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Studies on the roles of T helper type I and type II cytokines in HIV immunopathogenesis; role and regulation of interleukin-10

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Abstract

Infection of immune cells with human immunodeficiency virus (HIV) induces dysregulation of cytokines which may play a vital role in HIV pathogenesis. I analyzed the expression of Th1 [interferon-γ, (IFN-γ)] and Th2 [interleukin-4 (IL-4), IL-10] type cytokines in unstimulated and mitogen stimulated peripheral blood mononuclear cells (PBMC) from HIV seropositive (HIV^+) patients. It was determined that IFN-γ mRNA in unstimulated PBMC was significantly decreased and IL-10 mRNA as well as IL-10 protein was significantly increased in patients with <400 $CD4^+$ T cells/mm^3 (n=30) as compared to patients with >400 $CD4^+$ T cells/mm^3 (n=6) and normal controls (n=16).

Mitogen stimulation of PBMC revealed two groups of HIV^+, low and normal IL-10 producers. Production of IL-4 was reduced in HIV^+ individuals with < 400 $CD4^+$ T cells/mm^3 while it was comparable to that of HIV^- controls in those with >400 $CD4^+$ T cells/mm^3. However, ability to produce IFN-γ by mitogen stimulated PBMC and CD4 T^+ cells was not impaired in HIV^+ individuals. These results suggest that PBMC of HIV^+ exhibit dysregulation of Th2 type cytokines which may play a role in HIV immunopathogenesis.

In the next set of experiments, the IL-10 production was correlated with the levels of proliferative responses to recall antigens. Low IL-10 producers proliferated in response to recall antigens, and demonstrated enhanced recall antigen-induced proliferation upon addition of anti-IL-10 antibodies and/or IL-12. Conversely, normal IL-10 producers had PBMC that failed to proliferate to recall antigens, and did not demonstrate enhanced recall

antigen-induced proliferation upon addition of anti-IL-10 antibodies and/or IL-12. Source of the IL-10 production in PBMC of HIV^+ individuals was shown to be monocytes, while, in HIV^- controls, it was produced by both T cells and monocytes.

The molecular mechanisms underlying the production of IL-10 are not clear. I have demonstrated that monocytes/ macrophages are required for IL-10 production by normal activated T cells. IL-10 production was significantly downregulated in both T cell and monocyte depleted PBMC compared to undepleted PBMC, and IL-10 production could be restored following addition of monocyte conditioned medium (MCM), this suggested that IL-10 production by T cells is regulated by monokine(s) produced by activated monocytes. The monokine(s) responsible for IL-10 induction by T cells were further studied. Addition of IL-6 and IL-12 enhanced IL-10 production in monocyte depleted PBMC in a dose dependent and additive manner. With respect to regulation of IL-10 produced by monocytes, tumor necrosis factor α (TNF-α) was found to induce IL-10 production by resting purified monocytes. Taken together, these findings suggest that IL-10 production by human T cells and monocytes is differentially regulated. IL-12 and IL-6 induce the expression of IL-10 by PHA stimulated T cells whereas TNF-α induces IL-10 production by monocytes. Since IL-10 inhibits production of IL-6, IL-12 and TNF-α , these results may indicate a potential mechanism of negative feedback regulation of the immune system. Furthermore, mitogen stimulated PBMC from HIV^+ individuals produced significantly lower levels of IL-12 than did those from HIV- controls. A defect in IL-12 induction may partially cause IL-10 dysregulation in HIV infection.

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I would like to dedicate my thesis to my wife, Farzaneh

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AIDS	Acquired immunodeficiency syndrome
BSA	Bovine serum albumin
CMIR	Cell mediated immune responses
CM	Complete media
CPM	Count per minute
CTL	Cytotoxic T lymphocytes
DNA	Deoxy ribonucleic acid
DU	Densitometric Unit
ELISA	Enzyme linked immunosorbent assay
FBS	Fetal bovine serum
FLU	Influenza antigen
GM-CSF	Granulocyte-macrophage colony stimulating factor
HIV	Human immunodeficiency virus
HIV^-	HIV seronegative individual
HIV^+	HIV seropositive individual
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
min	Minutes
MCM	Monocyte conditioned media

NK	Natural killer
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMA	Phorbol myristate acetate
PMN	Polymorphonuclear cells
RT	Reverse transcriptase
SEM	Standard Error of the Mean
TGF	Transforming growth factor

Chapter I. Introduction

By the time you read two lines of this manuscript (every 13 seconds) one person becomes infected with the human immunodeficiency virus (HIV) and every nine minutes someone dies from it. It has also been estimated that by the year 2000, between 30 and 100 million people world-wide will be infected by HIV. Up until May 1994, more than 90,000 scientific articles have been published on HIV. HIV is perhaps one of the most intensively studied pathogens in history (Levy, 1994e). All aspects of medical sciences have been affected and altered by HIV infection.

In the last decade, investigations in the area of HIV infection have mainly targeted the virus itself. It is now fair to say that despite all advances, in terms of therapy, this strategy has had limited success. Recently, however, a series of complex immunological mechanisms such as immune activation, cytokine elaboration, defective antigen presentation and activation of apoptosis have been found to have critical roles in the pathogenesis of HIV infection (Fauci, 1993a and 1993b; Pantaleo et al. 1993b; Levy, 1994g; Ada, 1994; Pantaleo and Fauci, 1995).

This introduction will provide background information on structural aspects of HIV-1 (which is hereafter referred to as HIV), and immunopathogenesis of HIV infection, focusing particularly on the possible role of cytokines in the pathogenesis of HIV infection.

1. Overview of the human immunodeficiency virus (HIV)

HIV is a member of the genus Lentivirus, family *Retroviridae*. Infection with lentiviruses, in general, results in autoimmunity, pneumonitis, immunodeficiency, and brain and joint disorders (Levy, 1994b). Being host-species-specific, lentiviruses infect horses, sheep, goats, cows, cats, primates, and humans. Diseases caused by lentiviruses have a long incubation period, and are characterized by immune suppression that results in opportunistic infection (Levy, 1994b; Ada, 1994).

The HIV particle is cone-shaped, composed of an envelope and a capsid (p25). Inside the capsid there is a nucleoid which contains two identical RNA molecules. Two glycosylated proteins, gp41 and gp120, form the envelope, the RNA-dependent DNA polymerase (Pol or reverse transcriptase) and the nucleocapsid are associated with these RNA strands. To support the integrity of the virus, a myristoilated p17 core protein forms a matrix between the capsid and the envelope. The envelope glycoproteins have a precursor, gp160, which is cleaved into a transmembrane protein, gp41 and a surface protein, gp120. The production of the gag protein, p25, is 100 times more than envelope gp120 (Levy, 1994d).

2. Genomic organization of HIV

The HIV provirus includes regulatory sequences at both ends called long terminal repeats, and genes of structural (env, gag) and enzymatic proteins (pol). HIV has a genome of approximately 9.8 kb and its open reading frames code for gag, polymerase, protease,

integrase, envelope, tat, rev, nef, vif, vpr, vpu, vpx, and tev. HIV full-length mRNA is translated into gag and polymerase proteins. Gag is cleaved into p25, p17, p9 and p6 molecules, whereas cleavage of the polymerase results in the production of reverse transcriptase (RT), the protease, and the integrase proteins (Hahn, 1994). The protease is known to be responsible for the processing of gag and polymerase, while the integrase is involved in the integration of the viral DNA into the DNA of the cell. Subgenomic mRNAs are the products of splicing events; these subgenomic mRNAs give rise to other viral proteins. Rev, a product of multiply spliced mRNA, seems to determine the relative amounts of unspliced, singly and multiply spliced mRNAs. Tat, a transactivator, is known to up-regulate virus replication. Nef appears to have pleiotropic effects; involvement in interference of signal transduction, downregulation of expression of CD4 molecules and suppression of virus replication (Hahn, 1994; Ada, 1994; Levy, 1994e).

3. HIV tropism: Cells and tissues that become infected with HIV

Having a polytropic nature, HIV has been found in several tissues and cell types, however, $CD4^+$ T lymphocytes and monocytes/macrophages are the primary cell types that become infected. HIV infection of brain, bowel, heart, lung, kidney, and joints may be responsible for related pathologic findings in HIV infected patients. The highest rate of HIV replication has been reported in $CD4^+$ T cells and monocytes. Infection *in vitro* is dependent on the efficiency of viral entry as well as postpenetration steps within the cell. In $CD4^+$ T cells, HIV is cytopathic and may induce formation of syncytia (multinucleated cells). In contrast,

infection of macrophages is usually noncytopathic but affects functions of these cells. HIV infection impairs the ability of macrophages to present antigens (Levy, 1994e; Ada, 1994), perhaps, through effect of IL-10, a potent down-regulator of monocyte, which is inducted after HIV infection (Akridge et al, 1994).

The concentration of HIV in body fluids varies. HIV may be isolated from plasma, tears, saliva, semen and cerebrospinal fluid (Levy, 1994e). The amount of HIV is small in samples other than plasma or semen, which may partially explain the major routes of transmission such as sexual contact and inoculation with contaminated blood. By means of quantitative competitive PCR, and depending on the stage of the HIV infection, 50 to 110 million virions may be found in each millilitre of plasma (Levy, 1994e). The infectivity of HIV depends more on cell-associated virus than free virus, due to the fact that infected cells are more resistant to destruction by the immune system (Levy, 1994e; Ada, 1994).

In semen, about one million white cells are released per ejaculate, 0.01 to 5% of which are infected with the virus. This makes semen highly transmittable. The rate of HIV transmission is estimated to be 1 in 100 for anal intercourse, 1 in 1000 for male to female, and 1 in 1500 for female to male transmission. Those sexually transmitted diseases which result in ulcers may increase transmission rate of HIV. Major routes of infection are as follows (Levy, 1994e; Ada, 1994; Devita et al. 1997):

a. Blood: from infected blood or blood products

b. anal intercourse

c. vaginal intercourse

d. mother to fetus

4. Molecular steps in HIV infection of cells

The first step in HIV infection of a cell is the attachment of the viral envelope to the cell surface receptor (CD4 or alternative receptor). Consequently, after a conformational change, gp120 is displaced and the V3 loop is cleaved; this perhaps facilitates the interaction of the fusion domain (e.g. gp41) with a glycolipid on the cell surface. The next step is the pH-independent fusion of the virus with the cell. Viral RNA in association with the core will then enter the cytoplasm and reverse transcription will be initiated. Viral cDNA will be generated and become duplicated to make double-stranded DNA and then generation of its circular form. DNA copies of viral RNA make a preintegration complex which is associated with some viral core proteins (phosphorylated MA and Vpr) and transports to nucleus. HIV is able to enter the nucleus of nondividing cells such as differentiated macrophages. DNA then integrates into cellular chromosomes. After integration, proviral DNA is transcribed to produce viral mRNA and viral genomic RNA, the extent of which is determined by expression of HIV regulatory genes. Following production of viral proteins, viral genomic RNA is incorporated into the capsid and the processing of gag and gag-pol polyproteins takes place. Processed viral proteins are incorporated while the budding of the viral capsid occurs through the cell membrane (Levy, 1994e; Ada, 1994; Devita et al. 1997).

Early mRNA transcription events are suggested to be dependent on the interaction of the HIV long terminal repeat (LTR) with transcriptional factors such as nuclear factor kappa B (NF-κB), nuclear factor of activated T cells (NFAT), activation protein 1 (AP1), and the tat binding proteins (TBP). Induction of these proteins in HIV infected cells may trigger HIV

replication. $CD4^+$ and other cells in a resting stage may contain HIV in an unintegrated non-infectious form, or integrated but non-expressed form (Ada, 1994; Levy, 1994h).

5. Immune responses against HIV

Immune responses against HIV have been shown in HIV-exposed seropositive and in seronegative individuals (who have been exposed to virus with no sign of seroconversion). A variety of host responses, including cytotoxic T lymphocytes (CTL) responses, humoral responses, antigen processing, and cytokine production are generated, the nature of which are characteristics of the stage of HIV disease (Pantaleo and Fauci, 1995).

The complex interaction between HIV and host immune cells results in a state of profound immunodeficiency (Pantaleo et al. 1994). Following infection, immune defects including sequential loss of responsiveness to recall antigens, alloantigens and mitogens are observed (Clerici et al. 1989). A number of qualitative changes are also observed such as the disruption of the CD4-MHC class II (major histocompatibility complex), defective signal transduction, disruption of the normal cytokine network and a direct cytopathic effect on $CD4^+$ T cells (Fauci et al. 1991; Rosenberg and Fauci, 1991b; Pantaleo et al. 1993b; Fauci and Rosenberg, 1994; Levy, 1994b; Ada, 1994). In addition, infection of host T cells and macrophages with HIV induces the expression of certain cytokines such as TNF-α, TNF-β, granulocyte-macrophage colony stimulating factor (GM-CSF), and IL-6, which influences immune responses against HIV (Sher et al. 1992; Trial et al. 1995).

Upon infection with HIV, despite humoral and cellular responses against HIV, progression of the disease occurs in >95% cases (van Noesel et al. 1990; Levy, 1994e; Brix and Redfield, 1994). Failure of immune responses to clear HIV may be due to immunosuppressive activity among HIV antigens, which may be due to direct effects of viral products. Alternatively, HIV may induce factors, such as some cytokines, to exert immunosuppressive effects.

At the time of acute infection, a peak in plasma viremia is observed. This initiates a series of immune responses against the virus which, in turn, may increase levels of activated cells such as $CD4^+$ which are virus target cells. Increased expression of inflammatory cytokines such as IL-1, TNF-α, and IL-6 may also enhance the virus production (Romagnani et al. 1994a; Ada, 1994; Pantaleo et al. 1994).

Cellular and humoral immune responses to HIV-associated antigens develop one to three months after infection (Levy, 1994g; Pantaleo et al. 1993; Folks et al. 1989; Francis et al. 1992). A few weeks after the initial infection and right after a decrease in the level of HIV viremia, an antibody response is detectable which lasts for a variable amount of time before the development of AIDS. Among these antibodies are specific anti-HIV neutralizing antibodies against the infecting HIV. The relationship between the development of neutralizing antibodies to envelope glycoproteins and p25 antigens with disease progression remains controversial (Ada, 1994; Levy, 1994c). In spite of the presence of a large panel of antibodies such as anti-gp120/gp160, almost all patients progress to AIDS. This might be due to the fact that humoral responses are not able to clear the infection. Furthermore, HIV undergoes some antigenic variation to escape the humoral responses and the appearance of antibody-escape mutants of

HIV may completely fool the immune system to always invest in the production of antibodies for the earlier strains of HIV. Alternatively, some antibodies may compete with neutralizing anti-HIV antibodies (Folks et al. 1989; Francis et al. 1992; Pantaleo et al. 1993; Levy, 1994g).

As HIV disease progresses, loss of T helper function precedes and predicts the rate of decline in $CD4^+$ T cell numbers (Rosenberg and Fauci, 1991a; Sher et al. 1992; Fauci, 1993b; Levy, 1994c). T helper cells sequentially lose the ability to respond to viral antigens (to which T cells of same patients had previously responded), alloantigens, and finally to mitogens (Shearer and Clerici. 1993; Levy, 1994g; Ada, 1994). Because recall responses are dependent on memory cells, and there is a selective depletion of memory cells in HIV infection, improved Th functions may be more easily achieved for responses that are less dependent on memory cells (Levy, 1994e). Although antiretroviral drugs improve $CD4^+$ T cell numbers and some T helper functions, there is little effect on responses involving antigen presentation by monocytes.

In a preliminary study on patients on antiviral therapy, an improvement in Th function was observed without any changes in $CD4^+$ T cell counts (D'Andrea, 1992), which suggests improvement in T cell function is independent of $CD4^+$ T cell number. It was also shown that a defect in the proliferative responses to recall antigens, alloantigens and mitogens occurs even before a substantial drop in $CD4^+$ number, suggesting that a different helper subset may contribute to the improvement of immune responses as opposed to an increase in the number of $CD4^+$ cells.

A few years after infection, CTL responses are detectable in infected individuals and remain detectable until the development of AIDS. In spite of the presence of CTL responses, HIV continues to replicate. Some infected cells may escape the CTL response by being

sequestered in immunoprivileged sites such as the brain or epididymis. Some infected cells may not express the right T cell determinants or the MHC antigens. CTL specific for env, pol, and gag gene products may confer resistance to HIV disease progression but their activity decreases with progression of disease (Levy, 1994i; Ada, 1994). CTL responses against some HIV proteins become reduced while such responses to CMV and HSV are maintained, this may be due to selection of CTL escape mutant among HIV mutants. It is also possible that syncytium inducing (SI) strains may predominate in lymphoid organs, favoring more rapid viral replication, mutation and increased rate of virus recombination, which could be an important factor in the emergence of viral strains that become resistant to cytotoxic T cells, and neutralizing antibodies, and in the development of resistance to antiviral drugs. Despite the fact that there has been a correlation between the CTL response and persistence of asymptomatic stage of HIV infection, CTL may also contribute to the depletion of CD4 cells (Levy, 1994i; Ada, 1994). Cytotoxic $CD4^+$ T cell clones against HIV envelope proteins produce both transmembrane and secreted forms of TNF (Liu et al. 1992).

6. Immunopathogenesis of HIV infections

The hallmarks of HIV infection are immunosuppression and the onset of opportunistic infections. HIV induces dysfunction and eventual depletion of $CD4^+$ T cells (Pantaleo and Fauci, 1995). Interaction of HIV with $CD4^+$ cells involves an array of complex processes which include the direct cytopathic effect of HIV on $CD4^+$ T cells, syncytia formation, disruption of the critical interaction between MHC proteins and CD4 molecules, defective

signal transduction in $CD4^+$ T cells, induction of autoimmune phenomena that may result in $CD4^+$ T cell destruction, and interference with the normal cytokine network (Poli et al. 1993; Pantaleo et al. 1994; Pantaleo and Fauci, 1995). Both MHC molecules and gp120 bind to the same ligand CD4. Homologies among gp120 and MHC class I and II has been reported (Devita et al. 1997). Interaction of CD4 with gp120 has been shown to initiate humoral and cellular responses against $CD4^+$ cells, such as antibody-dependent cellular cytotoxicity (Gallagher, 1991). Induction of autoimmune responses in HIV^+ individuals has been reported in both humoral and cellular levels (Gallagher, 1991; Levy, 1994c). Interaction of $CD4^+$ cells with gp120 was also shown to induce unresponsiveness of these cells to further stimulation (Leigler et al. 1994). HIV-gp120 has been shown to bind $p56^{lak}$. The retention of $p56^{lak}$ has been shown to interfere with signal transduction of $CD4^+$ cells and may lead to induction of PKC and enhanced HIV replication (Levy, 1994; Devita et al. 1997). Infection of monocytes and T cells has also been shown to induce a change in cytokine production which in part is responsible for the enhanced virus replication and may result in inhibition of immune responses required for the clearance of the infection (Akridge et al. 1994; Fauci, 1997). It is not certain what contribution each of these processes has in the disruption of the immune system, but the role of each probably varies considerably depending upon the stage of infection. High levels of HIV in lymph nodes (LN), and low viral load in peripheral blood mononuclear cells (PBMC) during clinical latency, suggest that viral replication in LN cells and direct $CD4^+$ T cell killing by HIV play significant roles in the pathogenesis of AIDS (Poli et al. 1993; Pantaleo et al. 1993a; Fauci, 1993b; Pantaleo et al. 1994; Pantaleo and Fauci, 1995).

The mechanism of $CD4^+$ T cell depletion in AIDS remains elusive (Fauci, 1993a; Pantaleo et al. 1993b; Pantaleo and Fauci, 1995). There is evidence that HIV infection causes $CD4^+$ T cell depletion by indirect mechanisms, such as the inhibition of immunoregulatory cytokines and by apoptosis (Fauci, 1993a; Pantaleo et al. 1993b; Romagnani et al. 1994b; Oyaizu et al. 1995; Del Prete et al. 1995; Cossarizza et al. 1995), which may be more important than direct T cell killing.

The role of lymphoid organs in HIV pathogenesis has recently been investigated (Pantaleo et al. 1993a; Levy, 1994a). Following primary infection, HIV tends to localize more in the lymphoid organs than in the peripheral lymphocytes. The number of cells harboring HIV DNA and RNA is 5 to 10 times higher in LN than in PBMC (Pantaleo et al. 1993a), and during periods of clinical latency most of the HIV load is located in lymphoid organs (Pantaleo et al. 1993a).

HIV can infect both resting and activated $CD4^+$ T cells *in vitro* equally well, but its ability to replicate in T cells is dependent on the state of cell activation (Poli et al. 1993; Pantaleo et al. 1993b; Fauci, 1993a; Pantaleo et al. 1994; Pantaleo and Fauci, 1995). The proportion of activated $CD4^+$ T cells in LN, which is higher (25-50%) than in PBMC (5-10%), could also explain the higher HIV load in lymphoid organs (Pantaleo et al. 1993a).

7. Factors affecting pathogenesis of HIV infection

$CD4^+$ T cells and monocytes play critical roles in the development and maintenance, and regulation of immune responses against pathogens and malignant tumors. By targeting

$CD4^+$ lymphocytes and monocytes, HIV may potentially affect all of the above mentioned mechanisms. Thus, utilization of the CD4 molecule by HIV is a central key in the pathogenesis of HIV infection (Miedema et al. 1990; Fauci, 1993a; Levy, 1994d; Fauci and Rosenberg, 1994).

HIV is highly heterogeneous in molecular, biological and structural aspects. Different strains of HIV may vary in genome structure, cellular tropism, replication kinetics, cytopathic effects and the ability to form syncytia, and so forth (Levy, 1994a). HIV heterogeneity influences the pathogenesis of infection in a variety of ways (Levy, 1994d). The cytopathology of different strains also appears to be different; some have the characteristic of cell killing and induction of syncytium formation while others do not. The kinetics and the level of HIV replication varies among strains and is the basis of definition of "rapid/high" and "slow/low" strains (Levy, 1994a). Perhaps, affinity of virus for reçeptor attachment, rate of virus entry into the cell, and the influence of intracellular factors, all of which might be different for different strains, influence the kinetics of HIV replication (Levy, 1994a). $HIV\text{-}_{SF2}$ isolates from asymptomatic (early isolates) resulted in limited cytopathogenicity, whereas $HIV\text{-}1_{SF13}$ from an AIDS patient (late isolates) caused the formation of syncytia, characteristic of virulent strains (Tersmette et al. 1989; Levy, 1994a). Lack of proofreading by the viral and cellular enzymes results in about 10^{-4} per site per generation error and thus generation of variants (Devita et al. 1997). HIV has the ability to change its biological and antigenic characteristics over time in the same host. Strains with different biological properties were isolated from different AIDS patients (Tersmette et al. 1988; Tersmette et al. 1989). These changes might be a reflection of viral escape from immune responses (Levy, 1994a).

Some HIV strains with T cell tropism preferentially infect T cells and others infect both T cells and monocytes. In a majority of cases, HIV infection of monocytes is associated with low-level replication. However, this might be due to infection by a non-macrophage tropic strain since co-culturing with T cells induces virus replication (Levy, 1994a; Schrier et al. 1990). This effect may be via the involvement of cellular factors such as cytokines.

There are other factors that influence the infectivity of the virus and consequently affect pathogenesis of HIV infection including: the structure, the conformation and the charge of viral envelope, amount of viral envelope glycoproteins, number of envelope spikes, extent of gp120 shedding, interaction of intracellular factors with the viral LTR, and extent of expression of viral regulatory proteins (Levy, 1994a).

Some external stimuli such as cytokines also induce the expression of HIV. TNF-α, IL-1 and perhaps IL-12, for example, induce PKC_t activation which in turn may facilitate binding of NF-κB to the LTR and enhance viral replication (Fauci and Rosenberg, 1994; Brix and Redfield, 1994; Ada, 1994; Levy, 1994d; Daftarian et al. 1995c). TNF-α expression by monocytes in HIV infected individuals was reported to be involved in the induction of apoptosis in $CD4^+$ cells (Levy, 1994a).

