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Khaled Hassan Abd Kader
AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.Sc. (Microbiology and Immunology)
GRADE / DEGREE

Department of Biochemistry, Microbiology and Immunology
FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

Disruption of the Jak/Stat Cytokine Signaling Pathway in T Cells from HIV-Infected Patients

TITRE DE LA THÈSE / TITLE OF THESIS

Dr. Marko Kryworuchko
DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

Dr. Francisco Diaz-Mitoma
CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

EXAMINATEURS (EXAMINATRICES) DE LA THÈSE / THESIS EXAMINERS

Dr. Jonathan Angel

Dr. Lisheng Wang

Gary W. Slater
Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

DISRUPTION OF THE JAK/STAT CYTOKINE SIGNALING PATHWAY IN T CELLS FROM HIV-INFECTED PATIENTS

Khaled Abd kader

A thesis submitted to the Faculty of Graduate and Postdoctoral Studies in partial fulfillment of the requirements for the Master of Science degree.

Microbiology and Immunology Program

University of Ottawa
Ottawa, Ontario, Canada.

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Abstract

HIV infection leads to a profound T cell dysfunction well before the clinical onset of AIDS. This study investigated whether alterations in the Janus kinase (Jak)/signal transducer and activator of transcription (STAT) pathway in CD4+ and CD8+ T cells from HIV-infected patients induced by the growth promoting cytokines IL-2, IL-4, IL-7, IL-10, and IL-15 may be involved. IL-2 and IL-15 induced STAT5 phosphorylation (P-STAT5) in CD4+ T cells but there was a clear separation of cells into P-STAT5- and P-STAT5+ populations to similar proportions in HIV- and HIV+ patients. In response to IL-7, the proportion of P-STAT5- cells was significantly higher in HIV+ patients compared to HIV- controls. The IL-7 dependent P-STAT5- CD4+ T cells appeared to correlate with a lack of IL-7Rα chain expression. However, the P-STAT5- cells observed did not correlate significantly with other cytokine receptor components. Cytokine-induced P-STAT5 in the CD8+ T cells from HIV+ patients was clearly detected. However, IL-2 and IL-15 stimulation revealed a P-STAT5- CD8+ T cell population, which was not detected in HIV- subjects. P-STAT5- CD8+ T cells were observed in all HIV+ patients studied in response to IL-2 and IL-15, irrespective of HAART. Defects in the IL-2 and IL-15 mediated signaling pathways appeared to exist downstream of cytokine receptor expression. In response to IL-7, P-STAT5- cells were observed in both HIV+ and HIV- patients. However, their proportion was higher in HIV+ patients and returned towards that of HIV- controls under HAART and correlated inversely with IL-7Rα chain expression. The presence of P-STAT5- CD8+ T cells did not correlate with the expression of other cytokine receptor chains. The P-STAT5- T cells observed in response to these cytokines may represent cells at distinct differentiation stages and functional capacities that arise in response to chronic HIV infection and may contribute to the eventual insufficiency of the cell mediated immune response.

Dedicated to the children and babies born with HIV/AIDS, to everyone living with and dying from AIDS, and to all the people in the world who are trying to make a difference.

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A further thanks goes to all the individuals with whom I have worked and collaborated.

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LIST OF ABBREVIATIONS

HIV	Human Immunodeficiency Virus
AIDS	Acquired Immuno-Deficiency Syndrome
Jak	Janus kinase
EBV	Epstein-Barr virus
STAT	Signal Transducer and Activator of Transcription
P-STAT	tyr-phosphorylated STAT
CTLs	cytotoxic T lymphocytes
HAART	highly active antiretroviral therapy
MHC	Major Histocompatibility Complex
HLA	human leukocyte antigen
TCR	T cell receptor
T_h	T helper
IL	Interleukin
IL-2Rα, -β	IL-2 receptor[alpha, beta]
IL2Rγ or γc	IL2R gamma or common gamma chain.
RT	reverse transcription
APC	antigen presenting cells
PBMCs	peripheral blood mononuclear cells
EMSA	electrophoretic mobility shift assays
Abs	antibodies
PBS	phosphate buffered saline
FBS	fetal bovine serum
BSA	bovine serum albumin
MEOH	methanol
PFA	paraformaldehyde
IMDM	Iscove's Modified Dulbecco's Medium
STDev	standard deviation
r^2	coefficient of determination
BMLF1, BZLF1	EBV early lytic genes

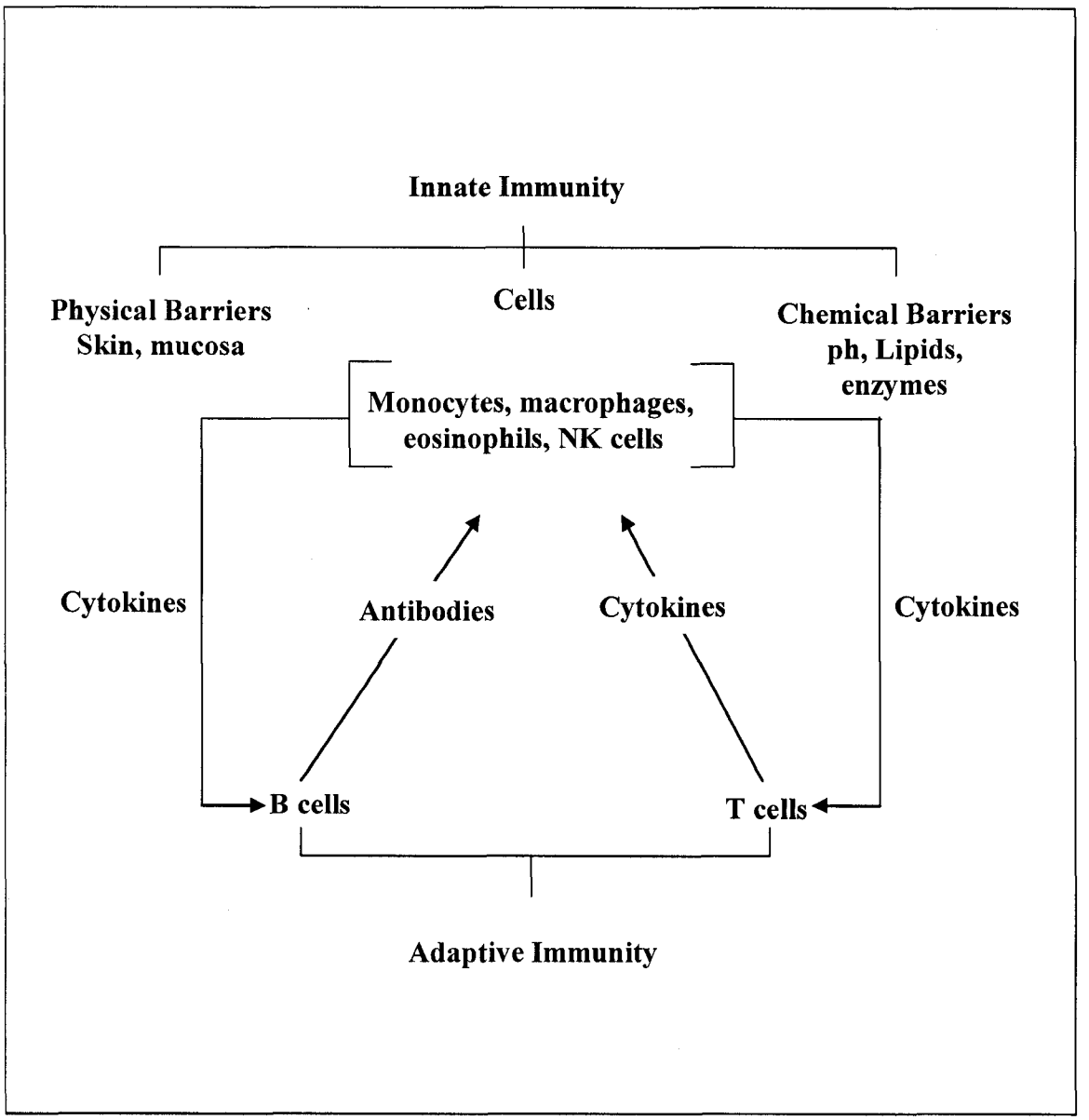
I. Introduction

I.1. Overview of immune system components

The human immune system is a dynamic arrangement of components that do not operate independently of each other (1-3). It has been categorized into innate and adaptive immunity (1-3). Innate immunity (antimicrobial peptides, phagocytes, natural killer cells, and complement) acts rapidly and has an important role in the initial control of acute viral infections (4). For example, humans with a defective natural killer cell response develop severe infections with the herpes family of viruses (5). In addition, cells of the innate immune system, such as dendritic cells, are necessary to activate adaptive immunity (2,3). Adaptive immunity consists of B cell (antibody production) and T cell (cytotoxic, helper) responses to specific antigens (Figure 1). B cells arise from bone marrow while T cell maturation occurs in the thymus. When mature T lymphocytes leave the thymus, they are considered naive cells until exposed to antigenic peptides in a Major Histocompatibility Complex (MHC)-restricted context. Clonal expansion and differentiation of antigen-specific lymphocytes results in a strong and selective capacity to recognize and eliminate an extremely diverse range of pathogens.

There exists a complex communication network between innate and adaptive immunity, which is essential for proper functioning of the immune response (Figure 1). This interaction occurs through the action of soluble signaling molecules called cytokines (6). Cytokines are low molecular weight, soluble proteins that are produced in response to antigen and function like chemical messengers for regulating the innate and adaptive immune response. These mediators act in pleiotropic, antagonistic, and synergistic

Figure 1. Overview of immune system components. Flow diagram emphasizing the fact that immune system components do not operate independently of each other. Communication is elicited through secretion of small signalling molecules called cytokines.



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manners. Cytokines are especially important for regulating inflammatory and immune responses. They transmit signals to enhance or suppress immune responses by directing the differentiation, proliferation, and survival of immune cells. A thorough comprehension of the power that cytokines hold over the immune response is necessary for understanding and advancing our battle against infectious diseases such as Human Immunodeficiency Virus (HIV).

In adaptive immune responses, the cytotoxic T lymphocytes represent a subset of CD8+ cells that can eliminate virus-infected cells by recognizing viral protein fragments (peptide epitopes) on the infected cell surface presented by MHC I (8,9). Similarly, CTLs are able to lyse tumour cells (9). Briefly, viral proteins are processed intracytoplasmically by the proteasome complex of the infected cell and peptides are transported to the endoplasmic reticulum where they bind nascent MHC class I molecules. The MHC-peptide complexes are then presented on the cell surface. The process by which CTL recognize and lyse target cells is specific and restricted by the interaction of the T cell receptor (TCR) with the MHC molecule/epitope/β_2-microglobulin complex. This recognition event leads both to the lysis of the infected cells and to the release of cytokines as described in section I.3.3. CTLs exert their effector function through multiple mechanisms (9). Two involve direct cell–cell contacts between the effector CTL and the target cell leading to target cell death. Another is mediated by their secretion of cytokines such as IFN-γ and tumor necrosis factor-α, following TCR engagement (10,11). Tumor necrosis factor-α engages its receptor on the target cell and triggers target-cell apoptosis (10). IFN-γ induces transcriptional

activation of the MHC class I antigen presentation pathway leading to enhanced presentation of endogenous peptides by MHC class I, and enhanced cytotoxicity (12). Target cell cytotoxicity is known to be mediated by two different mechanisms (13). Briefly, one involves interaction between the TNF/TNF receptor family molecules Fas/FasL. Fas ligand is expressed on the surface of CTLs and binds to the Fas receptor (Fas, CD95) on the target cell. This binding triggers apoptosis through the classical caspase cascade. The other pathway involves the exocytosis of cytotoxic molecules from the CTL (perforin, granzymes) which bring about the apoptosis of the target cell.

T helper (T_h) or CD4+ T cell immune responses can be subdivided according to which type of T helper cell is induced (14). Type-1 helper responses are critical for controlling intracellular infections via CD8 T cell-mediated mechanisms whereas type-2 helper responses mainly protect against pathogens neutralized by Abs. These CD4+ T cells produce distinct sets of cytokines that promote the growth and differentiation of different effector cells of the immune system. The temporal balance between cytokines produced in response to infection is thought to govern the type of immune response elicited and eventually serves to turn off the immune response once the pathogen is eliminated. In several infectious diseases including HIV, the profile of the cytokines produced may influence progression of the disease. Type I cytokines include interleukin (IL)-2, IFNγ, and IL-12, which are necessary for inducing cellular immunity, Type II cytokines include IL-4, IL-10, IL-5, and IL-13, promote B cell activation, differentiation and antibody synthesis (14). Since cytokines are critical in mobilizing the immune

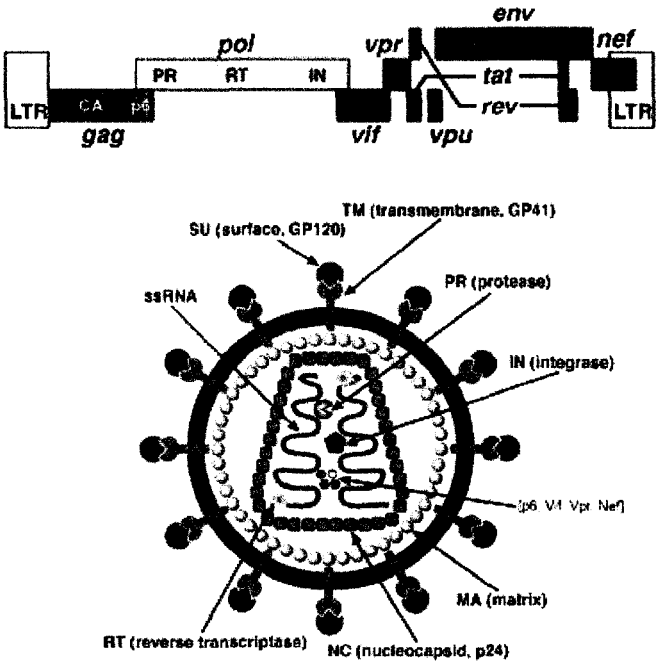
response against HIV but also have the ability to up and down regulate HIV replication, their role in HIV pathogenesis continues to be intensively investigated (15).

I.2. Human Immunodeficiency Virus

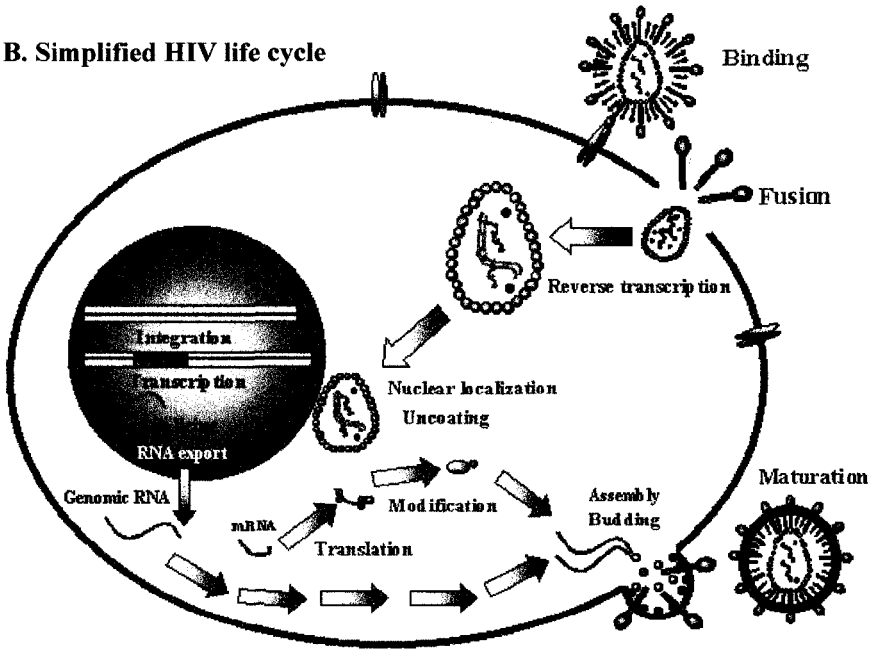
HIV is genetically related to the lentivirus genus of the Retroviridae family (16). Lentiviruses are characterized by structural genes: gag, pol, env, and a complex combination of additional non-structural genes including tat, rev, nef, vif, vpr, (Figure 2A). The typical genome of the lentivirus genus is 9 kilobases long. The Gag proteins drive the assembly and release of virus particles and encapsidate the viral RNA genome. The *pol*-encoded enzymes, including the protease, reverse transcriptase and integrase, cleave the Gag and GagPol polyprotein precursors at the time of virus release, convert the viral RNA genome to double-stranded DNA following virus entry, and catalyze the integration of viral DNA into the host cell chromosome, respectively. The envelope glycoproteins direct virus binding to the target cell and also fusion between viral and cellular membranes. In addition to the products of the gag, pol and env genes, HIV-1 and other lentiviruses encode several novel regulatory and accessory proteins, the functions of which are unique to this group of retroviruses. Each of the virally encoded proteins is exquisitely adapted to take advantage of the host cell to promote viral propagation. The main targets of HIV are the CD4+ T cell and monocytes/macrophages; however, HIV has been shown to be capable of infecting other cell types such as CD8+ T cells, B cells, and dentritic cells (17,18). HIV enters CD4+ T cells through interaction of viral glycoprotein gp120 with CD4 receptor found on the surface of these cells, in conjunction with chemokine co-receptors. The life cycle consists of several stages: viral entry, generation

Figure 2. HIV genome, virion structure and life cycle.

A. HIV Genome and Virion Structure



B. Simplified HIV life cycle



Adapted from University of South Carolina, Medical school, website
http://pathmicro.med.sc.edu/lecture/hiv2000.htm

of the viral DNA by reverse transcription (RT), integration of the viral DNA in the nucleus, synthesis of viral RNA, translation and assembly of viral proteins into virions, and release of viral particles for further rounds of infection. A simplified description of the HIV replicative cycle is presented in Figure 2B.

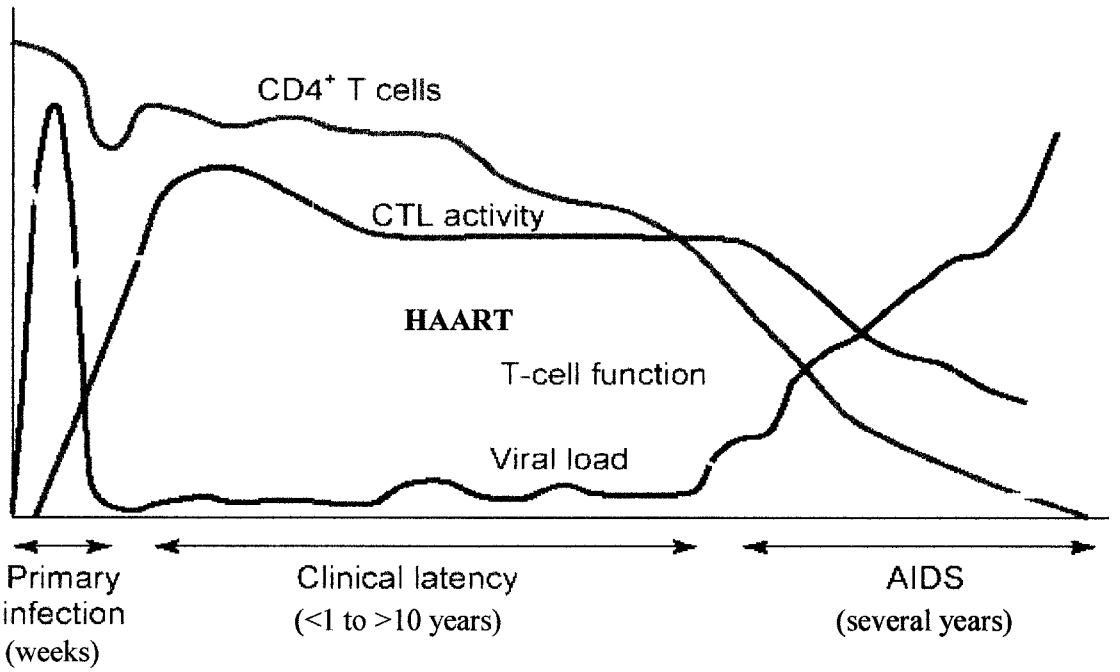
I.3. Pathogenesis of HIV

HIV targets the immune system and causes the fatal disease, AIDS (Acquired Immune Deficiency Syndrome) (19). There are currently over 38.6 million people living with HIV, including 4.1 million adults and children who became newly infected in the last year alone, making AIDS the worst tragedy of modern times (20). Unfortunately, despite more than 20 years of intensive studies, many aspects of HIV pathogenesis are still unclear. Current therapeutic strategies mainly involve a combination of three or more antiretroviral agents from three major categories (nucleoside reverse-transcriptase inhibitors, non-nucleoside reverse-transcriptase inhibitors and protease inhibitors), referred to as highly active antiretroviral therapy (HAART). HAART has become the standard regimen since the late 1990s bringing about a dramatic improvement in the number of patients attaining undetectable viral loads, increased CD4 counts, and survival. However, the genetic variability of HIV makes resistance to drugs and vaccines an important problem (21). In addition, many aspects of the virus and its ability to impair the cellular immune response, and disrupt the cytokine network are not yet completely understood (15,19).

