on the membranes of human monocytes and DCs was up-regulated, whereas CD86 was down-regulated. In contrast to other T_R cells from the $CD8^+$ and $CD4^+$ subsets that exert their inhibitory effects *via* IL-10, $CD8^+CD28^-$ T_S cells do not produce IL-10 (2, 10, 11). Therefore, the up-regulation of ILT3 and ILT4 induced in APCs by $CD8^+CD28^-$ T_S cells is not unique to these cells, but more likely is a feature that is shared with other inhibitors of antigen-specific T cell responses.

The plasticity of DCs and their capacity to polarize T cells toward functionally distinct subsets seems to be central to the regulation of the immune response (42). The data we present here suggest that the modulation of ILT3 or ILT4 expression on APCs may permit the development of tolerogenic or immunogenic vaccines.

E. Supplemental Experimental Discussion

The upregulation of ILT3 and ILT4 induced in monocytes and dendritic cells by CD8+CD28- T_s is not a unique property of these cells but a feature shared with other inhibitors of antigen specific T cell responses. For example, treatment of APC with IL-10, vitamin D3 analogs or CD4+CD25+ regulatory T cells also induces upregulation of ILT3-ILT4 rendering these APC tolerogenic.

This finding implies the following:

(1) Agents that have the capacity of rendering dendritic cells tolerogenic can be easily identified by testing their ILT3-ILT4 enhancing activity.

(2) The discovery of methods for generating tolerogenic APC has immediate application in clinical organ transplantation. "Passenger" APC of donor origin are always present in solid organ transplants. These "passenger" APC are the imitators of allograft recognition and rejection, stimulating recipient T cell responses. Pre-treatment of the transplant with tolerogenic agents will not only prevent the "passenger" APC from eliciting T cell reactivity in the host, but by inducing ILT3 and ILT4 upregulation they render these APC capable to anergize T cells with the corresponding TCR. Hence, T cells which recognize Major Histocompatibility Complex (MHC) antigens on donor APC will become tolerant. This phenomenon can be defined as blocking of the direct recognition pathway.

Apoptotic donor dendritic cells (DC) will be captured and processed by recipient DC in the lymph nodes. Recipient T cells recognize donor MHC /peptide complexes, expressed on the membrane of immature host dendritic cells, will render T cells anergic blocking the indirect pathway of allorecognition. Therefore, specific tolerance to organ allografts can be induced by pre-treating the graft with tolerogenic agents.

(3) Tolerogenic agents have the potential of increasing substantially the availability of donors for bone marrow transplantation.

Currently, bone marrow or umbilical cord stem cell transplants are performed only when there is complete matching for HLA-A, B, DR and DQ antigens between the recipient and the donor. In

the absence of HLA-identical donor from the family (sibling) the likelihood of finding a suitable donor is less than 1 in 1,000,000. For this reason, the cost of HLA-typing in search of a donor, stem cell preservation and transplantation is outrageously high. Furthermore, graft-versus-host disease will occur in about 50% of the recipients, leading to high mortality.

The discovery that overexpression of ILT3 and ILT4 on APC renders these cells capable to anergize T cells implies the possibility of using HLA-mismatched stem cell donors for transplanting recipients pre-treated by use of tolerogenic agents. If host APC become tolerogenic, donor T cells will be anergized rather than activated, avoiding the Graft versus Host Disease.

By analogy Host versus Graft reactions in solid organ transplantation could be avoided by treatment of recipient with tolerogenic DC from the donor or from an unrelated individual sharing HLA antigens with the donor.

(4) Patients with AIDS have an increased number of CD4+ and CD8+ T cells which upon stimulation (with mitogens) produce IL-10 and TNF-alpha (J. Acquir Immune Defic Synd 20001, Dec. 15, 28(5) 429-438). These two cytokines increase the expression of ILT3 and ILT4 on monocytes and dendritic cells. Not only do patients with AIDS display a greater than 10 fold increase in the level of ILT4 expression on APC, but their sera also increase ILT3 and ILT4 expression on APC from healthy blood donors.

It results that APC from AIDS patients, which overexpress ILT3 and ILT4, present peptides derived from the processing of pathogens in a tolerogenic form. To prevent T cell tolerization

by self-APC, ILT3 and ILT4 interaction with patient's T cells must be blocked. Receptor blockade is generally accomplished either by treatment with "blocking" antibodies or by treatment with a soluble form of the ligand. While the ligand for ILT3 is not known as yet, the ligand for ILT4 is known to be HLA-A, B and G. Hence, treatment of patients with soluble HLA-G may prevent the interaction between the T cell surface ligand of ILT4 with the ILT4-receptor on APC thus preventing the transduction of inhibitory signals.

(5) Another field of clinical immunology that may greatly benefit from our discovery resides in the treatment of autoimmune diseases.

The current dogma is that autoimmunity results from cross-priming the patients' T cells by dendritic cells which present tissue or organ-specific peptides, derived from cells undergoing necrosis under inflammatory conditions. To prevent progression of the autoimmune response it may be sufficient to treat the patient with autologous dendritic cells that have processed *ex vivo* apoptotic cells of the target organ (for example, pancreatic islets, thyroid cells, etc). Treatment with vitamin D3, IL-10 or other tolerogenic agents may render these antigen-pulsed dendritic cells tolerogenic. This will permit blocking of the autoimmune disease.

Direct administration of tolerogenic agents or *ex vivo* manipulation of patients' dendritic cells may accomplish this purpose.

(6) It has been hypothesized that progression of malignancies is caused by the inefficiency of the immune response against tumor-specific peptides/MHC class I complexes. Notoriously, numerous tumors display a decrease in the level of expression

of some (but not all MHC antigens) and may thus escape recognition by antigen-specific T cells. It is conceivable that in the absence of inflammatory cytokines, patients' APC will be unable to cross prime the T cell response against the tumor. To create conditions that optimize "licensing" of APC to stimulate an immune response versus tolerization of APC that will inhibit an immune response, it may be necessary to block the capacity of patients' APC to transcribe ILT3-ILT4.

This may be accomplished by depletion of CD8+CD28CD27+ T suppressor cells and of CD4+CD25+ T regulatory cells or by specific inhibition of ILT3 and ILT4 transcription.

In conclusion, the discovery of the inhibitory function of ILT3 and ILT4 receptors concerns the central control mechanisms of the immune response which must be inhibited to induce specific tolerance in transplantation and autoimmune diseases and augmented in AIDS and Cancer.

Modulation of ILT3 or ILT4 expression on APC may permit the development of tolerogenic or immunogenic vaccines. *Ex vivo* manipulation of dendritic cells to express high levels of ILT3-ILT4 or conversely, to express low levels of these molecules will result in the generation of APC which elicit tolerance or immunity, respectively.

Second Series of Experiments

Study of ILT3 and ILT4 in monocytes from HIV-infected patients and non-infected individuals.

A. Introduction

It has been shown that the number of $CD8^+$ $CD28^-$ T cells increases massively during HIV infection and progression to AIDS (21-23). These cells have impaired cytolytic function which is associated with persistent expression of CD27 (22) and inhibitory NK receptors (iNKRs) (24-26). The fact that HIV infected patients have expanded $CD8^+$ $CD28^-$ T cell population provided the opportunity to examine the consequences of this expansion on APC phenotype and function.