8. Role of CD28-B7 interaction in inducing unresponsiveness

Antigen-induced activation of T cells and IL-2 production is mediated by at least two distinct surface molecules comprised of the antigen-specific T cell receptor complex and

co-stimulatory molecules (Bluestone, 1995; Thompson, 1995). T cell interaction with accessory cells via CD28-B7 complex provides one such example of co-stimulatory molecules (Allison, 1994; Bluestone, 1995; Thompson, 1995). CD28 is a 44 kilodalton homodimeric glycoprotein expressed on most T cells. CD28 is the receptor for B7/BB-1, a membrane glycoprotein induced upon B cell, monocyte and dendritic cell activation (Allison, 1994). CD28-B7 recognition provide a secondary, but essential, co-stimulatory signal to T cells, since antigen-MHC-TCR interaction in the absence of co-stimulatory signals leads to unresponsiveness or apoptosis (Allison, 1994; Bluestone, 1995; Thompson, 1995). At the molecular level, CD28 stimulation increases IL-2 secretion both by increasing the IL-2 gene enhancer activity and by stabilizing IL-2 mRNA (Bluestone, 1995). The possible role of other costimulatory pathways and whether they can compensate for CD28/B7 is not clear.

Antigen-presenting cells in lymph nodes have been shown to be productively infected with HIV (Schuurman et al. 1988; Pantaleo et al. 1994; Pantaleo and Fauci, 1995; Weissman et al. 1995a), which might directly interfere with the expression of crucial co-stimulatory molecules such as HLA class II molecules, with CD28-B7/BB1 interactions and with cytokine release (Petit et al. 1987; Caruso et al. 1994; Vingerhoets et al. 1995). Replication of HIV in the thymus of HIV-infected persons may cause dysregulation of the expression of co-stimulatory molecules (Schnittman et al. 1991). Monocytes/macrophages and dendritic cells from HIV-infected individuals have been shown to have decreased expression of HLA class II molecules and accessory cell function (Petit et al. 1987). In addition, CD28 expression has also been documented to be significantly decreased on T cells infected with HIV (Caruso et al. 1994; Vingerhoets et al. 1995). In HIV^+ individuals, dysregulated IL-10 production is

associated with alterations in the CD28-B7 pathway (Kumar et al. 1996), and CD28-induced proliferation is inversely correlated with IL-10 produced by PHA-stimulated PBMC from HIV^+ individuals (Kumar et al. 1996). Whether expression of these molecules is modulated directly by the virus itself or indirectly by various regulatory cytokines is not known.

There are also other costimulatory molecules such as 4-1BB and little is known about their role especially in HIV infection.

9. Cytokine network in HIV infection

The interaction of the human immune system with HIV is very complex. The hallmark of AIDS is the infection of $CD4^+$ T cells and monocytes/macrophages with HIV, which results in a progressive loss of effective immune responses (Ada, 1994). HIV employs a variety of mechanisms to undermine the effectiveness of the immune system. Perhaps, one of the strategies used by HIV to defeat the immune system is dysregulation of the cytokine network.

9. a. Cytokine involvement in the regulation of HIV replication

Cytokines may directly or indirectly influence HIV replication (Fauci and Rosenberg, 1994; Ada, 1994). A series of cytokines can enhance virus replication such as TNF--α, IL-1, and IL-6. Others can inhibit HIV replication ie IL-10 and IFN--α. There are also other cytokines that are bifunctional such as IL-4 and IFN-γ (Fauci, 1996). Activation of $CD4^+$ cells

promotes HIV replication via a number of pathways such as induction of expression of NF-κB, IL-2, TNF-α, IL-6, GM-CSF, IL-7 and to a lesser degree IFN-γ, IL-12, IL-4, transforming growth factor (TGF)-β, and IL-3. Some of these effects are restricted to certain cell lines or experimental situations (Poli and Fauci, 1992b; Fauci and Rosenberg, 1994; Foli et al. 1995; Smithgall et al. 1996). Infection of host T cells and monocytes/macrophages with HIV also induces the expression of various cytokines such as TNF-α, TGF-β, GM-CSF, IL-4 and IL-6 (Fauci and Rosenberg, 1994; Biswas et al. 1994; Emilie et al. 1994). Some of these cytokines, also known as HIV-inductive cytokines, enhance HIV replication, while a variety of other soluble factors have been shown to exert suppressive effects on HIV replication. Certain cytokines may have different effects in different cell lines or culture conditions (Fernie et al. 1991; Boue et al. 1992; Fauci and Rosenberg, 1994). For instance, IL-4, IFN-γ, and TGF-β have bifunctional effects since they either enhance or suppress HIV replication depending on the state of infection, cell type and the presence or absence of other cytokines (Fauci and Rosenberg, 1994). IFN-α, IFN-γ, , IL-13 and IL-10 inhibit HIV replication in macrophages and monocytoid cell lines, mitogen-stimulated PBMC and chronically infected cell lines (Fernie et al. 1991; Shirazi and Pith, 1992; Fauci and Rosenberg, 1994; Mikovits et al. 1994; Masood et al. 1994; Akridge et al. 1994; Autran et al. 1995). IL-10 has also been reported to enhance HIV replication in the monocyte/macrophage population (Angel et al. 1995; Weissman et al. 1995a). A group of cytokines is capable of regulating HIV expression from latent to active viral replication. IL-1, IL-3, IL-6, TNF-α, IFN-γ, GM-CSF, and M-CSF stimulate HIV replication in chronically infected monocytic cell lines and in primary cultures of cells of the same lineage. TNF-α increases HIV expression in chronically infected T cell lines. In

summary, several cytokines are able to regulate virus production, and strategies aimed at controlling the production of these cytokines or their effects on cells infected by HIV could prove therapeutically beneficial.

9. b. Induction of cytokines by HIV

The disruption of cell communication through systematic perturbation of the cytokine network by HIV may play an essential role in HIV pathogenesis. Infection by HIV may induce changes in the profile of cytokine expression in different infected cells as well as in bystander cells (Molina et al. 1989; Rieckmann et al. 1991b; D'Addario et al. 1992; Levy, 1994; Kinter et al. 1995). In general, the expression of proinflammatory cytokines TNF-α, IL-6, and IL-1β is shown to be increased in PBMC and also in the lymph nodes of HIV^+ individuals (Fauci, 1996). In addition, the expression of IFN-γ is also enhanced in the lymph nodes of HIV^+ individuals (Fauci. 1996). Induction of TNF-α synthesis has been shown to occur in human PBMC upon infection by HIV (Navikas et al. 1995). However, chronic infection of THP-1, a monocytic cell line, does not result in enhanced IL-1β or TNF-α expression even after LPS stimulation. Nevertheless, enhanced IL-1-β and TNF-α expression is observed upon acute infection of THP-1 cells by HIV (Molina et al. 1989). Chronic infection by HIV_{MB} (a monocyte-tropic strain of HIV) enhanced the expression of IL-1β in phorbol myristate acetate (PMA)-stimulated myelomonoblastic cell line (D'Addario et al. 1992). Other studies have shown that prostaglandins, IL-1, TNF-α, and IL-6 were induced after HIV infection of

macrophages or incubation of gp120 with macrophages (Gallagher et al. 1991). The overexpression of TGF-β in PBMC from HIV infected individuals has been reported and may deactivate monocytes (Navikas et al. 1994). Recombinant gp120 was shown to induce TNF-α and enhance immunoglubulin secretion in B cells from HIV infected persons (Rieckmann et al. 1991b). HIV infection has been shown to downregulate the expression of IL-2 in peripheral T cells, which may result in the suppression of protective cell mediated immune responses (CMIR) (Fan et al. 1993). Recombinant gp160 was shown to suppress IFN-γ and IL-2 production and induce IL-4 expression (Hu et al. 1994). However, in supernatants of infected PBMC or plasma from HIV-infected individuals, the expression of IFN-γ has been shown to be decreased (Rossol et al. 1989; Hu et al. 1994) or increased, respectively (Navikas et al. 1994).

There is increasing evidence (see below) to support the existence of a polarized T cell response, as originally reported in mice (Mosmann et al. 1991; Romagnani, 1994; Romagnani, 1995). According to this hypothesis, intracellular pathogens should ideally and predominantly induce a T helper (Th)1 response which is suitable for the induction of CMIR. Th2 responses are responsible for the generation of humoral responses, for example, during helminthic and allergic reactions (Romagnani, 1995). Human Th2 type clones are shown to express higher levels of CD30, a member of the TNF receptor family. IL-12 and IFN-γ are involved in the generation of Th1 type response, while IL-4 and perhaps IL-10 favor induction of a Th2 response (Romagnani, 1995). In HIV infection, a strategy to evade protective immune response may be the induction of a switch from Th1 to Th2 type responses which facilitates the persistence of the infection.

10. T Helper responses in HIV infection

Classification of cytokines into Th1 and Th2 types has helped in the elucidation of the mechanisms of resistance or susceptibility to infections. In both humans and mice, Th1 and Th2 cells have been described with different functional properties. Th1 cells produce IL-2, IFN-γ, and TNF-β, and Th2 cells mainly produce IL-4, IL-5, IL-10, and very low levels of IL-2. (Mosmann et al. 1991; Romagnani, 1994). Th0 cells produce both Th1 and Th2 cytokines. According to the original hypothesis, preferential induction of CMIR, such as CD4 functions pertaining to CTL, DTH, activation of macrophages and NK cells, by intracellular pathogens including viruses, bacteria, and protozoa, and induction of humoral immune responses by extracellular pathogens such as free viruses, extracellular bacteria, and helminths, may be dependent upon the selective induction of Th1 or Th2 cell subsets respectively (Mosmann et al. 1991; Romagnani, 1994; Romagnani, 1995). Consistent with this hypothesis, by producing IL-2 and IFN-γ, Th1 cells induce CMIR, while by producing IL-4, IL-5 and IL-10, Th2 cells induce humoral and, in particular, allergic responses (Romagnani, 1994; Mosmann et al. 1991; Romagnani, 1995; Leung, 1995). IL-4, a Th2 cytokine, enhances IgG1 and IgE while IL-5 and IL-6 enhance IgA production. However, IFN-γ, a Th1 cytokine, enhances IgG2a production and is essential for the generation of opsonizing and complement activating antibodies which act against extracellular pathogens (Muller, 1995).

Th1 and Th2 responses are reciprocally controlled; Th1 cells inhibit the development of Th2 cells and vice versa (Romagnani and Maggi, 1994; Fishman and Perelson, 1994; Romagnani, 1995). IL-4 inhibits Th1 responses and IFN-γ production, and IL-10 inhibits Th1

responses by suppressing synthesis of IL-2, IFN-γ and IL-12 production. On the other hand, IFN-γ inhibits the production of IL-4. It has also been shown that IL-10, IL-4 and IL-13, another Th2 cytokine, inhibit CTL and DTH responses (Abbas, 1994).

In experimental leishmaniasis, in mice, and leprosy, in human, there is a significant correlation between resistance or susceptibility and Th1 or Th2 responses, respectively (Mosmann et al. 1991; Romagnani, 1994; Romagnani, 1995; Muller, 1995). It has been hypothesized HIV infection also may follow the same pattern, and the gradual replacement of Th1 by Th2 responses will be associated with disease progression (Maggi et al. 1994; Romagnani et al. 1994; Ameglio et al. 1994; Barcellini et al. 1994; Hu et al. 1994; Romagnani et al. 1994; Clerici and Shearer, 1994; Vigano et al. 1995; Del Prete et al. 1995; Muller, 1995; Romagnani, 1995; Meyaard et al. 1994).

In HIV infection, sequential loss of immune responses to recall antigens, alloantigens and mitogens has been hypothesized to be associated with diminished Th1 type and enhanced Th2 type cytokine production. In HIV^- individuals, the functions of $CD4^+$ cells were reduced even before a significant decrease in $CD4^+$ number. For instance, IL-2 production upon exposure to recall antigens (influenza A and tetanus) was decreased, allogeneic responses were inhibited, and reduced responses to PHA were observed in some cases (Clereci et al. 1989; Shearer et al. 1991). It was shown that IL-4 and IL-10 in response to mitogens were enhanced in HIV^+ individuals comparing to those of HIV^- controls. IL-10 production was also shown to be associated with the disease progression and unresponsiveness of $CD4^+$ cells to antigens negatively correlated with IL-2 production in HIV^+ individuals (Clerici and Shearer,

1993; Clerici and Shearer, 1994; Clerici et al. 1994b; Hooper et al. 1994; Mannhalter et al. 1995).

I have previously shown that humoral immune responses to sulfamethoxazole, an antibacterial agent commonly used in HIV infected patients, are significantly higher in this population than in seronegative patients who receive this drug (Daftarian et al, 1995). This suggests an increase in Th2 responses in HIV^+ individuals compared to the HIV^- persons. A strong humoral response to gp120 has been demonstrated after immunization with protein or DNA of gp120, as vaccine, while CMIR is inhibited with gp120 (Rieckmann et al. 1991b; Fuller and Haynes, 1994; Levy, 1994b).

Apoptosis is known to be an important element in the HIV immunopathogenesis (Muro-Cacho et al. 1995; Oyaizu et al. 1995; Gougeon, 1995; Martin and Green, 1995). In HIV infection, an imbalanced Th1/Th2 response is suggested to be involved in the induction of apoptosis (Cordiali Fei et al. 1994; Clerici et al. 1994a). Type 1 cytokines, IL-2, IFN-γ, and IL-12, inhibit apoptosis, whereas IL-4 and IL-10, type 2 cytokines, enhance apoptosis (Cordiali Fei et al. 1994; Clerici et al. 1994).

The mechanism of a possible switch from Th1 to Th2 is not understood. Cells infected by HIV may enter an anergic state which consequently leads to unresponsiveness to recall antigens. It has been shown that when normal $CD4^+$ cells were exposed to gp120-antibody they become unresponsive to further *in-vitro* stimulation (Devita et al. 1997). It has been hypothesized that such anergy may occur preferentially in Th1 cells resulting in increased cell death of such cells and consequent predominance of Th2 cells (Meyaard et al. 1993; Devita et al. 1997). This hypothesis has been underscored by the fact that Th0 and Th2 cells are

susceptible to HIV infection more than Th1 cells (Meyaard et al. 1993; Romagnani, 1995). Alternatively, such a switch may be due to defect in antigen presentation upon HIV infection that favors development of Th2 responses.

It has recently become clear that immunoregulatory cytokines such as IL-4, IL-10 and IL-12 induce differentiation of naive T helper cells (Th0) into Th1 and Th2 cells (Romagnani, 1994; Muller, 1995; Germann and Rude, 1995). IL-12 deficient mice show deficiency in generation of Th1 responses, while IL-5 deficient mice show deficiency in generation of Th2 responses (Muller, 1995). There is ample evidence to suggest that the commitment of Th0 cells could be driven either to Th1 or Th2, depending upon: (i) the early induction of cytokines such as IFN-γ, IL-12, IL-4 and IL-10 (Trinchieri et al. 1992; Paganin et al. 1995; Romagnani, 1995; Germann and Rude, 1995), (ii) the nature and concentration of antigens (Romagnani, 1995), and,(iii) the antigen processing or presentation by the host cells (Romagnani, 1995). In allergic humans, enhanced IL-4 production was a result of antigen presentation by B cells while such presentation by monocytes resulted in downregulation of IL-4 and enhanced IFN-γ, suggesting that antigen processing by B cells may lead to Th2 responses, whereas processing by macrophages induces Th1 type responses (Secryst et al. 1995). It has also been shown that IL-4 enhances antigen presentation by B cells whereas IFN-γ enhances antigen presentation by monocytes (Abbas et al. 1994). Addition of IL-4 and IL-10 has been shown to lead to predominantly Th2 responses whereas TGF-β, IL-2, IFN-γ and IL-12 may induce Th1 responses (Trinchieri et al. 1992; Paganin et al. 1995; Romagnani, 1995; Germann and Rude, 1995). Moreover, certain antigens induce Th1 cell anergy exclusively, which could enhance IL-4 and IL-10, thereby resulting in predominantly Th2 type responses. Although the type of

Th response generated in response to HIV antigens is not well characterized, alterations in the crossregulation of Th1 and Th2 class responses may play a critical role in HIV immunopathogenesis (Clerici and Shearer, 1993; Rook et al. 1993; Romagnani et al. 1994). It also has been shown that healthy virus positive individuals display a Th1 response and produce IL-2 in response to HIV antigens(Clerici and Shearer, 1993). Survival of IL-4 deficient mice infected with MAIDS virus (murine AIDS) has underscored this hypothesis (Kanagawa et al. 1993). Fauci et al, however, when examining cytokine in biopsied lymph nodes, failed to show a distinct Th1 or Th2 cytokine production in HIV infected individuals, instead, they have shown the production of both IFN-γ and IL-4 (Fauci et al. 1994; Fauci 1997). Romagnani et al. also have shown the induction of both IFN-γ and IL-10 in the PBMC of HIV^+ individuals after mitogenic stimulation and both reports have suggested a tendency toward Th0 (Romagnani et al. 1994; Fauci, 1997). It may also, depend on the stage of HIV infection, degree of immunodeficiency, coexisting infections and other experimental differences.

HIV infection has cytopathic effects on $CD4^+$ T cells and results in their destruction. HIV preferentially replicates in Th2 and Th0 clones (Maggi et al. 1994), which may result in their destruction *in vivo*. Also, exposure of Th1 cells to Th2 cytokines may induce destruction of Th1 cells (Clerici and Shearer, 1994; Del Prete et al. 1995). It has been demonstrated that Th function during HIV infection is affected before a decrease in CD4 cell count occurs (Levy, 1994i; Ada, 1994). The initial Th defect involves the loss of proliferative response to recall antigens (Clerici et al. 1990) and may be due to selective loss of Th memory cells (van Noesel et al. 1990; Fauci and Rosenberg, 1994; Levy, 1994d) or due to the production of immunosuppressive, soluble factors (Fauci and Rosenberg, 1994; Levy, 1994c) or due to

anergy (Meyaard et al. 1993). Gradual loss of CMIR has been correlated with disease progression in HIV^+ individuals (Ada, 1994; MacDonell et al. 1990; Pantaleo and Fauci, 1994). In addition, enhanced IL-10 production by PHA-stimulated PBMC of HIV^+ individuals has been correlated with impaired ability to secrete IL-2 following stimulation with recall antigens, alloantigens and PHA (Romagnani, 1995). Presence of a Th1 type response against HIV antigens is reported among seronegative HIV-exposed persons. Interleukin-2 was elevated upon exposure of PBMC of these individuals to HIV antigens (Clerici and Shearer, 1993). Thus, certain elements of HIV pathogenesis may dictate an inappropriate class of immune response in HIV infection.

11. Role of HIV regulatory genes in cytokine expression

HIV contains at least three regulatory genes (tat, rev, nef) that operate both positively and negatively to regulate virus expression (Levy, 1994b). The HIV tat protein is a potent transactivator of gene transcription (Levy, 1994a). In contrast, the nef gene is nonessential for virus growth and remains without a precisely defined function (Levy, 1994a). It has been suggested that the nef gene exerts negative regulatory effects on virus expression (Ada, 1994; Levy, 1994e). In addition to their regulatory roles in viral replication, tat and nef gene products have been suggested to exert some effects on the expression of host cellular genes, particularly on cytokine genes (Masood et al. 1994; Sharma et al. 1995). The tat gene has been shown to suppress antigen but not mitogen-induced proliferation of PBMC and may contribute to the development of immunodeficiency (Viscidi et al. 1989). This biological

activity of *tat* is due to its inhibition of IL-2 production and may have clinical relevance since lymphocytes from HIV-infected individuals show a loss of proliferation to recall antigens (Subramayam et al. 1993). Similarly, the nef gene has also been demonstrated to downregulate the expression of at least two cellular proteins, namely CD4 and IL-2 (Baur et al. 1994; Levy, 1994e), while it could stimulate B-cell (Chirmule et al. 1994).

12. Role of IL-10 and IL-12 in the pathogenesis of HIV infection

12. a. IL-10 and its biological characteristics

IL-10 was first cloned from murine Th2 cells and, since it inhibited cytokine synthesis by Th1 clones, it originally was designated as cytokine synthesis inhibitory factor (CSIF) (Fiorentino et al. 1989). Human IL-10 was later cloned and was shown to have 73% homology at the amino acid level with the murine protein (Viera, et al. 1991) and 83% homology to the product of an open reading frame of Epstein-Barr virus (EBV) genome known as BCRF1 or viral IL-10 (Moore et al. 1990; Hsu et al. 1990). Human IL-10 is produced by Th0 and Th2-like CD^+ T cell clones, B cell lines derived from AIDS patients and patients with Burkitt's lymphoma, activated monocytes and peripheral blood T cells, Th2-like $CD8^+$, $CD4^+$ $CD45RA^+$ naive, and $CD4^+$ $CD45RA^-$ memory T cells (Go et al. 1990; de Waal Malefyt, et. al., 1992; Le Gros and Erard, 1994; Smith et. al., 1994; Mosmann, 1994; Banchereau, 1995). The major cellular source of IL-10 is monocytes and T cells. Small quantities of IL-10, however, are produced by Th1 cells (Romagnani, 1994; Romagnani,

1995). The receptor for human IL-10 is a transmembrane glycoprotein and closely resembles the IFN-γ receptor (Liu, et. Al. 1994; Banchereau, 1995).

IL-10 is a pleiotropic molecule possessing a broad spectrum of biological activities. IL-10 may contribute to the polyclonal B cell proliferation and hypergammaglobulinemia observed in HIV infection, as it is a growth factor for normal and EBV transformed human B cells (Romagnani, 1995). Moreover, the EBV-negative B cell lymphomas observed in patients with AIDS secrete IL-10, which may further aggravate immunosuppression (Emilie et al. 1992). These observations show a correlation between the IL-10 production and disease progression.

IL-10 is a promoter of proliferation of IL-2 activated cytotoxic T cells (Le Gros and Erard, 1994; Banchereau, 1995). IL-10 has potent inhibitory functions on T cell proliferation which act partially via downregulation of MHC II expression and inhibition of production of cytokines from monocytes (Banchereau, 1995; Fiorentino et al. 1991). Human IL-10 inhibits antigen-specific proliferation of Th0, Th1, and Th2 cells, as well as antigen-stimulated cytokine synthesis by PBMC and natural killer (NK) cells in a macrophage/monocyte dependent manner (De Waal Malefyt et. al., 1991; De Waal Malefyt et al. 1993; Enk et al. 1993). These effects of IL-10 might be at least in part a result of the downregulation of expression of class II MHC and expression of co-stimulatory molecules that are necessary for T cell and NK cell activation (de Waal Malefyt et. al., 1991; Yssel et al. 1992; Howard and O'Garra 1992; de Waal Malefyt et al. 1993; Romagnani, 1995).

IL-10 is a strong modulator of monocytes and downregulates the production of prostaglandin E2 as well as production of pro-inflammatory cytokines such as TNF-α, IL-1

and IL-6 (Yssel et al. 1992; de Waal Malefyt et al. 1993; Romagnani, 1995). IL-10 inhibits the expression of B7 and ICAM-1 molecules while it enhances the expression of soluble TNF receptors (Romagnani, 1995). IL-10 has been associated with the immunopathogenesis of a number of diseases including septic shock, lymphoproliferative disorders and autoimmune diseases (Romagnani, 1995). Induction of IL-10 expression in certain parasitic and EBV infections is suggested to be a strategy employed by these pathogens to evade the host immune system (Hsu et al. 1990; Sher et al. 1992). There is growing evidence to believe in the existence of such a strategy for HIV as well (Romagnani, 1995; Clerici and Shearer, 1993; Clerici and Shearer, 1994; Clerici et al. 1994b; Hooper et al. 1994; Mannhalter et al. 1995). Independent observations have indicated a strong association between enhanced mitogen stimulated IL-10 production and HIV disease progression which has led to the controversial suggestion that a Th1 to Th2 switch may correlate with disease progression (Clerici and Shearer, 1993; Mannhalter et al. 1995; Clerici and Shearer, 1994; Clerici et al. 1994b; Hooper et al. 1994). On the other hand, IL-10 production upon HIV infection may be part of immune mechanisms to suppress HIV replication (Akridge et al. 1994) or inhibit immune activation leading to apoptosis (Gougeon, 1995a).