Chronic HIV infection is characterized by a progressive decline in CD4+ T cells, an increase in CD8+ T cells numbers and progressive immune dysfunction, eventually leading to AIDS (19). Primary infection is a period of rapid viral replication that immediately follows exposure to HIV and lasts for few weeks (22-28). As illustrated in Figure 3, during the primary infection with HIV, there is an initial rise in viral load and CD4+ T cell numbers decline. Concurrent with a rise in CTL response against HIV, virus replication is controlled. However, almost invariably the immune response is unable to clear the virus, chronic infection is established, and a period of clinical latency ensues. During chronic infection, CD4+ T cells numbers and T cell function continue to progressively decline, eventually leading to opportunistic infections and AIDS. As mentioned, the advent of HAART has dramatically prolonged the clinical latency period and slowed progression to AIDS, but this is not a cure. Eventually, as a result of complex and incompletely understood pathogenic mechanisms the immune system is overwhelmed leading to AIDS and death.

Investigators in the HIV/AIDS field have struggled with two sets of apparently contradictory findings (29). On one hand, early observations that HIV targets, infects and kills CD4+ T cell as it disseminates throughout the lymphoid tissues during disease, led to the hypothesis that CD4+ T cell destruction results in the loss of immune effectors and regulatory functions, and is a key marker for disease progression. This suggested that the pathogenesis of AIDS was mainly related to the direct, virus-mediated killing of CD4+ T cells. On the other hand, the long period of time between infection and development of

Figure 3. Course of progressive HIV infection, describing the changes in plasma HIV viral load (blue line), peripheral blood $CD4^+$ T cell count (green line), and CTL responses (red line).



Adapted from Wolthers, K.C. and Mediema, F., 1998 Trends Microbiol 6: 144-147

(30)

AIDS, a time when only a small fraction of total CD4+ T cells appear to become infected and the elevated death rates observed in both CD4+ and CD8+ T cells support a pathogenic model in which other 'indirect' factors are involved. These include the disruption of the cytokine network, anergy and apoptosis of T cells (31-33).

Evidence has surfaced recently that even despite seemingly effective HAART, T cell responses in HIV infection have been associated with defects in cytokine production (19), perforin expression (34-36), alterations in phenotypic maturation (37,38), as well as the decreased proliferative capacity of HIV-specific CD8+ T cells (36,39). Defects in T cell function arising during chronic HIV infection are not well understood. One potential mechanism involves the action of viral proteins, released into the intracellular milieu, on uninfected bystander cells. For instance Nef and Vpr have been shown to induce cell death in uninfected cells (40).

I.3.1. CD8+ T cells

As mentioned, CD8+ T cells represent a major arm of the cellular immune response and their differentiation into effector cells usually requires T_h cell stimulation (41-43). The importance of CTLs in clearing virus infections is well established and has been demonstrated for several viruses (9). Evidence of the critical role of CD8+ lymphocytes in the control of HIV-1 replication was first reported in 1986 with the observation that isolated PBMC from asymptomatic infected individuals produced virus after removal of CD8+ T cells (44). These CD8+ T cells suppressed HIV-1 replication in

a dose-dependent manner when added back to the cell culture in vitro. Studies using the rhesus macaque animal model have shown that CD8+ T cell depletion from Simian Immunodeficiency Virus (SIV)-infected macaques resulted in a dramatic increase in viral load (45,46). Early on, it was established that HIV+ patients have very high levels of activated HIV-1-specific CTLs (19). Therefore, the main role of CD8+ T cells in the context of HIV infection is to suppress viral replication and clear virally-infected cells. However, CD8+ T cell responses are insufficient to prevent the establishment of chronic HIV infection and are eventually overwhelmed, leading to AIDS in the majority of patients (19). Several reasons for this have been proposed. First, depletion of essential helper CD4+ T cells by HIV may be an important cause for the progressive loss of CTL function (47). These cells are critical for maintaining optimum virus specific CTL responses. A direct correlation between the HIV-specific CTL response and helper response has been reported (48). Defects in CTL function induce a reduction in perforin and IFN-γ expression have also been reported (34,49,50). It has also been shown that alterations in the maturation of HIV-specific CTLs may limit their effectiveness (37,51). Thirdly, CTLs are not able to maintain complete control over HIV replication as a result of the continual generation of viral escape mutants (52-55). Moreove, Viral proteins such as Nef are known to downregulate MHC I infected cells, which may allow the virus to escape CD8+ T cell recognition (56,57).

I.3.2. CD4+ T Cells

As mentioned, CD4+ T_h cells are crucial in coordinating cellular and humoral immune responses against pathogens (58). It is clear that HIV replicates in activated

human CD4+ T cells *in vitro,* that the viral envelope protein utilizes CD4 as its principle receptor, and that circulating CD4+ T cells decrease in number as disease progresses (47). A central, yet incompletely resolved issue of HIV disease is the mechanism responsible for CD4+ T cell loss. Several mechanisms have been proposed to explain HIV-mediated depletion of CD4+ T cells (59). One involves the loss of CD4+ T cells directly due to viral replication. However indirect mechanisms involving HIV proteins released into the extracellular environment such as gp120, Tat, Nef and Vpr have been shown to induce anergy and cell death in uninfected cells (57). Soluble gp120 induces apoptosis through interaction with cellular receptors such as CD4, CXCR4 and CCR5, via both Fas-dependent (upregulation of Fas/FasL, decreased FLIP) and Fas-independent (increased Bax, decreased Bcl-2) apoptotic pathways (60). Tat, is endocytosed by neighbouring cells and upregulates caspase 8 and FasL to induce apoptosis (61). Moreover, Vpr, or its C terminus, can cause membrane disruption and induce cell death in CD4+ T cells (40).

The potential role of alterations in the cytokine expression profile during the course of HIV infection has been intensively investigated (14,62-64). Early studies in HIV infection suggested an overproduction of type 2 cytokines (IL-4, IL-10, IL-5, IL-13) and decreased production of type 1 cytokines (IL-2, IFNγ, and IL-12) (62). However, there are contradictory observations regarding production of type 2 cytokines (14,63). Nevertheless, There appears to be a general agreement that IL-12 production is decreased in HIV infection (14). In one study cells from peripheral blood and lymph nodes of HIV-infected individuals were isolated at different stages of disease (65). Expression of

IFNγ and IL-4 mRNA was barely detectable whereas IL-10 was increased in CD4+ T cells and the level of IL-10 mRNA was inversely associated with IFNγ throughout the course of infection (65). A similar pattern of cytokine expression was observed after antigenic or mitogenic stimulation of purified CD4+ T cell populations obtained from HIV+ patients at different stages of disease with HIV envelope synthetic peptides (Env) *in vitro* (66,67). In a recent study, impaired production of cytokines was found to be a predictor of mortality in HIV+ patients and the preserved capacity to produce cytokines was associated with prolonged survival (68). This does suggest a clinical relevance to impaired cytokine production in HIV pathogenesis. Another study has demonstrated that a balanced type1/type2 response is associated with long term non progressive HIV infection (69).

The role of CD4+ T cells in the development of a CD8+ T cell response to viral infection, tumours, and autoimmune diseases has been well documented (70-72). CD4 helper T cells are required for the generation, maintenance, and survival of CD8+ T cell and by their secretion of specific cytokines including IL-2 promote CD8+ T cell expansion and enhances their production of cytotoxic molecules (73,74). Under conditions of diminishing T cell help and chronic antigenic stimulation, CD8+ T cells are thought to degenerate into an anergic and preapoptotic state characterized by alterations in their differentiation phenotype, and impaired function (34,37,75-78). This loss of the T cell response may lead to immunodeficiency, leaving infected individuals more susceptible to opportunistic infections and development of AIDS. Despite intense investigation, the mechanisms responsible for the impairment of lymphocyte function by

HIV are still not well understood. Disruption of the cytokine network regulating CD8+ and CD4+ T cell immune responses may be involved (79).

I.3.3. Cytokines

In response to several infections including HIV, the profile of cytokines produced may influence the progression of the disease (80). Cytokines not only activate the cells of the immune system, but also influence the expression of various critical molecules on the cell surface and modulate HIV replication in infected cells (15,79). Studies conducted mostly in animal models indicate that maintenance of the peripheral T cell pool is largely dependent on 3 cytokines: interleukin (IL)–2, IL-7, and IL-15 (81). The functions of these cytokines have been shown to overlap, particularly in situations in which the host is lymphopenic (82), at least in part due to their shared receptor subunits and signaling pathways (83,84). Therefore, they are attractive and vulnerable targets for viruses such as HIV.

Interleukin (IL)-2

IL-2 plays a central role in the development and regulation of different components of the immune response (85-87). It is secreted mainly by Ag-activated T cells, mediates their clonal expansion, upregulates perforin expression, and promotes the differentiation of CD8+ T cells into armed effector cells capable of lysing virus infected targets. IL-2 mediates its biological effects via interaction with its cell surface receptor consisting of three components, the IL-2Rα, β and γ chains (85-89). In humans, growth signals are transduced via the α, β, γ trimolecular complex or high affinity receptor and β,

γ heterodimers making up the intermediate affinity receptor (86). IL-2R subunit expression has been evaluated in human peripheral blood mononuclear cell (PBMC) subsets (90). Unstimulated CD8+ T cells express low levels of IL-2R β, γ_c and undetectable levels of α chain. All three chains are upregulated upon Ag or mitogenic activation as is their IL-2 responsiveness. Deficiencies within the IL-2 system that have long been recognized in HIV infection, including poor reactivity to this cytokine (75,78,91), may contribute to the eventual failure of HIV-specific CD8+ T cells in HIV infection and lead to AIDS progression. It has been demonstrated that the CD8+ T cells derived from a subset of patients with high virus load expressed higher levels of all three IL-2R components relative to HIV- controls yet were defective in their response to IL-2 at the level of cell cycle entry, and may be indicative of defective IL-2-dependent signal transduction (75,78). In patients undergoing successful HAART, the CD8+ T cells expressed IL-2R levels approaching that of HIV- controls, yet exhibited a strong capacity to enter into cycle following stimulation with IL-2 (78). In addition, HIV specific CD4+ T cell proliferation defects were found to be associated with reduced production of IL-2, which could be partially corrected in vitro by the addition of IL-2 (92,93). The presence of CD4+ T lymphocytes that secrete IL-2, or a subset of CD4+ T lymphocytes that secrete both IFN-γ and IL-2, is associated with good clinical outcome in HIV+ patients (94-96). Thus, production of IL-2 by CD4+ T cells is important for the maintenance of a functional immune system in infected individuals.

Interleukin-15:

IL-15, a product of antigen-presenting cells such as monocytes/macrophages and dendritic cells but not T cells, utilizes a distinct α chain but shares the IL-2Rβ and γ_c chains for its receptor (97). IL-15 shares certain biological activities with IL-2, such as exerting important growth promoting effects on memory and effector CD8+ T cells, including the enhancement of CD8+ memory cell survival, upregulation of perforin, Granzyme A, B and IFN-γ expression, and inhibition of T cell apoptosis (98-102). Its production is reduced in the PBMC of untreated HIV-infected patients (103). However, evidence generated to date does not support a role for defective responsiveness to this cytokine. IL-15 has been reported to be more potent than IL-2 in activating and stimulating the proliferation of naive and memory CD8+ T cells as well as memory CD4+ T cells in PBMCs from HIV+ patients. Some data have indicated that IL-15 may be useful in expansion of CTL in SIV-infected macaques (104) as well as CD8+ T cells (76) and NK/CD4+ lymphocytes (105) in HIV-1-infected subjects. In a recent study investigating its effect on HIV-specific CTLs, it was demonstrated that such cells respond to IL-15 *ex vivo* and during prolonged *in vitro* culture (106). It was shown that IL-15 inhibited spontaneous Fas-induced apoptosis, upregulated the antiapoptotic molecule Bcl-2, enhanced the survival of HIV-specific CTLs in culture and induced their activation, IFN-γ production and cytotoxic activity.

Interleukin-7:

The cytokine IL-7 is constitutively produced by stromal cells from the bone marrow and thymus, and plays a crucial role in T cell homeostasis (107). IL-7 is under

intense investigation in the context of HIV due to its importance in the development, regeneration and function of T cells (108). It is able to support the growth of CD4+ and CD8+ T cells, thymocytes as well as lymphoid progenitors through signal transduction via the IL-7Rα and shared γ_c chains (109,110). It has also been shown to be an important component for the establishment and maintenance of T cell memory (111-114). IL-7 levels are elevated in patients with progressive disease and correlated inversely with both CD4+ and CD8+ counts but regressed to below detection limits in patients that responded to HAART (108). IL-7Rα (CD127) expression has been evaluated on CD8+ T cells from HIV+ patients (115,116). In HIV- controls, 65% of CD8+ T cells expressed the receptor. Receptor expression was downregulated (21%) in untreated HIV+ patients but returned towards that of normal controls in patients undergoing effective HAART, correlating inversely with virus load (115). The downregulation of IL-7Rα expression in CD8+ effector T cells may impact on their responsiveness to this cytokine as was suggested by the finding that the sensitivity to IL-7 is reduced in both CD8+ and CD4+ T cells derived from HIV+ patients (116). In another study, IL-7Rα expression was evaluated in T-cell subsets from HIV+ patients (117). The loss of IL-7Rα was analysed in relation to CD4+ T cell counts and IL-7 concentrations. IL-7Rα was down regulated on T cells and correlated with depletion of CD4+ T cells and with increased concentration of serum IL-7 (117). Decreased IL-7Rα expression was associated with low Bcl-2 expression and with reduced T cell survival, when cultured *in vitro* in the presence of IL-7. Particularly, T cells with a memory phenotype showed a decreased IL-7Rα expression in association with CD28 down-regulation (117).

Interleukin-10:

IL-10 is a pleuripotent cytokine produced by a variety of cells including T cells, B cells and monocytes, and is capable of mediating both immunosuppressive and stimulatory effects (118). Importantly, it exhibits these dual effects on human CD8+ T cells (118). IL-10 suppresses their alloantigen-induced proliferative response indirectly through inhibition of the co-stimulatory functions of antigen presenting cells (APC). In contrast, it displays direct growth promoting effects on activated CD8+ T cells when added in combination with low doses of IL-2. In keeping with these effects, when injected just after a booster vaccination in a murine anti-tumor vaccine model, it was able to boost anti-tumor immunity by potentially enhancing the number of responding tumor-specific CD8+ T cells (119). IL-10 has been studied in the context of HIV infection extensively and, owing to its immunosuppressive function, it may play an important role in HIV immune unresponsiveness (65,66,120,121). For example, IL-10 was found to be upregulated in the PBMC of HIV+ patients with <400 $CD4^+$ cells/μl compared to those with >400 $CD4^+$ cells/μl and uninfected controls (66). Furthermore, antigen responses of lymphocytes from HIV+ patients can be enhanced in vitro by neutralization of IL-10 (67,122). However, a detailed study on the growth promoting and inhibitory effects of IL-10 or the status of its receptor subunits (IL-10R1 and R2) on CD8+ T cells from HIV+ patients has not been undertaken. Moreover, IL-10 prevents Fas-mediated apoptosis of CD8+ T cells from HIV-infected persons while having no preventive effect on CD4+ T cell death. It has been proposed that in HIV+ patients, IL-10 may cause detrimental effects by contributing to decreased production of T_h1 cytokines such as IL-2 and IL-12,

and by inhibiting antigen-presenting cell function and cell-mediated immune responses (118,123-127)

Interleukin-4:

The effect of IL-4 on the development of cytolytic function appears to be variable, both *in vitro* and in models of tumor or viral immunity *in vivo* (128,129) . Some work in which IL-4 has been ablated by gene targeting or delivered exogenously, for example using recombinant viruses, indicated that IL-4 reduced CTL activity and/or viral clearance (129,130). Adoptive transfer of type 1- or 2-polarized CD8+ T cells (termed Tc1 or Tc2, respectively) also showed that IL-4-producing Tc2 cells were less efficient than Tc1 cells in tumor rejection (128,131). By contrast, other studies have suggested that IL-4 either had no effect or enhanced the CTL response and viral or tumor clearance *in vivo* (132). IL-4 produced by CD4+ T cells has multiple immune response–modulating functions, including induction of IgE production in B lymphocytes and the differentiation of precursor T helper (Th) cells toward the Th2 subset that promotes humoral immunity (14). With respect to HIV infection, IL-4 differentially regulates 2 major HIV-1 coreceptors, the chemokine receptors CCR5 and CXCR4 (133-135). CCR5 is a coreceptor used by nonsyncytia-inducing/macrophage-tropic/R5 viruses, which are more likely to be transmitted through sexual contact and are present early after infection in nearly all HIV-1–infected persons (136). In contrast, CXCR4 is a coreceptor used by syncytia-inducing T cell line–tropic/X4 variants, which are usually present in the latter stages of infection (137). IL-4 down-regulates CCR5 expression and thus inhibits

replication of R5 HIV in human T cells and macrophages (133,134), while it up-regulates the expression of CXCR4 and enhances the replication of X4 variant (135).

Interleukin-12:

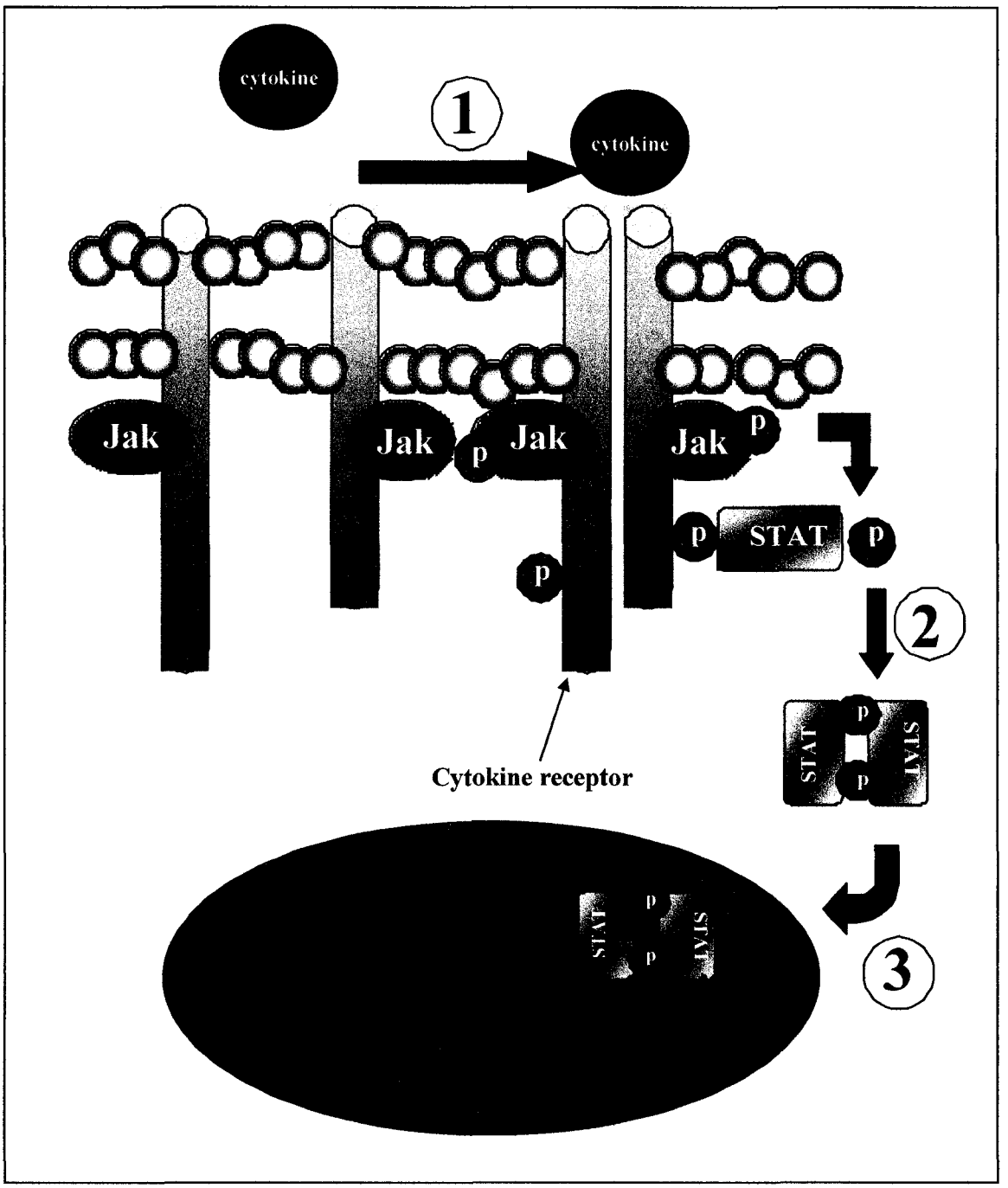
IL-12 is a 70 kDa heterodimeric cytokine produced primarily by cells of the monocyte/macrophage lineage and exerts a profound positive impact on the development of cell-mediated immunity (138,139). The biological activity of IL-12 is mediated via interaction with its high affinity receptor composed of IL-12R-β1 and β2 subunits that are expressed primarily on T cells and NK cells (138). IL-12 is a powerful inducer of Th1 responses, whereas it inhibits Th2 responses. It has been shown that IL-12 synthesis was markedly reduced following cellular activation as was the responsiveness to this cytokine in the PBMC of HIV-infected patients as well as in SIV-infected macaques (120,121,140,141). However *in vitro*, it considerably boosted HIV-specific CTL function in the PBMC of HIV+ patients (142). In another study, the CD8+ T cells from HIV+ patients had a reduced sensitivity to IL-12 yet IL-12R expression was elevated in these cells (116), suggestive of signal transduction defects, similar to what has been observed in IL-2 (75,78).