B. Results

First, the relationship between $CD8^+$ $CD28^-$ T cells and ILT3/ILT4 expression on monocytes from immunologically deficient, HIV infected patients were analyzed. This study included a population of 18 HIV-infected and 15 uninfected healthy, individuals. Phenotypic characterization of peripheral blood T lymphocytes showed that HIV-infected individuals had a significantly higher frequency of $CD8^+$ $CD28^-$ (Fig. 7a), $CD28^+$ $CD28^-$ $CD27^+$, and $CD8^+$ $CD28^-$ $CD94^+$ T cells compared to non-infected individuals (Table 2), in agreement with other investigators data (21, 24-26). The percentage of monocytes expressing ILT4 was also significantly increased in patients (Fig. 7a, 7b, and Table 2). Furthermore, the mean channel of fluorescence intensity was significantly higher (103.11 ± 71.13) in HIV infected than in healthy non-infected individuals (48.10 ± 24.27) ($p<0.006$), as illustrated in Fig 7a and 7b.

Table 2. Phenotypic Characteristics of T Cells and Monocytes from HIV-infected and Non-Infected Individuals

Cell Type	Healthly Controls (Mean ± S.D.)	HIV+ Patients (Mean ± S.D.)	P-value
%CD45+CD3+	72.40 ±10.26	73.44 ± 11.57	N.S.
%CD45+CD3+CD4+	51.20 ±8.16	15.88 ±6.64	0.0001
%CD45+CD3+CD8+	19.00 ±3.80	54.11 ±12.98	0.0001
%CD8+CD28-	24.60 ±12.42	63.22 ±16.36	0.0006
%CD8+CD28-CD27+	7.66 ±3.72	21.05 ±10.41	0.0049
%CD8+CD28-CD94+	11.56 ±7.30	27.37 ±8.72	0.0029
%CD14+ILT4+	20.20 ±11.22	46.88 ±20.03	0.0007

Consistent with these results, ILT4 mRNA level measured by semiquantitative RT-PCR in $CD14^+$ cells was 3-5 fold higher in HIV infected individuals than in healthy controls (Fig 7c). There was a direct correlation between the frequency of $CD8^+$ $CD28^-$ T cells and the percentage of $ILT4^+$ monocytes in the population of HIV infected patients (Fig. 8), suggesting the possibility that these two phenomena are inter-related. Analysis of ILT3 cell surface expression and mRNA levels in monocytes from patients and controls showed no quantitative differences.

To determine whether increased expression of ILT4 in $CD14^+$ cells from HIV infected patients is associated with impaired APC function the MLC stimulatory capacity of patients' monocytes were compared with that of normal controls using as responders PBMC from healthy blood donors. Monocytes from HIV infected individuals, failed to induce T cell alloreactivity. Since the expression of HLA class II antigens and CD86 molecules on APC from HIV and control individuals did not differ, it appears that the impaired MLC stimulatory capacity of patients' monocytes is related to upregulation of ILT4.

C. Discussion

To study the biological relevance of the ILT3 and ILT4 molecules induced by Ts in APC, the expression of these inhibitory receptors were analyzed in HIV-infected patients, known to exhibit an increased population of $CD8^+$ $CD28^-$ T cells (21-23). The frequency of $CD14^+$ monocytes expressing ILT4, as well as the mean channel of fluorescence was significantly higher in HIV-infected patients than in healthy controls.

Although it has not been directly determined that the noncytotoxic $CD8^+$ $CD28^-$ T cells seen in patients with HIV infection (21-23) have suppressor function and are responsible for the increased level of ILT4 expression on monocytes, there is found a direct correlation between the frequency of the $CD8^+$ $CD28^-$ T cells and that of $CD14^+$ $ILT4^+$ cells. This correlation suggests the possibility that Ts, induced during the immune response to viral or microbial antigens, may be responsible for upregulation of ILT4 expression in HIV-infected patients.

The increased expression of ILT4 in monocytes from HIV-infected patients may be responsible for their lack of allostimulatory activity. This suggests that $ILT4^+$ monocytes from HIV infected individuals may inhibit Th activation and proliferation.

Third Series of Experiments

A. Introduction

Patients infected with Hepatitis C Virus (HCV) have an impaired response against HCV antigens while maintaining immune competence for other antigens. Although some patients exhibit acute self-limited infection the majority of them (70%) display persistent infection and chronic hepatitis with a strong risk for the development of hepatocellular carcinoma. T cell responses against viral antigens are vigorous in individuals who have cleared HCV after acute infection or after treatment with alpha interferon, while patients who fail to respond to therapy exhibit poor reactivity.

B. Results

The impairment of T cell reactivity in patients with chronic infection may be secondary to virus induced alterations of APC function. The level of expression of the inhibitory receptors ILT3 and ILT4 were tested on monocytes from 13 patients with chronic infection and from 8 patients that have resolved infection (as determined by a negative PCR test of their serum). In all patients with chronic infection the level of ILT3 and ILT4 expression was significantly higher (more than 50% positive monocytes) than in patients who have resolved infection or healthy controls (less than 25% positive monocytes) ($P<0001$). Furthermore, monocytes from patients with chronic infection displayed low allostimulatory capacity in conjunction with high ILT3/ILT4 expression indicating impaired antigen presenting function. Their stimulatory capacity, however, was restored in cultures containing anti-ILT3 and ILT4 antibodies.

C. Conclusion

These data suggest that quantitation of ILT4 expression on patients' monocytes provides an excellent parameter for assessing their immunologic competence. Furthermore, it appears that blockade of inhibitory receptors ILT3 and ILT4 on APC is required for restoring APC function and implicitly T cell immunity in patients with chronic HCV infection.

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What is claimed is:

1. A composition comprising at least two of a CD4+CD25+ cell, IL-10, a CD8+CD28- cell and a vitamin D_3 analog, in prophylactic or therapeutic amounts.

2. The composition of claim 1, further comprising a pharmaceutically acceptable carrier.

3. The composition of claim 1, wherein the CD8+CD28- and CD4+CD25+ cells and IL-10 are human.

4. A method for generating a tolerogenic antigen-presenting cell which comprises contacting the cell with an effective amount of IL-10, a CD4+CD25+ cell and/or a vitamin D_3 analog.

5. A method for increasing the expression of ILT3 and/or ILT4 by an antigen-presenting cell which comprises contacting the cell with an effective amount of IL-10, a CD4+CD25+ cell and/or a vitamin D_3 analog.

6. The method of claim 4 or 5, wherein the antigen-presenting cell is a human antigen-presenting cell.

7. The method of claim 4 or 5, wherein the contacting is performed *in vivo*.

8. The method of claim 4 or 5, wherein the contacting is performed *ex vivo*.

9. The method of claim 4 or 5, wherein the contacting is performed *in vitro*.

10. The method of claim 4 or 5, further comprising contacting the antigen-presenting cell with a CD8+CD28- cell.

11. The method of claim 4 or 5, wherein the antigen-presenting cell is a dendritic cell or a monocyte.

12. A method for inhibiting the onset of rejection of an antigenic substance in a subject, which comprises administering to the subject a prophylactically effective amount of IL-10, a CD4+CD25+ cell, and/or a vitamin D_3 analog.