12. b. Biological activity of IL-12

Natural killer cell stimulatory factor, or IL-12, is a heterodimeric disulfide-linked polypeptide comprised of a heavy chain (p40) and a light chain (p35) (Trinchieri et al. 1992; Trinchieri, 1994; Germann and Rude, 1995). The heavy chain is inducible following stimulation, but IL-12 biological activity is limited to the dimeric polypeptide (Trinchieri,

1994; Chehimi et al. 1994; Trinchieri et al. 1992; Germann and Rude, 1995). IL-12 was first isolated from EBV-positive B cell lines (Trinchieri et al. 1992; Germann and Rude, 1995). Monocytes/macrophages seem to be the major producers of IL-12 *in vivo* (Trinchieri et al. 1992; Chehimi et al. 1994; Germann and Rude, 1995). It is produced a short time after stimulation with various antigens, such as bacterial antigens (Trinchieri et al. 1992; Chehimi et al. 1994; Germann and Rude, 1995).

IL-12 is involved in the regulation of cytokine production, proliferation and cytotoxic activity of T cells and natural killer cells, and stimulates IFN-γ production (Trinchieri et al. 1992; Germann and Rude, 1995). IL-12 participates in the development of $CD8^+$ T cells and Th1 cells (Chehimi et al. 1994; Germann and Rude, 1995). IL-12 has been implicated in protective immune responses to a number of parasitic infections such as leishmania major and in antitumor and antimetastatic activities (Trinchieri et al. 1992; Chehimi et al. 1994; Germann and Rude, 1995).

The fact that IL-12 induces differentiation of Th0 cells into cells with Th1 phenotypes (Trinchieri et al. 1992; Germann and Rude, 1995; Paganin et al. 1995) makes this cytokine a candidate for therapeutic purposes especially in diseases in which a cellular response is needed for protection, such as cancer and AIDS (Germann and Rude, 1995). The role of the immunoregulatory cytokines IL-10 and IL-12 in the pathogenesis and loss of immune function in HIV infection is not well understood.

13. Unresponsiveness in HIV infection

The loss of CMIR in HIV infection has been attributed to impaired memory T cell function (van Noesel et al. 1990; Mannhalter et al. 1995). In some HIV infected individuals, memory T cells are unresponsive to the TCR-triggering by recall antigens and anti-CD3 antibodies (Schellekens et al. 1990; Levy, 1994d; Pantaleo and Fauci, 1995). However, the cellular basis for this unresponsiveness is not well understood. Various mechanisms have been suggested to explain the loss of immune functions including the immunosuppressive effects of viral proteins such as gp120, *nef* and *tat* proteins (Fauci and Rosenberg, 1994; Liegler and Stites, 1994; Pantaleo and Fauci, 1995), programmed cell death following defective stimulation of $CD4^+$ T cells by antigen presenting cells and ligation of CD4 molecules by gp120 antigen (Gougeon, 1995b; Martin and Green, 1995; Corbeil and Richman, 1995; Muro-Cacho et al. 1995; Fauci and Rosenberg, 1994); and defective expression of T cell co-stimulatory molecules (Vingerhoets et al. 1995; Levy, 1994d; Ada, 1994; Fauci and Rosenberg, 1994) which are essential for T cell activation.

PBMC proliferation in response to recall antigens in many HIV infected individuals can be restored by exogenous administration of IL-2, indicating the impaired ability of lymphocytes to be activated by antigen (Clerici and Shearer, 1994). Perhaps at early times after HIV infection the signalling mechanisms of PBMC from HIV^+ persons are not disturbed. It has been shown that the uninfected T cell clones derived from HIV infected individuals exhibited normal early cell signalling mechanisms, such as Ca^{2+} influx and protein kinase C activation (Kinter et al. 1990; Gibellini et al. 1994).

As mentioned earlier, IL-10 has been shown to induce antigen-specific unresponsiveness *in vivo* (Gallo et al. 1994; Doherty, 1995; Banchereau, 1995; Bluestone, 1995), however, the contribution of IL-10 to the unresponsiveness to HIV antigens and other recall antigens in HIV infection remains to be investigated.

14. The regulation of IL-10 expression in monocytes and T cells

The regulation of IL-10 expression is not well understood. TNF-α has been shown to induce IL-10 expression in monocytes (Wanidworanun and Strober, 1993). However, regulation of IL-10 expression in T cells is not clear. Based on my original observations (to be discussed later), I hypothesized that T cells from HIV^+ individuals gradually lose the ability to produce IL-10 and consequently the primary source of IL-10 is monocytes. Thus, after HIV infection, a switch in IL-10 production from "T cells and monocytes" to "monocytes" is observed (Diaz-Mitoma et al. 1995; Daftarian et al. 1995a). This switch may result from the overall dysregulation of cytokine production following HIV infection. Therefore, it is necessary to study the regulation of IL-10 production by T cells, and to clarify which cytokines are required for T cells to produce IL-10, and which of these cytokines demonstrate altered expression in HIV infected individuals. Thus, the regulation of IL-10 production by T cells and monocytes must be addressed in HIV^- as well as HIV infected individuals.

Cell-mediated and humoral immune responses are often mutually exclusive and associated with Th1 type and Th2 type cytokines, respectively (Mosmann and Moore, 1991). Th1 and Th2 cells are negatively cross regulated through their cytokines which are responsible

for the reciprocal suppression of cell mediated and humoral immune responses (Mosmann and Moore, 1991). IFN-γ and IL-12 induce Th1 type responses, whereas humoral immune responses are mediated by IL-4, IL-6 and IL-10 as these cytokines induce B cell proliferation and antibody production (Romagnani, 1995; Trinchieri et al. 1992).

IL-6, a multifunctional cytokine, plays a vital role in antibody production as it is a potent B cell stimulatory and differentiation factor (Akira et al. 1993). The role of IL-6 in humoral immune responses was demonstrated recently in IL-6 deficient mice (Kopf et al. 1994) showing a profound inhibition of specific antibody production following T cell dependent stimulation of B cells *in vivo*. In addition, IL-6 regulates T cell activation, growth and differentiation, IL-2 production and haemopoiesis (Akira et al. 1993).

IL-10 exerts multiple effects in various facets of the immune response including inhibition of macrophage-antigen presenting cell dependent cytokine synthesis by Th1 cells, co-stimulation of mast cell growth, and co-stimulation of thymocyte growth in the presence of IL-2 and/or IL-4 (Go et al. 1990; Howard and O'Garra, 1992; Banchereau, 1995).

Recent evidence suggests that IL-10 is immunoregulatory in nature as it inhibits antigen driven activity of both Th1 and Th2 subsets (de Waal Malefyt et al. 1991; Banchereau, 1995). Studies on the kinetics of cytokine production have revealed that IL-6 and IL-12 along with IL-1, IL-2, tumor necrosis factor-α (TNF-α), granulocyte-monocyte colony stimulating factor (GM-CSF), G-CSF and IFN-γ are the first cytokines synthesized following activation of PBMC by mitogens (de Waal Malefyt et al. 1991; Seeder and Paul, 1994). Moreover, IL-10 has been suggested to be involved in shifting the balance of an immune response away from cellular immunity to humoral immunity (Banchereau, 1995; Li et al. 1994; Mosmann and

Moore, 1991). Therefore, understanding the regulation of IL-10 production may help in devising strategies to modulate the immune response. However, the molecular mechanisms underlying the production of IL-10 are not clear.

IL-10 production by monocytes has been shown to be regulated by TNF-α (Wanidworanun and Strober, 1993). Since induction of Th1 and Th2 type immune responses can be negatively cross regulated by immunoregulatory cytokines, I reasoned that IL-10 production by T cells may be regulated by soluble factors produced by monocytes/macrophages.

15. Hypothesese

I. HIV infection may alter the balance of immunoregulatory T helper cytokines. Such an alteration may result in the emergence of a non protective class of immune response which may undermine the clearance of HIV infection.

II. HIV infection may result in the altered expression and production of IL-10 by T cells and monocytes. This altered regulation of IL-10 may be associated with the progression of HIV disease by contributing to T cell unresponsiveness to recall antigens and by inhibiting Th1 type responses.

III. Altered production of IL-10 by T cells and monocytes may be regulated by distinct immunoregulatory cytokines.

16. Aims and objectives

I. Analysis of the relative levels of Th1 and Th2 cytokines in HIV infection

In addition to the direct loss of $CD4^+$ T cells, immunosuppression may also result from the release of cytokines which inhibit the expression of growth factors essential for normal functions of immune effector cells. HIV may induce IL-10 (Ameglio et al. 1994), a cytokine synthesis inhibitory factor that inhibits essential elements of the anti-viral response, including IFN-γ and IL-2 synthesis (Banchereau, 1995; Le Gros and Erard, 1994). IL-10 may also contribute to the polyclonal B cell proliferation, hypergammaglobulinemia, and the EBV induced B cell lymphomas observed in HIV^+ patients as suggested by *in vitro* studies (Banchereau, 1995; Benjamin et al. 1992; Benjamin et al. 1994). These observations suggest that IL-10 is intimately associated with the pathogenesis of HIV. Similarly, enhanced expression of IL-4, another Th2 type cytokine has been observed in HIV^+ individuals (Clerici et al. 1994a; Clerici and Shearer, 1994). Furthermore, conflicting results have recently been obtained regarding the Th1-Th2 switch hypothesis in HIV disease progression (Graziosi et al. 1994). Therefore, to better understand the status of cytokine patterns in HIV infection, the first objective was to measure IL-10, IL-4, IL-12 and IFN-γ production *in vivo* and *in vitro* in unstimulated and mitogenically stimulated PBMC of HIV^+ patients compared to HIV^- individuals.

II. Effects of IL-10 and IL-12 on unresponsiveness of PBMC of HIV^+ individuals to recall antigens

To investigate the presence of a possible link between imbalanced IL10/IL-12 expression and unresponsiveness observed in HIV infection, I intended to examine the role of these cytokines in responses to recall antigens.

IL-12 and IL-10 are known for the generation and suppression of Th1 responses, respectively. Therefore, the relationship of their expression with recall-antigen responses was the focus of the second objective. It was hypothesized that in HIV infection, a gradual loss of responses to recall antigens is associated and correlated with increased IL-10 expression. It was also hypothesized that there is a deficiency of IL-12 production in HIV infection. Therefore, the effects of IL-10 and IL-12 on the proliferation responses of PBMC from HIV^+ individuals to recall antigens were evaluated.

III. Monokine(s) required for the regulation of IL-10 production by T cells and monocytes in HIV^- and HIV^+ individuals

The fact that IL-10 is a suppressor factor for the Th1 responses, an inducer of Th2 responses, a suppressor of production of IL-12 and IFN-γ, and an inducer of anergy, makes it an excellent candidate for the studies of HIV immunopathogenesis. My initial observations revealed that in HIV^+ individuals, regulation of IL-10 expression is different from that of HIV^-

persons. I have shown that monocytes are required for IL-10 production by HIV^- T cells. I have also shown that HIV^+ T cells do not produce IL-10. It is possible that factor(s) from monocytes are required by T cells to produce IL-10. After HIV infection, a defect in production of such factors may contribute to IL-10 dysregulation. Therefore, the regulation of IL-10 production in T cells and monocytes was further studied. The third objective was then to clarify factor(s) from monocytes that are required for IL-10 production in T cells and monocytes.

Chapter II. Materials and methods

1. HIV$^+$ Patients and Controls

Blood samples were collected following approval of the protocol by the Ethics Review Committee of the Ottawa General Hospital, University of Ottawa, Ottawa, Ontario, Canada. Blood was obtained for mononuclear cell isolation from healthy adult volunteers and HIV$^+$ individuals. Clinical information was obtained for each of the HIV$^+$ patients, including CD4$^+$ and CD8$^+$ T cell counts. In a series of experiments HIV$^+$ patients were divided into two groups, patients with <400 CD4$^+$ T cells/ mm^3 and patients with >400 CD4$^+$ T cells/mm^3 {range 88-517 cells/mm^3}. All patients were EBV seropositive but had no clinical manifestations of infectious mononucleosis or B cell lymphomas. None of the patients had clinical evidence of bacterial or fungal infection at the time of specimen collection. We used the revised CDC classification to stage HIV disease in this population (Levy, 1994c). All HIV$^+$ individuals had recently been vaccinated against influenza virus, whereas HIV- controls population included both vaccinated and unvaccinated.

In order to study the regulation of IL-10 expression by T cells, blood was obtained for isolation of PBMC from healthy volunteers that were recruited by the Canadian Red Cross, Ottawa, Canada. The blood was tested for presence of antibodies against a panel of pathogens including hepatitis B, CMV, and HIV; the blood was used only if negative for these antibodies.

2. Reagents

Human(h) IL-1α , IL-1ß, GM-CSF and M-CSF were purchased from Genzyme, Montreal, Canada. Human IL-6, neutralizing antibodies to hIL-6, IL-10, neutralizing antibodies IL-10, IFN-γ , IL-4, neutralizing antibodies IL-4 and TNF-α were obtained from R&D Systems, Minneapolis MN. IL-12 and neutralizing antibody to IL-12 were generously provided by Dr. Maurice Gately, Hoffman La Roche, Nutley, NJ. GM-CSF and G-CSF were kindly provided by Dr. L. G. Filion (Department of Microbiology and Immunology, University of Ottawa, Ottawa). Phytohemagglutinin-M (PHA) was purchased from Sigma, St. Louis, MO and pokeweed mitogen (PWM) from GIBCO Laboratories, Grand Island, NY.

Influenza antigen (heat-inactivated A/Hong Kong/1/68) was generously provided by Dr. Earl Brown (University of Ottawa, Ottawa, Ontario). Anti-CD3 antibodies were obtained from supernatants of the OKT-3 hybridoma (American Type Culture Collection, Rockville, MD). HIV antigens p25 and gp120 were kindly provided by the National Institutes of Health, AIDS Research and Reference Reagents Program, (Bethesda, MD).

3. Isolation and culture of PBMC

Standard methods were used for cell preparation and fractionation (Coligan et al. 1992). In brief, PBMC were isolated by density gradient centrifugation using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). In 50-ml polystyrene tubes (Corning, Oxnard, CA), 33 ml of blood was layered on 17 ml of Ficoll-Hypaque. Tubes were centrifuged for 30 minutes at 400

× g at 18°C. The cell layer consisting mainly of mononuclear cells was collected and washed three times in phosphate buffered saline (PBS). Cell viability was determined by trypan blue dye exclusion. PBMC were resuspended in complete media (CM) for biological assays. The media (CM) used for all cultures was complete Iscove's Modified Dulbecco's Medium (IMDM, Sigma Chemical Company, St. Louis, MO) supplemented with 10% Fetal Bovine Serum (FBS, GIBCO Laboratories, Grand Island, NY), 100 U / ml penicillin, 100 μg / ml gentamicin, 10 mM HEPES and 2mM glutamine, unless otherwise specified.

For proliferation assays, cells were cultured in 200 μl / well, at a concentration of 2 × 10^6 cells/ml in 96-well tissue culture plates (Falcon Labware, Oxnard, CA). In order to perform biological assays, cells were cultured at a concentration of 2 x 10^6 cells / ml in 24-well tissue culture plates (Falcon Labware, Oxnard, CA). All cultures were incubated at 37° C in 5% CO_2 in a humidified incubator unless otherwise mentioned.

4. Stimulation of PBMC for analysis of cytokines

To determine the ability of PBMC from HIV^+ individuals and controls to produce cytokines, the cells were cultured at a concentration of $2x10^6$ cells / ml in 24 well tissue culture plates (Falcon Labware, Oxnard, CA). PBMC were stimulated with PHA at a final dilution of 1:50, anti-CD3 antibodies at a final dilution of 1:200 and pokeweed mitogen (PWM, GIBCO) at a final dilution of 1:100. The supernatants were harvested after 24, 48 and 72 hours and frozen at -70° C. Supernatants were thawed once at the time of analysis of cytokine production by ELISA. Thawing of supernatants once, compared to not freezing, did not

influence measured cytokine concentration. To determine the ability of unstimulated PBMC from HIV^+ individuals to produce cytokines, the cells were cultured in a similar fashion in 24 well tissue culture plates (Falcon Labware, Oxnard, CA) in the absence of stimuli.

5. Measurement of IL-10 by ELISA

IL-10 was measured by a sandwich ELISA using two different monoclonal antibodies (mAb) which recognize distinct epitopes as described previously (Mosmann et al. 1990). Briefly, 96-well plates (Nunc Immunomodules, Denmark) were coated overnight at 4^0 C with purified anti-IL-10 mAb, JES3-9D7 (Rat IgG1 obtained from Pharmingen, San Diego, CA) 100 µl / well at a concentration of 3 µg / ml in the coating buffer (0.1 M $NaHCO_3$, pH 8.2). The plates were washed with PBS-Tween 20 and blocked with PBS-10% FBS. IL-10 was detected by employing a second biotinylated mAb, 18562D (Rat IgG2a obtained from PharMingen) at a concentration of 3 µg/ml in PBS-10% FBS. Streptavidin-peroxidase was used at a final dilution of 1:1000 (Jackson Immuno Research). The color reaction was developed by O-phenylenediamine dihydrochloride (OPD, Sigma) and hydrogen peroxide, and optical density was read at 490 nm. Recombinant human IL-10 (R&D Systems) was used as a standard.

The sensitivity of the IL-10 ELISA was 16 pg/ml. The IL-10 stimulation index (SI) was calculated as a ratio of IL-10 levels (pg/ml) produced by PHA stimulated PBMC to IL-10 levels produced by unstimulated PBMC.

6. Measurement of IL-12 by ELISA

The active form of IL-12 is a heterodimer molecule composed of p35 and p40. P40 may be expressed in excess and a p40 dimmer may act as regulator of IL-12 production (Trinchieri, 1994). A sandwich ELISA for measurement of IL-12 p40 was performed as described by Dr. M. Gately (Hoffman La Roche) using two different mAb that recognize distinct epitopes on IL-12 p40. Briefly, plates were coated with 100 µl / well of 2.5 µg/ml of 2-4A1 antibody (rat anti-hIL-12 p40, IgG2b, Hoffman La Roche) in coating buffer (0.015 M sodium carbonate, 0.035 M sodium bicarbonate pH 9.6) overnight at 4^0C. Wells were blocked with 0.5% bovine serum albumin in PBS. The wells were incubated at room temperature for two hours with 100 µl / well of culture supernatants to be analyzed for IL-12 p40. IL-12 was detected by a second peroxidase conjugated mAb 4D6 (rat anti-hIL-12 p40, IgG1, Hoffman La Roche) at a concentration of 0.5 µg / ml. The color reaction was developed by OPD and hydrogen peroxide and optical density was read at 490 nm. IL-12 p40 was used as a standard. Sensitivity of the IL-12 ELISA was 8 pg/ml.

7. Measurement of IFN-γ by ELISA

A sandwich ELISA was employed to quantify the IFN-γ produced by PBMC. Two different mouse monoclonal antibodies recognizing two distinct IFN-γ epitopes were used; 100µl / well of mAb 3-11.1 (American Type Culture Collection, Rockville, MD) was used at a concentration of 5 µg/ml in PBS for coating the binding 96-well plates (Nunc Immuno-

modules, Denmark) at 4° C overnight. Plates were blocked with 5% skimmed milk in PBS-Tween 20 (0.05%). IFN-γ was detected by a second mAb B-24 (UBI-Olympus, Lake Success, NY) which was biotinylated as described (Coligan et al. 1992) at a concentration of 5 μg/ml in PBS-Tween 20. Streptavidin conjugated with horseradish peroxidase (Jackson Immuno Research, West Grove, PA) was used at a final dilution of 1:1000. Color reaction was developed by OPD and hydrogen peroxide which was measured at 490 nm. Recombinant human IFN-γ (BioSource International, Camarillo, CA) was used as a standard. The sensitivity of assay was 16 pg/ml.

8. Measurement of IL-4 by ELISA

Human IL-4 was quantified by sandwich enzyme immunoassay using the human IL-4 QuantikineTM kit (R&D Systems) as described by the manufacturer. Recombinant human IL-4 provided in the kit was used as a standard. Ninety six-well microtiter plates were coated with a murine monoclonal antibody against IL-4 and a polyclonal antibody against IL-4, conjugated to horseradish peroxidase, was provided as secondary antibody. Sensitivity of the assay was 16 pg/ml. The kinetics of IL-4 expression by PBMC were studied, and the peak of IL-4 secretion after PHA stimulation was shown to be 48 hours. Thereafter, IL-4 was measured in the culture supernatants harvested 48 hours after stimulation with PHA.

9. Measurement of cytokines by semi-quantitative reverse transcriptase-based polymerase chain reaction (RT-PCR)

9. a. RNA isolation and detection of IL-10 and IFN-γ mRNAs

The RT-PCR employed for the measurement of relative levels of β-actin, IL-10 and IFN-γ mRNA was according to Gendelman et al. 1990; Svetic et al. 1991, Peterson et al. 1994. An internal housekeeping gene, β-actin, was used as standard. Such an endogenous standard permits the detection of relative differences in the integrity of individual mRNA samples. Because the degree of RNA degradation may vary from sample to sample, the method is semiquantitative and has limitations.

Total cellular RNA was extracted from equivalent numbers of PBMC obtained from both HIV- and HIV^+ individuals using a monophase solution containing guanidine isothiocyanate and phenol (Tri Reagent solution, Molecular Research Centre, Inc., Cincinnati, OH) as described by the manufacturer. The RT-PCR technique was employed to assess levels of IL-10 and IFN-γ mRNA expression. cDNAs were generated by reverse transcription from 1 μg of total RNA using the Perkin Elmer thermal cycler 9600 and Perkin Elmer Gene Amp RT-PCR kit (Perkin Elmer, Norwalk, CT) as described by the manufacturers. Briefly, the reverse transcription was performed using 2.5 mM $MgCl_2$, 1 mM each of the nucleotides (dNTP), 1 U/ml RNase inhibitor and 2.5 mM random hexamers in a final 10 ml volume. The reaction was carried out under the following conditions: 42°C for 15 min followed by 94°C for 5 min and 5°C for 5 min. Equal aliquots of cDNA (5 μl) were subsequently amplified for IL-10, IFN-γ and β-actin using 2.5 units of AmpliTaq DNA polymerase, 150 pMol each of the appropriate 5'

and 3' primers, 1mM of each dNTP and 4 mM $MgCl_2$ in a total volume of 100 ml. The oligonucleotide primers for IL-10 (Van Voorhis, 1992), IFN-γ (Gray and Goeddel, 1982) and β-actin (Iijima, 1985) were obtained from Strategene, La Jolla, CA.. and the conditions for amplification were optimized for each of them. For IL-10, the first cycle consisted of denaturing at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for one min and primer annealing and extension at 60°C for one min. The last extension was at 60°C for 7 min. For IFN-γ and β-actin, the first cycle consisted of denaturation at 94°C for 5 min followed by primer annealing at 60°C for 5 min. The PCR reaction consisted of 35 cycles, with denaturation at 95°C for 45 sec followed by primer annealing at 60°C for 45 sec and extension at 72°C for 1.5 min. Finally, the last cycle was at 72°C for 10 min. PCR products (20 µl) were resolved by electrophoresis on 2% agarose gels. To ensure the detection of specific amplified cDNA, the PCR products were transferred onto Zeta probe filters (BioRad Laboratories, Richmond, CA) according to manufacturer's specifications. The filters were then hybridized to ^{32}P-labelled cDNA probes specific for the amplified products (Prime IT II kit, Stratagene, La Jolla, CA).