I.3.4. JAK-STAT signaling pathway

The Janus Kinase (Jak)/ Signal Transducer and Activator of Transcription (STAT) pathway represents one of the main and best studied pathways involved in cytokine responses (143). Studies have demonstrated an essential role played by the Jak/STAT signaling cascade in driving multiple biological and cellular functions in response to

cytokines such as differentiation, proliferation, and apoptosis (144). There are currently four Jaks (Jak1, Jak2, Jak3 and Tyk2) that have been identified in mammals. Jaks are a family of cytosolic tyrosine kinases that are constitutively associated with many cytokine receptors. The STAT family has seven identified members (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6), and their recruitment is a critical component to the induction of cytokine responses. As illustrated in Figure 4, the interaction of a cytokine with its receptor triggers within minutes Jak/STAT signal transduction cascade, which involves the phosphorylation of Jak kinases that are constitutively associated with the cytoplasmic tail of the cytokine receptor complex, subsequent receptor phosphorylation, STAT recruitment to the phosphorylated receptor, STAT phosphorylation by Jaks, STAT dimerization, nuclear translocation and transactivation of target genes containing GAS (IFN-γ activated site) DNA motifs. However, only a few studies have investigated Jak kinases and STATs in HIV-positive patients. Recent investigations have suggested that defects within the Jak/STAT signaling pathway may be implicated in the dysregulation of cytokine responses and HIV pathogenesis. It has been demonstrated that STAT5a, STAT5b and STAT1α expression was reduced in CD3 T cells purified from HIV+ patients. However, the functional status of these proteins was not addressed in this study (145). Another study showed that STAT1 and a C-terminal truncated, potentially dominant negative mutant of STAT5 was constitutively activated primarily in the CD4+ T cell compartment of most HIV+ patients studied (146). It has also been shown that CD8+ T cells from a subset of HIV+ patients naïve to therapy were unable to functionally activate STAT5a and STAT5b proteins in response to *ex vivo*

Figure 4. The sequence of events in the JAK/STAT signaling pathway. (1) Upon cytokine binding, receptor subunits dimerize bringing the Jaks, which are constitutively associated with the cytoplasmic tails of receptors, into close proximity. The Jaks transphosphorylate each other and receptor chains on conserved tyrosine residues. Cytoplasmic STAT proteins are recruited through their SH2 domain to the phosphorylated tyrosine residues on receptors and are phosphorylated by the Jaks on C-terminal tyrosine residues. (2) Activated STATs dissociate from the receptor chains and dimerize through interactions between a phosphorylated tyrosine on one and an SH2 domain on the other. (3) STAT dimers then translocate to the nucleus where they initiate transcription of genes containing STAT responsive elements.



Adapted from Sant, N. (147)

stimulation with IL2 (75). This defect was not the result of alterations in IL-2R expression but correlated with impaired Jak-3 activation, the upstream kinase in this pathway. Furthermore, this defect appeared to be restored following HAART.

II. Rationale and Objectives

Whether Jak/STAT defects arise in response to cytokines other than IL-2 critical in the growth and differentiation of CTL, particularly those sharing the common γ_c chain receptor (IL-2, IL-15, IL-7, and IL-4) has not been investigated. Also, whether cytokine signaling defects arise in the CD4+ T cell compartment of HIV+ patients remained to be evaluated.

Unfortunately, standard molecular methods to investigate signal transduction are difficult to carry out mainly due to the limitation on the number of cells obtainable from patient blood samples. Furthermore, such techniques (immunoblotting, immunoprecipitation) are essentially impossible to perform in rare PBMC populations such as virus-specific CTLs (< 1% PBMC). These facts lead to the following specific objectives.

1. Optimization of a sensitive and specific assay to detect Jak/STAT signaling within PBMC sub-populations (CD8, virus-specific CTLs and CD4) at the single cell level.
2. Evaluation of cytokine-induced JAK/STAT signaling in the PBMC subsets from HIV-infected patients.
3. Phenotypic characterization and studies into the mechanism responsible for HIV-induced alterations in cytokine-dependent Jak/STAT signaling.

III. Materials and Methods

III.1. Patient Study Groups

Previous work by *Kryworuchko et al.,* (75) has documented IL-2 dependent STAT5 signaling defects in CD8+ T cells from a subset of HIV+ patients naïve to therapy and their apparent restoration under HAART. Therefore, a similar set of patients was studied. Patient stratified into three groups: Group 1 included 8 patients that had discontinued therapy for more than 6 months and had high viral loads (mean, 1.09 x 10^5 copies/ml [range, 0.59-2.92 x 10^5]) and low CD4+ T cell counts (<300 [100-289] cells/μl). Group 2 included 8 patients on sustained HAART (>1 year). At the time of sampling, group 2 subjects had a low viral load (<50 copies/ml) and relatively higher CD4+ counts (527 [489-725]). Group 3 included 9 HIV-negative controls. Patient samples were obtained from the Immunodeficiency Clinic at the Ottawa Hospital in collaboration with Dr. Jonathan Angel.

III.2. Antibodies and reagents

All monoclonal antibodies (Abs) used for flow cytometry unless otherwise indicated were mouse Abs purchased from BD Pharmingen, BD Biosiences, or Transduction Laboratories (San Diego, CA.). Anti-CD4 PerCP (SK3; 2μg/mL) (antibody clone; final concentration used), anti-CD8 PE CY5 (HIT8A; 1μg/mL), anti-CD28 PE (CD28.2; 3μg/mL) [BD Biosiences], anti-IL-2R[alpha] (M-A251; 2μg/mL), anti-IL-2R[beta] (MIK-β2; 2μg/mL), anti-IL-15R[alpha] (151303; 2μg/mL), anti-IL7R[alpha] (40131; 2μg/mL) (Immunotech, Marseilles, France), anti-IL-2R[gamma] (TUGH4; 2μg/mL) (Pharmingen) were used. Isotype control Abs included mouse IgG1 (MOPC-

31C) rat IgG2a and IgG2b (Pharmingen) used at the appropriate concentration. Abs used for intracellular staining included anti-phospho-(P)STAT1 (Y701) Alexa Fluor® 488 (4a;0.09 μg/mL), anti-pSTAT5 (Y694; 0.048 μg/mL) Alexa Fluor® 488 (47; 0.09 μg/mL), and anti-P-STAT6 (Y641) PE (18; 0.09 μg/mL). Stat3 (Y705) Alexa Fluor® 488 (49/p-Stat3; 0.09 μg/mL)

III.3. Cell Isolation, Culture and Stimulation

Peripheral blood mononuclear cells (PBMCs) were isolated from blood by density gradient centrifugation over Ficoll-Paque™ (Amersham Biosciences, Piscataway, NJ), as previously described (75). Briefly, heparinized blood was obtained from HIV+ and HIV- donors, approximately 50 ml, and layered onto Ficoll-Paque™ at a ratio of 11:4 (blood:Ficoll-Paque). Next, centrifugation was performed at 400g for 30 minutes with the brake off in a Megafuge centrifuge, (Baxter Scientific Products, Minneapolis). Subsequently, the interphase containing PBMCs was collected and washed three times in phosphate buffered saline (PBS) (Sigma-Aldrich). PBMCs were subsequently cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Invitrogen/Life Technologies, Grand Island, NY), penicillin (100 U/mL), gentamicin (100 μg/mL), and HEPES (100 mM). PBMCs ($4x10^5$) were stimulated for 15 minutes at 37°C and 5% CO_2 with IL-2 (34 ng/mL), IL-4 (4 ng/mL), IL-7 (1.5 ng/mL), IL-15 (2 ng/mL), or IL-10 (1 ng/mL), in parallel with unstimulated cells. All recombinant human cytokines were purchased from R&D Systems Inc (Minneappolis, MN).

III.4. Flow Cytometry

III.4.1. Cell surface staining

The cell surface expression of cytokine receptors in CD8+ and CD4+ T cells was determined by flow cytometry. Briefly, PBMCs ($2x10^5$) were resuspended in 200 µL PBS and stained with the indicated anti-cytokine receptor chain specific or isotype-matched control Abs at the concentrations indicated in section II.2. Following a 10 min incubation at room tempture, lineage specific anti-CD4-PerCP, anti-CD8 PE Cy5 were added for a further 10 min incubation. The cells were subsequently washed with PBS containing 0.1% bovine serum albumin (PBS-BSA) and analyzed by flow cytometry using a FACScan Flow Cytometer (BD Bioscience) and fluorescence data analysed using CellQuest software (BD Bioscience) or the WinMDI version 2.8 software package (Joseph Trotter, BD Bioscience). For standardization and calibration of the instrument, fluorescent Calibrite™ beads (BD Biosciences) were used.

III.4.2. Intracellular staining

The intracellular phosphoprotein staining procedure described by *Krutzik et al.*, (148) was adapted for use in this study to analyze STAT activation in response to growth promoting cytokines in CD4+ and CD8+ T cells from HIV+ patients and HIV- controls. Briefly, stimulated and unstimulated PBMCs were fixed with formaldehyde (1.5 %) for 10 minutes and permeablized with cold methanol (MEOH) (EM Science), vigorously mixing and incubated for further 10 minutes at 4 °C. Cells were washed twice with PBS-BSA, and anti-P-STAT Abs were added at a final concentration 0.09 µg/mL for all

STATs Abs except STAT5, which was added at 0.048 μg/mL. After a 5 minutes incubation at room temperature in the dark, anti-CD8 PE Cy5 or anti-CD4 PerCP Abs were added to the appropriate tubes and incubated for a further 10 minutes under the same conditions. The cells were then washed with PBS-BSA, pelleted and resuspended in 2 % PFA for subsequent flow cytometric analysis as above.

III.4.3. Detection of cytokine induced STAT-activation in virus-specific CTL populations.

HIV+ patients were typed for the expression of human leukocytes antigen (HLA) A2 and HLA B8 alleles using anti-HLA-A2 and HLA-B8 specific Abs by surface staining as described in section III.4.1. HLA A2+ and HLA B8+ patients were subsequently screened for the presence of CTL specific for the following viral epitopes. The EBV CTL epitopes included BMLF1 (GLCTLVAML), and BZLF1 (RAKFKQLL) (149). HIV epitopes included gag (SLYNTVATL) (150), and nef (AFHHVAREL) (37). HLA-A2 BMLF1 and gag tetramers were purchased from Beckman Coulter. HLA-B8 BZLF1 and nef tetramer were kindly provided by Dr. Victor Appay (Lausanne, Switzerland). PBMCs ($4x10^5$) from HIV+ patients and HIV- donors expressing HLA-A2 or HLA-B8 epitopes were stimulated for 15 min at 37°C and 5% CO_2 with the appropriate cytokine in parallel with unstimulated cells. HLA-A2 or -B8 tetramers were added for a further 15 min incubation. Subsequently, to detect P-STAT induction the protocol followed was the same as in section III.4.2.

It should be noted that the overlay of flow cytometry histograms and the observation of a reproducible population shift in log fluorescence intensity were the parameters used to determine the significance of any changes in expression level reported.

III.5. Statistical analysis

Means were compared using the two tailed Student's T test. Results are expressed as mean ± standard deviation (STDev). The correlation between cytokine receptor expression and STAT activation was determined by linear regression analysis using the Sigma Plot version 9.0 software package.

IV. Results

IV.1. Optimization of a sensitive and specific flow cytometric assay to detect Jak/STAT signaling within PBMC sub-populations at the single cell level.

As mentioned, current molecular methods such as western blot, immunoblot and electrophoretic mobility shift assays (EMSA) require relatively large numbers of purified cells to be feasible, making it difficult or often impossible for them to be applied to *ex vivo* signaling studies. Flow cytometry offers many advantages over these techniques, provided the sensitivity and specificity for the intended investigations are maintained. Most importantly, flow cytometry does not require large numbers of cells and permits the analysis and quantification of multiple populations and parameters simultaneously. This allows for the analysis of rare cell populations without prior purification steps. Therefore, the first objective of this study was to optimize a flow cytometry based technique that uses Abs specific for tyrosine-phosphorylated (activated) STAT (P-STAT) proteins to detect their intracellular induction in a cytokine-dependent manner. As described in the Materials and Methods Section III.4.2 the procedure described by *Krutzik et al.,* (148) was adapted for this purpose.

As it is generally established in the literature which STATs are activated by which cytokines and in what cell type (144), it was important to ensure that the relevant STAT proteins are activated in response to the appropriate cytokines by a flow cytometry. PBMCs isolated from volunteer HIV- donors were subjected to stimulations with IL-2, IL-4, IL-7, IL-15 and IL-10, in parallel with unstimulated controls and stained with anti-P-STAT1, 3, 5 or 6 and anti-CD8 or anti-CD4 Abs, as described in the materials and

methods section III.4.2. Flow cytometric analysis was conducted and histogram overlays were then plotted for stimulated and unstimulated cells.

Overall, the results indicated that the anti-P-STAT Abs used were specific according to the above criteria. That is, the appropriate STAT proteins were activated in response to the appropriate cytokines. Representative data is shown in (Figure 5). In CD8+ T cells, IL-2, IL-7, and IL-15 induced STAT5 tyrosine phosphorylation (P-STAT5) in contrast to IL-10 (Figure 5A). However, IL-10 stimulation did mediate STAT3 and marginally STAT1 tyr-phosphorylation (Figure 5B, C). IL-4 specifically induced STAT6 tyr-phosphorylation while IL-10 did not (Figure 5D). Figure 5B also shows that there was no STAT1 activation detected in response to IL-4 stimulation.

Specificity in CD4+ T cells was demonstrated with all cytokines in a similar fashion and found to correlate with what has been reported in the literature (Figure 6) (144). In brief, IL-2, IL-7, and IL-15 specifically induced P-STAT5. IL-4 induced P-STAT6, while IL-10 induced P-STAT3. IL-4 was also able to induce P-STAT5, although marginally. A summary of the results for cytokine-dependent STAT activation detected in both CD8+ and CD4+ T cells is provided in Table 1.

IV.2 Evaluation of cytokine-induced Jak/STAT signaling in CD8+, CD4+ T cells and virus specific-CTLs from HIV-infected patients.

IV.2.1. STAT activation in CD8+ T cells from HIV+ patients

Figure 5. Reactivity and specificity of P-STAT Abs in CD8 T cells. PBMCs were stimulated for 15 minutes with IL-2 (34 ng/mL), IL-7(1.5 ng/mL), IL-15 (2 ng/ml), IL-4 (1.5 ng/mL) and IL-10 (1 ng/mL) in parallel with unstimulated controls. Cells were stained with each anti-P-STAT antibody to determine which STATs where activated in response to which cytokines. Within the CD8+ cell gate, 5000 events were acquired. Histograms were plotted using WinMDI2.8 software (events on y-axis, fluorescence intensity on x-axis) and comparison between STAT activation in stimulated and unstimulated cells was carried out. (A) STAT5 activation in unstimulated compared to IL-2, IL-7, IL-15 and IL-10 stimulated CD8+ T cells (B) STAT1 activation in unstimulated compared to IL-10 and IL-4 stimulated CD8+ T cells. (C) STAT3 activation in unstimulated compared to IL-4 and IL-10 stimulated CD8+ T cells. (D) STAT6 activation in unstimulated compared to IL-10 and IL-4 stimulated CD8+ T cells. Results are shown for one representative experiment out of at least three performed.

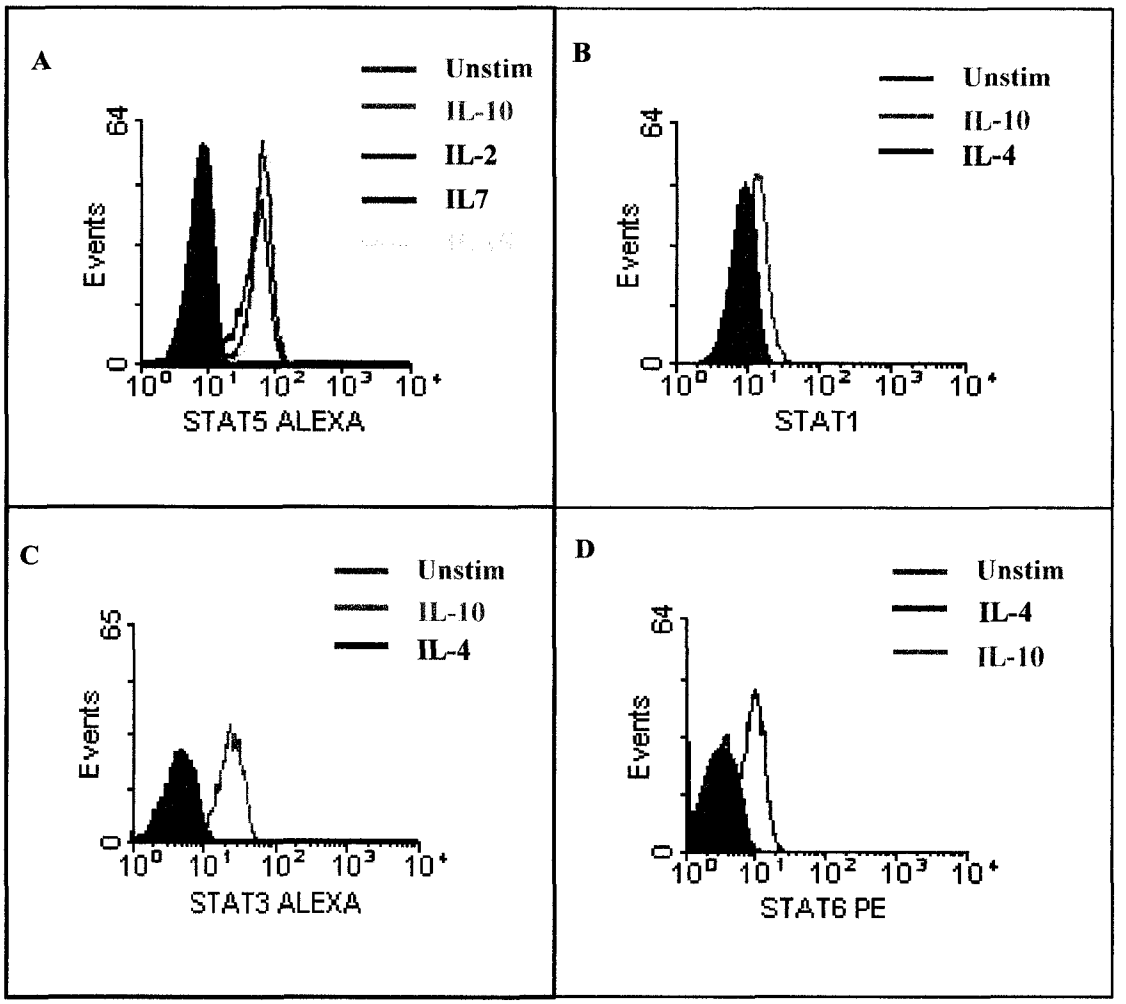


Figure 6. Cytokine-induced STAT activation in CD4+ T cells. PBMCs were stimulated for 15 minutes with IL-2 (34 ng/mL), IL-4 (4 ng/mL), IL-7 (1.5 ng/mL), IL-15 (2 ng/mL) and IL-10 (1 ng/mL), with the unstimulated control performed in parallel. Within the CD4+ cell gate, 5000 events were acquired. Data is presented as fold change for each of the STAT proteins. Comparing geometric mean of fluorescence intensity in stimulated *vs* unstimulated cells, values were obtained from histogram plots of log fluorescence intensity (WinMDI2.8) in stimulated (STIM) and unstimulated (UNSTIM) cells. Results are shown for one representative experiment out of three performed.