13. A method for treating the rejection of a antigenic substance in a subject, which comprises administering to the subject a therapeutically effective amount of IL-10, a CD4+CD25+ cell, and/or a vitamin D_3 analog.

14. The method of claim 12 or 13, wherein the antigenic substance is a transplanted cell, tissue or organ.

15. The method of claim 12 or 13, wherein the antigenic substance is xenogeneic.

16. The method of claim 12 or 13, wherein the antigenic substance is allogeneic.

17. The method of claim 12 or 13, wherein the antigenic substance is a prosthetic device.

18. The method of claim 12 or 13, wherein the subject is human.

19. The method of claim 12 or 13, wherein the method further comprises administering a CD8+CD28- cell to the subject.

20. A method for inhibiting the onset of an autoimmune disease in a subject, which comprises administering to the subject a prophylactically effective amount of IL-10,

a CD4+CD25+ cell, a CD8+CD28- cell and a vitamin D_3 analog.

21. A method for treating autoimmune disease in a subject, which comprises administering to the subject a therapeutically effective amount of IL-10, a CD4+CD25+ cell, and/or a vitamin D_3 analog.

22. The method of claim 20 or 21, wherein the disease is selected from the group consisting of autoimmune encephalomyelitis, lupus, rheumatoid arthritis, multiple sclerosis, myasthenia gravis, psoriasis and Crohn's disease.

23. The method of claim 20 or 21, wherein the method further comprises administering a CD8+CD28- cell to the subject.

24. The method of claim 20 or 21, wherein the subject is human.

25. A composition of matter comprising an agent that specifically binds to ILT3 and/or ILT4.

26. The composition of claim 25, wherein the agent is an anti-ILT3 or ILT4 antibody, or antigen-binding portion thereof.

27. A composition comprising the composition of claim 25 and a pharmaceutically acceptable carrier.

28. A method for decreasing the expression of ILT3 and/or ILT4 by an antigen-presenting cell which comprises contacting the cell with the composition of claim 25.

29. The method of claim 28, wherein the antigen-presenting cell is a human antigen-presenting cell.

30. The method of claim 28, wherein the contacting is performed *in vivo*.

31. The method of claim 28, wherein the contacting is performed *ex vivo*.

32. The method of claim 28, wherein the contacting is performed *in vitro*.

33. The method of claim 28, wherein the antigen-presenting cell is a dendritic cell or a monocyte.

34. A method for inhibiting the onset of AIDS or cancer in a subject, which comprises administering to the subject a prophylactically effective amount of the composition of claim 27.

35. A method for treating AIDS or cancer in an afflicted subject, which comprises administering to the subject a therapeutically effective amount of the composition of claim 27.

36. The method of claim 34, wherein the subject is human.

37. The method of claim 35, wherein the subject is human.

38. A method for inhibiting the onset of a Hepatitis C-related disorder in a subject infected with the Hepatitis C virus, which comprises administering to the subject a prophylactically effective amount of the composition of claim 27.

39. The method of claim 38, wherein the subject is human.

40. A method for treating a Hepatitis C-related disorder in a subject infected with the Hepatitis C virus, which comprises administering to the subject a therapeutically effective amount of the composition of claim 27.

41. The method of claim 40, wherein the subject is human.

42. A method for determining the degree to which a subject is immunocompromised, which comprises determining the expression level of ILT3 and/or ILT4 in antigen-presenting cells of the subject and comparing the expression level so determined to the ILT3 and/or ILT4 expression level in antigen-presenting cells of a subject whose immune system is normal or compromised to a known degree.

43. The method of claim 42, wherein the subject is human.

44. The method of claim 42, wherein the antigen-presenting cells are monocytes or dendritic cells.

45. The method of claim 42, wherein determining the expression level of ILT3 and/or ILT4 comprises determining the level of mRNA encoding same.

46. The method of claim 42, wherein determining the expression level of ILT3 and/or ILT4 comprises determining the level of ILT3 and/or ILT4 protein.

47. A method for determining the likelihood that a subject's immune system will reject an antigenic substance if

introduced into the subject, which comprises determining the expression level of ILT3 and/or ILT4 in the antigen-presenting cells of the subject, and comparing the expression level so determined to the expression level of ILT3 and/or ILT4 determined in antigen-presenting cells of a subject whose immune system has a known likelihood for rejecting the antigenic substance.

48. The method of claim 47, wherein determining the expression level of ILT3 and/or ILT4 comprises determining the level of mRNA encoding same.

49. The method of claim 47, wherein determining the expression level of ILT3 and/or ILT4 comprises determining the level of ILT3 and/or ILT4 protein.

50. The method of claim 47, wherein the subject is human.

51. The method of claim 47, wherein the antigen presenting cells are monocytes or dendritic cells.

52. The method of claim 47, wherein the antigenic substance is a transplanted cell, tissue or organ.

53. The method of claim 47, wherein the antigenic substance xenogenic.

54. The method of claim 47, wherein the antigenic substance is allogenic.

55. The method of claim 47, wherein the antigenic substance is a prosthetic device.

56. A method for determining whether an agent is an immunosuppressant or an immunostimulant which comprises (a) contacting the agent with an antigen-presenting cell and (b) determining the resulting expression level of ILT3 and/or ILT4 in the cell, an increase of ILT3 and/or ILT4 expression resulting from step (a) indicating that the agent is an immunosuppressant, and a decrease of ILT3 and/or ILT4 expression resulting from step (a) indicating that the agent is an immunostimulant.

57. The method of claim 56, wherein determining the expression level of ILT3 and/or ILT4 comprises determining the level of mRNA encoding same.

58. The method of claim 56, wherein determining the expression level of ILT3 and/or ILT4 comprises determining the level of ILT3 and/or ILT4 protein.

59. The method of claim 56, wherein the antigen-presenting cell is human.

60. The method of claim 56, wherein the antigen-presenting cell is a monocyte or dendritic cell.

61. A method for determining whether an agent is an immunosuppressant or an immunostimulant which comprises (a) administering the agent to a subject and (b) determining the resulting expression level of ILT3 and/or ILT4 in the subject's antigen-presenting cells, an increase of ILT3 and/or ILT4 expression resulting from step (a) indicating that the agent is an immunosuppressant, and a decrease of ILT3 and/or ILT4 expression resulting from step (a) indicating that the agent is an immunostimulant.

62. The method of claim 61, wherein determining the expression level of ILT3 and/or ILT4 comprises determining the level of mRNA encoding same.

63. The method of claim 61, wherein determining the expression level of ILT3 and/or ILT4 comprises determining the level of ILT3 and/or ILT4 protein.

64. A kit for practicing the method of claim 42, 47, 56, or 60, comprising (a) an agent useful for quantitating ILT3 and/or ILT4, or nucleic acid encoding same, and (b) instructions for use.

65. The kit of claim 64, wherein the agent comprises an antibody specific for ILT3 and/or ILT4.

66. The kit of claim 64, wherein the agent comprises a nucleic acid that specifically hybridizes to a nucleic acid encoding ILT3 and/or ILT4.