9. b. Primers and probes

The following primers for IL-10, IFN-γ and β-actin were used. IL-10: sense 5'-ACA GGA TCC TAT GCA CAG CTC AGC ACT GCT C- 3'; antisense 5' -TAG GAT CCT CAC CTG GCT TTA ATT GTC ATG TAT GC- 3'. The primers for IL-10, () which amplified a

531 bp product and were specific for human IL-10 (Stratagene, La Jolla, CA). Oligonucleotide primers for IFN-γ amplified a PCR product of 501 bp and were provided by Stratagene (La Jolla, CA): sense 5' -ATG AAA TAT ACA AGT TAT ATC TTG GCT TT- 3'; antisense 5' -GAT GCT CTT CGA CCT CGA AAC AGC AT- 3'. The primers for β-actin were as follows: sense 5' -TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA- 3' and antisense 5' -CTA GAA GCA TTG CGG TGG ACG ATG GAG GG- 3' and amplified a PCR product of 610 bp. The human IL-10 cDNA probe was obtained from Dr. Kevin Moore, DNAX Research Institute of Molecular and Cellular Biology, CA.

9. c. Semiquantitation of IL-10 and IFN-γ mRNA by RT-PCR analysis

The quantitation of specific mRNA for IL-10 and IFN-γ expression in PBMC by semiquantitative RT-PCR analysis involved comparing the signals for IL-10 and IFN-γ with respect to β-actin. Equal amounts of cDNA obtained following reverse transcription of cellular RNA from PBMC of patients and normal individuals were PCR amplified for IL-10, IFN-γ and β-actin. The autoradiographs obtained following hybridization were scanned for the signals by densitometry, analyzed by image analyzing software and assigned arbitrary units. The bands specific for IL-10, IFN-γ and β-actin mRNA were measured by densitometry with the help of an image software program (Image-Pro Plus, Media Cibernetics, Silver Spring, MD).

Density values for IL-10 and IFN-γ were provided as a ratio of the cytokine to the β-actin signal to normalize the expression of cytokines to β-actin. The relative expression of IL-

10 and IFN-γ was analyzed by comparing the ratio of the densitometric units for IL-10 to β-actin, and IFN-γ to β-actin respectively.

For the statistical analysis, means of ratios were compared by the two-tailed Student's T test. To evaluate the reproducibility the coefficient of variance of different points was calculated.

10. Preparation of anti-CD3 antibodies

Anti-CD3 antibodies were prepared from supernatants from the OKT-3 hybridoma (American Type Culture Collection, Rockville, MD). Ten ml of PBS containing 1.5 g of A-sepharose CL4B (protein-A immobilized on sepharose CL-4B, protein-A from Staphylococcus aureus binds FC portion of IgG) was incubated for one hour at room temperature, the solution was loaded into a chromatography column and stored at 4° C. Ten ml of the OKT-3 hybridoma cell supernatant were diluted with an equal amount of PBS and then filtered through the column at a flow rate of 30 ml/hour. The column was washed with PBS until there was no protein detected by UV spectrophotometer at 280 nm.

Bound antibodies were eluted with glycine-HCL buffer, pH 2.8. The pH of the antibody solution was then titrated to 7.0 by NaOH/Tris and antibody solution was dialyzed against PBS overnight at 4° C .

11. Cell proliferation assays

PBMC ($1x10^6$ cells/well) were stimulated with PHA (1:50 final dilution), or anti-CD3 antibodies (1:200 final dilution) for 48 hours, or with influenza antigen (1 μg/ml) or p25 antigen (1 μg/ml) for 7 days, in a final volume of 200 μL in triplicate in 96 well plates (Falcon, Becton Dickinson Labware, Lincoln Park NJ). The cells were stimulated in the presence or absence of neutralizing anti-IL-10 antibodies (R & D Systems, 5 μg/ml) and/or IL-12 (Hoffmann La Roche, 170 U/ml). Cells were pulsed with 0.5 μCi [^{3}H] thymidine (Amersham, Arlington Heights, IL) and cultured for a further 16 hours followed by cell harvesting (Horvester 96, Conecc.) and measurement of [^{3}H] thymidine incorporation (liquid scintillation counter, 1450 Microbeta, Wallac, Turku, Finland). The stimulation index (S.I.) was calculated as a ratio of [^{3}H] thymidine incorporation (cpm) by PBMC stimulated in the presence of antigen/mitogen to that of PBMC cultured in the absence of antigen/mitogen.

12. Depletion of T and B cells in PBMC

T cells and B cells were depleted from PBMC using anti-CD2 antibody and anti-CD19-antibodies coated immunobeads (Dynal, Lake Success, NY), respectively according to manufacturer description. Briefly, PBMC were incubated with immunobeads at a bead to target cell ratio of 20:1 at 4° C for 20 min with gentle rotation. The cells bound to the immunobeads were separated using a magnetic field.

The remaining unbound cells were washed and analyzed for the presence of T cells and B cells by flow cytometry using fluorescein isothiocyanate (FITC) conjugated anti-CD3 antibodies (Becton Dickinson), phycoerythrin conjugated anti-CD20 antibodies (Becton Dickinson), respectively. Anti-CD2 antibody-depleted PBMC and anti-CD19 antibody-depleted PBMC contained less than 2% T cells and B cells, respectively.

Depletion of T and B cells was also evaluated by measuring cell proliferation in response to PHA, and the combination of anti-IgM antibodies coated sepharose beads (BIO-RAD, Richmond, CA) and IL-4, respectively. Cell proliferation was measured by incorporation of [^{3}H] thymidine. Cells were pulsed with 0.5 μCi [^{3}H] thymidine (Amersham, Arlington Heights, IL) and cultured for a further 16 hours followed by cell harvesting and measurement of thymidine incorporation. A liquid scintillation counter (microbeta PLUS from Wallac) was used for the measurement of thymidine incorporation. This system normally gives a cpm lower than some other methods. T cell depleted PBMC did not proliferate following stimulation with PHA (cpm of this fraction when stimulated with PHA were comparable to background levels, 2460 ± 720 *versus* 2770 ± 582, while cpm of undepleted PBMC in response to PHA were 38,910 ± 3,750). B cell depleted PBMC, on the other hand, failed to proliferate in response to stimulation with anti-IgM antibody coated beads plus IL-4 (cpm of this fraction when stimulated were not significantly different from background level, 1065 ± 120 *versus* 770 ± 440 cpm; the thymidine incorporation of undepleted PBMC was 6,390 ± 375 cpm).

13. Depletion of monocytes in PBMC

PBMC were depleted of monocytes using anti-CD14 coated immunobeads (Dynal, Lake Success, N.Y.) as described above for T and B cell depletion. CD14 negative PBMC contained less than 2% $CD14^+$ monocytes as determined by flow cytometric analysis for contaminating monocytes using phycoerythrin conjugated anti-CD14 antibody (Becton Dickinson). Alternatively, monocytes and natural killer cells were removed by L-leucine methyl ester (L-LME) as described (Coligan et al. 1992). Phagocytic and cytotoxic cells will take up L-LME. Lysosomal enzymes in lysosomes then make a toxic product, L-leucyl-L-leucyl methyl ester, which eliminates monocytes, NK cells and cytotoxic T cells. Briefly, PBMC (3×10^6 cells/ml) were incubated with 0.05 M L-LME in serum free medium at room temperature for 35 min. L-LME was neutralized by the addition of FCS at a final concentration of 10%. LME treated population showed < 2% monocytes after staining with anti-CD14 and analysis by flow cytometry. The cells were washed three times and assayed for T cell proliferation by thymidine incorporation in response to PHA and anti-CD3 antibodies. L-LME-treated PBMC proliferated in response to PHA and anti-CD3; however, [^{3}H] thymidine incorporation of this fraction compared to that of PBMC was partially reduced (27±4 %). Data from flow cytometric analysis (presence of < 2% monocytes) and reduced proliferation after PHA stimulation show that depletion of monocytes was successful but not complete.

14. Isolation of pure $CD4^+$ and $CD8^+$ T cells from PBMC

$CD4^+$ and $CD8^+$ T cells were isolated from PBMC by positive selection using anti-CD4 antibody and anti-CD8 antibody coated immunobeads (Dynal), as described above. Briefly, PBMC were incubated with immunobeads at a bead to target cell ratio of 4:1 at 4° C with gentle rotation for 20 min. The cells attached to the immunobeads were washed six times and separated using a magnetic field.

To deplete $CD4^+$ monocytes bound to the anti-CD4 antibody conjugated immunobeads, $CD4^+$ cell fraction was treated with L-LME as described above. $CD4^+$ T cells and $CD8^+$ T cells bound to the immunobeads were detached by DetachaBeads (Dynal), a polyclonal antibody raised against the Fab fragment of mouse immunoglobulins, as described by the manufacturer. Briefly, cells were cultured at a concentration of $5 \times 10^6/100$ µl culture medium with 1 unit of DetachaBeads for 1 hour at room temperature. The detached cells were separated from the beads with a magnet followed by three washings with IMDM containing 1% FCS. The detached $CD4^+$ T cells and $CD8^+$ T cells were washed three more times with IMDM, 1% FCS, and subsequently analyzed for $CD4^+$ T cells and $CD8^+$ T cells by flow cytometric analysis using phycoerythrin-conjugated anti-CD4 antibodies (Becton Dickinson) and phycoerythrin-conjugated anti-CD8 antibodies (Becton Dickinson), respectively. $CD4^+$ T cells and $CD8^+$ T cells thus obtained were >98% pure.

15. Isolation of monocytes from PBMC

Monocytes were isolated from PBMC by positive selection using anti-CD14 antibody coated immunobeads (Dynal) with a bead to target cell ratio of 4:1. CD14 is a receptor for

LPS which is expressed on the surface of monocytes. Cells attached to the immunobeads were separated using a magnetic field as described above. Isolated $CD14^+$ monocytes contained less than 2% T cells and B cells following analysis by flow cytometry. $CD14^+$ monocytes were not detached from the immunobeads and were cultured in 24 well plates for 24 hours in subsequent stimulation experiments. The presence of anti-CD14 antibody conjugated immunobeads bound to monocytes did not influence cytokine production as the supernatants collected from unstimulated monocytes depleted with anti-CD14 did not contain detectable levels of IL-1α, IL-1ß, IL-6, IL-10, IL-12 or TNF-α as determined by ELISA (R&D Systems).

16. Preparation of monocyte conditioned medium (MCM)

Monocyte conditioned medium was prepared by stimulating a T-cell depleted fraction of PBMC with PHA at a final dilution of 1:100. After 24 hours of incubation, cells were washed after 24 hours and re-cultured in fresh medium for another 48 hours. The supernatants were harvested and analyzed for IL-10 production. The supernatants (MCM) thus collected did not contain IL-10 as determined by ELISA.

17. Flow cytometry analysis

Cells (1×10^6 per reaction) were washed with cold PBS containing 1% sodium azide and incubated with specified concentrations of FITC-conjugated antibodies for 30 minutes.

After three washes, cells were analyzed for fluorescence intensity after excitation with a 488 nm Argon laser FACS (Becton Dickinson). The Instrument was calibrated daily by using non-fluorescent and FITC-conjugated beads as fluorescence controls for flow cytometry (Laboratory Center for Disease Control, Ottawa, Canada). The green fluorescence was filtered through a 530/30 band pass absorption filter. The conventional scatter gating method was used to exclude debris and dead cells from the analysis. The threshold of positivity for the green fluorescence intensity was arbitrarily set based on the negative controls.

18. Generation of EBV immortalized B cells

18. a. Preparation of Epstein-Barr Virus containing culture supernatant

EBV infected B cells were used as a positive control as IL-10 producing cells for optimization of ELISA and in RT-PCR. Exponentially growing EBV-transformed B95/8 B cells were incubated at a density of 1×10^6 cells per ml in a humidified 37° C, 5% CO_2 incubator. After three days of incubation, the EBV-containing culture was centrifuged for 10 minutes at 300×g at 4° C. To avoid cellular contamination, the supernatant was then filtered through a 0.45 μm filter, aliquoted and stored in liquid nitrogen.

18. b. Infection of target B cells with EBV

Ten million PBMC in 2.5 ml of RPMI-10% FCS were incubated with 2.5 ml of EBV-containing supernatant in a humidified 37°C, 5% CO_2 incubator. After two hours of

incubation, 5 ml of RPMI-10% FCS containing 1 μg/ml of cyclosporin A were added and cells were transferred to a 25-cm^2 tissue culture flask (Falcon). Cells were incubated for three more weeks after which they were diluted 1:2 with complete RPMI-10% FCS and transferred to a new 25-cm^2 tissue culture flask. Culture for 4 weeks was necessary for the generation of immortalized B cells, after which B cell lines were maintained by splitting them 1:3 in complete RPMI-10% FCS once weekly and culturing in a humidified 37° C, 5% CO_2 incubator. Immortalized cells were cryopreserved in liquid nitrogen.

19. Statistical analysis

For the discription of data means, Standard Errors (SE), and Standard Deviations (SD) were used. For the comparison of group populations a Chi-Square and for the comparison of groups with normal distributions of data a Student's t-test was used. For the comparison of IL-10 production in supernatants of unstimulated PBMC of HIV^+ and HIV- individuals, a Chi-Square used. In the figures, for the comparison of HIV^+ and HIV^- populations, the mean ± SEM is shown while for the reproducibility experiment mean ± SD are shown. In the studies of test reproducibility (RT-PCR) Coefficient of Variation (CV) has been also shown in the legend.

Chapter III. Results

Analysis of cytokines and their role in HIV immunopathogenesis

A switch from Th1 to Th2-type responses has been suggested to be associated with HIV disease progression. Such an alteration in T helper responses may be reflected in an alteration in the profile of cytokine expression of PBMC from HIV^+ individuals. In this study, we measured Th1 (IFN-γ and IL-12) and Th2 (IL-4 and IL-10) cytokine production by PBMC from HIV^+ individuals. The measurement of cytokines in the supernatant of unstimulated and mitogenically-stimulated PBMC of HIV^+ and HIV^- individuals was carried out by means of ELISA and measurement of mRNA of cytokines was performed by semi-quantitative RT-PCR.

1. Measurement of IL-10, IL-4 and IFN-γ production in the culture supernatants of unstimulated PBMC by ELISA

To evaluate whether PBMC from HIV^+ individuals produce relatively higher levels of IL-10 and consequently lower amounts of IFN-γ compared to cells from HIV^- individuals, I measured IL-10 by ELISA in the supernatants of unstimulated PBMC cultured for 24, 48 and 72 hours. IL-10 at levels of more than 16 pg/ml (detection limit of IL-10 ELISA) was

produced by unstimulated PBMC from a significantly higher number of HIV-infected as compared to HIV uninfected individuals when PBMC were cultured either for 24 hours (eight out of 11 HIV^+ *versus* two out of 10 normal individuals), 48 hours (seven out of 11 HIV^+ *versus* three out of 12 normal individuals), or 72 hours (43 out of 45 HIV^+ *versus* three out of 12 normal individuals) (Table 1). However, the absolute amounts of IL-10 produced by PBMC from HIV^+ and HIV^- individuals producing more than 16 pg/ml were not statistically significant (Table 1). These results indicate spontaneous production of IL-10 by PBMC from HIV^+ individuals which was not observed in HIV^- individuals.

The levels of IL-4 and IFN-γ production in supernatants collected from unstimulated PBMC from more than 80% of 28 HIV^+ as well as 100% of 8 HIV^- individuals were found to be below the detection limits of ELISA (<16 pg/ml).

Optimization of RT-PCR

Since quantification of cytokines by this method involved measuring relative expression of IL-10 and IFN-γ with respect to β-actin, the PCR technique was optimized for reproducibility and accuracy (Diaz-Mitoma et al. 1995). Various concentrations of mRNA obtained from PBMC of a normal individual ranging from 25 to 100 ng were reverse transcribed. The cDNA was amplified for 25, 30 and 35 cycles using β-actin primers. Measurement of the signals by densitometric analysis revealed that amplification of β-actin was directly proportional to the amount of cDNA used for amplification. Furthermore, dose

Table 1. Production of IL-10 by unstimulated PBMC from HIV^+ and HIV^- controls

Subjects (*n*)	Supernatant collection at:	Number of individuals secreting IL-10*			
		>16 pg/ml	(mean ± s.e.m.)	<16 pg/ml	*P* value
HIV^+(11)	24 Hours	8	45.34 ± 15.07	3	
HIV^-(12)	24 Hours	2	35.00 ± 2.00	10	<0.006
HIV^+(11)	48 Hours	7	50.50 ± 16.11	4	
HIV^-(12)	48 Hours	3	50.00 ± 11.90	9	<0.05
HIV^+(45)	72 Hours	43	44.28 ± 14.16	2	
HIV^-(12)	72 Hours	3	47.50 ± 6.20	9	<0.001

PBMC from HIV^+ individuals with less than 400 $CD4^+$ T cells/mm^3 and normal controls were cultured at a concentration of 2×10^6/ml for 24, 48 and 72 hours without stimulation. Supernatants were harvested and assayed for IL-10 production by a sandwich ELISA.

* Means of only those samples that secreted more than 16 pg/ml of IL-10 were calculated.

† *p*-value was calculated by χ^2 test which is a comparison between the number of HIV^+ and HIV^- individuals secreting IL-10.

n The number of samples examined.

dependent amplification was not observed as the number of amplifying cycles was increased beyond thirty. Therefore, in subsequent experiments, for β-actin PCR analysis, 30 amplifying cycles was employed.

To test reproducibility in the amplification of cDNA, reverse transcribed cDNA from PBMC of four HIV^+ individuals and EBV transformed B-95/8 B cells were amplified. Four aliquots from the same sample, each containing 25 ng of cDNA, were amplified for β-actin and IL-10 at different times under similar conditions. Similar values were obtained following densitometric scanning of IL-10 and β-actin expression signals in two out of four samples going through the same system. Two samples, however, showed high Coefficient of Variations (CV). Furthermore, the ratio of densitometric values of IL-10 to β-actin expression signals for two samples were also similar (CV, 25%, and 18%) (Fig. 1). In order to reduce variabilities, the RT-PCR for all samples in any one experiment (HIV^+ plus HIV^- controls) were, therefore, performed simultaneously. All figures showing RT-PCR results of HIV^+ and HIV^- individuals are presented as ratios of densitometric units of IL-10 to β-actin or IFN-γ to β-actin.

Measurement of cytokine mRNA by RT-PCR

Spontaneous production (in unstimulated PBMC) of IFN-γ by PBMC from 30 HIV^+ and 16 normal individuals was assessed by subjecting equal amounts (5μl of a total of 50 μl) of

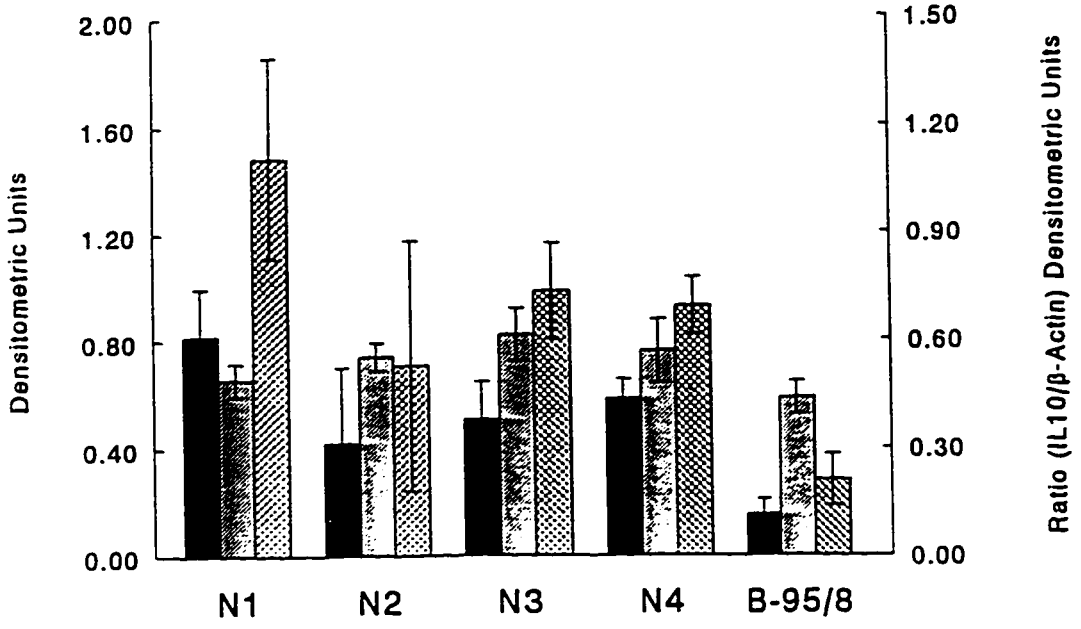

2.00
1.60
1.20
0.80
0.40
0.00
Densitometric Units
1.50
1.20
0.90
0.60
0.30
0.00
Ratio (IL10/β-Actin) Densitometric Units
N1
N2
N3
N4
B-95/8

Figure 1. Reproducibility of semiquantitative RT-PCR analysis. Total RNA from PBMC from four HIV^+ Individuals and one EBV-transformed B cell line, B-95/8, were reverse transcribed. Four aliquots of equal amounts of cDNA were amplified for IL-10 and β-actin at four different times under identical conditions. The signals obtained on the autoradiographs for IL-10 and β-actin were scanned by densitometry. The ratios of the densitometry scanning of the autoradiographs assigning arbitrary densitometric units (DU) of IL-10 to β-actin was calculated. The results are expressed as the means of values for the four aliquots in each patient ± (standard deviation) SD, ■ IL-10 densitometric unit (DU); ▨ β-actin DU; ▩ , ratio of IL-10 to β-actin.

cDNA produced from 1 μg RNA to semi-quantitative PCR for IFN-γ and β-actin. The ratios obtained from densitometry scanning of the autoradiographs assigning arbitrary densitometric units (RDU) demonstrated that the ratio of IFN-γ to β-actin signals from HIV-infected individuals with <400 $CD4^+$ T cells/mm^3 (RDU= 0.24 ± 0.07) was significantly lower than the HIV^- controls [DU= 1.41 ± 0.60; $p< 0.06$ (Fig. 2A, B)]. Furthermore, the ratios of IFN-γ / β-actin mRNA amplified from PBMC from 60% of HIV^+ patients with <400 $CD4^+$ T cells/mm^3 were 10 fold lower than the HIV^- controls. Although a trend towards lower levels of IFN-γ was observed in the PBMC of patients with > 400 $CD4^+$ T cells/mm^3 (RDU= 0.36 ± 0.15) compared to HIV^- controls (RDU= 1.41 ± 0.60), the difference was not statistically significant (Fig. 2A, B). IFN-γ has been shown to be regulated by IL-10 (Fishman and Perelson, 1994).