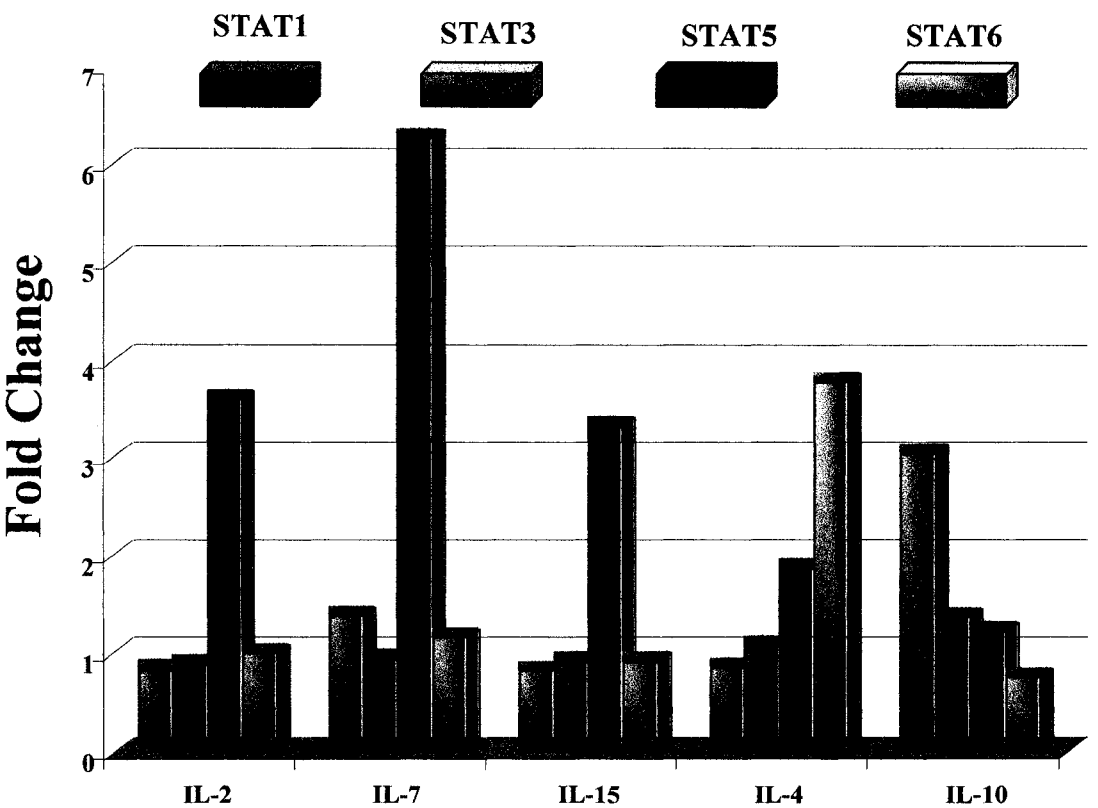


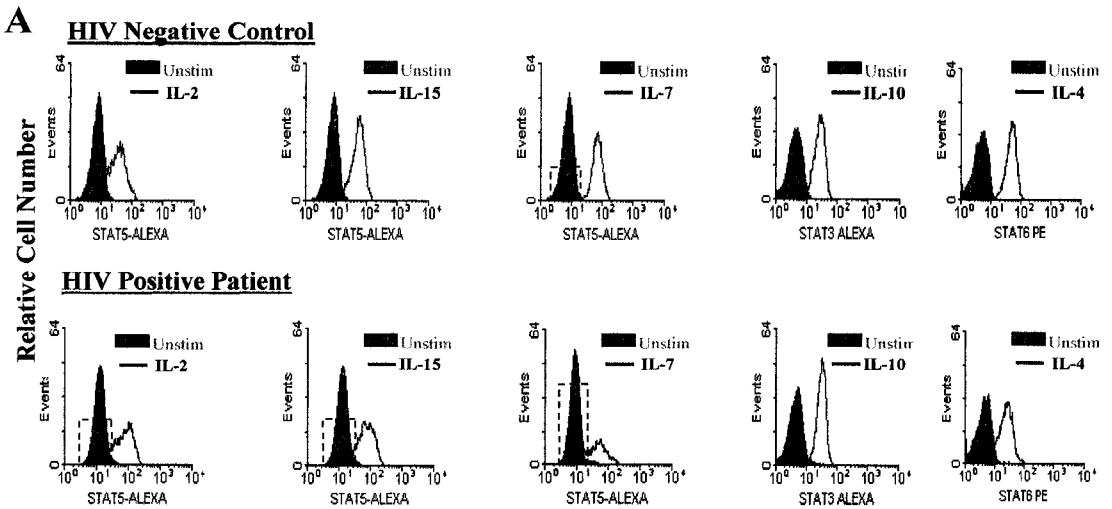
Table 1. Summary of cytokine dependent STAT activation detected in CD8+ and CD4+ T ells *

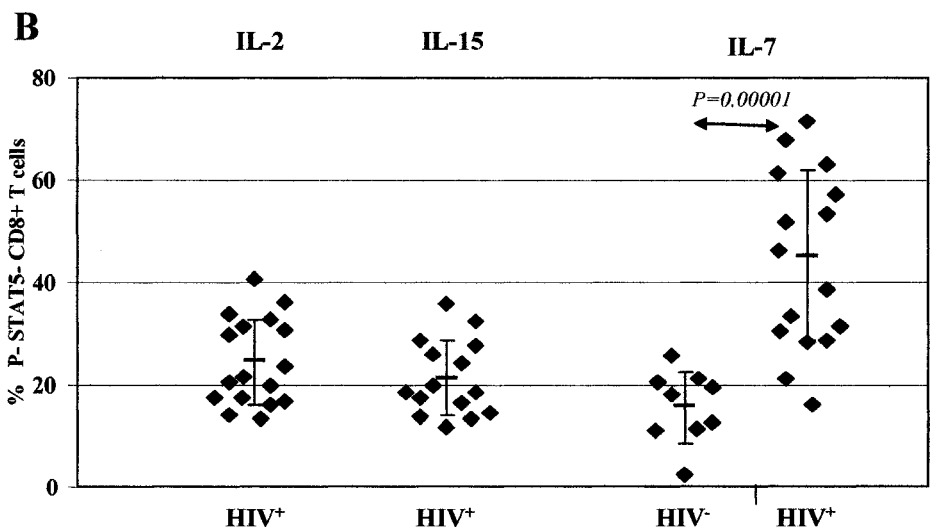
		IL2	IL4	IL7	IL15	IL10
CD8	**STAT1**	-	-	-	-	+/-
	STAT3	-	-	-	-	+
	STAT5	+	-	+	+	-
	STAT6	-	+	-	-	-
CD4	**STAT1**	-	-	-	-	+
	STAT3	-	-	-	-	-
	STAT5	+	+/-	+	+	-
	STAT6	-	+	-	-	-

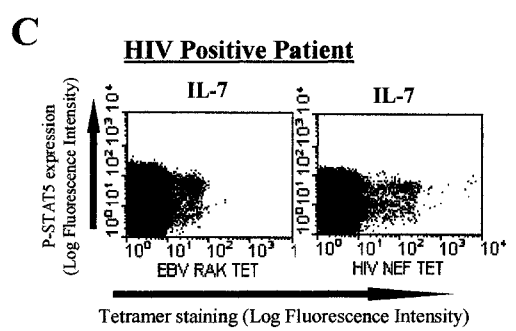
* + indicates a substantial induction, - indicates no appreciable induction, +/- indicates a marginal induction.

It has been previously shown that CD8+ T cells from a subset of HIV+ patients naïve to therapy were unable to functionally activate STAT5a and STAT5b proteins in response to *ex vivo* stimulation with IL-2 (75). This defect was not the result of alterations in IL-2R expression but correlated with impaired Jak-3 activation, the upstream kinase in this pathway. However, this defect appeared to be restored following HAART. Whether such defects arise in response to other cytokines critical in their growth and differentiation, particularly those sharing the common γ_c chain receptor (IL-2, IL-15, IL-7, and IL-4) has not been investigated. Furthermore, whether there are differences at this level in virus-specific CTL populations is not known. Therefore, STAT activation was measured in response to the appropriate cytokine stimulation in CD8+ T cells from study group patients. PBMCs from HIV+ as well as HIV- donors ($4x10^5$) were stimulated with IL-2 (34 ng/mL), IL-4 (4 ng/mL), IL-7 (1.5 ng/mL), IL-15 (2 ng/mL), and IL-10 (1 ng/mL) for 15 min, followed by staining CD8+ T cells with the relevant anti-P-STAT Abs. Flow cytometric analysis revealed that the CD8+ T cells from all subjects could activate the relevant STAT proteins in response to the appropriate cytokine stimulation (Figure 7). Specifically, P-STAT5 was activated in response to IL-2, IL-15, and IL-7. P-STAT6 and P-STAT3 were observed in response to IL-4 and IL-10, respectively. Strikingly however, a distinct population of CD8+ T cells (P-STAT5-) was revealed in HIV+ patients that were unable to activate STAT5 in response to IL-2 and IL-15 (Figure 7A). This population was not detected in HIV- controls. P-STAT5- cells were observed in response to IL-7 in both HIV+ and HIV- patients. In Figure 7B, the proportion of P-STAT5- CD8+ T cells was graphed for each patient studied. The P-STAT5- CD8+ T cell population was observed in all HIV+ patients studied in response to

Figure 7. STAT activation in CD8 T cells from HIV-infected patients. PBMCs ($4x10^5$) from HIV+ and HIV- patients were stimulated for 15 minutes with IL-2 (32 ng/ml), IL-7 (1.5 ng/mL), IL-15 (2 ng/mL), IL-4 (4 ng/mL), or IL-10 (1 ng/mL). (A) Histogram overlays for P-STAT expression on gated CD8 T cells is shown for one HIV+ and one HIV- subject. Note the bimodal cytokine-induced P-STAT5 expression pattern (positive and negative) in the HIV+ subject. The P-STAT5- CD8+ T cell populations are demarcated by the dashed box. (B) The % of P-STAT5-negative CD8 T cells detected in response to IL-2, IL-15 or IL-7 was plotted. Each graphed symbol represents the value obtained after 15 min of cytokine stimulation for a given patient. The mean % P-STAT5 negative (horizontal bars) for each group is plotted along with standard deviation (vertical bar). C) IL-7-induced P-STAT5 in EBV BZLF1 (RAKFKQLL) and HIV nef (AFHHVAREL) HLA-B8 restricted epitope specific CTLs. Staining for one B8+ HIV+ donor is shown.







IL-2, IL-15 and comprised (24 ± 8%; Mean ± Standard deviation), (21 ± 7%) respectively, of total CD8+ T cells. The proportion of P-STAT5- CD8+ T cells in response to IL-7 in HIV+ patients (45 ± 16%) on average was significantly higher ($p= 0.00001$) than in HIV-controls (15 ± 6%). Interestingly, this stratification was not observed in response to the other cytokines and STATs measured namely, IL-10 and IL-4 (Figure 7A).

IV.2.2. STAT activation in virus-specific CTL

In view of the phenotypic and functional differences that have been reported to distinguish HIV-specific CTLs from those directed against other viruses (EBV, CMV) (34,37), it was of interest to study P-STAT5 activation in virus specific-CTLs. Therefore, HIV+ patients were typed for the expression of HLA-A2 and HLA-B8 alleles using anti-HLA-A2 and HLA-B8 specific Abs by flow cytometry (data not shown). HLA-A2+ and HLA-B8+ patients were then screened using MHC class I-peptide tetramers for the presence of CTL specific for the different HIV and EBV epitopes, as described in materials and methods section III.4.3. EBV CTL epitopes included BMLF1 (GLCTLVAML), BZLF1 (RAKFKQLL). HIV epitopes includes gag (SLYNTVATL), nef (AFHHVAREL). Similar to bulk CD8+ T cell P-STAT5+ and P-STAT5- cells were detected in HIV+ patient CTLs specific for HIV nef and EBV BZLF1 epitopes in response to IL-7. IL-7 induced P-STAT5 expression in one patient out of five tested is shown (Figure 7C).

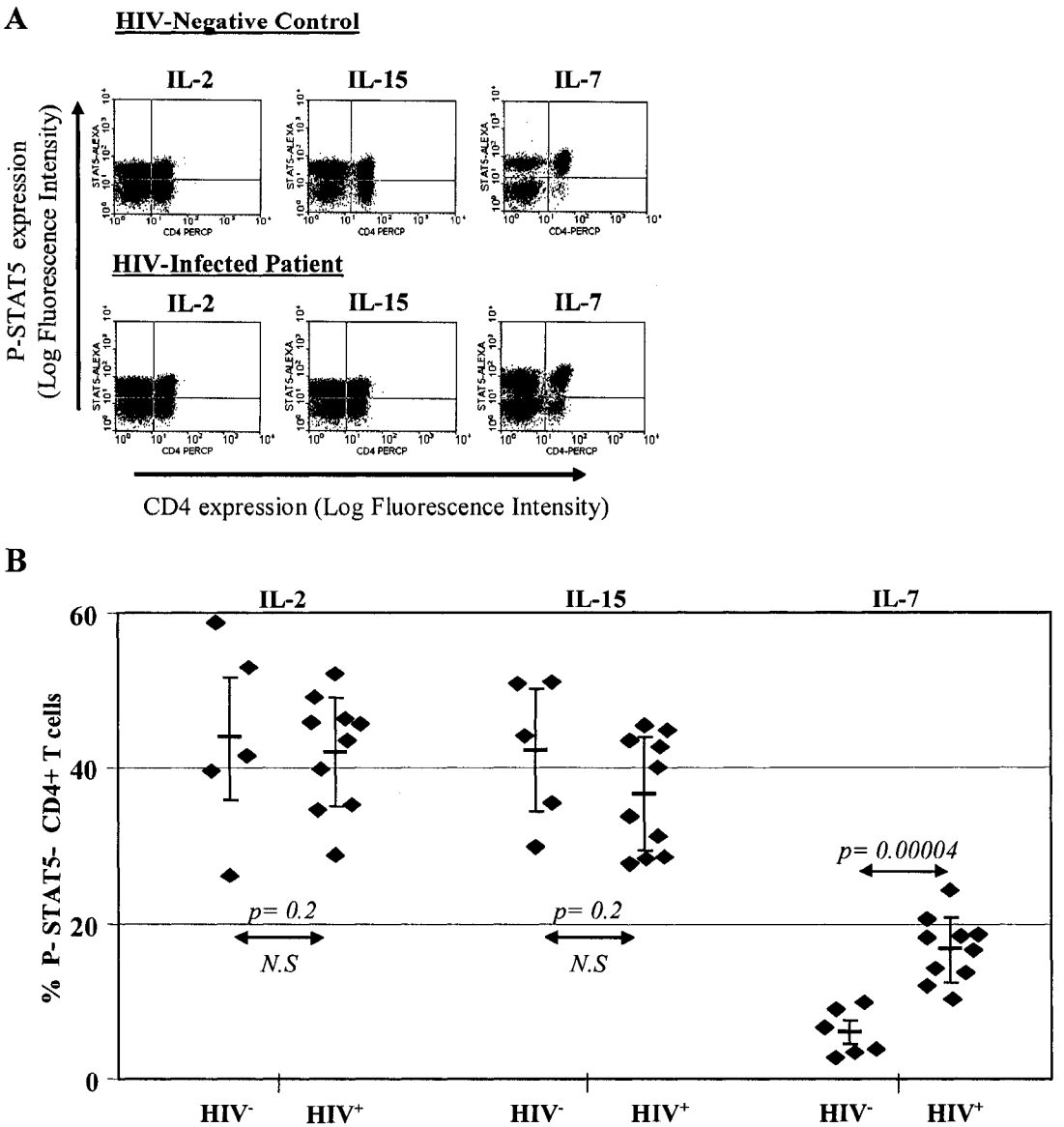
IV.2.3 STAT activation in CD4+ T cells from HIV+ patients

A study by *David et al.,* (78) suggested that IL-2 dependent signaling defects may also exist in the CD4+ T cell compartment of HIV+ patients with high viral load. Also as mentioned, STAT1 and a C-terminal truncated, potentially dominant negative mutant of STAT5 was constitutively activated primarily in the CD4+ T cell compartment of most HIV+ patients studied (146). Therefore, this study aimed to determine if cytokine-dependent STAT activation in the CD4+ T cell compartment of HIV+ patients was impaired. PBMCs from HIV+ as well as HIV- patients were stimulated with the above mentioned cytokines for 15 min, followed by fixation, permeabilization and staining with anti-P-STAT and anti-CD4 specific Abs in a fashion similar to that of CD8+ T cells. Flow cytometric analysis revealed that CD4+ T cells from all subjects studied could activate the relevant STAT proteins in response to the appropriate cytokine stimulation (Figure 8). Specifically, STAT5 was activated in response to IL-2, IL-15, and IL-7. However, P-STAT5- and P-STAT5+ populations emerged in both HIV+ and HIV- donors in response to these cytokines. The percentage of P-STAT5- CD4+ T cells was graphed for each patient studied. The proportion of P-STAT5- cells in response to IL-7 in HIV+ patients (17 ± 4%) was significantly higher (*p=0.00004*) compared to HIV- controls (6 ± 1.5%). The proportion of P-STAT5- cells detected in response to IL-2 (43 ± 7% in HIV- and 42 ± 7% in HIV+) and IL-15 (42 ± 7% HIV- and 36 ± 7% in HIV+) were not significantly different.

IV.2.4. Impact of HAART on P-STAT5- CD4+ and CD8+ T cells

To determine the impact of HAART on the observed phenomena, the data was stratified to compare the proportion of P-STAT5 negative CD4+ and CD8+ T cells in G1

Figure 8. STAT activation in CD4 T cells from HIV-infected patients. PBMCs ($4x10^5$) were stimulated for 15 minutes with IL-2 (32 ng/ml), IL-7 (1.5 ng/mL), IL-15 (2 ng/mL) IL-4 (4 ng/mL), or IL-10 (1 ng/mL), . HIV infected patients are compared to HIV-negative controls. A total of 5000 events were obtained in the CD4+ cell gate. (A) P-STAT5 expression in CD4+ T cells in one HIV+ and one HIV- volunteer is shown (B) The percentage of P-STAT5- CD4+ T cells in response to the indicated cytokines in each study subject is plotted. Each symbol represents a different donor. Horizontal bar represents the mean which is plotted along with standard deviation (vertical bar).
NS, not significant.



(off therapy >6 months) and G2 (sustained HAART > 1 year) patients. Interestingly, the proportion of P-STAT5- CD8+ T cells detected in response to IL-7 was significantly higher (*p=0.008*) in G1 patients (56%, ± 14%) compared to G2 patients (34%, ± 13%) (Figure 9A). However, there were no significant differences revealed in the population of P-STAT5- CD4+ T cells in response to IL-7 (Figure 9B). In contrast to IL-7, no difference in the percentage of P-STAT5- cells was observed in response to IL-2 and IL-15 between the two groups of patients in neither the CD8+ nor the CD4+ T cells (Figure 9A, B). This suggested that in the case of IL-2 and IL-15 these P-STAT5- cells were present despite seemingly effective HAART. In the case of IL-7, the % of P-STAT5- CD8+ T cells under HAART appeared to be returning towards that of HIV- controls but remained higher (*p=0.003*) in the patients studied.

IV.3 Phenotypic characterization and studies into the mechanism responsible for HIV-induced alterations in cytokine-dependent Jak/STAT signaling.