FIGURE 1A

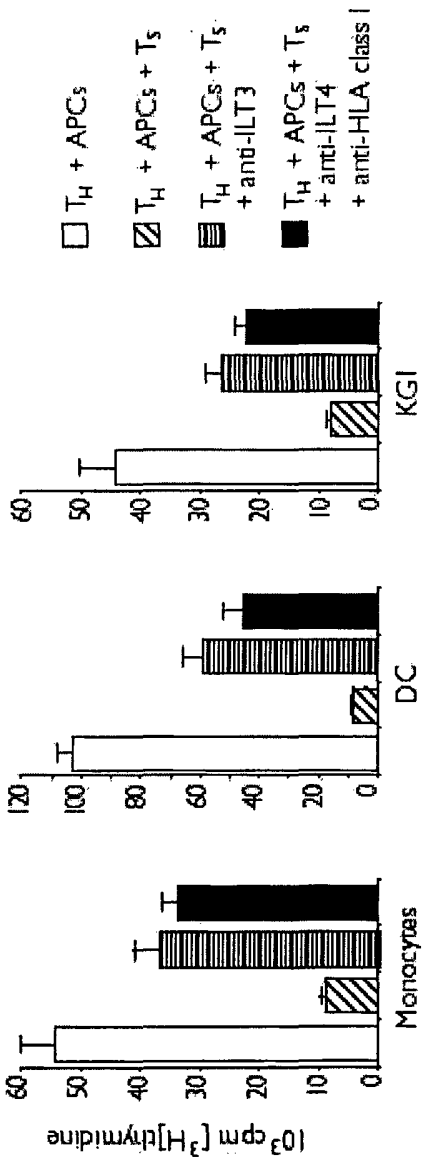
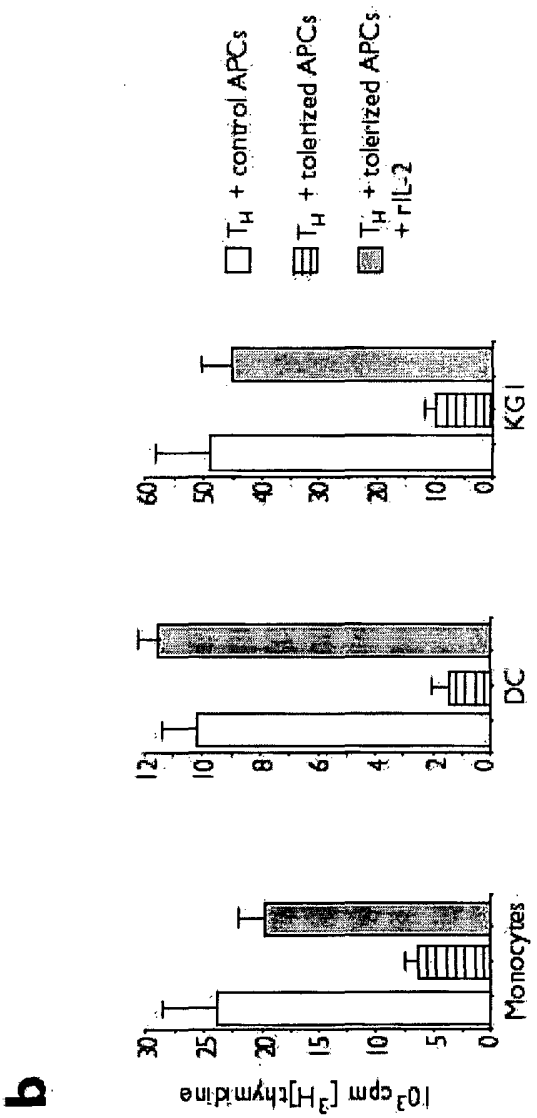

10^3 cpm [^{3}H]thymidine
Monocytes
DC
KG1
T_H + APCs
T_H + APCs + T_S
T_H + APCs + T_S + anti-ILT3
T_H + APCs + T_S + anti-ILT4 + anti-HLA class I

FIGURE 1B


b
10^3cpm [^{3}H]thymidine
Monocytes
DC
KG1
T_H + control APCs
T_H + tolerized APCs
T_H + tolerized APCs + rIL-2