To study whether reduced expression of IFN-γ in unstimulated PBMC of HIV^+ patients is associated with enhanced expression of IL-10, PBMC obtained from both HIV^+ patients and controls were assayed for IL-10 expression by semiquantitative RT-PCR analysis. Equal amounts of cDNA were amplified for IL-10 and β-actin. The ratio of arbitrary units obtained by densitometry from IL-10 and β-actin autoradiographs revealed significantly higher expression of IL-10 in HIV^+ patients (n = 13) with < 400 $CD4^+$ T cells/mm^3 (RDU= 2.82 ± 1.04) than in HIV^+ patients (n = 17) with > 400 $CD4^+$ T cells/mm^3 (RDU = 0.57 ± 0.27, $p< 0.05$) and normal controls [RDU= 0.322 ± 0.082; $p< 0.01$ (Fig 2A, B)]. However, significant differences between the expression of IL-10 in HIV^+ individuals with more than 400 $CD4^+$ T cells (RDU= 0.573 ± 0.271) and normal controls (RDU= 0.322 ± 0.082) were not observed. An inverse correlation in the expression of IL-10 and IFN-γ was observed in 25 out of 30

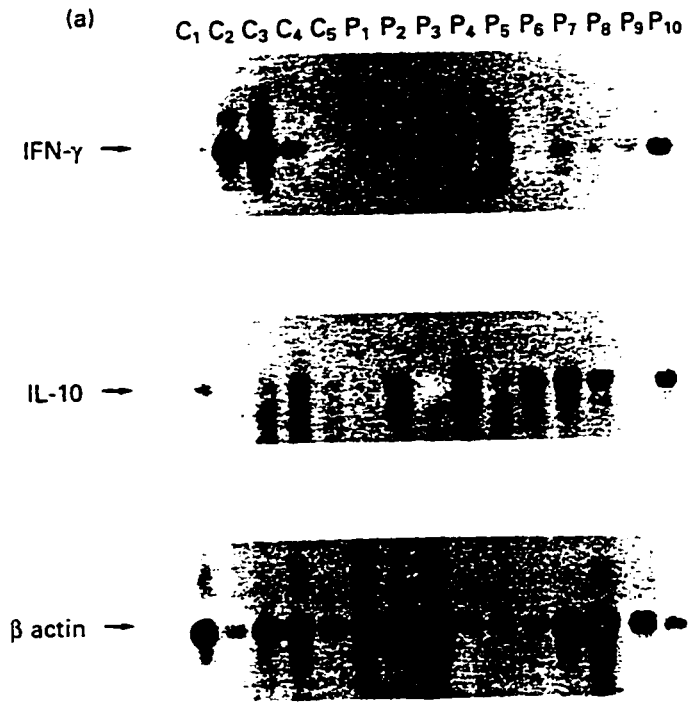

(a)
C1 C2 C3 C4 C5 P1 P2 P3 P4 P5 P6 P7 P8 P9 P10
IFN-γ
IL-10
β actin

Figure 2A. Expression of IFN-γ and IL-10 by PBMC from a representative group of 10 HIV^+ and 5 HIV^- individuals by semiquantitative RT-PCR. Total RNA was reverse transcribed and equal amounts of cDNA were amplified by PCR using specific oligonucleotide primers as described in Materials and methods. PCR amplified products were analyzed by electrophoresis followed by hybridization using cDNA probes specific for IFN-γ, IL-10 and β-actin. C_1 to C_5 denote HIV^- individuals. P_1 to P_{10} denotes HIV^+ individuals.

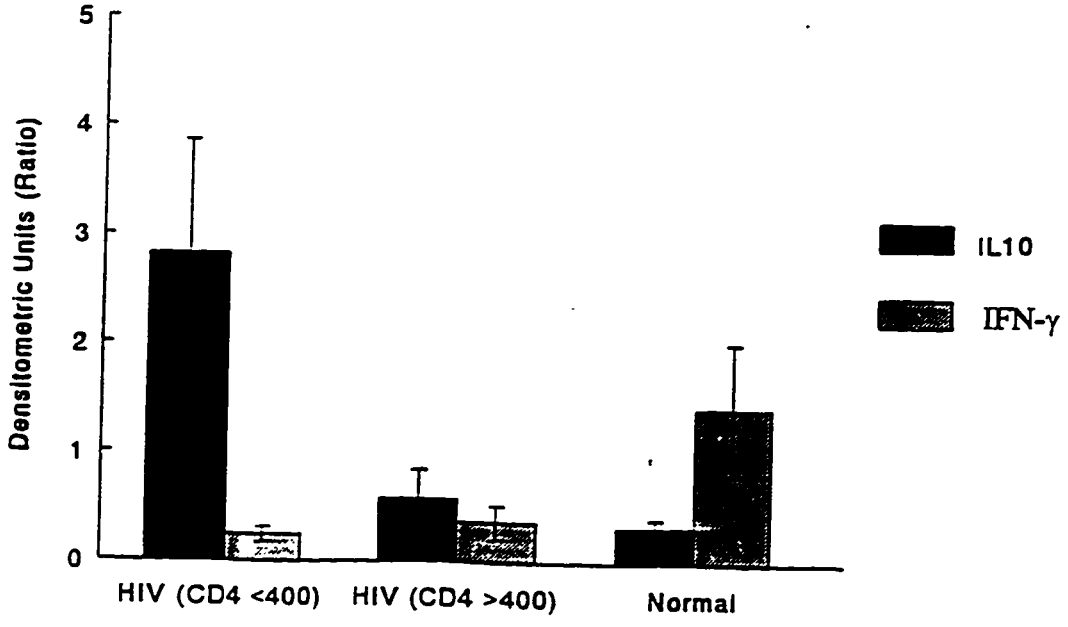

Densitometric Units (Ratio)
5
4
3
2
1
0
HIV (CD4 <400)
HIV (CD4 >400)
Normal
IL10
IFN-γ

Figure 2B. Differential expression of IFN-γ and IL-10 in HIV^+ (n = 30) and HIV^- (n = 16) individuals. IL-10, IFN-γ and β-actin PCR amplified bands as demonstrated by autoradiography were analyzed by densitometry and assigned arbitrary units. For comparison, IL-10 and IFN-γ expression was normalized with respect to β-actin as a ratio of values for IL-10 and β- actin, and IFN-γ and β-actin respectively. Ratios are expressed as means of densitometric values ± SEM.

patients (Fig 2A) and in all the normal controls. IL-10 expression was observed to be more than 100-fold higher in 16 HIV^+ individuals and more than 10-fold in 6 HIV^+ patients with respect to IFN-γ expression. However, inverse correlation between IL-10 and IFN-γ was not observed in HIV^+ patients with advanced HIV disease characterized by very low CD4 counts and lower CD4/CD8 ratio. These results suggest that expression of IL-10 and IFN-γ in PBMC is tightly and inversely regulated in normal individuals, and that in HIV infection, the relative level of expression of these two cytokines is reversed but continues to be inversely regulated.

2. Measurement of IL-10, IL-4 and IFN-γ by mitogen-stimulated PBMC

Enhanced expression of IL-10 and its inverse relationship with IFN-γ expression in unstimulated PBMC from HIV^+ individuals may also be reflected in the PBMC stimulated with mitogens. To determine the ability of PBMC to secrete IL-10, IFN-γ and IL-4, the supernatants of PBMC stimulated with PHA or anti-CD3 antibodies were harvested at 24, 48 and 72 hours and analyzed by ELISA. Two groups of HIV^+ individuals were observed based on the level of IL-10 production by PBMC (of 48 hours PHA stimulation)(table 2). PBMC from one subgroup of HIV^+ individuals did not produce IL-10 ($n = 25$). The stimulation index of IL-10 was calculated as a ratio of IL-10 produced after 48 hrs of PHA stimulation over spontaneous production of IL-10 in complete media. This group was designated as low IL-10 producers based on an arbitrary value of the IL-10 stimulation index (S.I) of <3.PBMC from the second group of HIV^+ individuals ($n = 17$) produced high levels of IL-10 (IL-10 S.I. ≥ 3)

Table 2. Production of IL-10 by PBMC from HIV^+ and HIV^- controls following mitogenic stimulation.

Subjects (n)	Stimulating agent	
	Anti-CD3	PHA
	IL-10 (pg/ml± SEM)	
HIV^+		
Low IL-10 producers(25)	22.26± 6.06(1)*	91.33± 22.69(3.3)
Normal IL-10 producers(17)	82.25± 24.72(4)	412 ± 128.11(20.5)
HIV^- controls		
Normal IL-10 producers(16)	143.8 ± 34.63(10.6)	550.25± 83.16(40.4)

PBMC (2×10^6/ml) from HIV^+ individuals and normal controls were stimulated either with anti-CD3 (final dilution 1:200) or PHA (final dilution 1:50) for 48 hours. Supernatants were collected and assayed for IL-10 production by a sandwich ELISA.

* Figures in parentheses indicate IL-10 stimulation index (SI).
† Statistically significant ($p < 0.001$) compared to normal IL-10 producers.
n The number of samples examined.

and amounts were comparable to that produced by PBMC from normal individuals (Table 2). However, IFN-γ production by PBMC from HIV^+ individuals varied over the time. IFN-γ production by PHA-stimulated PBMC was significantly lower at 48 hours in HIV^+ individuals (n = 15) than in HIV^- controls (n = 16) (Table 3). The levels of IFN-γ produced by PBMC stimulated with PHA increased significantly after 72 hours in HIV^+ individuals (n = 27) compared to HIV^- controls (n=31, Table 3). Conversely, IFN-γ levels were not different (p<0.05) in anti-CD3 antibody stimulated PBMC of HIV^+ and normal controls (Table 3). These results show that the kinetics of IFN-γ production were slower in HIV^+ individuals but levels of IFN-γ at 72 hours after stimulation with PHA were higher than those from HIV^- controls and comparable to levels of IFN-γ in HIV^- controls at 48 hrs of culture. The level of IFN-γ after culturing in the media alone was not detectable by ELISA.

IFN-γ is generally produced by $CD4^+$ T cells, and $CD8^+$ T cells and natural killer cells. Because of controversies about the status of IFN-γ production in HIV^+ individuals (Cleleri, et al. 1993; Fauci et al. 1994), these results were confirmed at the level of mRNA. To determine the ability of $CD4^+$ cells to secrete IFN-γ following stimulation, $CD4^+$ cells purified from both HIV^+ (n = 6) and HIV^- individuals (n = 27) were stimulated with PHA for 48 hours and analyzed for IFN-γ mRNA by RT-PCR as described in Materials and methods (Fig 3). There was no difference in the expression levels of IFN-γ in HIV^+ and normal individuals (Fig 3).

To further determine Th2 type cytokines in HIV^+ individuals, PHA stimulated PBMC were analyzed for IL-4 production in the supernatants harvested after 48 hours of culture. The results show significantly lower levels of IL-4 in HIV^+ individuals with less than 400 $CD4^+$ cells (n = 15) as compared to the normal controls (p<0.001) (Fig. 4). The low levels of IL-4 in

Table 3. Production of IFN-γ by PBMC from HIV^+ and HIV^- controls following mitogenic stimulation.

Subjects (n)	Day of supernatants collection	Stimulating agent: Anti-CD3	Stimulating agent: PHA
		IFN-γ (pg/ml± SEM)	
HIV^+ (15)	1	39.28 ± 6.6	37.5 ± 5.22
HIV- (16)	1	42.6 ± 7.06	54.8 ± 7.82
HIV^+ (15)	2	48.25 ± 16.8	48.6 ± 3.6*
HIV- (16)	2	41.4 ± 4.7	96.5 ± 20.6
HIV^+ (27)	3	81.4 ± 29.8	97.5 ± 20.1
HIV- (31)	3	55.7 ± 9.8	31.32 ± 7.74

PBMC (2×10^6/ml) from HIV^+ individuals and normal controls were stimulated either with anti-CD3 antibodies or PHA for 24, 48, or 72 hours. Supernatants were collected and assayed for IFN-γ production by a sandwich ELISA.

* Statistically significant ($p < 0.05$).

n The number of samples examined.

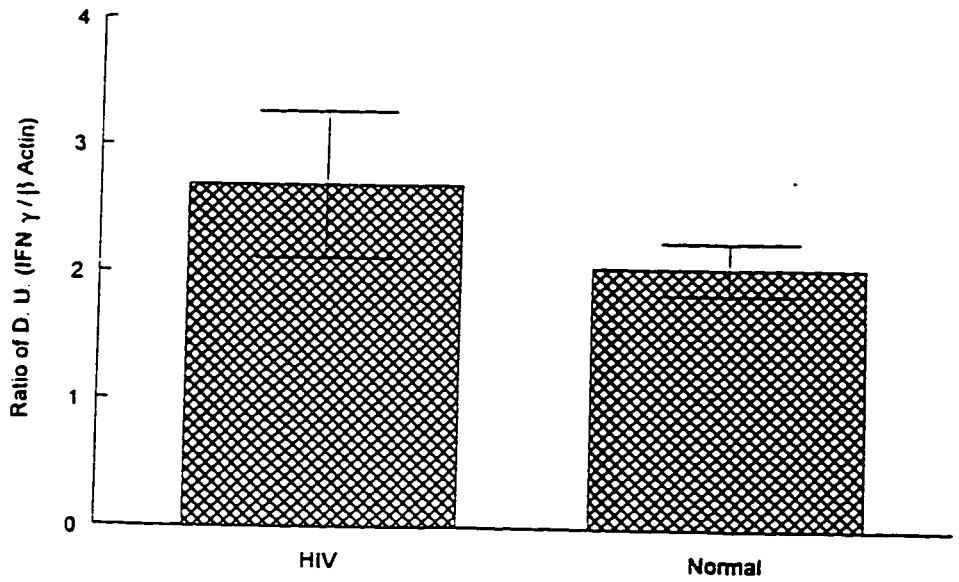

Ratio of D. U. (IFN γ / β Actin)
4
3
2
1
0
HIV
Normal

Figure 3. IFN-γ expression by $CD4^+$ cells from PBMC of six HIV^+ and eight normal individuals by semiquantitative RT-PCR analysis. Positively selected $CD4^+$ cells from PBMC of HIV- infected individuals and normal controls were stimulated with PHA for 48 hours. The cells were harvested and analyzed for IFN-γ expression by semiquantitative RT-PCR as described in the legend to Figure 2.

IL-4 ELISA for PHA stimulated PBMC

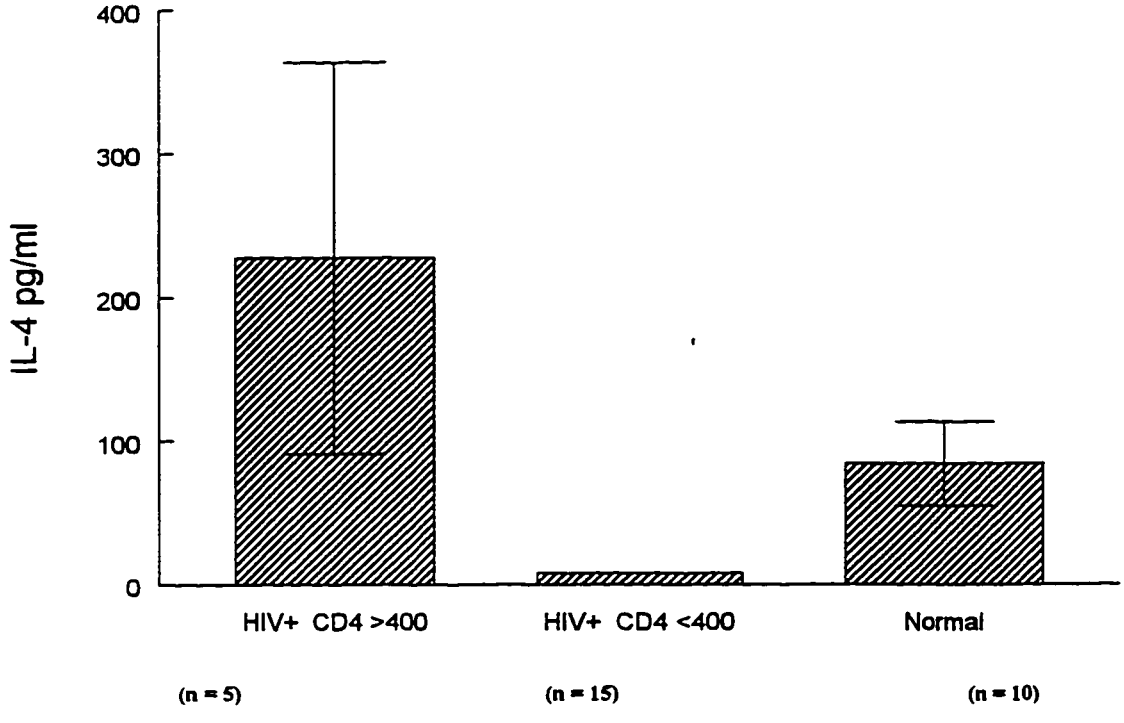


Figure 4. IL-4 production by PBMC of HIV infected individuals and HIV^- controls. PBMC stimulated with PHA (1:50 final dilution) for 48 hours were analyzed for IL-4 production by ELISA as described in Materials and methods. Results are shown as means ± SEM.

HIV^+ individuals with less than 400 $CD4^+$ cells may be a reflection of reduced $CD4^+$ number as these cells are the only producers of IL-4. Alternatively, this may be due to selective destruction of Th2 cells. On the contrary, there was no difference in IL-4 production in HIV^+ individuals with more than 400 $CD4^+$ cells (n = 5) and normal individuals (n = 10) (Fig 4). These observations, however, fail to confirm a switch from Th1 to Th2. Separation of HIV^+ individuals into those with more than 400 $CD4^+$ cells and with less than 400 $CD4^+$ cells did not give additional information in the case of IL-10 or IFN-γ after mitogen stimulation.

3. Further evaluation of normal and low IL-10 producers among HIV^+ individuals

It has previously been demonstrated that IL-10 expression is upregulated in unstimulated PBMC of HIV^+ individuals with < 400 $CD4^+$ T cells/mm^3 (Diaz-Mitoma et al. 1995). Production of different levels of IL-10 in subsets of patients provided a model to study the role of IL-10 in unresponsiveness to recall antigens. It was also possible that the levels of IL-10 expression in the HIV^+ individuals undergo a shift from low IL-10 producers towards normal IL-10 producers as disease progress.

To further study the IL-10 levels in mitogenically stimulated PBMC from HIV^+ individuals, PBMC from 65 HIV^+ individuals were stimulated with PHA or anti-CD3 antibodies and supernatants were harvested after 48 hours of culture. HIV^+ individuals could be grouped into two groups (Fig. 5) of low and normal IL-10 producers based on the amount

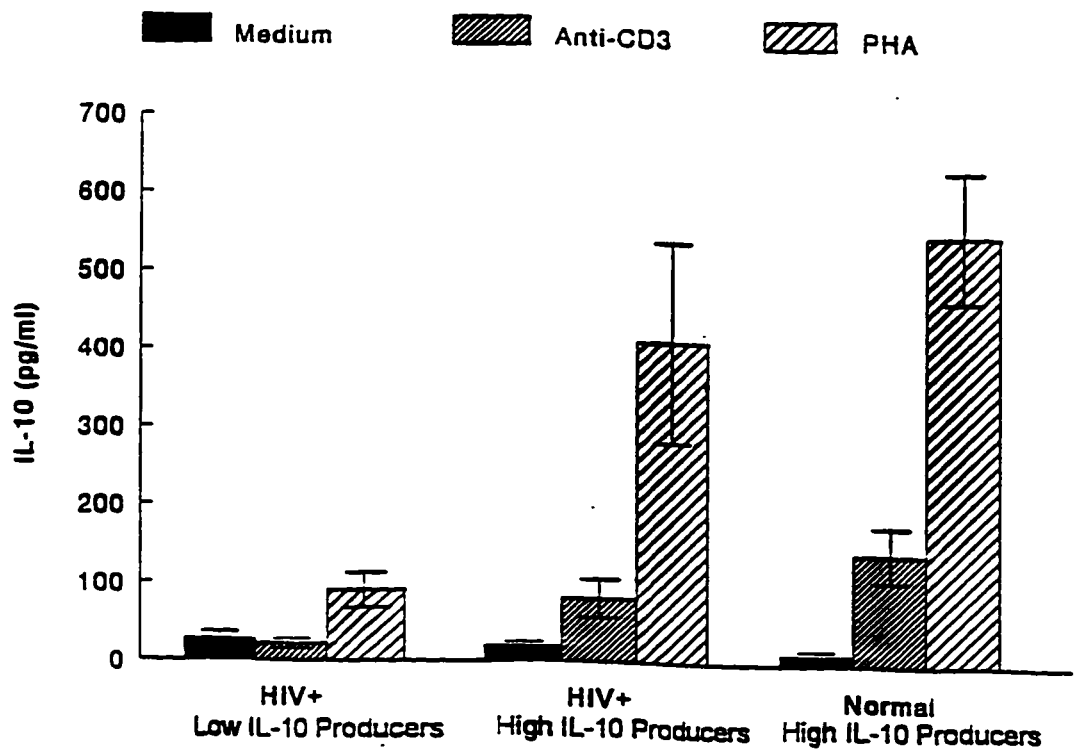

Medium
Anti-CD3
PHA
IL-10 (pg/ml)
700
600
500
400
300
200
100
0
HIV+
Low IL-10 Producers
HIV+
High IL-10 Producers
Normal
High IL-10 Producers

Figure 5. IL-10 production by PBMC from HIV^- and HIV^+ individuals following stimulation with PHA (1:50 dilution) or anti-CD3 antibodies (1:200 dilution) for 48 hours. Supernatants were analyzed for IL-10 production by ELISA as described in Materials and Methods. Low IL-10 producers (n = 38) and normal IL-10 producers (n = 27) were defined as individuals showing a less than or more than threefold increase in IL-10 production following mitogenic stimulation, respectively, compared with unstimulated PBMC (IL-10 SI of <3 or ≥ 3, respectively). Solid bars, unstimulated PBMC; closely hatched bars, PBMC stimulated with anti-CD3; widely hatched bars, PBMC stimulated with PHA. Values are means ± SEM.

of IL-10 produced following stimulation with PHA (Fig 5). Low IL-10 producers (n = 38) were those whose PBMC exhibited less than a three fold increase in IL-10 following PHA stimulation (IL-10 stimulation index (S.I.) < 3) compared with unstimulated PBMC. Conversely, normal IL-10 producers (n = 27) were those whose PBMC exhibited more than a three fold increase in IL-10 (IL-10 S.I. > 3, Fig 5). PBMC from HIV$^-$ individuals always demonstrated more than a three-fold increase in IL-10 production following stimulation with either anti-CD3 antibodies or PHA compared to unstimulated PBMC and levels of IL-10 produced by PHA stimulated PBMC from normal IL-10 producers and normal individuals were not significantly different. The lower levels of IL-10 produced by low IL-10 producers were not due to differences in IL-10 production over time, as low levels of IL-10 were consistently detected in supernatants harvested after 24, 48 and 72 hours of culture (Fig 6).

Role of IL-10 and IL-12 in HIV immunopathogenesis

4. Analysis of proliferation of PBMC from normal IL-10 producers and low IL-10 producers in response to recall antigens

Since IL-10 inhibits IFN-γ and IL-2 synthesis by T cells and alters the expression of costimulatory molecules on antigen-presenting cells (Banchereau, 1995), altered IL-10 production by PBMC of HIV$^+$ individuals may affect their proliferative responses to recall

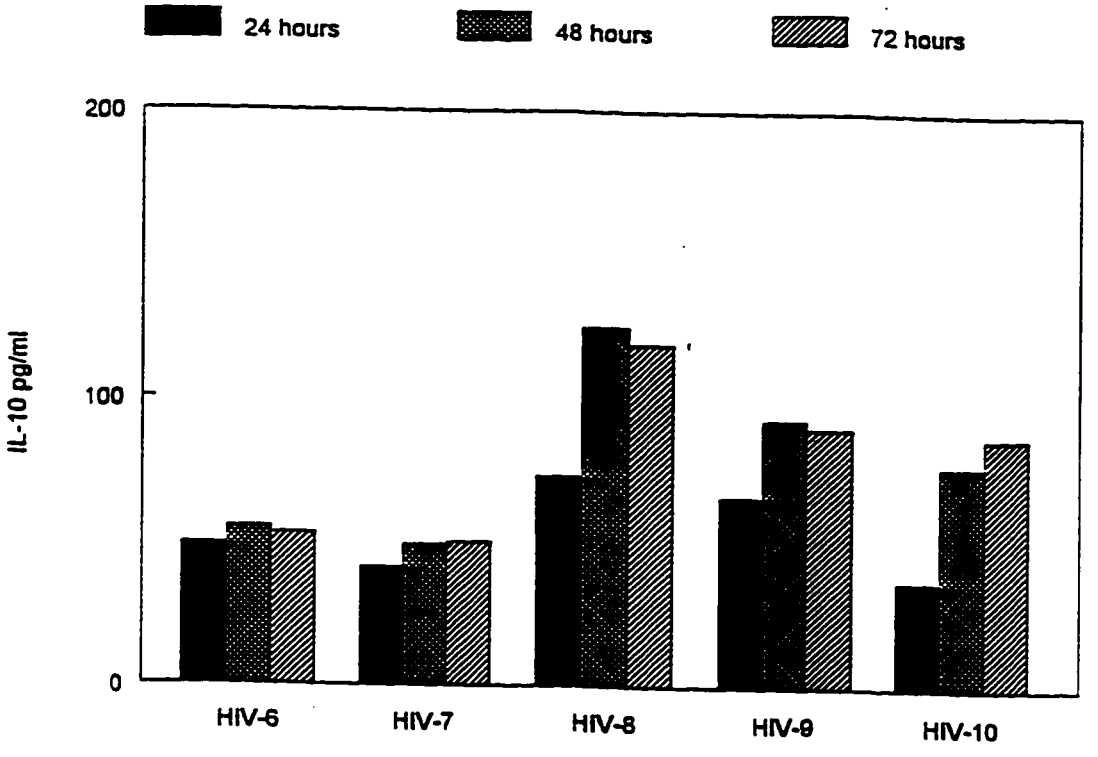

24 hours
48 hours
72 hours
200
100
0
IL-10 pg/ml
HIV-6
HIV-7
HIV-8
HIV-9
HIV-10

Figure 6. Kinetics of IL-10 production by PBMC from low IL-10 producers. PBMC from five low IL-10 producers were stimulated by PHA and supernatants were harvested at 24, 48, and 72 hours for IL-10 production.

antigens. Therefore, I analyzed the proliferative responses of PBMC from 39 of the above-mentioned 65 HIV^+ individuals to HIV p25 and influenza antigen (FLU). PBMC were stimulated with FLU and HIV p25 antigens for 7 days. The cells were pulsed with [^{3}H] thymidine and measured for thymidine incorporation 16 hours later. A three-fold increase in thymidine incorporation compared to that of media alone (S.I. ≥ 3) was considered to be a positive response. PBMC from 36 of 39 HIV^+ individuals did not proliferate in response to gp120 antigens (PBMC from three HIV^+ individuals proliferated against gp120 with stimulation indexes of 2.5, 4, and 5). However, distinct patterns of proliferative responses to the recall antigens p25 and FLU by PBMC from low and normal IL-10 producers were observed. PBMC from 18 out of 22 low IL-10 producers proliferated in response to p25 and influenza antigens, whereas PBMC from 15 out of 17 normal IL-10 producers did not (Figs. 8A and B). The proliferative responses of five representative low IL-10 producers and five normal IL-10 producers are shown in Fig.7. As expected, PBMC from HIV^- individuals (n = 5) did not proliferate in response to HIV p25 antigens; the results from one representative HIV^- individual are also shown in Fig 7. PBMC from unvaccinated HIV^- individuals (n = 5) did not proliferate in response to FLU antigen, while those of vaccinated (n = 2) proliferated, SI>3 (compared to media alone). Representatives of vaccinated and unvaccinated HIV^- controls are shown in in Figs 7 A and B.