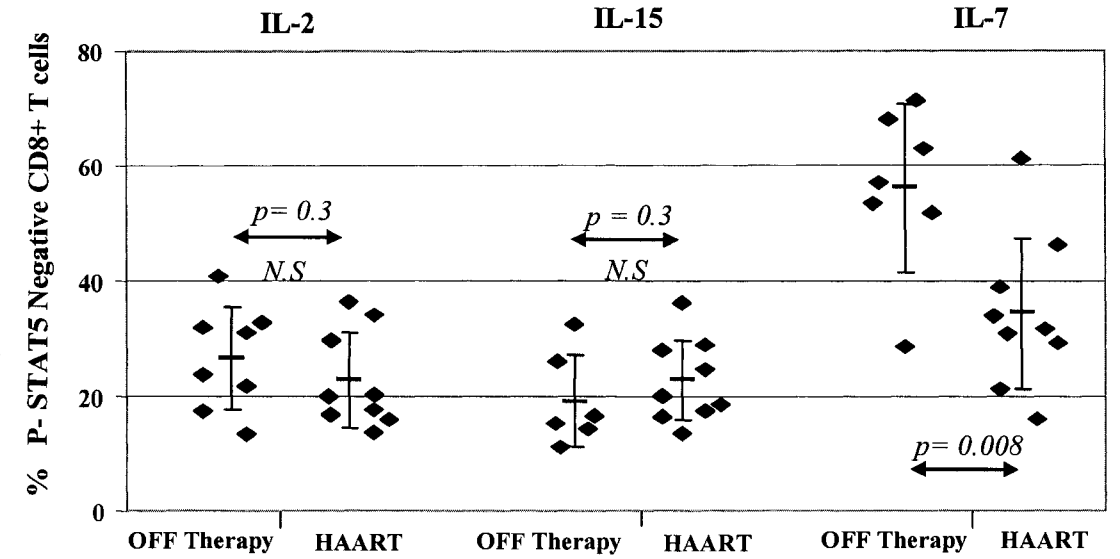
It was of great interest to further characterize the P-STAT5- cells which were detected in response to IL-2, IL-15 and IL-7 in order to gain insight into their nature and potential molecular mechanism responsible for their generation.

IV.3.1. Cytokine receptor expression in CD8+ T cells

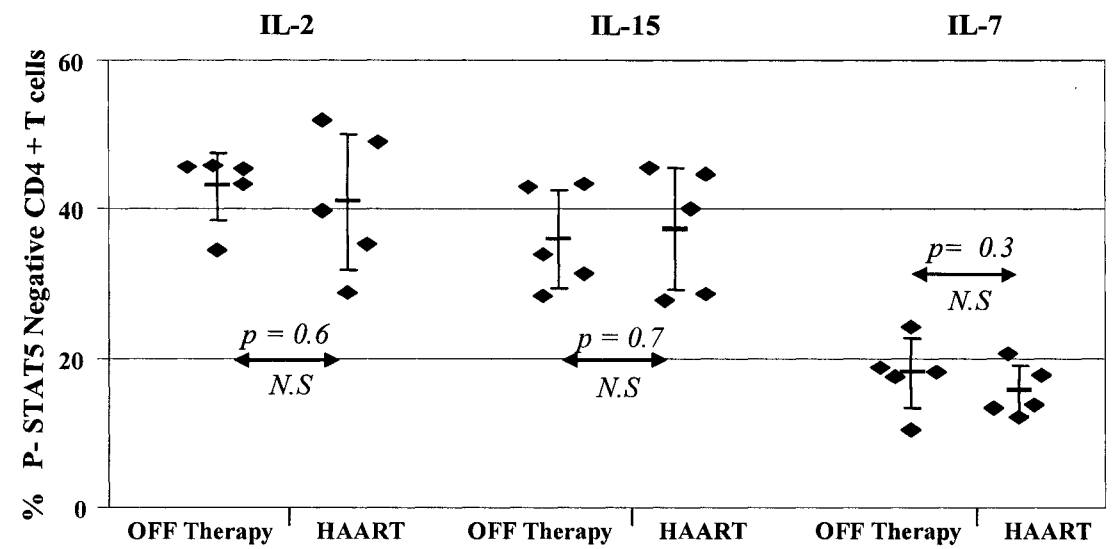
The stratification of CD8+ T cells into P-STAT5+ and P-STAT5- cells in response to IL-2, IL-15 and IL-7 could be the result of differences in cytokine receptor chain expression levels. This hypothesis was investigated by evaluating cell surface

Figure 9. Effect of HAART on cytokine induced P-STAT5- CD8+ and CD4+ T cells from HIV+ patients. PBMCs ($4x10^5$) were stimulated for 15 minutes with IL-2 (32 ng/mL), IL-7 (1.5 ng/mL), or IL-15 (2 ng/mL). A total of 5000 events were obtained in the CD4+ or CD8+ cell gate and analyzed for P-STAT5 expression by flow cytometry. The graph shows the percentage of P-STAT5- cells in the two groups of patients studied (off therapy > 6 month and those on sustained HAART) in response to the indicated cytokines. Each symbol represents a different patient. The horizontal bar represents the mean and is plotted along with standard deviation (vertical bar).

A



B



expression levels of IL-2Rα, IL-2Rβ, γc, IL-7Rα and IL-15Rα chains by flow cytometry in CD8+ T cells from the same group of patients. PBMC were stained with the relevant cytokine receptor chain- and CD8-specific as well as isotype control Abs, followed by flow cytometric analysis, as described in materials and methods, gating on CD8+ T cells. Results obtained from one HIV- and one HIV+ patient sample are shown in (Figure 10). It appeared that the % of IL-7Rα+ CD8+ T cells was reduced in certain HIV+ patients (Figure 10A). IL-2Rα chain expression was very low/undetectable in total CD8+ T cells from both HIV- and HIV+ patients compared to the isotype control Ab (Figure10B). However, IL-2Rβ and γc were expressed in CD8+ T cells from both HIV- and HIV+ patients. Notably, expression above that of isotype control Ab was detected as a single population of cells (Figure 10B). IL-15Rα expression was not detectable to any appreciable extent on CD8+ T cells (Figure 10B). No significant differences between HIV- and HIV+ patients were observed at the level of IL-2Rβ, γ_c and IL-15Rα chain expression. Further analysis plotting the % P-STAT5+ CD8+ cells obtained in response to IL-7 stimulation vs % IL-7Rα+ CD8+ T cells obtained in the same set of patients (Figure 11A) revealed a strong positive correlation (r^2 = 0.95) between these two parameters. This suggested that in the case of IL-7, the population of P-STAT5- CD8+ T cells observed was likely due to the lack of IL-7Rα chain expression on these cells. Therefore, the presence of P-STAT5- CD8+ T cells in response to IL-2, IL-7 or IL-15 did not correlate by this method with IL-2Rα, IL-2Rβ, γc or IL-15Rα chain expression (Fig. 10B, and data not shown).

Figure 10. Cytokine receptor expression in CD8+ T from HIV-infected patients. PBMCs ($2x10^5$) were surface stained with the indicated receptor-specific Abs. A total of 5000 events were obtained in the CD8+ cell gate and analyzed by flow cytometry, as mentioned in material and methods. The expression of IL-7Rα in one HIV+ compared to one HIV- patient is plotted. (A) Histogram overlays showing the indicated cytokine receptor (thin line) and isotype control antibody (shaded histogram) in one HIV+ and one HIV- patient (B).

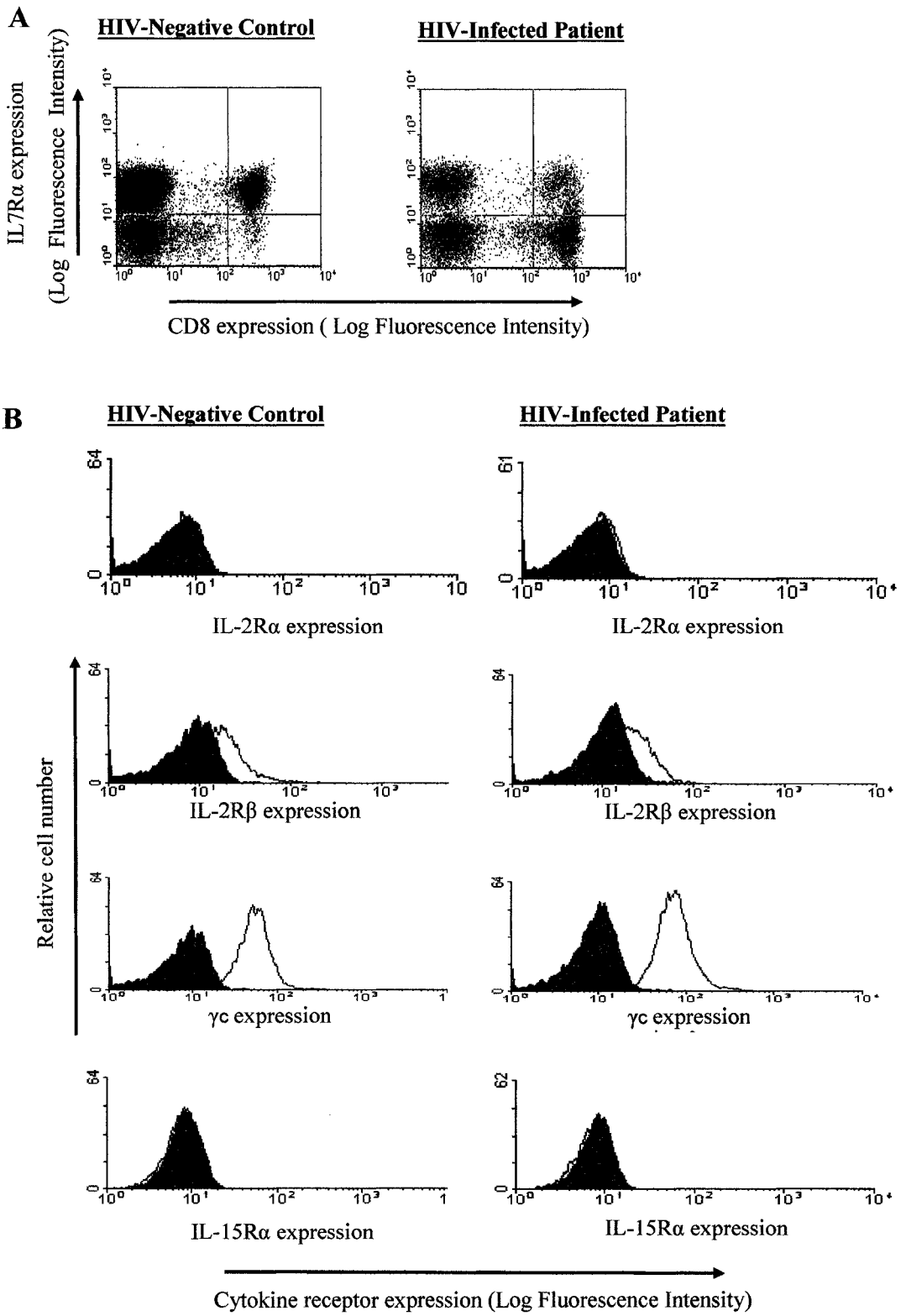
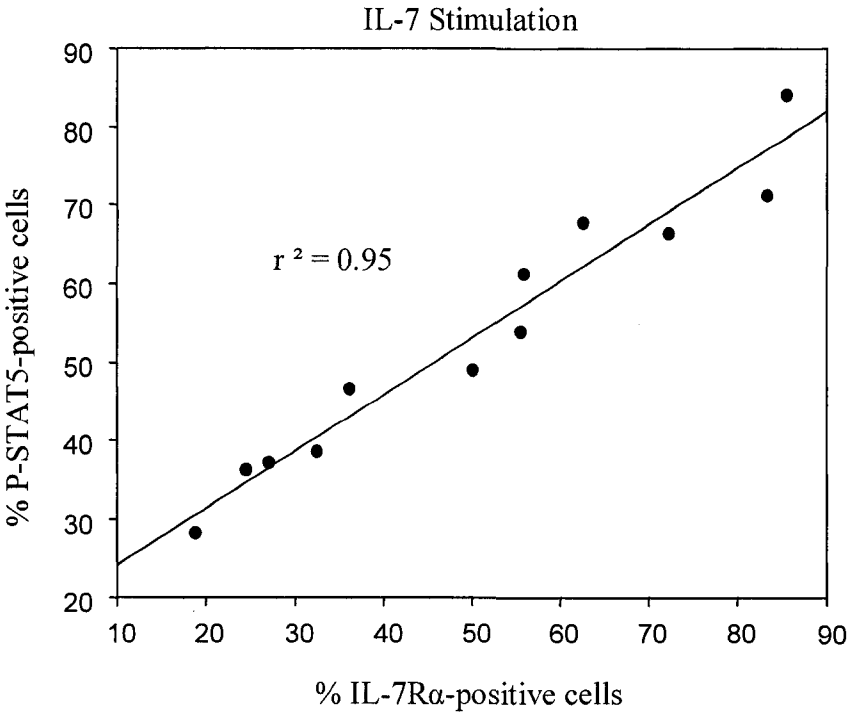


Figure 11. Comparison of IL-7-induced P-STAT5 expression *vs* IL-7Rα expression in CD8 T cells from HIV+ patients. The % P-STAT5+ cells vs. % IL-7Rα + cells was plotted and analyzed by simple linear regression (Sigma Plot). r^2 = coefficient of determination

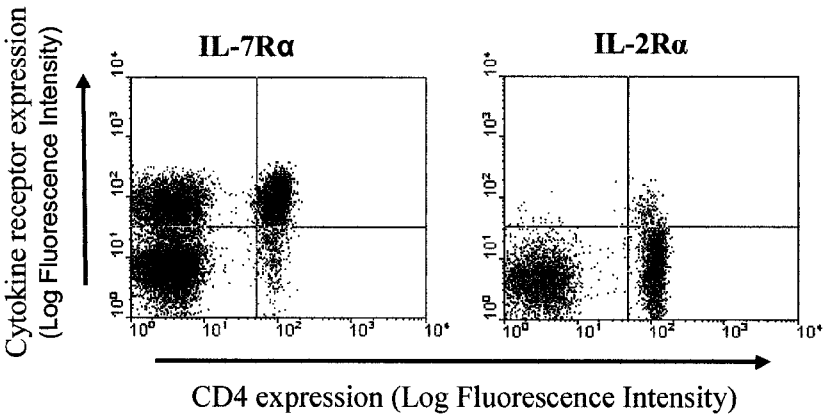


IV.3.2. Cytokine receptor expression in CD4+ T cells

Similar investigations were conducted to determine whether differences in the expression of cytokine receptor chains in CD4+ T cells could explain the stratification of CD4+ T cells into P-STAT5- and P-STAT5+ cells in response to IL-2, IL-7 and IL-15. The expression of cytokine receptor chains IL-2R α, β, γ_c, IL-7Rα and IL15Rα was determined by flow cytometry on CD4+ T cells from HIV+ patients. PBMC were stained with the relevant cytokine receptor chain- and CD4-specific Abs as well as isotype control Abs. Flow cytometyric analysis in one HIV+ patient sample is shown in (Figure 12). A small proportion of IL-2Rα+ cells was detectable in CD4+ T cells (Figure 12A) representing most likely the IL2Rα+ (CD25) CD4+ regulatory T cells. However, IL-2Rβ and γc expression in CD4+ T cells in HIV+ patients was detected as a single population of cells above that of isotype control (Figure 12B). IL-15Rα was also not detectable on CD4+ T cell (Figure 12B). The % IL-7Rα+ vs % P-STAT5+ CD4+ T cells obtained in response to IL-7 stimulation was plotted in patients for which samples were available, and revealed a positive albeit weaker correlation (r^2 = 0.65) between these two parameters compared to CD8+ T cells (Figure 13). This suggested that in the case of IL-7, and similar to CD8+ T cells, the population of P-STAT5- CD4+ T cells observed may also be due to a lack of IL-7Rα chain expression on these cells. However, additional patient samples will have to be included to strengthen this correlation. No such correlation was observed for IL-2Rα, β, γ_c and IL-15Rα expression (data not shown). As mentioned, IL-2Rβ and γc expression in CD4+ T cells was detectable as a single population of cells. Therefore, the presence of P-STAT5- and P-STAT5+ CD4+ T cells

Figure 12. Cytokine receptor expression in CD4+ T cells. PBMCs ($2x10^5$) were unstimulated and surface stained with different receptor chain-specific Abs. A total of 5000 events were acquired in the CD4+ cell gate and analyzed by flow cytometry. One HIV+ patient is represented. The expression of IL7Rα and IL-2Rα is represented in dot plots (A), and IL2Rβ, γ_c, IL15Rα are represented as histogram overlays showing cytokine receptor (thin line) and isotype control antibody (shaded histogram) staining (B).

A



B

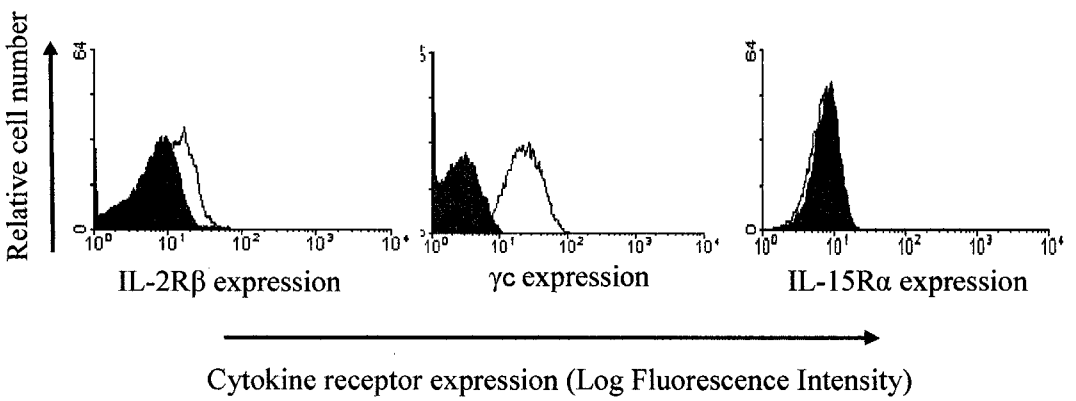
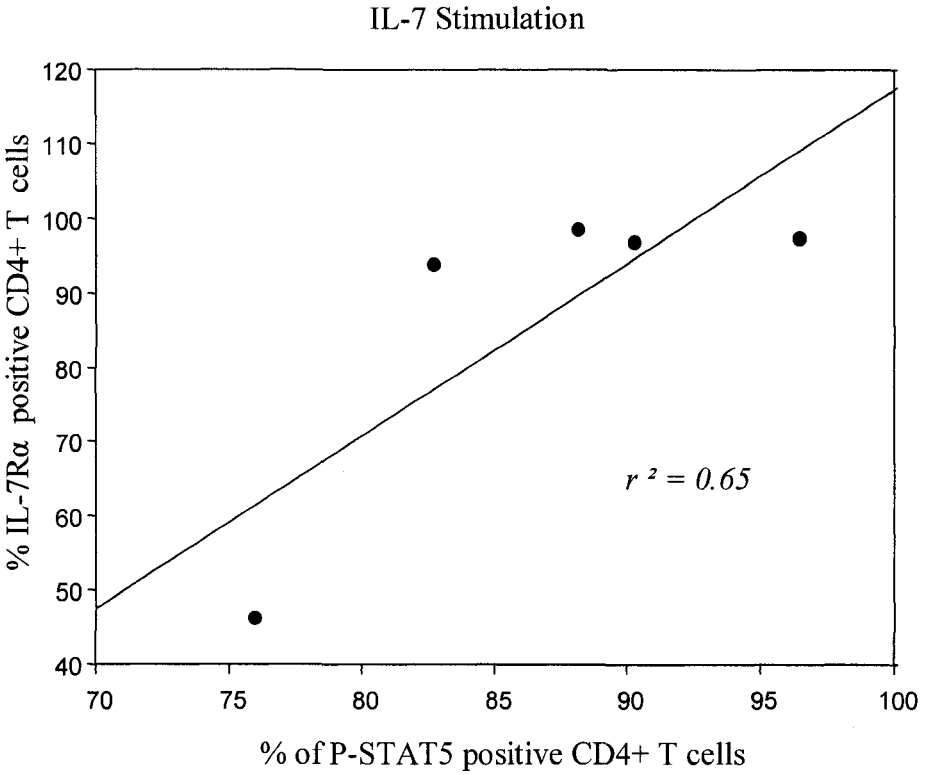


Figure 13. Plot of % IL-7Rα+ *vs* % P-STAT5+ CD4 T cells as evaluated by flow cytometry. The plot was analyzed by simple linear regression (Sigma Plot). r^2 = coefficient of determination



in response to IL-2 or IL-15 did not appear to be related, by this method, to the expression levels of IL-2Rα, IL-2Rβ, γc or IL-15Rα chains (Figure 12B and data not shown).