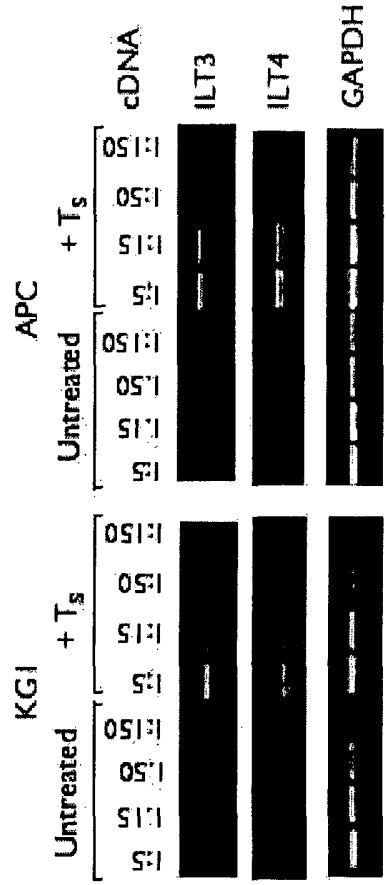


FIGURE 2A

FIGURE 2B

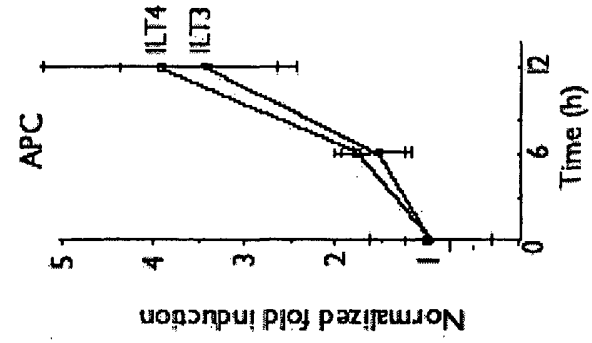


FIGURE 2C

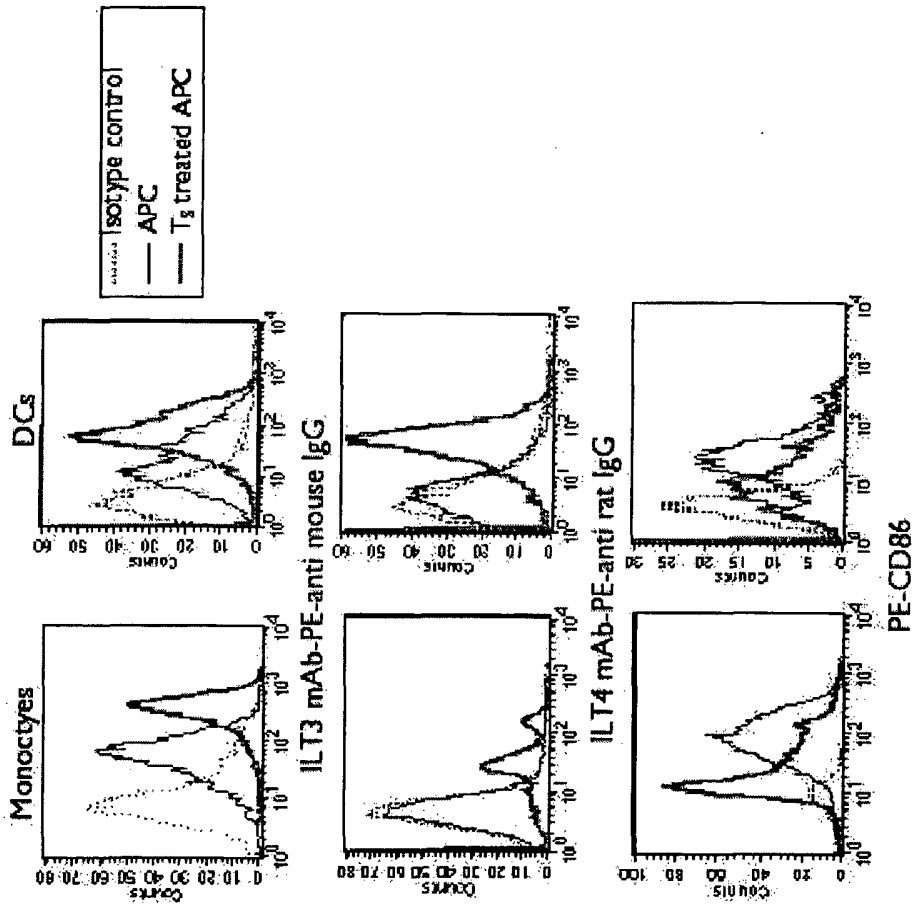
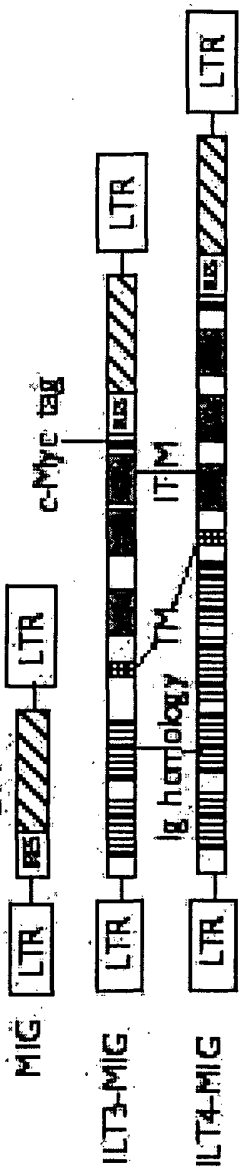


FIGURE 3A


MIG
LTR
GFP
LTR
ILT3-MIG
LTR
Ig homology
TM
c-Myc tag
ITIM
LTR
ILT4-MIG
LTR
LTR

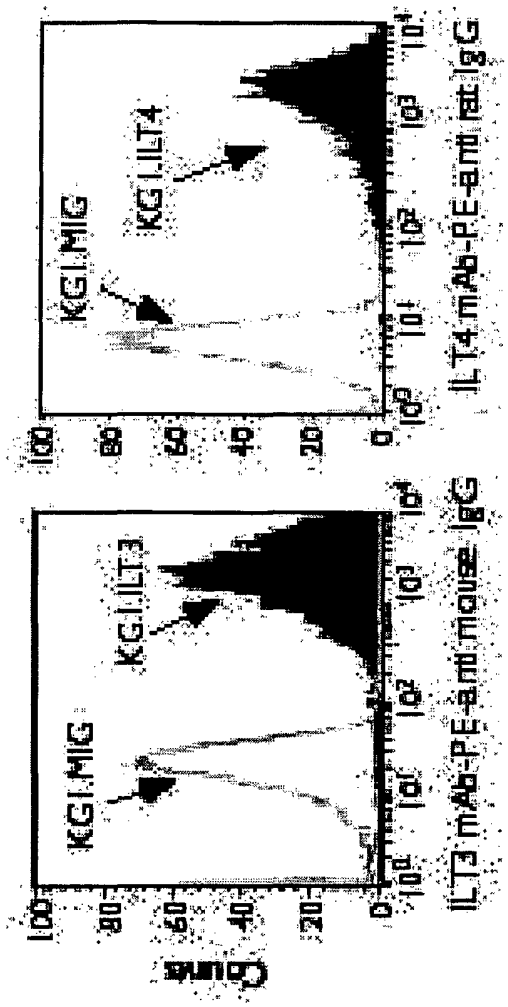

KG1.MIG
KG1.ILT3
KG1.ILT4
ILT3 mAb-PE-amMouse IgG
ILT4 mAb-PE-amMouse IgG
Count
0
20
40
60
80
100
10^0
10^1
10^2
10^3
10^4

FIGURE 3B

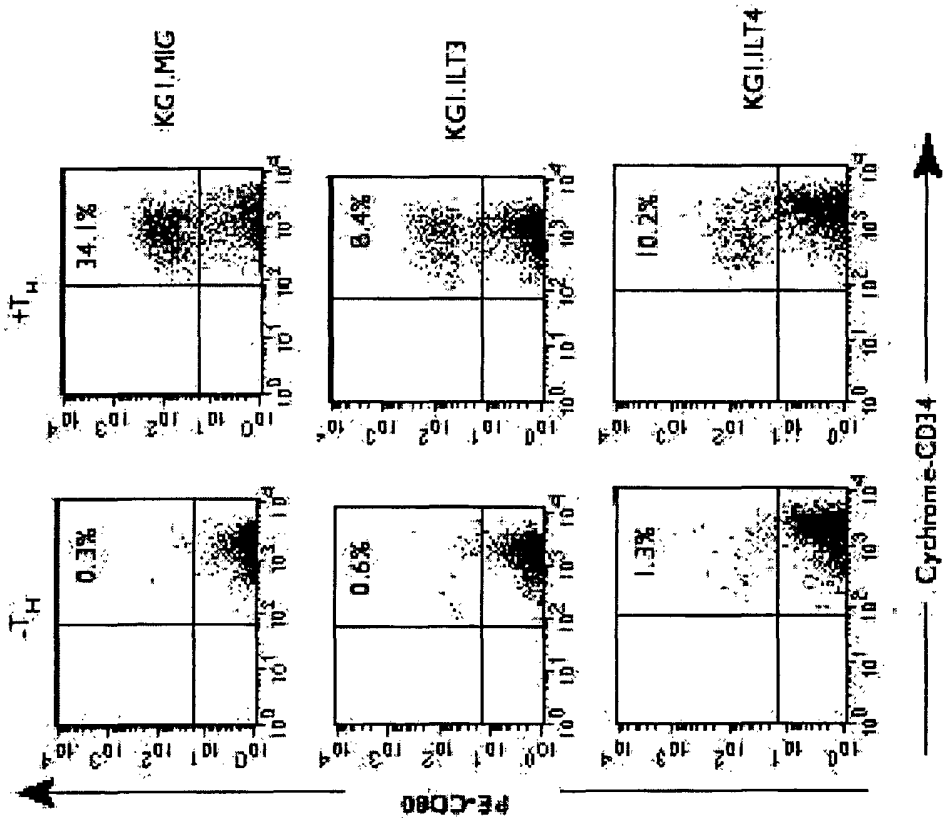

KG1.MIG
KG1.IL13
KG1.IL14
+T_H
-T_H
34.1%
8.4%
10.2%
0.3%
0.6%
1.3%
PE-CD80
Cychrome-CD34