The correlations between IL-10 production status and proliferation of PBMC from all individuals in response to p25 and FLU are shown in Figs. 8 A and 8B.

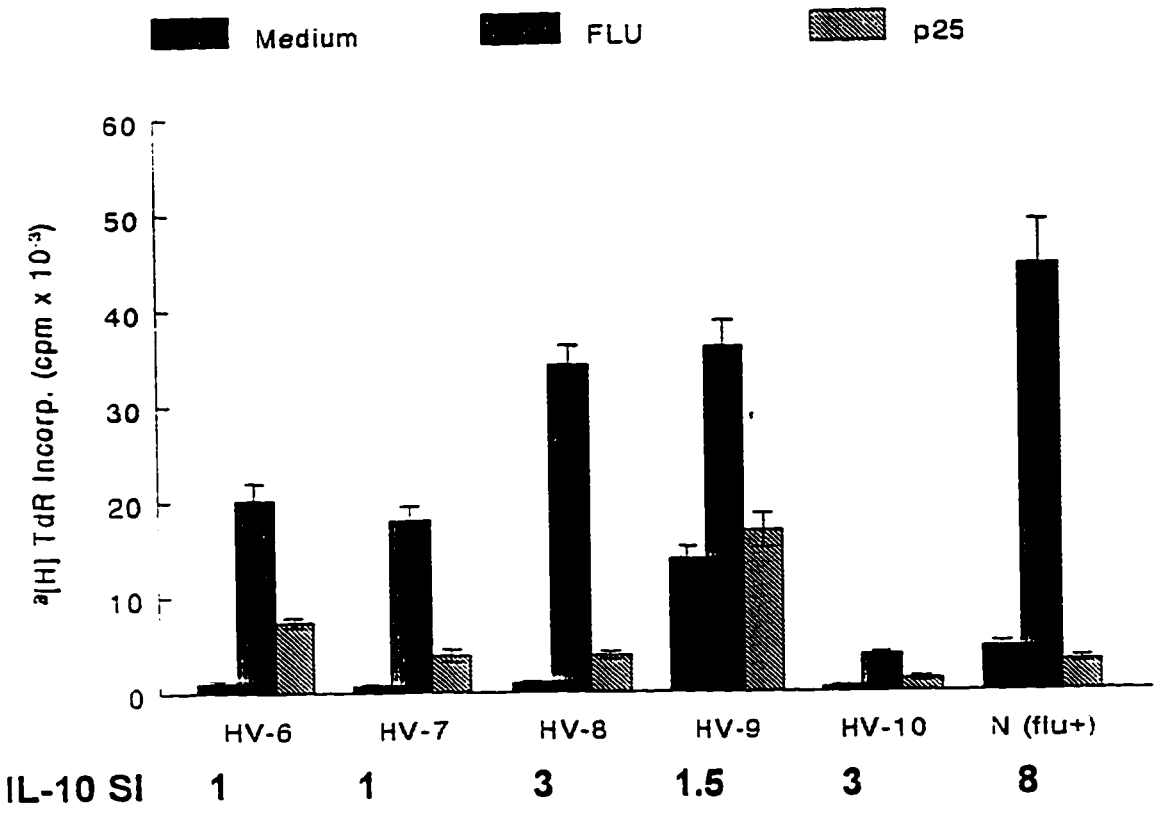
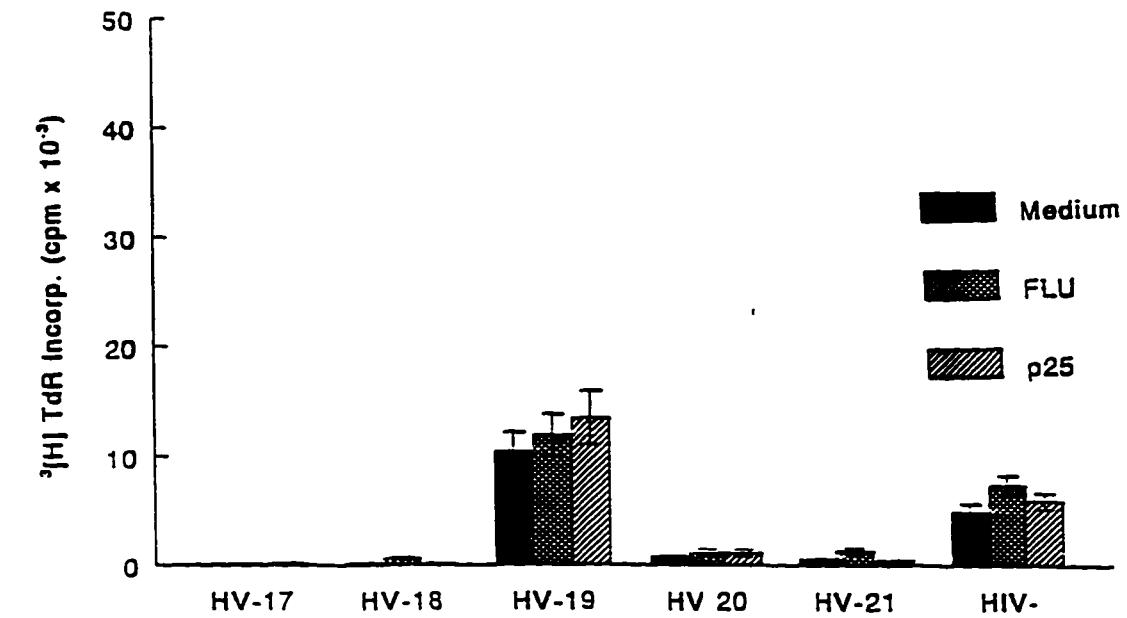

Medium
FLU
p25
60
50
40
30
20
10
0
³[H] TdR Incorp. (cpm x 10⁻³)
HV-6
HV-7
HV-8
HV-9
HV-10
N (flu+)
IL-10 SI
1
1
3
1.5
3
8

Figure 7A. Proliferative responses of PBMC from HIV^+ individuals exhibiting low IL-10 production in response to recall antigens. Five representatives of a total of 22 low IL-10 producers are shown 18 of which had positive response to FLU and to HIV p25 antigen (SI>3). PBMC were stimulated with FLU and HIV p25 antigens for 7 days. The cells were pulsed with [^{3}H] thymidine and measured for thymidine incorporation 16 hours later. Cell proliferation was correlated with their IL-10 stimulation index (IL-10 S.I.). IL-10 S.I. was calculated as a ratio of IL-10 levels (pg/ml) produced by PHA stimulated PBMC to IL-10 levels produced by unstimulated PBMC. The proliferative response of one representative HIV^- individual, with a positive response to FLU, is also shown in the Figure 7A. Proliferation assays were done in triplicate. Results represent means ± SEM of triplicate experiments.



Normal IL-10 Producers

Figure 7B. Proliferative responses of PBMC from HIV^+ individuals exhibiting normal IL-10 production, and HIV^- controls in response to recall antigens. Five representatives of a total of 17 normal IL-10 producers are shown 15 of which had no response to FLU or to HIV p25 antigen (SI<3). PBMC were stimulated with FLU and HIV p25 antigens for 7 days. The cells were pulsed with [^{3}H] thymidine and measured for thymidine incorporation 16 hours later. Cell proliferation was correlated with their IL-10 stimulation index (IL-10 S.I.). The proliferative response of one representative HIV^- individual (with no reaction to FLU) is shown. Proliferation assays were done in triplicate. Results represent means ± SEM of triplicate wells.

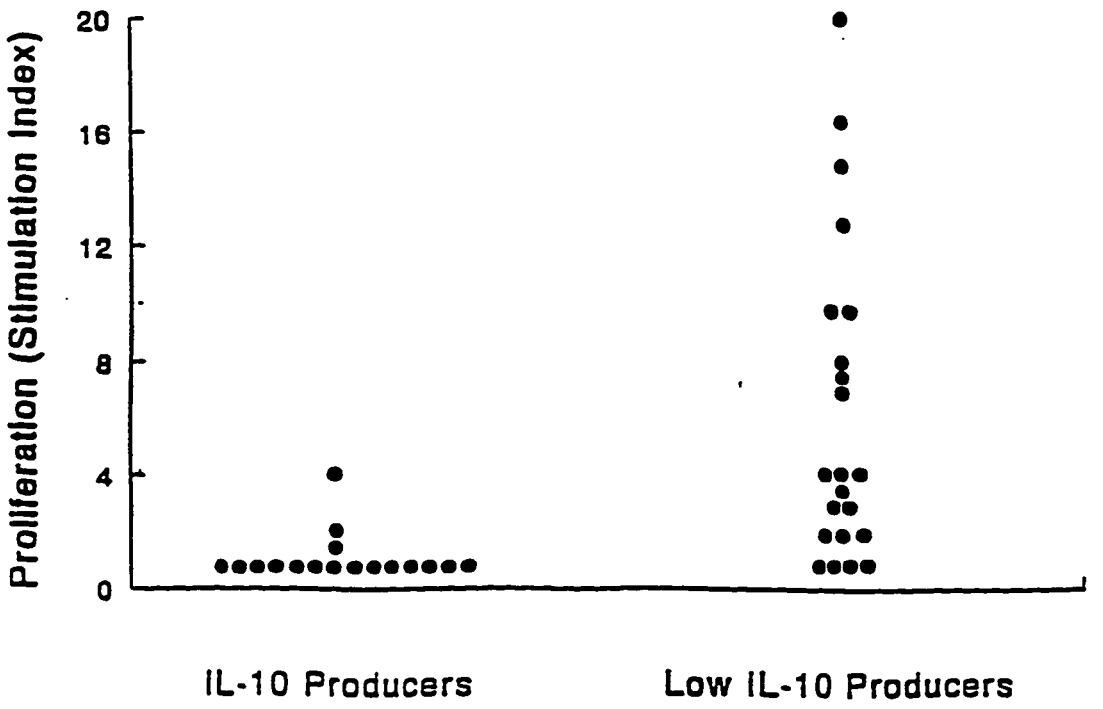

Proliferation (Stimulation Index)
20
16
12
8
4
0
IL-10 Producers
Low IL-10 Producers

Figure 8 A. Correlation of IL-10 production by PHA-stimulated PBMC from HIV^+ individuals with proliferative response to HIV p25 antigen. PBMC from HIV^+ individuals were stimulated with HIV p25 antigen. Increase in thymidine incorporation with a count of less than 1000 cpm by antigen-stimulated PBMC was not deemed significant. The stimulation index (ratio of [^{3}H] thymidine incorporation in the presence of antigen to that observed in the absence of antigen) was correlated with IL-10 stimulation index. Low IL-10 producers n = 17; Normal IL-10 producers, n = 22.

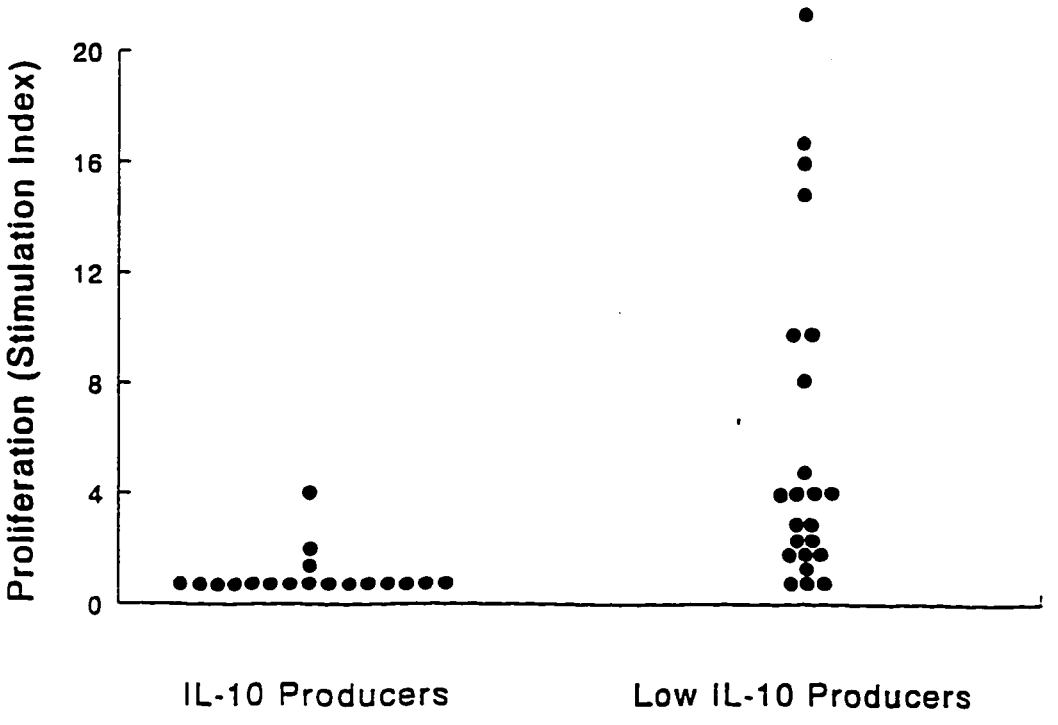
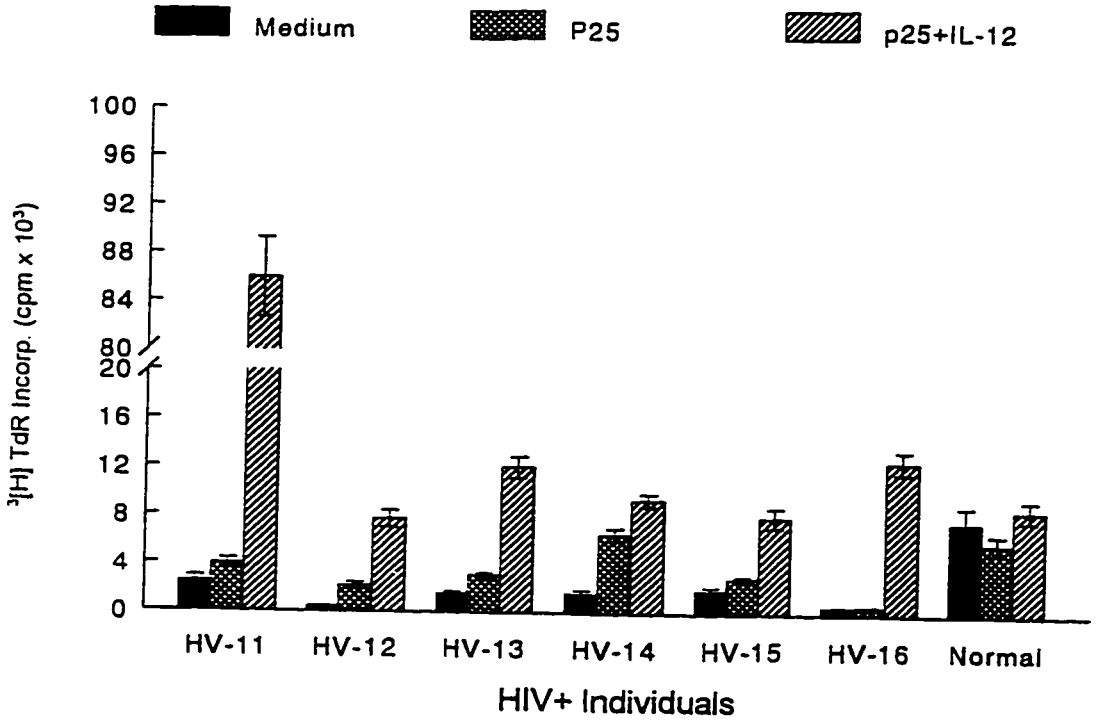

Proliferation (Stimulation Index)
20
16
12
8
4
0
IL-10 Producers
Low IL-10 Producers

Figure 8 B. Correlation of IL-10 production by PHA-stimulated PBMC from HIV^+ individuals with proliferative response to FLU antigen. PBMC from HIV^+ individuals were stimulated with FLU antigen. The stimulation index (ratio of [^{3}H] thymidine incorporation in the presence of antigen to that observed in the absence of antigen) was correlated with IL-10 stimulation index (ratio of IL-10 produced by PBMC stimulated with PHA to that produced by unstimulated PBMC). Low IL-10 producers n = 18; Normal IL-10 producers, n = 23.

5. Effect of neutralizing anti-IL-10 on proliferative responses of PBMC from low and normal IL-10 producers to P25 antigen

The proliferative responses of PBMC from low IL-10 producers to p25 antigen may have been due to a relative absence of the inhibitory effect of IL-10. To determine whether the proliferative responses to recall antigens were affected by endogenously produced IL-10, the proliferation of HIV^+ PBMC in response to p25 in the presence or absence of anti-IL-10 antibodies was measured. Anti-IL-10 antibodies enhanced (SI > 2)the proliferation of PBMC from 17 out of 22 low IL-10 producers only (Fig 9A) ($p < 0.01$). The proliferative responses of PBMC from seven representative individuals from the low IL-10 producers subset of HIV^+ patients are shown in Fig 9A. Enhanced proliferation was not due to the non-specific effect of Fc receptor-mediated stimulatory effect, as anti-IL-4' antibodies and isotype-matched control antibodies failed toenhance proliferation. Anti-IL-10 antibodies could not change the proliferative response of PBMC from normal IL-10 producers (2 out of 17) even when the antibodies were added at a high concentration of 25 μg/ml (Fig. 9B). Thymidine incorporation by PBMC from normal IL-10 producers did not exceed 1000 cpm, and anti-IL-10 antibodies and isotypically matched control antibodies did not alter proliferation.


Medium
P25
p25+IL-12
^{3}[H] TdR Incorp. (cpm x 10^3)
100
96
92
88
84
80
20
16
12
8
4
0
HV-11
HV-12
HV-13
HV-14
HV-15
HV-16
Normal
HIV+ Individuals

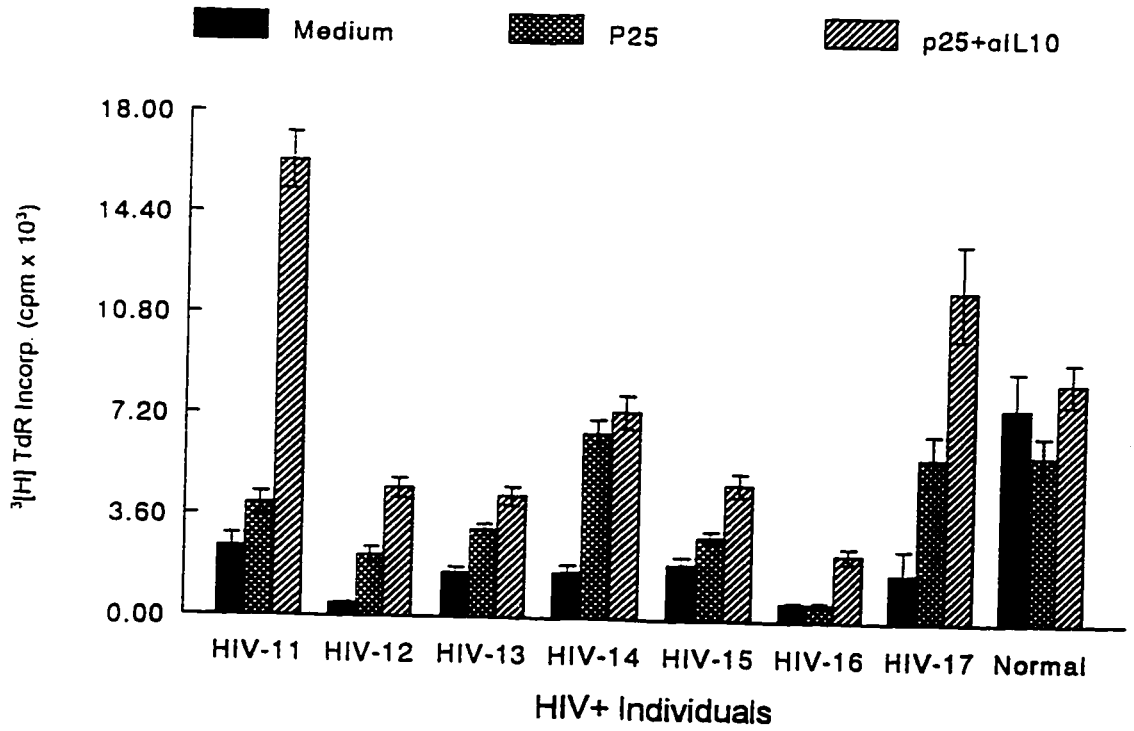
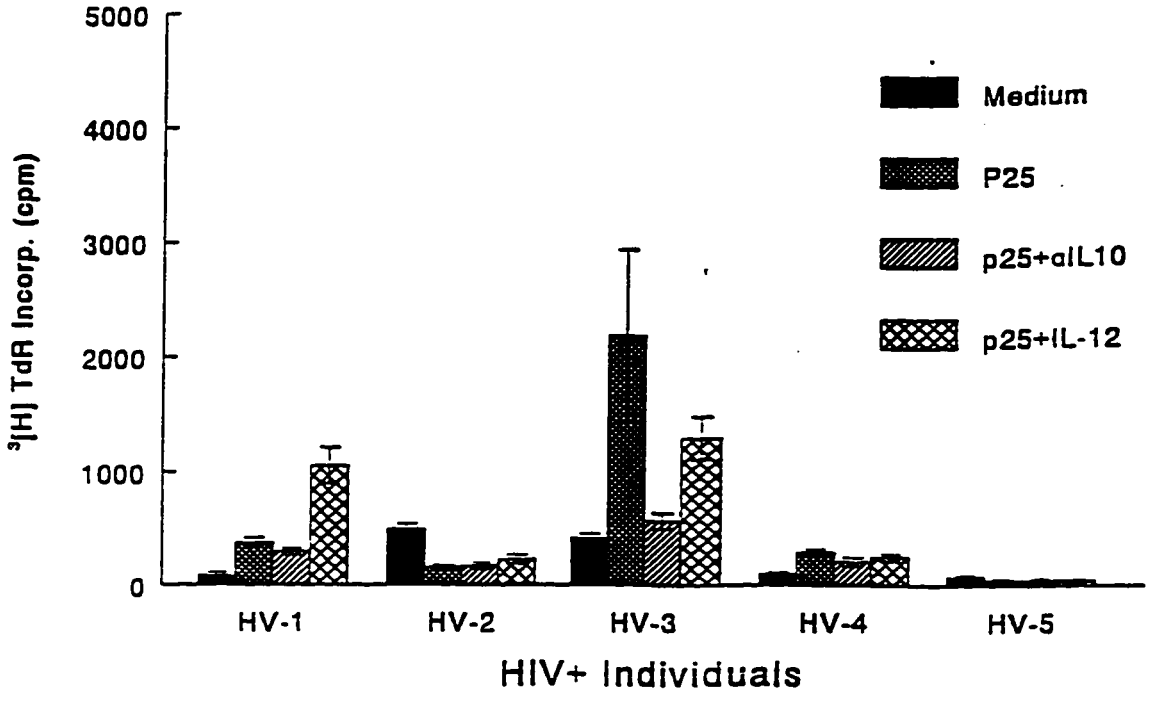

Medium
P25
p25+αIL10
^{3}[H] TdR Incorp. (cpm x 10^3)
18.00
14.40
10.80
7.20
3.60
0.00
HIV-11
HIV-12
HIV-13
HIV-14
HIV-15
HIV-16
HIV-17
Normal
HIV+ Individuals

Figure 9 A. Effect of anti-IL-10 antibodies (top) and IL-12 (bottom) on the proliferative response of PBMC from HIV^+ low IL-10 producers and one HIV^- controls to p25 antigen. Six representatives of a total of 22 low IL-10 producers are shown, 18 of whom responded to anti-IL_10 and to IL-12 treatment (SI>2). PBMC were stimulated with p25 antigen in the presence or absence of anti-IL-10 antibodies (5mg/ml) and/or IL-12 (170 U/ml) for 7 days. The cells were pulsed with [^{3}H] thymidine and measured for thymidine incorporation 16 hours later. Increase in thymidine incorporation of less than 1000 cpm following addition of anti-IL-10 antibodies by PBMC of low IL-10 producers was not deemed significant. Proliferation assays were done in triplicate. Results represent means ± SEM of triplicate wells. The SI of proliferations of low IL-10 producers in response to IL-12 with that of normal IL-10 producers were compared using a t-test ($p < 0.004$). Such comparison for anti-IL-10 showed a *p value* of less than 0.01.