III.3.3 CD28 expression in P-STAT5- and P-STAT5+ CD8+ T cells

It was hypothesized that the P-STAT5- CD8+ T cells may represent anergic or senescent cells with skewed differentiation phenotype and impaired function. Senescence is a state in which cells remain metabolically active but are incapable of further proliferation (151). One feature identifying senescent CD8+ T cells is the lack of the CD28 co-stimulatory molecule, expression of CD57 (37,152-154), and the inability of these CD8+ T cells to proliferate in response to antigen (155,156). Therefore, as a first step to evaluate whether P-STAT5- cells represent replicatively senescent cells, CD28 expression was measured on these cells from a subset of patients for which samples were available. PBMCs from HIV+ patients ($4x10^5$) were stimulated with IL-2 (34 ng/mL) for 15 min, followed by staining with anti-CD28, CD8 and P-STAT5 Abs. Flow cytometric analysis revealed that CD28+ and CD28- cells could both be found within the STAT5+ as well as the STAT5- cell gates (figure 14A) The % CD28- cells within the P-STAT5- and P-STAT5+ cell gates was compared in a total of 4 patients. There was no significant difference observed. (Figure 14B).

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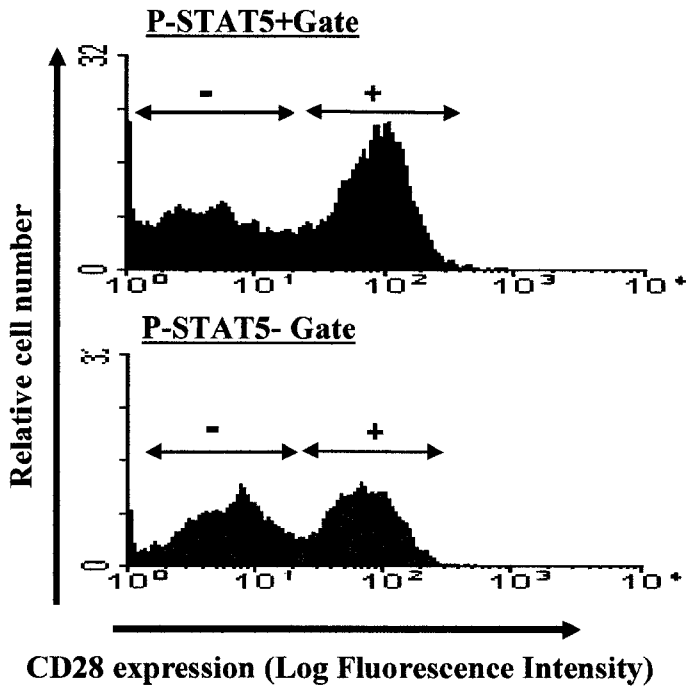
Figure 14. CD28 expression in P-STAT5+ and P-STAT5- CD8+ T cells from HIV-infected patients. PBMCs were stimulated for 15 minutes with IL-2 (32 ng/ml) and stained with CD8, P-STAT5 and CD28-specific Abs and subsequently analyzed by flow cytometry. A total of 5000 events were acquired in the CD8+ cell gate. A histogram plot is shown for the P-STAT5- and P-STAT5+ gate of CD8+ T cells in one patient (A) The % of CD28- cells detected within the P-STAT5- and P-STAT5+ CD8+ T cell gate was graphed for each patient. Each symbol represents a different patient (B). The horizontal bar represents the mean and is plotted along with standard deviation.

A



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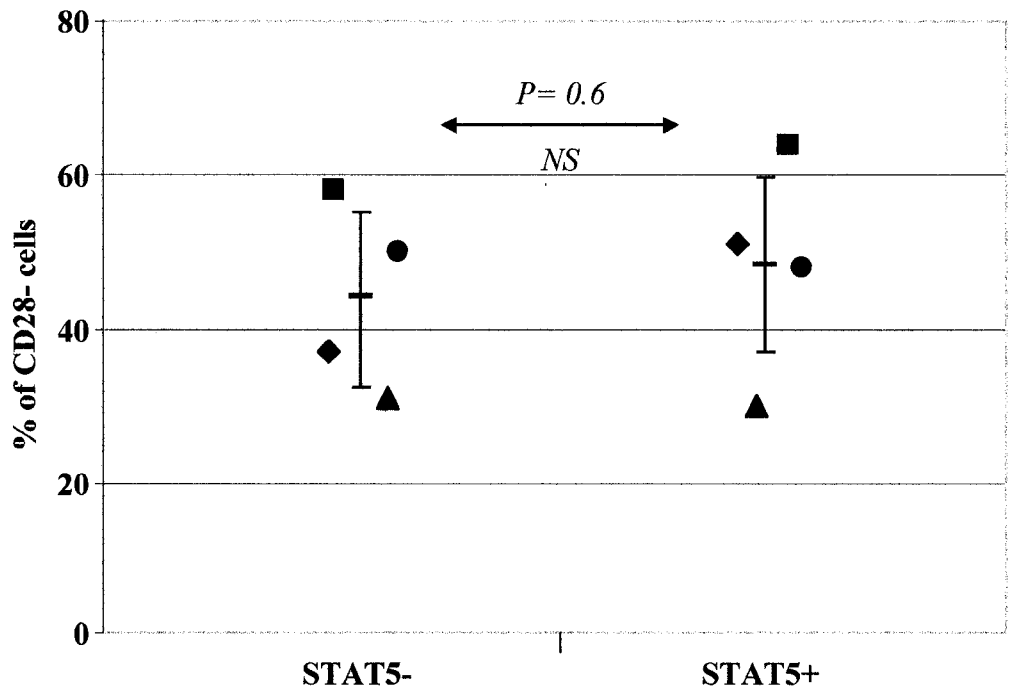

V. Discussion

The mechanisms by which HIV infection leads to immunodeficiency remains a critical unresolved issue (157). Despite mounting evidence that HIV-specific CTL are critical for suppressing HIV viral load, eventually CTL responses decline and lose control of virus replication, resulting in the progression to AIDS in almost all HIV+ patients (19). Due to the progressive HIV-induced CD4+ T cell depletion and resulting diminution of T cell help, as well as chronic antigenic stimulation, CD8+ T cells are thought to degenerate into a pre-apoptotic state characterized by an altered differentiation pattern and impaired functionality (76). Perturbations in cytokine signaling may have an important role to play in these defects (15,79). Previous studies have demonstrated deficiencies within the IL-2 system, including poor reactivity to this cytokine in T cells (78). CD4+ and CD8+ T cells derived from a subset of patients with high virus load failed to enter the cell cycle in response to higher or equivalent expression of IL-2 receptor components (α, β, γ) compared to HIV- controls (78). Under HAART, patient CD8+ and CD4+ T cells exhibited a strong capacity to enter the cell cycle in response to IL-2. The potential defects that arise in response to cytokines critical in their growth and differentiation, particularly those sharing the common γ_c chain receptor (IL-2, IL-15, IL-7, and IL-4) as well as IL-10 were the focus of this study. Specifically, the capacity of these cytokines to mobilize the Jak/STAT pathway, central to cytokine signaling, in CD4+ and CD8+ T cells derived from groups of patients off therapy and those on sustained HAART, was evaluated. Finally, the anomalies in P-STAT5 activation observed in response to IL-2,

IL-15, and IL-7 were pursued further to gain insight into the nature of these cells and potential molecular mechanisms involved in their generation.

In order to perform these studies, the difficulties associated with the application of standard biochemical techniques to rare PBMC sub-populations had to be circumvented. A flow cytometric approach adapated from *Krutzik et al.,* (148) was optimized and used to detect the rapid cytokine dependent intracellular STAT activation. In CD8+ T cells, IL-2 and IL-15 stimulation revealed a population unable to respond properly (P-STAT5-), which was not detected in HIV- subjects. This P-STAT5- population was observed in the CD8+ T cells from all HIV+ patients studied in response to IL-2 and IL-15, representing 24%, and 21%, respectively, of total CD8+ T cells on average. In response to IL-7, P-STAT5- cells were observed in both HIV+ and HIV- patients. However, their proportion was higher in HIV+ patients (45%) than in HIV- subjects (15%). Interestingly, similar to the bulk polyclonal CD8+ T cells, P-STAT5+ and P-STAT5- cells were detected in HIV+ patient CTLs specific for HIV nef and EBV BZLF1 epitopes. This data further supports the fact that chronic HIV infection leads to alterations in the activation and differentiation of CD8+ T lymphocytes (37). Altered cytokine responsiveness may be involved in this skewed maturation and impaired function of CD8+ T lymphocytes from HIV+ patients. It also points to a potentially novel mechanism by which defects in HIV+ patient CTLs arise. The underlying cause responsible for the generation of P-STAT5- CD8+ T cells in response to IL-2 and IL-15 is not understood. There are a number of plausible hypotheses which could explain the appearance of these cells. One of the potential mechanisms for the existence of P-STAT5- CD8+ T cells was the lack of cytokine receptor expression. IL-2Rα, β, γ_c, IL-15Rα and IL-7Rα chains were investigated in this

regard. In the case of IL-2 and IL-15 stimulation, no correlation was found between IL-2Rα, β, γ_c, IL-15Rα chain expression and the existence of P-STAT5- CD8+ T cells. This suggested that the appearance of P-STAT5- CD8+ T cells may involve an impairment of signaling molecules downstream of receptor expression, such as Jak-3 and/or Jak-1 known to mediate STAT5 activation. In support of this, IL-2 was unable to activate STAT5 in CD8+ T cells from a subset of untreated HIV+ patients (75). This defect was not the result of alterations in IL-2R expression but correlated with an impaired activation of the upstream kinase Jak-3. The activation status of Jak-1 and -3 kinase remains to be investigated in the P-STAT5- CD8+ T cells detected. Another possibility is that STAT5 activation may be downregulated by a reduction in the transcription or translation of the STAT5 gene. This possibility also remain to be investigated but could for example involve the methylation of STAT5A and STAT5B promoters, which has been shown by others to down regulate STAT5 transcription (158).

The P-STAT5- CD8+ T cells revealed after stimulation with IL-7 appear to be associated with a lack of IL-7Rα chain expression in these cells. These findings appear to be concordant with other studies. *Macpherson et al.* (115) have shown that IL-7Rα expression is diminished in CD8+ T lymphocytes from untreated HIV+ patients but returned towards that of normal controls in patients undergoing HAART, correlating inversely with virus load. *Vingerhoets* et al, (116) also suggested that sensitivity to IL-7 is reduced in both CD8+ and CD4+ T cells from HIV+ patients. Furthermore, a research study by *Paiardini et al,* (159) has demonstrated that in HIV+ patients with high viral load, an IL-7Rα- CD8+ T cell population with an effector phenotype is expanded. The

question of how IL-7Rα is down regulated is still unresolved but may involve transcriptional and/or post transcriptional regulatory mechanisms (160,161)

One of the characteristic features associated with the chronic state of immune activation observed in HIV disease is a high proportion of replicatively senescent and anergic T cells. Therefore, it was initially hypothesized that the P-STAT5- CD8+ T cells observed may represent such senescent or anergic cells. A senescent cell is a state in which cells remain metabolically active but are incapable of further proliferation (151). Replicative senescence seems to be distinct from anergy, a state of antigenic unresponsiveness induced in the absence of secondary co-stimulatory signals, despite the similar inability of both cell types to secrete IL-2 (155). The phenotypes associated with replicatively senescent CD8+ T cells in HIV infection are not well defined but have been associated with a lack of CD28, increased CD57 expression as well as shortening of telomeres (37,152-154,162,163). In one study, sequential samples from several individuals demonstrated progressive CD8+ T cell telomere shortening over time (163). In another study, the CD8+ T cells were further sorted, permitting the demonstration that within the CD8+ subset, all of the telomere shortening could be accounted for by the cells that lacked CD28 expression (162). A common phenotypic feature between anergic and senescent cells is the lack of CD28 surface expression (156,164). Therefore, CD28 expression in P-STAT5+ and P-STAT5- cells in response to IL-2 in HIV+ patients was evaluated. However, both CD28+ and CD28- cells could be detected within the P-STAT5- cell gate. This did not appear to support the hypothesis that P-STAT5- cells were senescent cells as defined by CD28 expression at least in response to IL-2. It is possible that these cells may not be classical senescent cells or be at some intermediate

stage towards senescence. In this regard, studies to characterize these cells further at the level of CD57 expression, telomere length and whether P-STAT5- cells are more prone to undergo apoptosis are warranted. Interestingly, these cells maintained their responsiveness to IL-10 and IL-4 at the level of STAT activation. This may be what maintains these cells in the periphery.

An intriguing and somewhat unexpected result of the present study was that in the case of IL-2 and IL-15 stimulation, HAART did not appear to have any impact on the generation of P-STAT5-negative CD8+ T cells. Upon initial consideration, it did not appeared that these observations were only partially consistent with previous work by *Kryworuchko et al.* (75) However, in the previous study, standard molecular techniques such as western blot and EMSA were used to detect STAT5 activation. These assays detect an "averaged" signal for the entire purified CD8+ T cell population. Unlike flow cytometry, these assays do not permit an effective stratification of the signal from sub-populations of cells. Furthermore, the patients studied here were somewhat different. In this study, patients off therapy for > 6months were compared to those on sustained HAART, while in the paper by *Kryworuchko et al.* (75) patients completely naïve to therapy were compared. It is possible that patients off therapy in our study have a substantially reduced proportion of IL-2 and IL-15-dependent P-STAT5-negative CD8+ T cells compared to patients completely naïve to therapy. This lead us to hypothesize that the detection of P-STAT5-negative cells (representing 24 % and 21 % of total CD8+ T cells, respectively) may not have been possible by the standard molecular methods employed by *Kryworuchko et al,* previously (75). This is consistent with the fact that only

a sub-population of patients naïve to therapy (6/11) had a detectable impairment in STAT5 activation by Western blotting and EMSA in the previous study. This possibility would require further substantiation by performing similar flow cytometric investigations in patients completely naïve to therapy. As mentioned, IL-7-induced STAT5 activation appeared to be a function of IL-7Rα expression and appeared to return towards that of HIV- controls under HAART.

In CD4+ T cells a very different P-STAT5 expression pattern was observed. Although IL-2 and IL-15 clearly induced P-STAT5 in CD4+ T cells, there was a clear separation of cells into P-STAT5- and P-STAT5+ populations to similar proportions in both HIV- and HIV+ patients. A similar stratification was observed in response to IL-7 but the proportion of P-STAT5- cells was significantly higher in HIV+ patients (17%) compared to HIV- controls (6%). In parallel, the cytokine receptor expression of the three IL-2 receptor chains (α, β, γ_c), IL-15Rα and IL-7Rα was determined. A small proportion of CD4+ T cells expressed IL2Rα chain in both HIV+ and HIV- patients. These cells most likely represent IL2Rα+ CD4+ regulatory T cells. Like CD8+ T cells, IL-15Rα chain was undetectable and no significant differences were observed at the level of IL-2Rβ, and the γ_c chains. However, in the case of IL-7 stimulation and similar to CD8+ T cells it appeared that P-STAT5- cells were likely present due to a concomitant lack of IL-7Rα chain expression. However, additional HIV+ samples need to be included to strengthen this conclusion. Preliminary investigations in HIV- controls aimed at the characterization of these two populations of CD4+ T cells yielded promising results. These cells may reflect their naïve (CD4+ CD45RA+) and memory cell (CD4+

CD45RO+) phenotypes. Specifically, *Sant, N. et al,* (147) showed that the majority of CD4+ CD45RO+ T cells activated STAT5 in response to both IL-2 and IL-15 stimulation. However, the P-STAT5- cells were found only in the CD4+ CD45RA+ T cell gate.

Also interesting was the observation that despite the fact that total CD4+ T cell counts were higher, HAART did not appear to significantly modulate the proportion of P-STAT5- and P-STAT5+ CD4+ T cells in response to IL-2, IL-15 or IL-7. Without more detailed investigations into the phenotype of these cells in HIV+ patients (naïve *vs* memory *vs* effector) it is difficult to speculate further as to the reason for this.

In conclusion, this is the first study in HIV+ patients to report a stratification of CD4+ and CD8+ T cells into P-STAT5+ and P-STAT5- populations in response to IL-2, -7, and -15 cytokine stimulation. Understanding, the mechanisms responsible for the generation of P-STAT5 negative CD8+ T cells in HIV+ patients, will further our understanding of how HIV infection may be able to overwhelm cellular immune responses and may provide important information leading the reversal of these effects.

VI References

1. Chaplin, D. D. (2003). 1. Overview of the immune response *J. Allergy Clin. Immunol.* **111,** S442-S459

2. Delves, P. J. and Roitt, I. M. (2000). The immune system. Second of two parts *N. Engl. J. Med.* **343,** 108-117

3. Delves, P. J. and Roitt, I. M. (2000). The immune system. First of two parts *N. Engl. J. Med.* **343,** 37-49

4. Medzhitov, R. and Janeway, C., Jr. (2000). Innate immunity *N. Engl. J. Med.* **343,** 338-344

5. Biron, C. A., Byron, K. S., and Sullivan, J. L. (1989). Severe herpesvirus infections in an adolescent without natural killer cells *N. Engl. J. Med.* **320,** 1731-1735

6. Janeway, C. A. et al. 2001. Immunobiology 5. The Immune System in Health and Disease. Chapter 8. T Cell-Mediated Immunity. Garland Publishing. New York, NY.

7. Filion L. (2004) Introduction To The Immune system. Basic Immunology Class MIC5124. Biochemistry, Microbiology and Immunology Program. Ottawa University, Ottawa, ON, Canada.