FIGURE 3C

FIGURE 3D

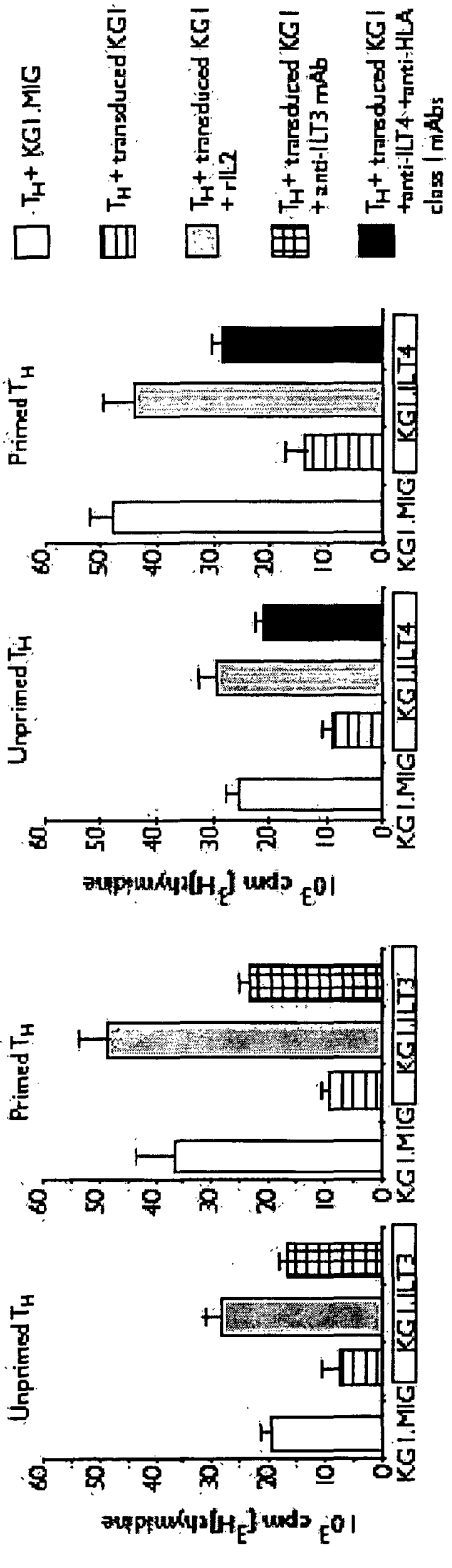

T_H+ KG1.MIG
T_H+ transduced KG1
T_H+ transduced KG1 + rIL2
T_H+ transduced KG1 +anti-ILT3 mAb
T_H+ transduced KG1 +anti-ILT4 +anti-HLA class I mAbs
Unprimed T_H
Primed T_H
10^3 cpn [^{3}H]thymidine
KG1.MIG
KG1.ILT3
KG1.ILT4
0
10
20
30
40
50
60

FIGURE 4A

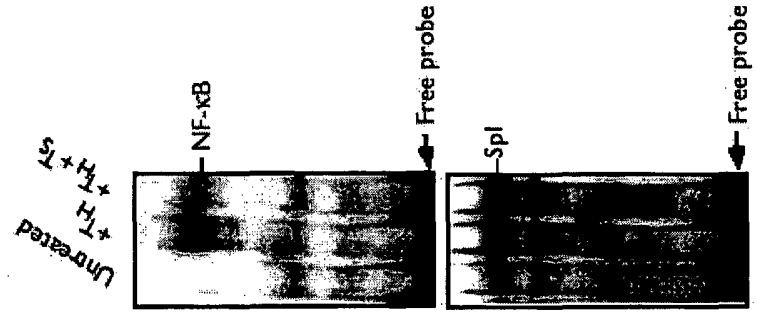


FIGURE 4B

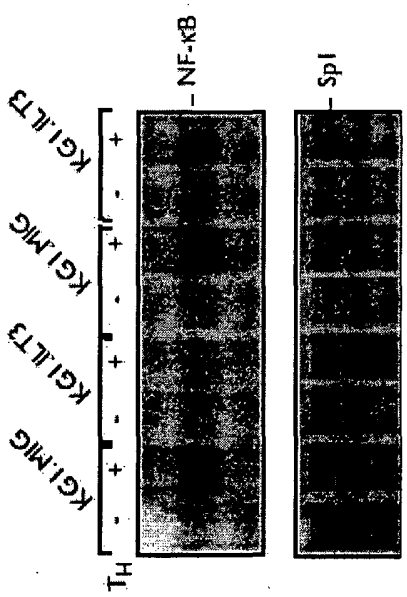


FIGURE 5A

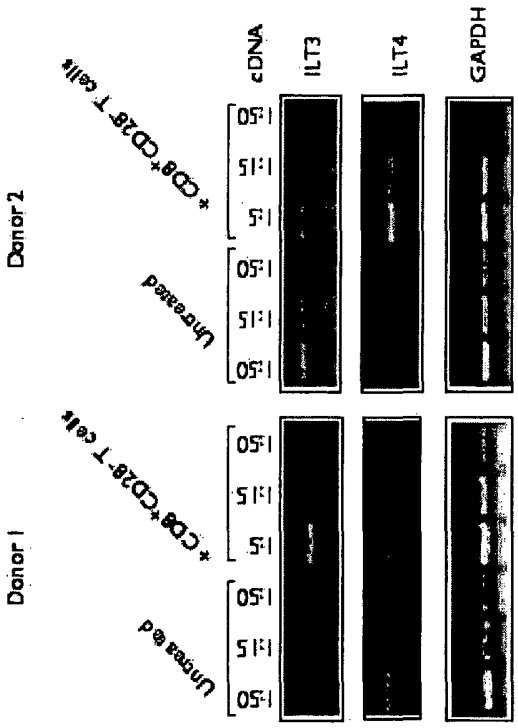

cDNA
ILT3
ILT4
GAPDH
Donor 2
* CD8+CD28- T cells
Untreated
Donor 1
* CD8+CD28- T cells
Untreated
1:50
1:15
1:5