6. Effect of IL-12 on proliferative responses to p25 antigen of PBMC from low IL-10 producers and normal IL-10 producers

Diminished proliferation of HIV^+ PBMC to recall antigens may be due to a defect in IL-12 production. If so, exogenous addition of IL-12 should restore proliferation of HIV^+ PBMC to recall antigens. To determine the effect of IL-12 on proliferative responses, low IL-10 producing and normal IL-10-producing PBMC were stimulated with HIV p25 antigen in the presence or absence of IL-12. IL-12 enhanced the proliferation of PBMC from low IL-10 producers (Fig 9A), whereas PBMC from normal IL-10 producers (15 out of 17) remained unresponsive (Fig 9B) ($p < 0.004$). Proliferative responses of PBMC from HIV^+ normal IL-10 producers were not affected by IL-12 even when high concentrations of IL-12 (500 U/ml) in addition to high concentrations of anti-IL-10 antibodies (25 μg/ml) were added to the cultures (stimulation index of proliferation < 2). However, anti-IL-10 antibodies synergized with IL-12 in enhancing the proliferation of low IL-10 producing PBMC of five HIV^+ individuals. Addition of IL-12 and anti-IL-10 neutralizing antibodies together enhanced [^{3}H] thymidine incorporation in response to P25 antigen by 24 ± 7% and to FLU antigen by 18±12% compared to that of IL-12 alone.



IL-10 S.I.	3.5	6	10	9	4

Figure 9 B. Effect of anti-IL-10 antibodies and IL-12 on the proliferative response of PBMC from HIV^+ normal IL-10 producers. Five representatives of a total of 17 normal IL-10 producers are shown, none of which responded to anti-IL_10 and to IL-12 treatment (SI>3). PBMC were stimulated with p25 antigen in the presence or absence of anti-IL-10 antibodies (5μg/ml) and/or IL-12 (170 U/ml) for 7 days. The cells were pulsed with [^{3}H] thymidine and measured for thymidine incorporation 16 hours later. Proliferation assays were done in triplicate. Results represent means ± SEM of triplicate experiments.

7. Impaired production of IL-12 by HIV^+ PBMC

Because the addition of IL-12 enhanced proliferation of PBMC from low IL-10 producers, IL-10 dysregulation may be associated with altered production of IL-12 which is central to CMIR. To measure IL-12 production by monocytes and B cells, PBMC were stimulated with PWM (as opposed to PHA that effects mainly T cells), supernatants were collected after 48 hours of culture and analyzed for the presence of IL-12. PWM-stimulation of PBMC from HIV^+ individuals did not increase IL-12 production, while PWM-stimulated PBMC of normal individuals produced IL-12 (Fig 10). In contrast, IL-10 production by PWM stimulated PBMC from HIV^+ individuals was comparable to that of normal individuals (Fig 10). Thus, PBMC from HIV^+ individuals exhibited specific impairment of IL-12 production although their ability to produce IL-10 by PWM was normal. PWM-stimulated PBMC from HIV^+ individuals produce predominantly IL-10, which might imply an altered balance of IL-10 and IL-12. Lower levels of IL-12 were not due to delayed production of IL-12, as low levels of IL-12 were consistently detected in supernatants harvested after 24, 48 and 72 hours of culture (Table 4). PWM did not induce IL-12 production in HIV^+ PBMC, indicating an impaired IL-12 production by monocytes and B cells.

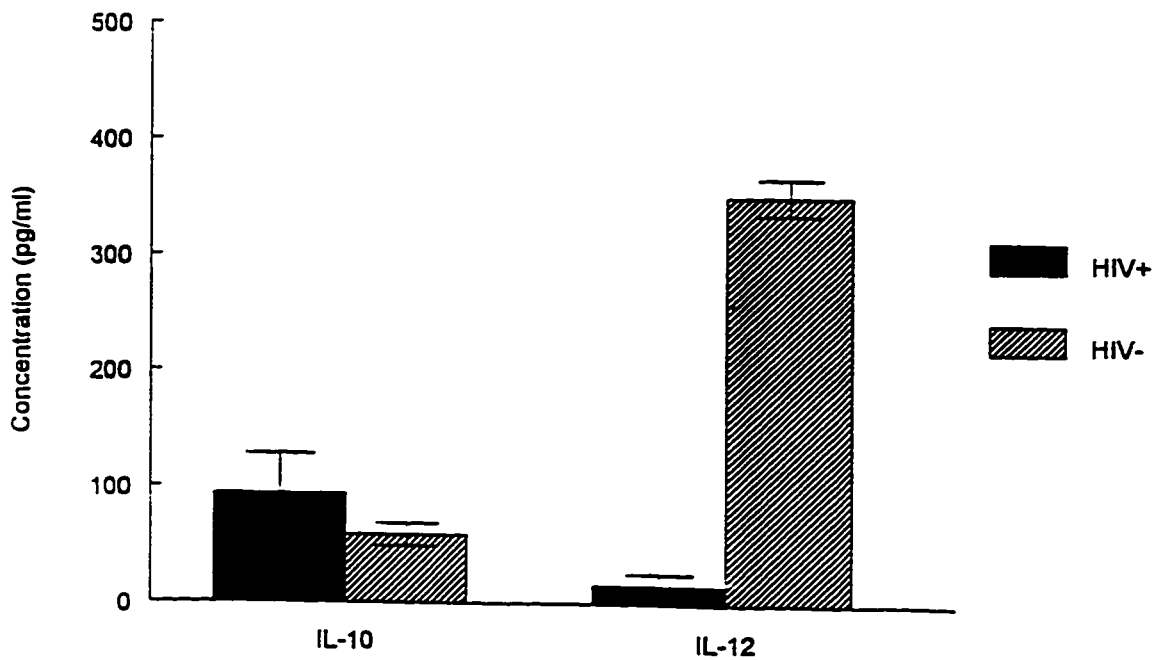

500
400
300
200
100
0
Concentration (pg/ml)
IL-10
IL-12
HIV+
HIV-

Figure 10. PBMC, from HIV^+ (n = 9) and normal controls (n = 12), were stimulated with PWM and supernatants were collected after 48 hours of culture and analyzed for the presence of IL-12 and IL-10. IL-12 was not detectable in the supernatant of unstimulated PBMC. Results are shown as means ± SEM.

Table 4. Kinetics of IL-12 production in the supernatant of stimulated PBMC in HIV^+ individuals.

	24 hours	48 hours	72 hours
HIV^- (n = 6)	362±44	349±36	350±28
HIV^+ (n = 9)	78±19	80±16	79±17

Kinetics of IL-12 production in the supernatant of stimulated PBMC from HIV^+ (n = 9) and HIV^- individuals (n = 6). Supernatants were collected after 24, 48, and 72 hours after PBMC were stimulated with PWM.

Regulation of IL-10 production in human monocytes and T cells

8. Characterization of the major source of IL-10 production in HIV^+ and HIV^- PBMC

In humans, IL-10 is produced by Th2 cells, monocytes and EBV transformed B cells, and to a lesser extent by Th1 cells (Romagnani, 1995). HIV infection has been proposed to cause an expansion of Th0/Th2-like cells (Hu et al. 1994; Romagnani et al. 1994a; Clerici and Shearer, 1994; Ameglio et al. 1994; Barcellini et al. 1994; Maggi et al. 1994; Meyaard et al. 1994; Muller, 1995; Romagnani, 1995; Del Prete et al. 1995; Vigano et al. 1995) which could constitute a major source of IL-10. I have shown that there is a defect in responses to FLU and p25 HIV antigens in normal IL-10 producers and not in low IL-10 producers. Although levels of IL-10 in this subset are comparable to those of HIV^- individuals, perhaps, due to the defective IL-2 and IL-12 production, in HIV^+ patients IL-10 takes over and its predominance in unstimulated and stimulated PBMC results partially in unresponsiveness. To analyze IL-10 production by various cell types, PBMC from HIV^+ individuals and HIV^- healthy adults were depleted of either B cells, T cells or monocytes, and stimulated with PHA. To determine the cell type producing IL-10 in PBMC of normal IL-10 producers, PBMC were depleted of B cells and T cells using anti-CD19 and anti-CD2 conjugated immunobeads, respectively. Monocytes were depleted by treatment of PBMC with L-leucyl-L-leucine methyl ester (L-LME). The depleted and undepleted cell populations were stimulated with PHA and anti-CD3

antibodies for 48 hours, and supernatants were harvested for analysis of IL-10 production by ELISA. Depleted cell populations contained less than 2% target cells as determined by flow cytometric analysis. Depleted T cell populations failed to proliferate in response to PHA, whereas B-cell depleted PBMC populations proliferated normally as compared to undepleted PBMC populations. The B-cell depleted populations failed to proliferate in response to anti-IgM coated sepharose beads, whereas undepleted PBMC populations proliferated normally (see below). In addition, monocyte depleted PBMC failed to proliferate in response to anti-CD3 antibodies and their proliferation in response to PHA was partially inhibited when compared with that of PBMC. Depletion of T and B cells was also evaluated by measuring cell proliferation in response to PHA, and the combination of anti-IgM antibodies coated sepharose beads (BIO-RAD, Richmond, CA) and IL-4, respectively. Cell proliferation was measured by incorporation of [^{3}H] thymidine. Cells were pulsed with 0.5 μCi [^{3}H] thymidine (Amersham, Arlington Heights, IL) and cultured for a further 16 hours followed by cell harvesting and measurement of thymidine incorporation. T cell depleted PBMC did not proliferate following stimulation with PHA (cpm of this fraction when stimulated with PHA was comparable with that of background levels, 2460 ± 720 *versus* 2770 ± 582, while cpm of undepleted PBMC in response to PHA was 38,910 ± 3,750). B cell depleted PBMC, on the other hand, failed to proliferate in response to stimulation with anti-IgM antibodies coated-beads plus IL-4 (cpm of this fraction when stimulated was not significantly different from that of background levels, 1065 ± 120 *versus* 770 ± 440 cpm; the thymidine incorporation of undepleted PBMC was 6,390 ± 375 cpm. These results indicate that depletion of T cells by anti-CD2 antibody coated sepharose immunobeads, depletion of B cells by anti-CD19 antibody coated sepharose

immunobeads and depletion of monocytes by L-LME was complete and specific as described in Materials and methods. Following T cell depletion, IL-10 production was abolished in HIV^- controls (n = 6), while, in HIV^+ individuals (n = 10) no significant change in IL-10 production was observed (Fig 11A). IL-10 production generally has a wide range, however, the majority of patients had IL-10 SI > 3 and were thus normal IL-10 producers. Monocyte depletion with L-LME abrogated IL-10 production by cells from both HIV- and HIV^+ individuals (Fig 11C). B cell depletion did not alter IL-10 production by cells from either HIV^- or HIV^+ individuals (Fig 11 B). The overall results are shown in Fig 11 D. Cell subset depletion results from three of representative HIV^+ individuals (a total of six) and one HIV^- individual (a total of six) are shown in Figs. 11A, 11B, and 11C for T cells, B cells and monocytes, respectively. These results suggest that in HIV^+ individuals, IL-10 is mainly produced by monocytes, whereas in HIV^- controls IL-10 is produced by both T cells and monocytes. In HIV- controls, the IL-10 production in PHA-stimulated T-cell-depleted-PBMC is significantly reduced, suggesting that T cells contribute to IL-10 production in these individuals. In some individuals, depletion of monocytes abrogates IL-10 production. These observations strongly suggest that PHA-stimulated monocyte-depleted PBMC from HIV^- individuals require the presence of T cells to produce IL-10, whereas PHA stimulated monocyte depleted PBMC from HIV^+ individuals do not. T cells contribute to IL-10 production after PHA stimulation in HIV^- and not in HIV^+ persons.

However, trace amounts of IL-10 produced by contaminating $CD2^+$ T cells and B cells cannot be excluded in these experiments.

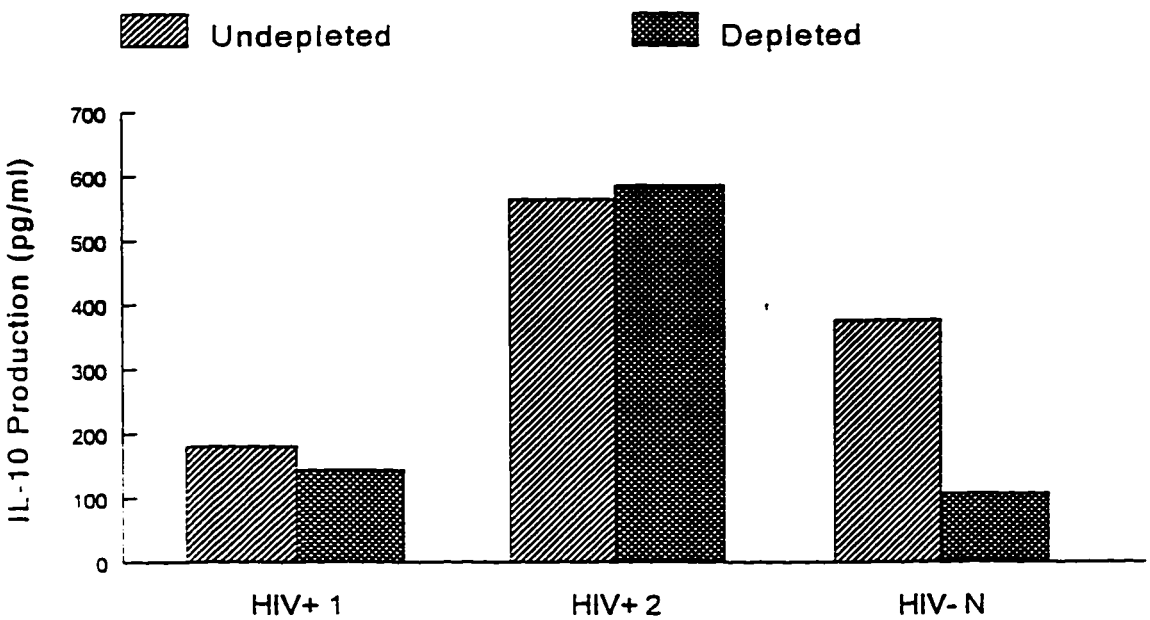

Undepleted
Depleted
IL-10 Production (pg/ml)
700
600
500
400
300
200
100
0
HIV+ 1
HIV+ 2
HIV- N

Figure 11 A. IL-10 production by T cell depleted PBMC. PBMC from HIV^+ and HIV^- individuals were depleted of T cells, stimulated with PHA (1:50) and after 48 hours of culture, the levels of IL-10 in the supernatants were measured by ELISA. Two representative of nine HIV^+ and one of six HIV^- individuals are presented. IL-10 levels in the media alone of all HIV^- and one HIV^+ individuals were not detectable. Trace amounts of IL-10 were detectable in the media alone of other HIV^+ individuals (30±4 pg/ml).

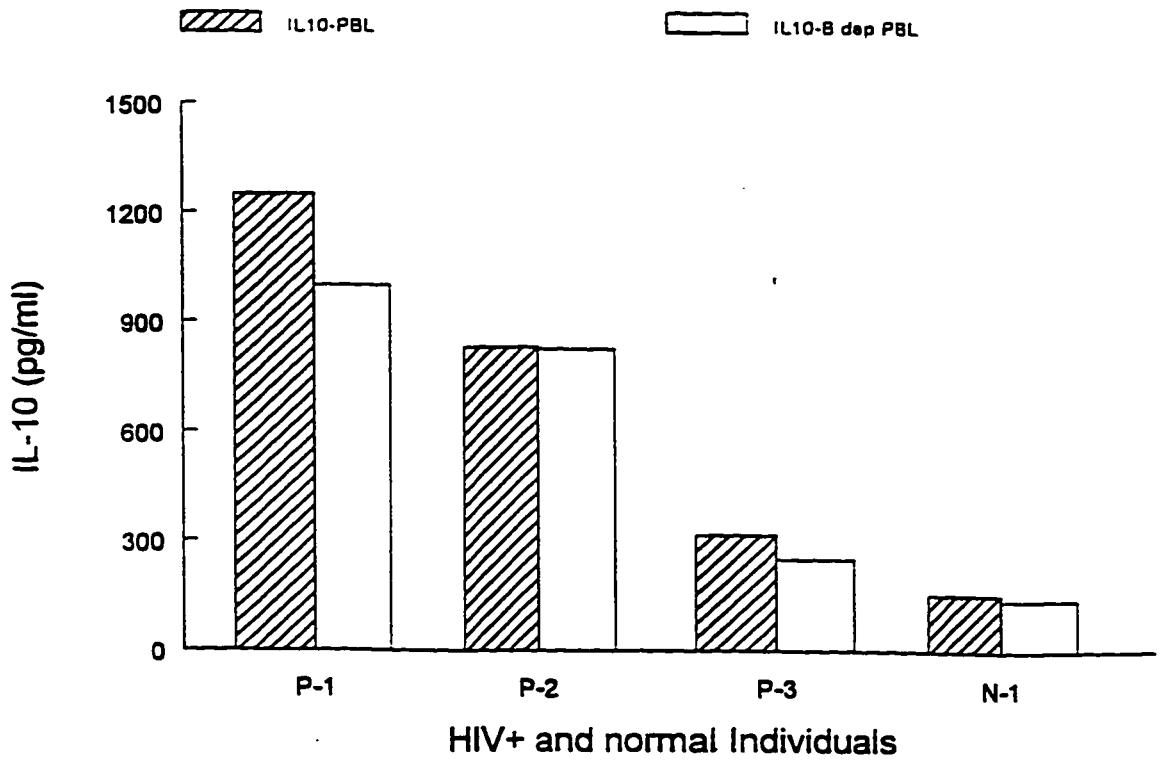

IL10-PBL
IL10-B dep PBL
1500
1200
900
600
300
0
IL-10 (pg/ml)
P-1
P-2
P-3
N-1
HIV+ and normal Individuals

Figure 11 B. IL-10 production by B-cell depleted PBMC. PBMC from HIV^+ (n = 3) and HIV^- (n = 1) individuals (subjects different from those shown in figure 11 A, C, and D) were depleted of B cells, stimulated with PHA and after 48 hours of culture, the levels of IL-10 in the supernatants were measured by ELISA.

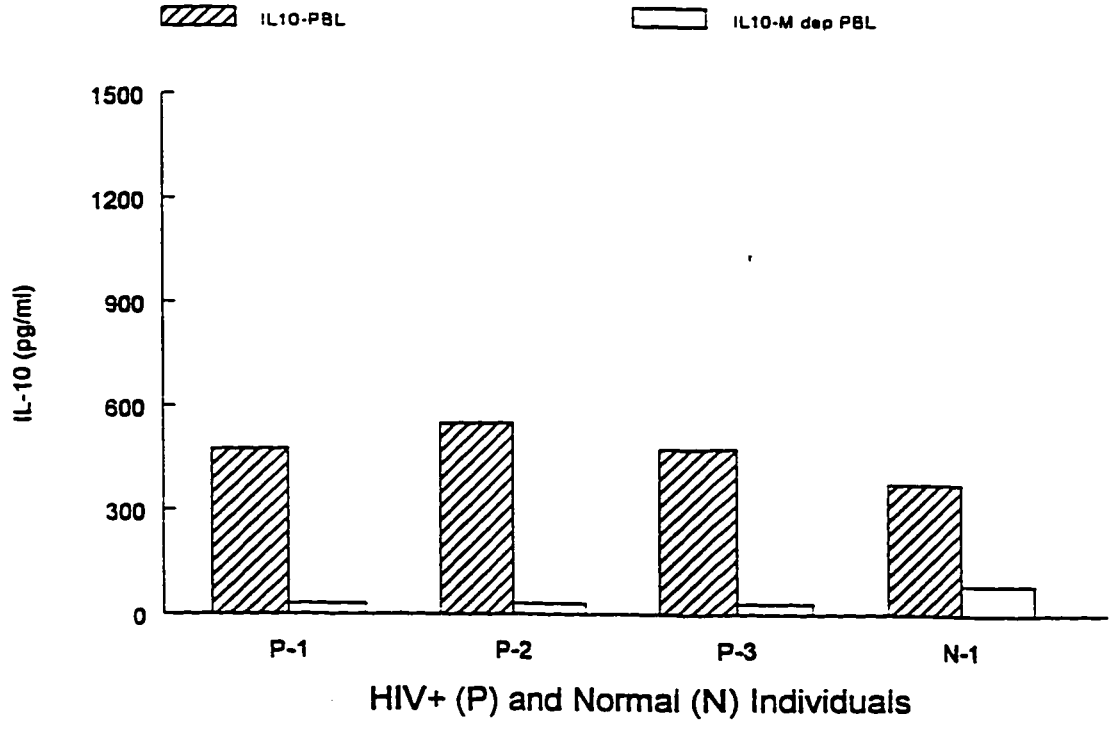

IL10-PBL
IL10-M dep PBL
1500
1200
900
600
300
0
IL-10 (pg/ml)
P-1
P-2
P-3
N-1
HIV+ (P) and Normal (N) Individuals

Figure 11 C. IL-10 production following depletion of monocytes. Using L-LME, PBMC from HIV^- (n = 3)and HIV^- (n =1) individuals were depleted of monocytes, stimulated with PHA and after 48 hours of culture, the levels of IL-10 in the supernatants were measured by ELISA. Subjects different from those shown in figure 11 A, B, and D.