8. Whitmire, J. K. and Ahmed, R. (2000). Costimulation in antiviral immunity: differential requirements for CD4(+) and CD8(+) T cell responses *Curr. Opin. Immunol.* **12,** 448-455

9. Henkart, P. A. and Catalfamo, M. (2004). CD8+ effector cells *Adv. Immunol.* **83,** 233-252

10. Slowik, M. R., Min, W., Ardito, T., Karsan, A., Kashgarian, M., and Pober, J. S. (1997). Evidence that tumor necrosis factor triggers apoptosis in human endothelial cells by interleukin-1-converting enzyme-like protease-dependent and -independent pathways *Lab Invest* **77,** 257-267

11. Yang, O. O. and Walker, B. D. (1997). CD8+ cells in human immunodeficiency virus type I pathogenesis: cytolytic and noncytolytic inhibition of viral replication *Adv. Immunol.* **66,** 273-311

12. Deng, Y., Yewdell, J. W., Eisenlohr, L. C., and Bennink, J. R. (1997). MHC affinity, peptide liberation, T cell repertoire, and immunodominance all contribute to the paucity of MHC class I-restricted peptides recognized by antiviral CTL *J. Immunol.* **158,** 1507-1515

13. Andersen, M. H., Schrama, D., Thor, S. P., and Becker, J. C. (2006). Cytotoxic T cells *J. Invest Dermatol.* **126,** 32-41

14. Lucey, D. R., Clerici, M., and Shearer, G. M. (1996). Type 1 and type 2 cytokine dysregulation in human infectious, neoplastic, and inflammatory diseases *Clin. Microbiol. Rev.* **9,** 532-562

15. Kinter, A., Arthos, J., Cicala, C., and Fauci, A. S. (2000). Chemokines, cytokines and HIV: a complex network of interactions that influence HIV pathogenesis *Immunol. Rev.* **177,** 88-98

16. Thomson, B. J. and Dalgleish, A. G. (1988). Human retroviruses *Blood Rev.* **2,** 211-221

17. Pantaleo, G., Graziosi, C., Butini, L., Pizzo, P. A., Schnittman, S. M., Kotler, D. P., and Fauci, A. S. (1991). Lymphoid organs function as major reservoirs for human immunodeficiency virus *Proc. Natl. Acad. Sci. U. S. A* **88,** 9838-9842

18. Gulzar, N. and Copeland, K. F. (2004). CD8+ T-cells: function and response to HIV infection *Curr. HIV. Res.* **2,** 23-37

19. McMichael, A. J. and Rowland-Jones, S. L. (2001). Cellular immune responses to HIV *Nature* **410,** 980-987

20. The Global HIV/AIDS pandemic, 2006 *MMWR Morb. Mortal. Wkly. Rep.* **55,** 841-844

21. Luber, A. D. (2005). Genetic barriers to resistance and impact on clinical response *MedGenMed.* **7,** 69

22. Cooper, D. A., Gold, J., Maclean, P., Donovan, B., Finlayson, R., Barnes, T. G., Michelmore, H. M., Brooke, P., and Penny, R. (1985). Acute AIDS retrovirus infection. Definition of a clinical illness associated with seroconversion *Lancet* **1,** 537-540

23. Ho, D. D., Sarngadharan, M. G., Resnick, L., Dimarzoveronese, F., Rota, T. R., and Hirsch, M. S. (1985). Primary human T-lymphotropic virus type III infection *Ann. Intern. Med.* **103,** 880-883

24. Henrard, D. R., Daar, E., Farzadegan, H., Clark, S. J., Phillips, J., Shaw, G. M., and Busch, M. P. (1995). Virologic and immunologic characterization of symptomatic and asymptomatic primary HIV-1 infection *J. Acquir. Immune. Defic. Syndr. Hum. Retrovirol.* **9,** 305-310

25. Daar, E. S., Moudgil, T., Meyer, R. D., and Ho, D. D. (1991). Transient high levels of viremia in patients with primary human immunodeficiency virus type 1 infection *N. Engl. J. Med.* **324,** 961-964

26. Clark, S. J., Saag, M. S., Decker, W. D., Campbell-Hill, S., Roberson, J. L., Veldkamp, P. J., Kappes, J. C., Hahn, B. H., and Shaw, G. M. (1991). High titers of cytopathic virus in plasma of patients with symptomatic primary HIV-1 infection *N. Engl. J. Med.* **324,** 954-960

27. Schacker, T. W., Hughes, J. P., Shea, T., Coombs, R. W., and Corey, L. (1998). Biological and virologic characteristics of primary HIV infection *Ann. Intern. Med.* **128,** 613-620

28. Lyles, R. H., Munoz, A., Yamashita, T. E., Bazmi, H., Detels, R., Rinaldo, C. R., Margolick, J. B., Phair, J. P., and Mellors, J. W. (2000). Natural history of human immunodeficiency virus type 1 viremia after seroconversion and proximal to AIDS in a large cohort of homosexual men. Multicenter AIDS Cohort Study *J. Infect. Dis.* **181,** 872-880

29. Gandhi, R. T. and Walker, B. D. (2002). Immunologic control of HIV-1 *Annu. Rev. Med.* **53,** 149-172

30. Wolthers, K. C. and Miedema, F. (1998). Telomeres and HIV-1 infection: in search of exhaustion *Trends Microbiol.* **6,** 144-147

31. Esser, M. T., Bess, J. W., Jr., Suryanarayana, K., Chertova, E., Marti, D., Carrington, M., Arthur, L. O., and Lifson, J. D. (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

32. Kryworuchko, M., Pasquier, V., and Theze, J. (2003). Human immunodeficiency virus-1 envelope glycoproteins and anti-CD4 antibodies inhibit interleukin-2-induced Jak/STAT signalling in human CD4 T lymphocytes *Clin. Exp. Immunol.* **131,** 422-427

33. Napolitano, L. A., Grant, R. M., Deeks, S. G., Schmidt, D., De Rosa, S. C., Herzenberg, L. A., Herndier, B. G., Andersson, J., and McCune, J. M. (2001).

Increased production of IL-7 accompanies HIV-1-mediated T-cell depletion: implications for T-cell homeostasis *Nat. Med.* **7,** 73-79

34. Appay, V., Nixon, D. F., Donahoe, S. M., Gillespie, G. M., Dong, T., King, A., Ogg, G. S., Spiegel, H. M., Conlon, C., Spina, C. A., Havlir, D. V., Richman, D. D., Waters, A., Easterbrook, P., McMichael, A. J., and Rowland-Jones, S. L. (2000). HIV-specific CD8(+) T cells produce antiviral cytokines but are impaired in cytolytic function *J. Exp. Med.* **192,** 63-75

35. Appay, V., Papagno, L., Spina, C. A., Hansasuta, P., King, A., Jones, L., Ogg, G. S., Little, S., McMichael, A. J., Richman, D. D., and Rowland-Jones, S. L. (2002). Dynamics of T cell responses in HIV infection *J. Immunol.* **168,** 3660-3666

36. Migueles, S. A., Laborico, A. C., Shupert, W. L., Sabbaghian, M. S., Rabin, R., Hallahan, C. W., Van, B. D., Kostense, S., Miedema, F., McLaughlin, M., Ehler, L., Metcalf, J., Liu, S., and Connors, M. (2002). HIV-specific CD8+ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors *Nat. Immunol.* **3,** 1061-1068

37. Champagne, P., Ogg, G. S., King, A. S., Knabenhans, C., Ellefsen, K., Nobile, M., Appay, V., Rizzardi, G. P., Fleury, S., Lipp, M., Forster, R., Rowland-Jones, S., Sekaly, R. P., McMichael, A. J., and Pantaleo, G. (2001). Skewed maturation of memory HIV-specific CD8 T lymphocytes *Nature* **410,** 106-111

38. Appay, V., Dunbar, P. R., Callan, M., Klenerman, P., Gillespie, G. M., Papagno, L., Ogg, G. S., King, A., Lechner, F., Spina, C. A., Little, S., Havlir, D. V., Richman, D. D., Gruener, N., Pape, G., Waters, A., Easterbrook, P., Salio, M., Cerundolo, V., McMichael, A. J., and Rowland-Jones, S. L. (2002). Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections *Nat. Med.* **8,** 379-385

39. Lichterfeld, M., Kaufmann, D. E., Yu, X. G., Mui, S. K., Addo, M. M., Johnston, M. N., Cohen, D., Robbins, G. K., Pae, E., Alter, G., Wurcel, A., Stone, D., Rosenberg, E. S., Walker, B. D., and Altfeld, M. (2004). Loss of HIV-1-specific CD8+ T cell proliferation after acute HIV-1 infection and restoration by vaccine-induced HIV-1-specific CD4+ T cells *J. Exp. Med.* **200,** 701-712

40. Azad, A. A. (2000). Could Nef and Vpr proteins contribute to disease progression by promoting depletion of bystander cells and prolonged survival of HIV-infected cells? *Biochem. Biophys. Res. Commun.* **267,** 677-685

41. Janssen, E. M., Lemmens, E. E., Wolfe, T., Christen, U., von Herrath, M. G., and Schoenberger, S. P. (2003). CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes *Nature* **421,** 852-856

42. Bennett, S. R., Carbone, F. R., Karamalis, F., Miller, J. F., and Heath, W. R. (1997). Induction of a CD8+ cytotoxic T lymphocyte response by cross-priming requires cognate CD4+ T cell help *J. Exp. Med.* **186,** 65-70

43. Benito, J. M., Lopez, M., and Soriano, V. (2004). The role of CD8+ T-cell response in HIV infection *AIDS Rev.* **6,** 79-88

44. Walker, C. M., Moody, D. J., Stites, D. P., and Levy, J. A. (1986). CD8+ lymphocytes can control HIV infection in vitro by suppressing virus replication *Science* **234,** 1563-1566

45. Schmitz, J. E., Kuroda, M. J., Santra, S., Sasseville, V. G., Simon, M. A., Lifton, M. A., Racz, P., Tenner-Racz, K., Dalesandro, M., Scallon, B. J., Ghrayeb, J., Forman, M. A., Montefiori, D. C., Rieber, E. P., Letvin, N. L., and Reimann, K. A. (1999). Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes *Science* **283,** 857-860

46. Jin, X., Bauer, D. E., Tuttleton, S. E., Lewin, S., Gettie, A., Blanchard, J., Irwin, C. E., Safrit, J. T., Mittler, J., Weinberger, L., Kostrikis, L. G., Zhang, L., Perelson, A. S., and Ho, D. D. (1999). Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques *J. Exp. Med.* **189,** 991-998

47. McCune, J. M. (2001). The dynamics of CD4+ T-cell depletion in HIV disease *Nature* **410,** 974-979

48. Kalams, S. A., Buchbinder, S. P., Rosenberg, E. S., Billingsley, J. M., Colbert, D. S., Jones, N. G., Shea, A. K., Trocha, A. K., and Walker, B. D. (1999). Association between virus-specific cytotoxic T-lymphocyte and helper responses in human immunodeficiency virus type 1 infection *J. Virol.* **73,** 6715-6720

49. Andersson, J., Behbahani, H., Lieberman, J., Connick, E., Landay, A., Patterson, B., Sonnerborg, A., Lore, K., Uccini, S., and Fehniger, T. E. (1999). Perforin is not co-expressed with granzyme A within cytotoxic granules in CD8 T lymphocytes present in lymphoid tissue during chronic HIV infection *AIDS* **13,** 1295-1303

50. Goepfert, P. A., Bansal, A., Edwards, B. H., Ritter, G. D., Jr., Tellez, I., McPherson, S. A., Sabbaj, S., and Mulligan, M. J. (2000). A significant number of human immunodeficiency virus epitope-specific cytotoxic T lymphocytes detected by tetramer binding do not produce gamma interferon *J. Virol.* **74,** 10249-10255

51. Kostense, S., Vandenberghe, K., Joling, J., Van, B. D., Nanlohy, N., Manting, E., and Miedema, F. (2002). Persistent numbers of tetramer+ CD8(+) T cells, but loss of interferon-gamma+ HIV-specific T cells during progression to AIDS *Blood* **99,** 2505-2511

52. Moore, C. B., John, M., James, I. R., Christiansen, F. T., Witt, C. S., and Mallal, S. A. (2002). Evidence of HIV-1 adaptation to HLA-restricted immune responses at a population level *Science* **296,** 1439-1443

53. Borrow, P. and Shaw, G. M. (1998). Cytotoxic T-lymphocyte escape viral variants: how important are they in viral evasion of immune clearance in vivo? *Immunol. Rev.* **164,** 37-51

54. Price, D. A., Goulder, P. J., Klenerman, P., Sewell, A. K., Easterbrook, P. J., Troop, M., Bangham, C. R., and Phillips, R. E. (1997). Positive selection of HIV-1 cytotoxic T lymphocyte escape variants during primary infection *Proc. Natl. Acad. Sci. U. S. A* **94,** 1890-1895

55. Goulder, P. J., Phillips, R. E., Colbert, R. A., McAdam, S., Ogg, G., Nowak, M. A., Giangrande, P., Luzzi, G., Morgan, B., Edwards, A., McMichael, A. J., and Rowland-Jones, S. (1997). Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS *Nat. Med.* **3,** 212-217

56. Collins, K. L., Chen, B. K., Kalams, S. A., Walker, B. D., and Baltimore, D. (1998). HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes *Nature* **391,** 397-401

57. Collins, K. L. and Baltimore, D. (1999). HIV's evasion of the cellular immune response *Immunol. Rev.* **168,** 65-74

58. Weyand, C. M., Brandes, J. C., Schmidt, D., Fulbright, J. W., and Goronzy, J. J. (1998). Functional properties of CD4+ *Mech. Ageing Dev.* **102,** 131-147

59. Moanna, A., Dunham, R., Paiardini, M., and Silvestri, G. (2005). CD4+ T-cell depletion in HIV infection: killed by friendly fire? *Curr. HIV. /AIDS Rep.* **2,** 16-23

60. Arthos, J., Cicala, C., Selig, S. M., White, A. A., Ravindranath, H. M., Van, R. D., Steenbeke, T. D., Machado, E., Khazanie, P., Hanback, M. S., Hanback, D. B., Rabin, R. L., and Fauci, A. S. (2002). The role of the CD4 receptor versus HIV coreceptors in envelope-mediated apoptosis in peripheral blood mononuclear cells *Virology* **292,** 98-106

61. Bartz, S. R. and Emerman, M. (1999). Human immunodeficiency virus type 1 Tat induces apoptosis and increases sensitivity to apoptotic signals by up-regulating FLICE/caspase-8 *J. Virol.* **73,** 1956-1963

62. Clerici, M. and Shearer, G. M. (1993). A TH1-->TH2 switch is a critical step in the etiology of HIV infection *Immunol. Today* **14,** 107-111

63. Romagnani, S., Maggi, E., and Del, P. G. (1994). An alternative view of the Th1/Th2 switch hypothesis in HIV infection *AIDS Res. Hum. Retroviruses* **10,** iii-iix

64. Romagnani, S., Parronchi, P., D'Elios, M. M., Romagnani, P., Annunziato, F., Piccinni, M. P., Manetti, R., Sampognaro, S., Mavilia, C., De, C. M., Maggi, E., and Del Prete, G. F. (1997). An update on human Th1 and Th2 cells *Int. Arch. Allergy Immunol.* **113,** 153-156

65. Kumar, A., Angel, J. B., Daftarian, M. P., Parato, K., Cameron, W. D., Filion, L., and Diaz-Mitoma, F. (1998). Differential production of IL-10 by T cells and monocytes of HIV-infected individuals: association of IL-10 production with CD28-mediated immune responsiveness *Clin. Exp. Immunol.* **114,** 78-86

66. Diaz-Mitoma, F., Kumar, A., Karimi, S., Kryworuchko, M., Daftarian, M. P., Creery, W. D., Filion, L. G., and Cameron, W. (1995). Expression of IL-10, IL-4 and interferon-gamma in unstimulated and mitogen-stimulated peripheral blood lymphocytes from HIV-seropositive patients *Clin. Exp. Immunol.* **102,** 31-39

67. Clerici, M., Sarin, A., Berzofsky, J. A., Landay, A. L., Kessler, H. A., Hashemi, F., Hendrix, C. W., Blatt, S. P., Rusnak, J., Dolan, M. J., Coffman, R. L., Henkart, P. A., and Shearer, G. M. (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

68. Ostrowski, S. R., Gerstoft, J., Pedersen, B. K., and Ullum, H. (2003). Impaired production of cytokines is an independent predictor of mortality in HIV-1-infected patients *AIDS* **17,** 521-530

69. Imami, N., Pires, A., Hardy, G., Wilson, J., Gazzard, B., and Gotch, F. (2002). A balanced type 1/type 2 response is associated with long-term nonprogressive human immunodeficiency virus type 1 infection *J. Virol.* **76,** 9011-9023

70. Maloy, K. J., Burkhart, C., Junt, T. M., Odermatt, B., Oxenius, A., Piali, L., Zinkernagel, R. M., and Hengartner, H. (2000). CD4(+) T cell subsets during virus infection. Protective capacity depends on effector cytokine secretion and on migratory capability *J. Exp. Med.* **191,** 2159-2170

71. Matsuzaki, J., Tsuji, T., Imazeki, I., Ikeda, H., and Nishimura, T. (2005). Immunosteroid as a regulator for Th1/Th2 balance: its possible role in autoimmune diseases *Autoimmunity* **38,** 369-375

72. Assudani, D. P., Horton, R. B., Mathieu, M. G., McArdle, S. E., and Rees, R. C. (2006). The role of CD4+ T cell help in cancer immunity and the formulation of novel cancer vaccines *Cancer Immunol. Immunother.*

73. Ostrowski, M. A., Justement, S. J., Ehler, L., Mizell, S. B., Lui, S., Mican, J., Walker, B. D., Thomas, E. K., Seder, R., and Fauci, A. S. (2000). The role of CD4+ T cell help and CD40 ligand in the in vitro expansion of HIV-1-specific memory cytotoxic CD8+ T cell responses *J. Immunol.* **165,** 6133-6141

74. Kalams, S. A. and Walker, B. D. (1998). The critical need for CD4 help in maintaining effective cytotoxic T lymphocyte responses *J. Exp. Med.* **188,** 2199-2204

75. Kryworuchko, M., Pasquier, V., Keller, H., David, D., Goujard, C., Gilquin, J., Viard, J. P., Joussemet, M., Delfraissy, J. F., and Theze, J. (2004). Defective interleukin-2-dependent STAT5 signalling in CD8 T lymphocytes from HIV-positive patients: restoration by antiretroviral therapy *AIDS* **18,** 421-426

76. Lieberman, J., Shankar, P., Manjunath, N., and Andersson, J. (2001). Dressed to kill? A review of why antiviral CD8 T lymphocytes fail to prevent progressive immunodeficiency in HIV-1 infection *Blood* **98,** 1667-1677

77. Shankar, P., Russo, M., Harnisch, B., Patterson, M., Skolnik, P., and Lieberman, J. (2000). Impaired function of circulating HIV-specific CD8(+) T cells in chronic human immunodeficiency virus infection *Blood* **96,** 3094-3101

78. David, D., Bani, L., Moreau, J. L., Treilhou, M. P., Nakarai, T., Joussemet, M., Ritz, J., Dupont, B., Pialoux, G., and Theze, J. (1998). Regulatory dysfunction of the interleukin-2 receptor during HIV infection and the impact of triple combination therapy *Proc. Natl. Acad. Sci. U. S. A* **95,** 11348-11353

79. Alfano, M. and Poli, G. (2002). The cytokine network in HIV infection *Curr. Mol. Med.* **2,** 677-689

80. Alfano, M. and Poli, G. (2001). Cytokine and chemokine based control of HIV infection and replication *Curr. Pharm. Des* **7,** 993-1013

81. Geginat, J., Lanzavecchia, A., and Sallusto, F. (2003). Proliferation and differentiation potential of human CD8+ memory T-cell subsets in response to antigen or homeostatic cytokines *Blood* **101,** 4260-4266

82. Schluns, K. S. and Lefrancois, L. (2003). Cytokine control of memory T-cell development and survival *Nat. Rev. Immunol.* **3,** 269-279

83. O'Connell, M. J. and McInerney, J. O. (2005). Gamma chain receptor interleukins: evidence for positive selection driving the evolution of cell-to-cell communicators in the mammalian immune system *J. Mol. Evol.* **61,** 608-619

84. Candotti, F., O'Shea, J. J., and Villa, A. (1998). Severe combined immune deficiencies due to defects of the common gamma chain-JAK3 signaling pathway *Springer Semin. Immunopathol.* **19,** 401-415

85. Smith, K. A. (1988). Interleukin-2: inception, impact, and implications *Science* **240,** 1169-1176

86. Minami, Y., Kono, T., Miyazaki, T., and Taniguchi, T. (1993). The IL-2 receptor complex: its structure, function, and target genes *Annu. Rev. Immunol.* **11,** 245-268

87. Bosco, M. C., Espinoza-Delgado, I., Rowe, T. K., Malabarba, M. G., Longo, D. L., and Varesio, L. (1997). Functional role for the myeloid differentiation antigen CD14 in the activation of human monocytes by IL-2 *J. Immunol.* **159,** 2922-2931

88. Jankovic, D. L., Rebollo, A., Kumar, A., Gibert, M., and Theze, J. (1990). IL-2-dependent proliferation of murine T cells requires expression of both the p55 and p70 subunits of the IL-2 receptor *J. Immunol.* **145,** 4136-4144

89. Kumar, A., Moreau, J. L., Gibert, M., and Theze, J. (1987). Internalization of interleukin 2 (IL-2) by high affinity IL-2 receptors is required for the growth of IL-2-dependent T cell lines *J. Immunol.* **139,** 3680-3684

90. David, D., Bani, L., Moreau, J. L., Demaison, C., Sun, K., Salvucci, O., Nakarai, T., de, M. M., Chouaib, S., Joussemet, M., Ritz, J., and Theze, J. (1998). Further analysis of interleukin-2 receptor subunit expression on the different human peripheral blood mononuclear cell subsets *Blood* **91,** 165-172

91. Hauser, G. J., Bino, T., Rosenberg, H., Zakuth, V., Geller, E., and Spirer, Z. (1984). Interleukin-2 production and response to exogenous interleukin-2 in a patient with the acquired immune deficiency syndrome (AIDS) *Clin. Exp. Immunol.* **56,** 14-17

92. Iyasere, C., Tilton, J. C., Johnson, A. J., Younes, S., Yassine-Diab, B., Sekaly, R. P., Kwok, W. W., Migueles, S. A., Laborico, A. C., Shupert, W. L., Hallahan, C. W., Davey, R. T., Jr., Dybul, M., Vogel, S., Metcalf, J., and Connors, M. (2003). Diminished proliferation of human immunodeficiency virus-specific CD4+ T cells is associated with diminished interleukin-2 (IL-2) production and is recovered by exogenous IL-2 *J. Virol.* **77,** 10900-10909

93. Younes, S. A., Yassine-Diab, B., Dumont, A. R., Boulassel, M. R., Grossman, Z., Routy, J. P., and Sekaly, R. P. (2003). HIV-1 viremia prevents the establishment of interleukin 2-producing HIV-specific memory CD4+ T cells endowed with proliferative capacity *J. Exp. Med.* **198,** 1909-1922