FIGURE 5B

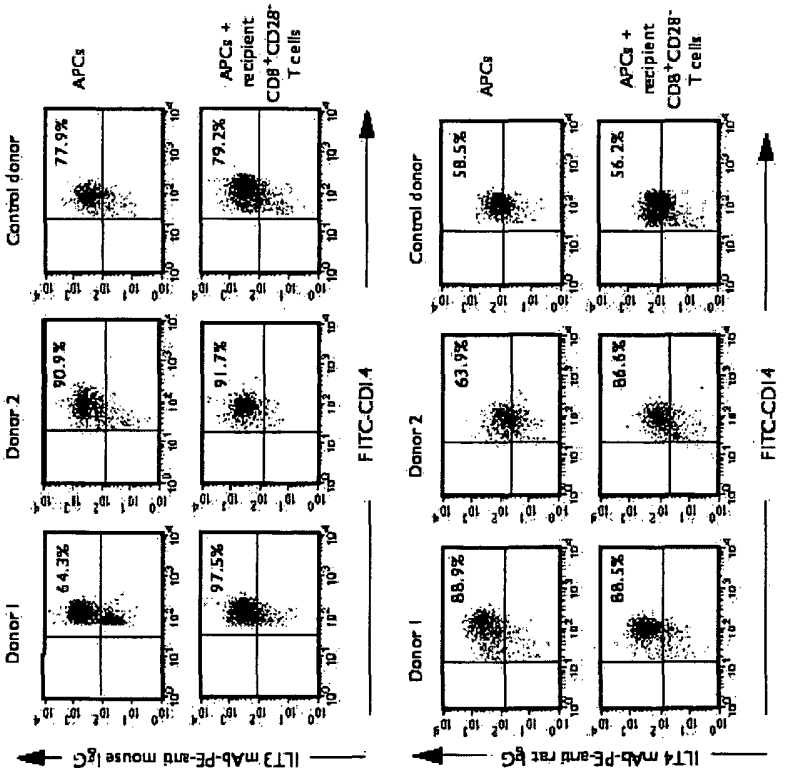

APCs
APCs + recipient CD8+CD28- T cells
Control donor
Donor 2
Donor 1
77.9%
79.2%
90.9%
91.7%
64.3%
97.5%
58.5%
56.2%
63.9%
86.6%
88.9%
88.5%
FITC-CD14
IL13 mAb-PE-anti mouse IgG
IL14 mAb-PE-anti rat IgG

FIGURE 5C

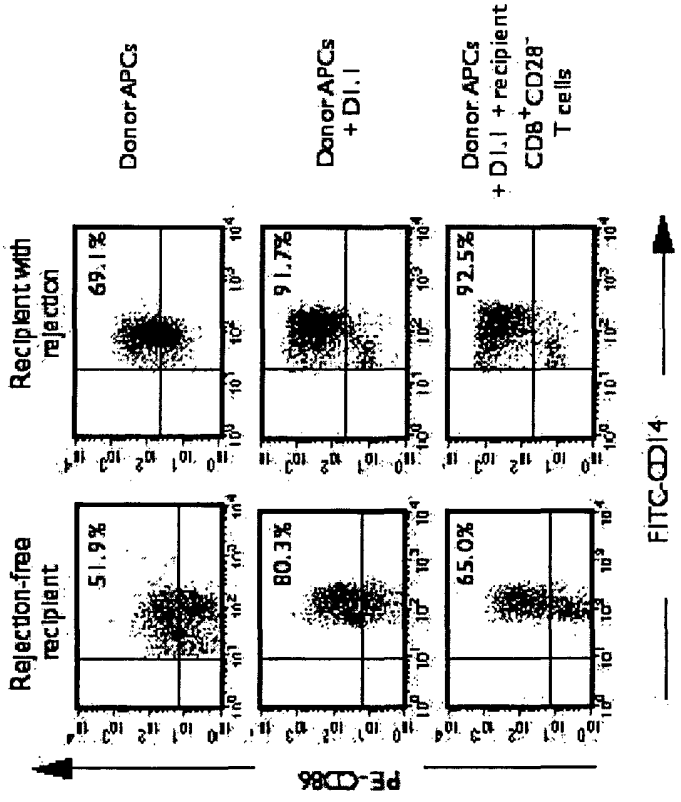

Donor APCs
Donor APCs + D1.1
Donor APCs + D1.1 + recipient CD8+CD28- T cells
Recipient with rejection
Rejection-free recipient
69.1%
91.7%
92.5%
51.9%
80.3%
65.0%
PE-CD86
FITC-CD14

FIGURE 6

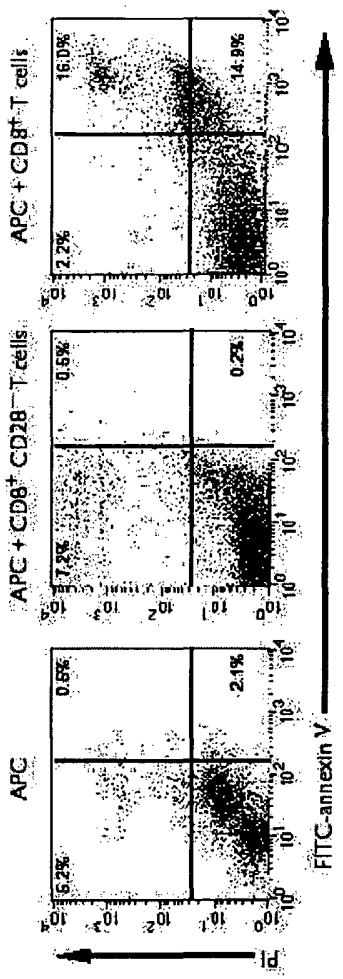

APC + CD8+ T cells
APC + CD8+ CD28− T cells
APC
16.0%
14.9%
2.2%
0.5%
0.2%
7.8%
0.8%
2.1%
6.2%
PI
FITC-annexin V

FIGURE 7A

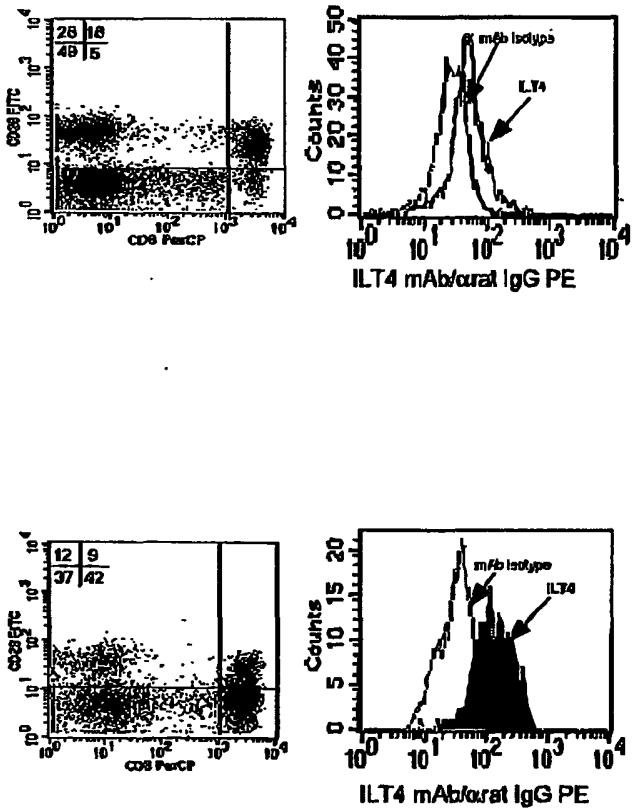

28
18
49
5
CD8 FITC
CD8 PerCP
mAb isotype
ILT4
Counts
ILT4 mAb/αrat IgG PE
12
9
37
42
CD8 FITC
CD8 PerCP
mAb isotype
ILT4
Counts
ILT4 mAb/αrat IgG PE

FIGURE 7B

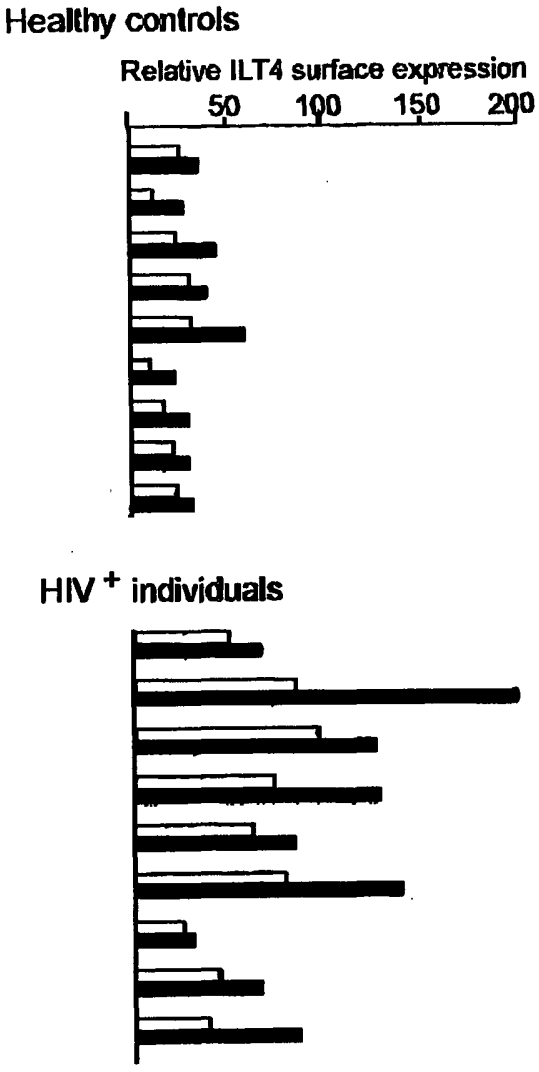
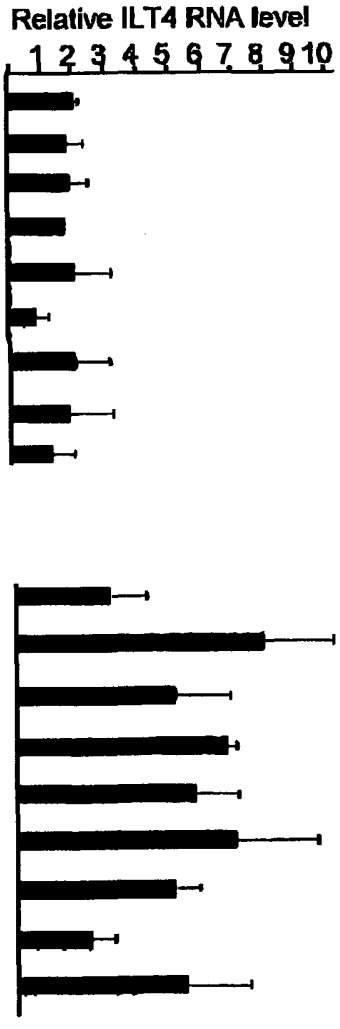


FIGURE 7C



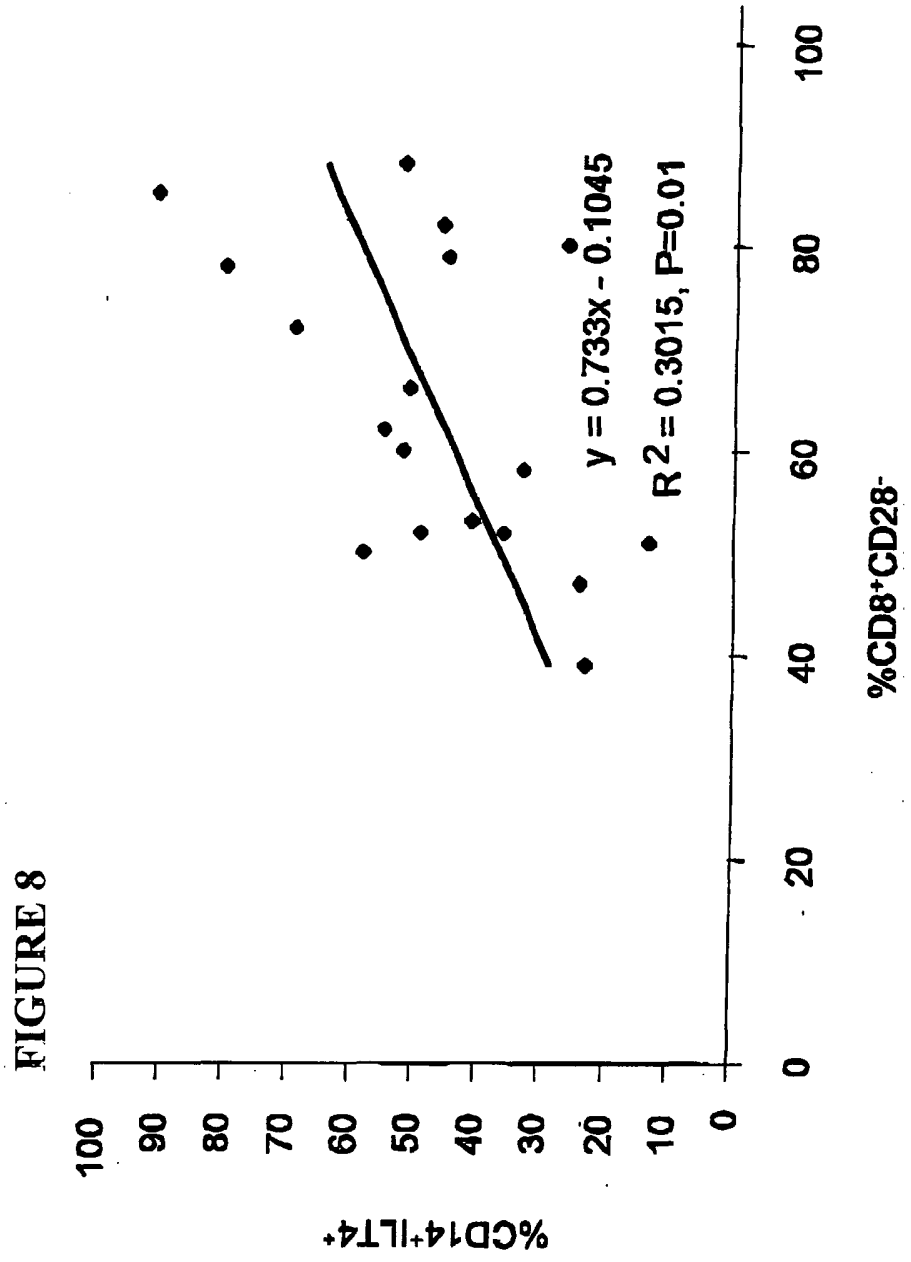

%CD14+ILT4+
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
%CD8+CD28-
y = 0.733x - 0.1045
R2 = 0.3015, P=0.01

FIGURE 8