94. Sieg, S. F., Bazdar, D. A., Harding, C. V., and Lederman, M. M. (2001). Differential expression of interleukin-2 and gamma interferon in human immunodeficiency virus disease *J. Virol.* **75,** 9983-9985

95. Sousa, A. E., Chaves, A. F., Loureiro, A., and Victorino, R. M. (2001). Comparison of the frequency of interleukin (IL)-2-, interferon-gamma-, and IL-4-producing T cells in 2 diseases, human immunodeficiency virus types 1 and 2, with distinct clinical outcomes *J. Infect. Dis.* **184,** 552-559

96. Boaz, M. J., Waters, A., Murad, S., Easterbrook, P. J., and Vyakarnam, A. (2002). Presence of HIV-1 Gag-specific IFN-gamma+IL-2+ and CD28+IL-2+ CD4 T cell responses is associated with nonprogression in HIV-1 infection *J. Immunol.* **169,** 6376-6385

97. Waldmann, T. A. (2006). The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design *Nat. Rev. Immunol.* **6,** 595-601

98. Zhang, X., Sun, S., Hwang, I., Tough, D. F., and Sprent, J. (1998). Potent and selective stimulation of memory-phenotype CD8+ T cells in vivo by IL-15 *Immunity.* **8,** 591-599

99. Tanchot, C., Lemonnier, F. A., Perarnau, B., Freitas, A. A., and Rocha, B. (1997). Differential requirements for survival and proliferation of CD8 naive or memory T cells *Science* **276,** 2057-2062

100. Kennedy, M. K., Glaccum, M., Brown, S. N., Butz, E. A., Viney, J. L., Embers, M., Matsuki, N., Charrier, K., Sedger, L., Willis, C. R., Brasel, K., Morrissey, P. J., Stocking, K., Schuh, J. C., Joyce, S., and Peschon, J. J. (2000). Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice *J. Exp. Med.* **191,** 771-780

101. Liu, K., Catalfamo, M., Li, Y., Henkart, P. A., and Weng, N. P. (2002). IL-15 mimics T cell receptor crosslinking in the induction of cellular proliferation, gene expression, and cytotoxicity in CD8+ memory T cells *Proc. Natl. Acad. Sci. U. S. A* **99,** 6192-6197

102. Vella, A. T., Dow, S., Potter, T. A., Kappler, J., and Marrack, P. (1998). Cytokine-induced survival of activated T cells in vitro and in vivo *Proc. Natl. Acad. Sci. U. S. A* **95,** 3810-3815

103. d'Ettorre, G., Forcina, G., Lichtner, M., Mengoni, F., D'Agostino, C., Massetti, A. P., Mastroianni, C. M., and Vullo, V. (2002). Interleukin-15 in HIV infection: immunological and virological interactions in antiretroviral-naive and -treated patients *AIDS* **16,** 181-188

104. Kanai, T., Thomas, E. K., Yasutomi, Y., and Letvin, N. L. (1996). IL-15 stimulates the expansion of AIDS virus-specific CTL *J. Immunol.* **157,** 3681-3687

105. Chehimi, J., Marshall, J. D., Salvucci, O., Frank, I., Chehimi, S., Kawecki, S., Bacheller, D., Rifat, S., and Chouaib, S. (1997). IL-15 enhances immune functions during HIV infection *J. Immunol.* **158,** 5978-5987

106. Mueller, Y. M., Bojczuk, P. M., Halstead, E. S., Kim, A. H., Witek, J., Altman, J. D., and Katsikis, P. D. (2003). IL-15 enhances survival and function of HIV-specific CD8+ T cells *Blood* **101,** 1024-1029

107. Lee, S. K. and Surh, C. D. (2005). Role of interleukin-7 in bone and T-cell homeostasis *Immunol. Rev.* **208,** 169-180

108. Mastroianni, C. M., Forcina, G., d'Ettorre, G., Lichtner, M., Mengoni, F., D'Agostino, C., and Vullo, V. (2001). Circulating levels of interleukin-7 in antiretroviral-naive and highly active antiretroviral therapy-treated HIV-infected patients *HIV. Clin. Trials* **2,** 108-112

109. Morrissey, P. J., Goodwin, R. G., Nordan, R. P., Anderson, D., Grabstein, K. H., Cosman, D., Sims, J., Lupton, S., Acres, B., and Reed, S. G. (1989). Recombinant interleukin 7, pre-B cell growth factor, has costimulatory activity on purified mature T cells *J. Exp. Med.* **169,** 707-716

110. Okazaki, H., Ito, M., Sudo, T., Hattori, M., Kano, S., Katsura, Y., and Minato, N. (1989). IL-7 promotes thymocyte proliferation and maintains immunocompetent thymocytes bearing alpha beta or gamma delta T-cell receptors in vitro: synergism with IL-2 *J. Immunol.* **143,** 2917-2922

111. Kaech, S. M., Tan, J. T., Wherry, E. J., Konieczny, B. T., Surh, C. D., and Ahmed, R. (2003). Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells *Nat. Immunol.* **4,** 1191-1198

112. Lenz, D. C., Kurz, S. K., Lemmens, E., Schoenberger, S. P., Sprent, J., Oldstone, M. B., and Homann, D. (2004). IL-7 regulates basal homeostatic proliferation of antiviral CD4+T cell memory *Proc. Natl. Acad. Sci. U. S. A* **101,** 9357-9362

113. Li, J., Huston, G., and Swain, S. L. (2003). IL-7 promotes the transition of CD4 effectors to persistent memory cells *J. Exp. Med.* **198,** 1807-1815

114. Schluns, K. S., Kieper, W. C., Jameson, S. C., and Lefrancois, L. (2000). Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo *Nat. Immunol.* **1,** 426-432

115. MacPherson, P. A., Fex, C., Sanchez-Dardon, J., Hawley-Foss, N., and Angel, J. B. (2001). Interleukin-7 receptor expression on CD8(+) T cells is reduced in HIV infection and partially restored with effective antiretroviral therapy *J. Acquir. Immune. Defic. Syndr.* **28,** 454-457

116. Vingerhoets, J., Bisalinkumi, E., Penne, G., Colebunders, R., Bosmans, E., Kestens, L., and Vanham, G. (1998). Altered receptor expression and decreased sensitivity of T-cells to the stimulatory cytokines IL-2, IL-7 and IL-12 in HIV infection *Immunol. Lett.* **61,** 53-61

117. Rethi, B., Fluur, C., Atlas, A., Krzyzowska, M., Mowafi, F., Grutzmeier, S., De, M. A., Bellocco, R., Falk, K. I., Rajnavolgyi, E., and Chiodi, F. (2005). Loss of IL-7Ralpha is associated with CD4 T-cell depletion, high interleukin-7 levels and CD28 down-regulation in HIV infected patients *AIDS* **19,** 2077-2086

118. Moore, K. W., de Waal, M. R., Coffman, R. L., and O'Garra, A. (2001). Interleukin-10 and the interleukin-10 receptor *Annu. Rev. Immunol.* **19,** 683-765

119. Fujii, S., Shimizu, K., Shimizu, T., and Lotze, M. T. (2001). Interleukin-10 promotes the maintenance of antitumor CD8(+) T-cell effector function in situ *Blood* **98,** 2143-2151

120. Angel, J. B., Kumar, A., Parato, K., Filion, L. G., Diaz-Mitoma, F., Daftarian, P., Pham, B., Sun, E., Leonard, J. M., and Cameron, D. W. (1998). Improvement in cell-mediated immune function during potent anti-human immunodeficiency virus therapy with ritonavir plus saquinavir *J. Infect. Dis.* **177,** 898-904

121. Daftarian, M. P., Diaz-Mitoma, F., Creery, W. D., Cameron, W., and Kumar, A. (1995). Dysregulated production of interleukin-10 (IL-10) and IL-12 by peripheral blood lymphocytes from human immunodeficiency virus-infected individuals is associated with altered proliferative responses to recall antigens *Clin. Diagn. Lab Immunol.* **2,** 712-718

122. Landay, A. L., Clerici, M., Hashemi, F., Kessler, H., Berzofsky, J. A., and Shearer, G. M. (1996). In vitro restoration of T cell immune function in human immunodeficiency virus-positive persons: effects of interleukin (IL)-12 and anti-IL-10 *J. Infect. Dis.* **173,** 1085-1091

123. Clerici, M., Wynn, T. A., Berzofsky, J. A., Blatt, S. P., Hendrix, C. W., Sher, A., Coffman, R. L., and Shearer, G. M. (1994). Role of interleukin-10 in T helper cell dysfunction in asymptomatic individuals infected with the human immunodeficiency virus *J. Clin. Invest* **93,** 768-775

124. Clerici, M., Hakim, F. T., Venzon, D. J., Blatt, S., Hendrix, C. W., Wynn, T. A., and Shearer, G. M. (1993). Changes in interleukin-2 and interleukin-4 production in asymptomatic, human immunodeficiency virus-seropositive individuals *J. Clin. Invest* **91,** 759-765

125. Chehimi, J., Starr, S. E., Frank, I., D'Andrea, A., Ma, X., MacGregor, R. R., Sennelier, J., and Trinchieri, G. (1994). Impaired interleukin 12 production in human immunodeficiency virus-infected patients *J. Exp. Med.* **179,** 1361-1366

126. Taoufik, Y., Lantz, O., Wallon, C., Charles, A., Dussaix, E., and Delfraissy, J. F. (1997). Human immunodeficiency virus gp120 inhibits interleukin-12 secretion by human monocytes: an indirect interleukin-10-mediated effect *Blood* **89,** 2842-2848

127. Schols, D. and De, C. E. (1996). Human immunodeficiency virus type 1 gp120 induces anergy in human peripheral blood lymphocytes by inducing interleukin-10 production *J. Virol.* **70,** 4953-4960

128. Tepper, R. I., Pattengale, P. K., and Leder, P. (1989). Murine interleukin-4 displays potent anti-tumor activity in vivo *Cell* **57,** 503-512

129. Stanford, M. M. and McFadden, G. (2005). The 'supervirus'? Lessons from IL-4-expressing poxviruses *Trends Immunol.* **26,** 339-345

130. Okada, H. and Kuwashima, N. (2002). Gene therapy and biologic therapy with interleukin-4 *Curr. Gene Ther.* **2,** 437-450

131. Hock, H., Dorsch, M., Kunzendorf, U., Qin, Z., Diamantstein, T., and Blankenstein, T. (1993). Mechanisms of rejection induced by tumor cell-targeted gene transfer of interleukin 2, interleukin 4, interleukin 7, tumor necrosis factor, or interferon gamma *Proc. Natl. Acad. Sci. U. S. A* **90,** 2774-2778

132. Kienzle, N., Buttigieg, K., Groves, P., Kawula, T., and Kelso, A. (2002). A clonal culture system demonstrates that IL-4 induces a subpopulation of noncytolytic T cells with low CD8, perforin, and granzyme expression *J. Immunol.* **168,** 1672-1681

133. Valentin, A., Lu, W., Rosati, M., Schneider, R., Albert, J., Karlsson, A., and Pavlakis, G. N. (1998). Dual effect of interleukin 4 on HIV-1 expression: implications for viral phenotypic switch and disease progression *Proc. Natl. Acad. Sci. U. S. A* **95,** 8886-8891

134. Wang, J., Roderiquez, G., Oravecz, T., and Norcross, M. A. (1998). Cytokine regulation of human immunodeficiency virus type 1 entry and replication in human monocytes/macrophages through modulation of CCR5 expression *J. Virol.* **72,** 7642-7647

135. Wang, J., Harada, A., Matsushita, S., Matsumi, S., Zhang, Y., Shioda, T., Nagai, Y., and Matsushima, K. (1998). IL-4 and a glucocorticoid up-regulate CXCR4 expression on human CD4+ T lymphocytes and enhance HIV-1 replication *J. Leukoc. Biol.* **64,** 642-649

136. Zhu, T., Mo, H., Wang, N., Nam, D. S., Cao, Y., Koup, R. A., and Ho, D. D. (1993). Genotypic and phenotypic characterization of HIV-1 patients with primary infection *Science* **261,** 1179-1181

137. Schuitemaker, H., Koot, M., Kootstra, N. A., Dercksen, M. W., de Goede, R. E., van Steenwijk, R. P., Lange, J. M., Schattenkerk, J. K., Miedema, F., and Tersmette, M. (1992). Biological phenotype of human immunodeficiency virus type 1 clones at different stages of infection: progression of disease is associated with a shift from monocytotropic to T-cell-tropic virus population *J. Virol.* **66,** 1354-1360

138. Sinigaglia, F., D'Ambrosio, D., Panina-Bordignon, P., and Rogge, L. (1999). Regulation of the IL-12/IL-12R axis: a critical step in T-helper cell differentiation and effector function *Immunol. Rev.* **170,** 65-72

139. Trinchieri, G. (1998). Interleukin-12: a cytokine at the interface of inflammation and immunity *Adv. Immunol.* **70,** 83-243

140. Marshall, J. D., Chehimi, J., Gri, G., Kostman, J. R., Montaner, L. J., and Trinchieri, G. (1999). The interleukin-12-mediated pathway of immune events is dysfunctional in human immunodeficiency virus-infected individuals *Blood* **94,** 1003-1011

141. Villinger, F., Bucur, S., Chikkala, N. F., Brar, S. S., Bostik, P., Mayne, A. E., Adams, J., Lee, M. E., Novembre, F. J., Gately, M. K., Ansari, A. A., and Hillyer, C. D. (2000). In vitro and in vivo responses to interleukin 12 are maintained until the late SIV infection stage but lost during AIDS *AIDS Res. Hum. Retroviruses* **16,** 751-763

142. Young, J. M., Ffrench, R. A., Clarkson, J. D., Stewart, G. J., Liang, T., Tideman, R. L., Packham, D., Fulcher, D. A., and Benson, E. M. (2001). In vitro HIV-specific CTL activity from HIV-seropositive individuals is augmented by interleukin-12 (IL-12) *AIDS Res. Hum. Retroviruses* **17,** 233-242

143. Leonard, W. J. and O'Shea, J. J. (1998). Jaks and STATs: biological implications *Annu. Rev. Immunol.* **16,** 293-322

144. Ortmann, R. A., Cheng, T., Visconti, R., Frucht, D. M., and O'Shea, J. J. (2000). Janus kinases and signal transducers and activators of transcription: their roles in cytokine signaling, development and immunoregulation *Arthritis Res.* **2,** 16-32

145. Pericle, F., Pinto, L. A., Hicks, S., Kirken, R. A., Sconocchia, G., Rusnak, J., Dolan, M. J., Shearer, G. M., and Segal, D. M. (1998). HIV-1 infection induces a selective reduction in STAT5 protein expression *J. Immunol.* **160,** 28-31

146. Bovolenta, C., Camorali, L., Lorini, A. L., Ghezzi, S., Vicenzi, E., Lazzarin, A., and Poli, G. (1999). Constitutive activation of STATs upon in vivo human immunodeficiency virus infection *Blood* **94,** 4202-4209

147. Sant, N. (2004) Analysis of STAT Activation in Natural Killer Cells and $CD4^+$ T Cells by Multi-parameter Flow Cytometry . B.Sc. Hon., Biochem. thesis, Department of Biochemistry, Microbiology, and Immunology. University of Ottawa.

148. Krutzik, P. O. and Nolan, G. P. (2003). Intracellular phospho-protein staining techniques for flow cytometry: monitoring single cell signaling events *Cytometry A* **55,** 61-70

149. Kuzushima, K., Hoshino, Y., Fujii, K., Yokoyama, N., Fujita, M., Kiyono, T., Kimura, H., Morishima, T., Morishima, Y., and Tsurumi, T. (1999). Rapid determination of Epstein-Barr virus-specific CD8(+) T-cell frequencies by flow cytometry *Blood* **94,** 3094-3100

150. Nixon, D. F., Townsend, A. R., Elvin, J. G., Rizza, C. R., Gallwey, J., and McMichael, A. J. (1988). HIV-1 gag-specific cytotoxic T lymphocytes defined with recombinant vaccinia virus and synthetic peptides *Nature* **336,** 484-487

151. Pommier, J. P., Gauthier, L., Livartowski, J., Galanaud, P., Boue, F., Dulioust, A., Marce, D., Ducray, C., Sabatier, L., Lebeau, J., and Boussin, F. D. (1997). Immunosenescence in HIV pathogenesis *Virology* **231,** 148-154

152. Kern, F., Ode-Hakim, S., Vogt, K., Hoflich, C., Reinke, P., and Volk, H. D. (1996). The enigma of CD57+ CD28- T cell expansion--anergy or activation? *Clin. Exp. Immunol.* **104,** 180-184

153. Hamann, D., Kostense, S., Wolthers, K. C., Otto, S. A., Baars, P. A., Miedema, F., and van Lier, R. A. (1999). Evidence that human CD8+CD45RA+CD27- cells are induced by antigen and evolve through extensive rounds of division. *Int. Immunol.* **11,** 1027-1033

154. Bandres, E., Merino, J., Vazquez, B., Inoges, S., Moreno, C., Subira, M. L., and Sanchez-Ibarrola, A. (2000). The increase of IFN-gamma production through aging correlates with the expanded CD8(+high)CD28(-)CD57(+) subpopulation *Clin. Immunol.* **96,** 230-235

155. Lewis, D. E., Tang, D. S., du-Oppong, A., Schober, W., and Rodgers, J. R. (1994). Anergy and apoptosis in CD8+ T cells from HIV-infected persons *J. Immunol.* **153,** 412-420

156. Lloyd, T. E., Yang, L., Tang, D. N., Bennett, T., Schober, W., and Lewis, D. E. (1997). Regulation of CD28 costimulation in human CD8+ T cells *J. Immunol.* **158,** 1551-1558

157. Levy, J. A. (2006). HIV pathogenesis: knowledge gained after two decades of research *Adv. Dent. Res.* **19,** 10-16

158. Crispi, S., Sanzari, E., Monfregola, J., De, F. N., Fimiani, G., Ambrosio, R., D'Urso, M., and Ursini, M. V. (2004). Characterization of the human STAT5A and STAT5B promoters: evidence of a positive and negative mechanism of transcriptional regulation *FEBS Lett.* **562,** 27-34

159. Paiardini, M., Cervasi, B., Albrecht, H., Muthukumar, A., Dunham, R., Gordon, S., Radziewicz, H., Piedimonte, G., Magnani, M., Montroni, M., Kaech, S. M., Weintrob, A., Altman, J. D., Sodora, D. L., Feinberg, M. B., and Silvestri, G. (2005). Loss of CD127 expression defines an expansion of effector CD8+ T cells in HIV-infected individuals *J. Immunol.* **174,** 2900-2909

160. Doan, L. L., Kitay, M. K., Yu, Q., Singer, A., Herblot, S., Hoang, T., Bear, S. E., Morse, H. C., III, Tsichlis, P. N., and Grimes, H. L. (2003). Growth factor independence-1B expression leads to defects in T cell activation, IL-7 receptor alpha expression, and T cell lineage commitment *J. Immunol.* **170,** 2356-2366

161. Park, J. H., Yu, Q., Erman, B., Appelbaum, J. S., Montoya-Durango, D., Grimes, H. L., and Singer, A. (2004). Suppression of IL7Ralpha transcription by IL-7 and other prosurvival cytokines: a novel mechanism for maximizing IL-7-dependent T cell survival *Immunity.* **21,** 289-302

162. Effros, R. B., Allsopp, R., Chiu, C. P., Hausner, M. A., Hirji, K., Wang, L., Harley, C. B., Villeponteau, B., West, M. D., and Giorgi, J. V. (1996). Shortened telomeres in the expanded CD28-CD8+ cell subset in HIV disease implicate replicative senescence in HIV pathogenesis *AIDS* **10,** F17-F22

163. Wolthers, K. C., Bea, G., Wisman, A., Otto, S. A., de Roda Husman, A. M., Schaft, N., de, W. F., Goudsmit, J., Coutinho, R. A., van der Zee, A. G., Meyaard, L., and Miedema, F. (1996). T cell telomere length in HIV-1 infection: no evidence for increased CD4+ T cell turnover *Science* **274,** 1543-1547

164. Sze, D. M., Giesajtis, G., Brown, R. D., Raitakari, M., Gibson, J., Ho, J., Baxter, A. G., Fazekas de St, G. B., Basten, A., and Joshua, D. E. (2001). Clonal cytotoxic T cells are expanded in myeloma and reside in the CD8(+)CD57(+)CD28(-) compartment *Blood* **98,** 2817-2827