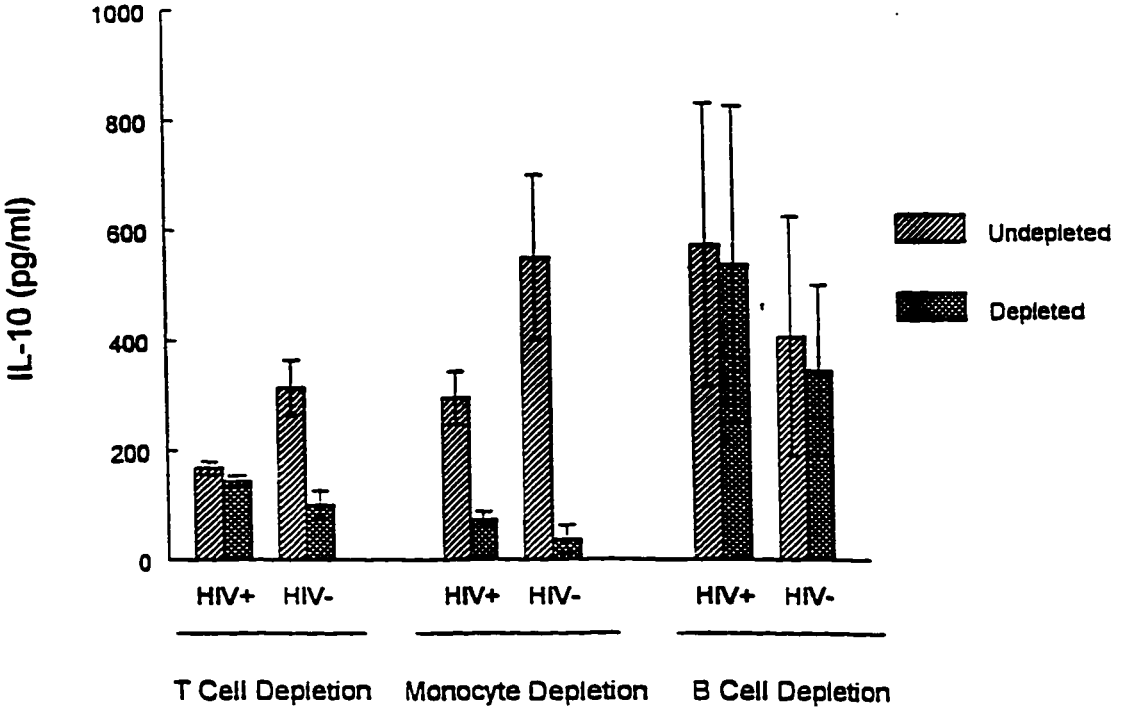

IL-10 (pg/ml)
1000
800
600
400
200
0
HIV+
HIV-
HIV+
HIV-
HIV+
HIV-
T Cell Depletion
Monocyte Depletion
B Cell Depletion
Undepleted
Depleted

Figure 11 D. The overall results of depletion studies of six HIV^+ and six HIV^- individuals. PBMC from six HIV^+ and six HIV^- individuals (subjects are different from those shown in Figures 11 A, B, and C) were depleted of either B cells or T cells or monocytes. Undepleted and depleted monocytes were stimulated with PHA for 48 hours, and supernatants were analyzed for IL-10 production by ELISA. Means ± SEM IL-10 production are shown. IL-10 production in T cell depleted PBMC from HIV^- ($p<0.001$), monocyte depleted PBMC from HIV^+ ($p<0.0003$)and HIV^-($p<0.0004$), are statistically different from those produced by undepleted fractions, respectively. The differences between IL-10 production in the T cell-depleted and undepleted fractions of HIV^+ individuals, B cell-depleted and undepleted fractions of HIV^+ individuals, and B cell-depleted and undepleted fractions of HIV^- controls were not statistically significant.

There was no significant difference in IL-10 production by unfractionated PBMC and PBMC depleted of B and T cells from HIV^+ individuals (Figs. 11A and 11B). However, depletion of monocytes by L-LME completely abrogated mitogen-induced IL-10 production (Fig. 11C). These results were confirmed by depletion of monocytes using anti-CD14 conjugated immunobeads (Table 5). PHA (Kurisu et al. 1980; Reiner et al. 1985) and PHA activated lymphocytes (Zwilling et al. 1994) have been shown by others to activate monocytes and induce monokine production.

9. Monocyte-conditioned medium (MCM) induces IL-10 production by monocyte depleted PBMC

The results from the previous section suggest that PBMC from HIV^- controls depleted of either T cells or monocytes fail to produce IL-10 following stimulation with PHA. To understand the regulation of IL-10 production by T cells and monocytes and to further confirm the above observations, these experiments were repeated on a larger scale among HIV^- controls.PBMC depleted of either T cells, B cells or monocytes were stimulated with PHA, and the supernatants harvested after 48 hours were analyzed for IL-10 production by ELISA. IL-10 production was significantly reduced in PBMC depleted of either T cells or monocytes compared to undepleted PBMC. However, depletion of B cells did not affect IL-10 production compared to undepleted PBMC.

Table 5. IL-10 production of PHA stimulated monocyte depleted PBMC.

	HIV-12	HIV-7	HIV-4	N-1	N-2	N-3
PBMC	372±15	194±11	410±14	559±16	405±12	380±16
Mn Dep.	56±9	32±7	35±4	60±17	57±10	38±7

PBMC from three representatives HIV^+ and HIV^- individuals were depleted of monocytes by means of magnetic beads coated with anti-CD14 antibodies. After 48 hour stimulation with PHA, IL-10 was measured in the supernatant of depleted and undepleted fractions. Mn Dep., monocyte depleted PBMC.

Table 6. Effect of T cell, B cell and monocyte depletion on IL-10 production by normal PBMC following stimulation with PHA.

Cells	IL-10 production (pg/ml) Exp-1	Exp-2
Undepleted PBMC	625 ± 29.75	750 ± 35.51
T cell depleted PBMC	190 ± 16.15	125 ± 20.75
Undepleted PBMC	1250 ± 52.27	625 ± 58.50
B cell depleted PBMC	1000 ± 33.48	500 ± 43.11
Undepleted PBMC	630 ± 49.92	700 ± 54.57
Monocyte depleted PBMC	30 ± 7.10	62.5 ± 12.40

Undepleted PBMC and PBMC depleted of either T cells, B cells or monocytes were stimulated with PHA for 48 hours as described in the Materials and methods. The supernatants were analyzed for IL-10 production by ELISA. The values are expressed as mean ± SEM of IL-10 production in triplicate wells.

IL-10 production data from two representative experiments out of six are shown in Table 6. These results suggested that both T cells and monocytes are required for maximal IL-10 production. To determine whether the cytokines produced by monocytes can induce IL-10 production by activated T cells, monocyte-conditioned medium (MCM; the supernatant containing factors produced by monocyte-enriched PBMC) was prepared by stimulating T cell-depleted PBMC with PHA. The cells were washed after 24 hours of stimulation to remove any IL-10 produced during this period from the supernatant.

The supernatant (MCM) harvested after an additional 48 hours of culture did not contain IL-10 as determined by ELISA (lower than the detection level of the ELISA). Addition of MCM to the monocyte-depleted PBMC restored IL-10 production following stimulation with PHA (Fig. 12). The levels of IL-10 thus produced were comparable to those of undepleted PBMC following stimulation with PHA (Fig 12). IL-10 production by monocyte depleted PBMC from HIV^- individuals was not usually completely abrogated (Fig. 11 C and Fig. 12) which may be due to the presence of contaminating monocytes or non-T non-B cells. These results indicated that factors produced by activated monocytes/macrophages regulate IL-10 production by activated T cells.

10. Characterization of monokines that induce IL-10 production by nonocyte depleted PBMC (HIV^-)

To determine the cytokines responsible for the induction of IL-10 by PHA-activated T cells, monocyte-depleted PBMC were stimulated with PHA in the presence of cytokines

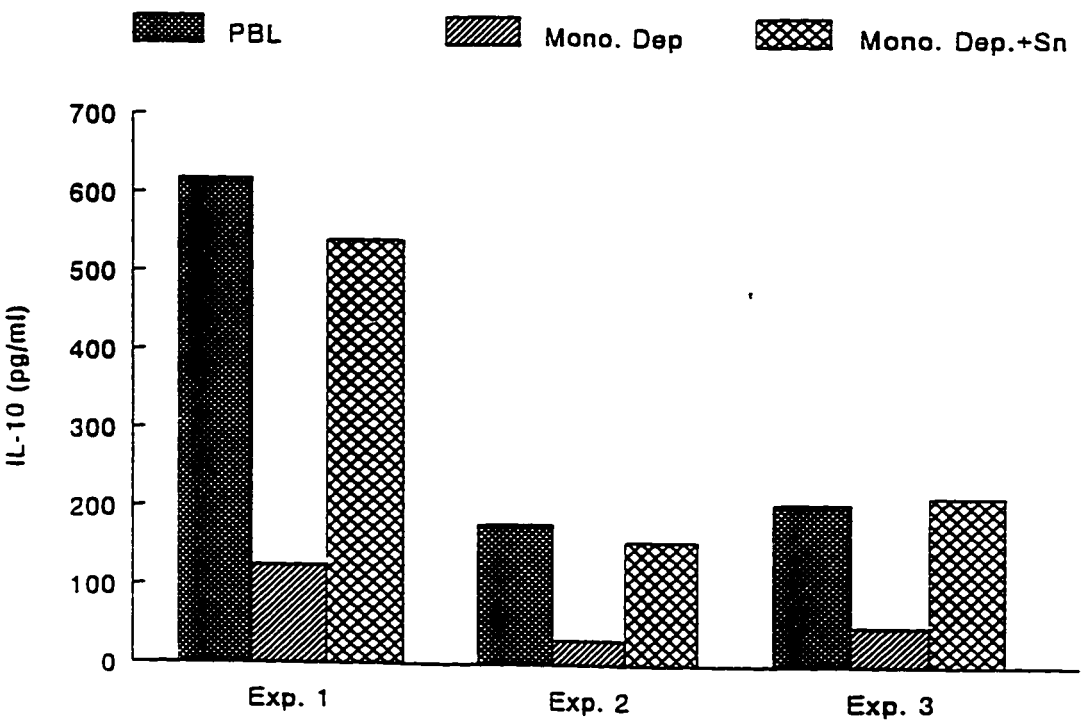

PBL
Mono. Dep
Mono. Dep.+Sn
IL-10 (pg/ml)
700
600
500
400
300
200
100
0
Exp. 1
Exp. 2
Exp. 3

Figure 12. Effect of monocyte-conditioned medium on IL-10 production by monocyte depleted PBMC following stimulation with PHA. Undepleted PBMC and monocyte-depleted PBMC were stimulated with PHA in the presence or absence of monocyte-conditioned medium (MCM, 25 % final concentration). The supernatants harvested after 48 hours of stimulation were analyzed for IL-10 production by ELISA as described in the Materials and methods. The results from three different experiments are expressed as means of triplicate wells. MCM was prepared by stimulating T cell depleted PBMC with PHA, as described in Materials and methods.

produced primarily by monocytes such as IL-1, IL-6, IL-12, TNF-α, GM-CSF and M-CSF. Enhancement of IL-10 production by monocyte-depleted PBMC was not observed with any concentration of IL-1, TNF-α, GM-CSF and M-CSF (base-line IL-10 levels). In contrast, addition of IL-6 and/or IL-12 to monocyte-depleted PBMC induced IL-10 production. The levels of IL-10 produced by monocyte depleted PBMC in the presence of either IL-6 or IL-12 was comparable to the levels of IL-10 produced in the presence of MCM (Table 7 and Fig 12). The effect of IL-6 and IL-12 on IL-10 production by monocyte depleted PBMC was dose-dependent (Table 8). However, IL-6 and IL-12 either alone or in combination did not induce IL-10 production by monocyte depleted PBMC in the absence of PHA (Table 7). The IL-10 production by monocyte depleted PBMC in the presence of MCM was further attributed to IL-6 and IL-12 as incubation of MCM with neutralizing anti-IL-6 and/or anti-IL-12 antibodies inhibited the IL-10 inductive effect (Fig 13).

Anti-IL-6 antibodies at a concentration of 5 μg/ml and 10 μg/ml, and anti-IL-12 antibodies at a concentration of 10 μg/ml significantly reduced the IL-10 inductive effect of MCM to levels comparable to the IL-10 produced by PHA stimulated monocyte depleted PBMC (Fig. 13). The inhibitory effect of neutralizing anti-IL-6 and anti-IL-12 antibodies was specific as isotype matched control antibodies did not affect IL-10 production by monocyte depleted PBMC (Fig. 13). These results suggested that IL-6 and IL-12 independently enhance IL-10 production by monocyte depleted PBMC stimulated with PHA. Furthermore, this effect of IL-6 and IL-12 on IL-10 production was additive (Table 7).

11. IL-12 and IL-6 induce IL-10 production by PHA stimulated $CD4^+$ T cells and $CD8^+$ T cells

IL-10 produced by PHA-stimulated monocyte depleted PBMC in the presence of IL-6 and/or IL-12 may possibly be attributed to $CD4^+$ T cells, $CD8^+$ T cells, NK cells or non-T non-B cells. To study the regulation of IL-10 production by T cells, pure $CD4^+$ and $CD8^+$ T cells (>98 %) isolated from PBMC were stimulated with PHA in the presence of various concentrations of IL-6 and/or IL-12. IL-10 production by these cells was assessed by semi-quantitative RT-PCR and ELISA.

Optimization of semi-quantitative RT-PCR analysis

Since quantification of IL-10 involved measuring relative expression of IL-10 with respect to β-actin expression, the RT-PCR technique was again optimized for the reproducibility and accuracy of these experiments as described earlier, with some modification (Diaz-Mitoma et al., 1995; Daftarian et al. 1996e). Total RNA (1μg) obtained from PHA stimulated PBMC was reverse transcribed. Equal amounts of cDNA were amplified for β-actin and IL-10 using 25, 30 and 35 amplifying cycles. Measurement of the signals by densitometric analysis revealed that amplification of β-actin and IL-10 was proportional to the amount of cDNA used for amplification. Since dose-dependent amplification was not observed as the number of amplifying cycles was increased beyond 30 for β-actin and 35 for IL-10.

Table 7. Effect of IL-6 and IL-12 on IL-10 production by monocyte-depleted PBMC following stimulation with PHA.

Stimulant	IL-10 Production (pg/ml)	
	Exp-1	Exp-2
Medium	15 ± 4.95	32 ± 9.89
Medium $^{+}$ IL-6	15 ± 3.82	32 ± 8.50
Medium $^{+}$IL-12	15 ± 6.10	32 ± 8.14
PHA	53 ± 11.31	124 ± 21.92
PHA $^{+}$ MCM	179 ± 21.92	340 ± 37.75
PHA $^{+}$ IL-6	166 ± 14.85	314 ± 27.57
PHA $^{+}$ IL-12	176 ± 17.67	317 ± 24.04
PHA $^{+}$ IL-6 $^{+}$ IL-12	218 ± 44.14	547 ± 48.08
PHA $^{+}$ IFN-γ	51± 8.48	13.32 ± 30.53
PHA $^{+}$ TNF-α	35 ± 4.95	10.9 ± 21.20
Undepleted PBMC		
Medium	35± 14.5	47± 17.5
PHA	364± 36.27	712± 68.72

PBMC depleted of monocytes were stimulated with PHA for 48 hours in the presence of monocyte conditioned medium (25% final concentration), IFN-γ (350 units/ml), TNF-α (100 units/ml), IL-6 (50 ng/ml), IL-12 (50 U/ml; 170 Units of IL-12 consisted of 1 ng of IL-12) or IL-6 (50 ng/ml) $^{+}$ IL-12 (50 U/ml). The supernatants were analyzed for IL-10 production by ELISA. The values are expressed as mean ± SEM of IL-10 production in triplicate wells.

Table 8. Dose dependent effect of IL-6 and IL-12 on IL-10 production by monocyte-depleted PBMC following stimulation with PHA

Stimulant	IL-10 Production (pg/ml)	
	Exp-1	Exp-2
Medium	15 ± 5.0	15 ± 4.87
PHA	174 ± 26.65	38 ± 7.99
PHA $^{+}$IL-6 (2.5 ng /ml)	270 ± 37.67	61 ± 14.56
PHA $^{+}$IL-6 (5.0 ng/ml)	507 ± 29.18	67 ± 12.02
PHA $^{+}$IL-6 (10.0 ng/ml)	631 ± 37.23	111 ± 14.14
PHA $^{+}$IL-6 (20.0ng/ml)	762 ± 25.62	167 ± 16.26
PHA $^{+}$IL-6 (40.0 ng/ml)	755 ± 47.90	166 ± 19.7
PHA $^{+}$IL-12 (50.0 u/ml)	645 ± 54.10	103 ± 15.38
PHA $^{+}$IL-12 (100.0 u/ml)	710 ± 37.83	140 ± 16.12
PHA $^{+}$IL-12 (200.0 u/ml)	1302 ± 62.14	189 ± 20.59
PHA $^{+}$IL-12 (400.0 u/ml)	1377 ± 58.47	177 ± 20.12

PBMC depleted of monocytes were stimulated with PHA for 48 hours in the presence of various concentrations of IL-6 (2.5-40 ng/ml) and IL-12 (50-400 units/ml; 170 units of IL-12 consisted of 1 ng of IL-12). The supernatants were analyzed for IL-10 production by ELISA. The values are expressed as mean ± SEM of IL-10 production in triplicate wells. The levels of IL-10 produced by undepleted cells were as follows: Unstimulated, 42 ± 16.25 and 35 ± 13.62 pg/ml in experiments 1 and 2 respectively; PHA stimulated, 1547 ± 72.21 and 270 ± 64.75 pg/ml in experiments 1 and 2 respectively.

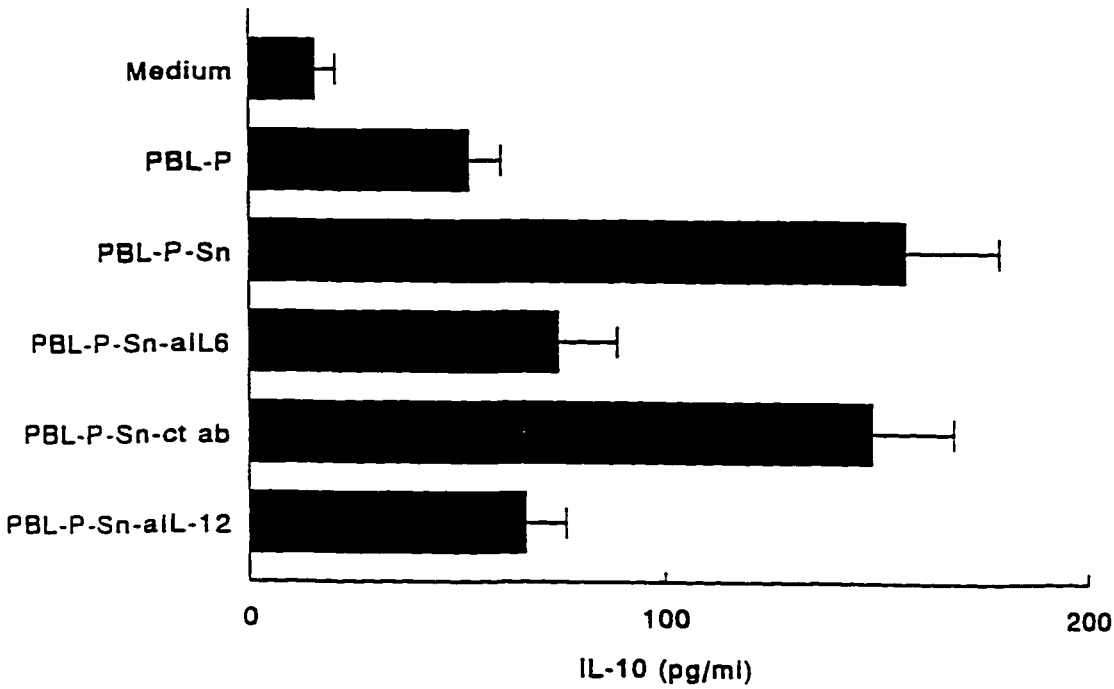

Medium
PBL-P
PBL-P-Sn
PBL-P-Sn-aIL6
PBL-P-Sn-ct ab
PBL-P-Sn-aIL-12
0
100
200
IL-10 (pg/ml)

Figure 13. Effect of anti-IL-6 and anti-IL-12 antibodies on the IL-10 inductive effect of monocyte-conditioned medium: Monocyte-depleted PBMC were stimulated with PHA in the presence of monocyte conditioned medium treated with anti-IL-6 neutralizing antibodies (final concentration 5 μg /ml), anti-IL-12 neutralizing antibodies (10 μg/ml) and isotype matched control antibodies (10 μg/ml). MCM was incubated with the antibodies at room temperature for 30 min. before addition to the PHA-stimulated monocyte-depleted PBMC. The supernatants harvested after 48 hours were analyzed for IL-10 production by ELISA. The results are expressed as mean ± SEM of IL-10 production in triplicate wells. M, medium; P, PHA; MCM, monocyte-conditioned medium; PBL, monocyte depleted PBMC.

Therefore, in the subsequent PCR, 30 amplifying cycles for β-actin and 35 cycles for IL-10 were employed.

To ensure non-saturating conditions in the amplification, equal volumes of various concentrations (5 μl) of cDNA (final dilution ranging from 1:2 to 1:256, which represent approximately 400 PBMC) were amplified for IL-10 and β-actin. PCR products were separated by electrophoresis and visualized with ethidium bromide staining followed by densitometric analysis (Fig. 14). Amplification of β-actin and IL-10 at dilutions of cDNA ranging from 1:2 to 1:128 was proportional to the amount of cDNA (Fig. 15). Furthermore, the ratio of densitometric units for IL-10 to β-actin remained constant (mean ± S.E., 1.17 ± 0.1) for each of the concentrations of cDNA used for amplification (Fig. 15). However, PCR products obtained following amplification of cDNA used at a dilution of 1:256 were not visible by ethidium bromide staining. These results suggested that the semiquantitative RT-PCR assay used in these studies was accurate. Subsequently, a PCR for serial dilutions of cDNA was performed in each and every experiment.

12. Measurement of IL-10 expression by $CD4^+$ and $CD8^+$ T cells by semiquantitative RT-PCR

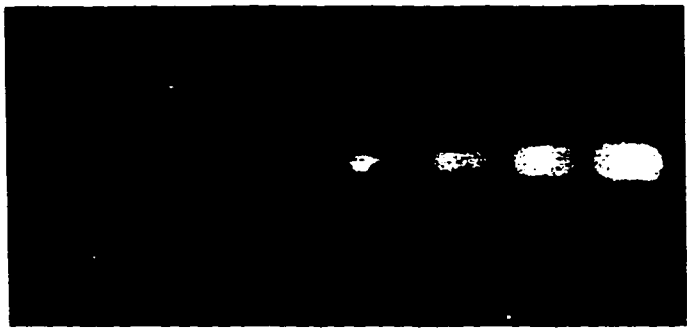
$CD4^+$ and $CD8^+$ T cells were purified (>98%) by $CD4^+$ and $CD8^+$ beads (Dynal) after which beads were removed by detachabeads (Dynal), as was explained in the Material and methods. $CD4^+$ and $CD8^+$ were stimulated with PHA and various cytokines for 24 hours then

cells were harvested for mRNA isolation and analyzed for IL-10 expression by semi-quantitative RT-PCR by comparing the IL-10 signals to β-actin, as described above. For semi-quantitative RT-PCR analysis of IL-10, cells were harvested after 24 hours of stimulation as maximal levels of IL-10 RNA were detected in PHA-stimulated PBMC at this time in the preliminary studies. IL-10 expression by unstimulated and PHA-stimulated $CD4^+$ and $CD8^+$ T cells was undetectable (Figs. 16 and 17). However, IL-12 and IL-6 were the major cytokines that independently enhanced significant IL-10 expression in PHA-stimulated $CD4^+$ and $CD8^+$ T cells. IL-12 induced more than a three fold increase in IL-10 expression in both $CD4^+$ and $CD8^+$ T cells compared to that of unstimulated and PHA-stimulated $CD4^+$ and $CD8^+$ T cells (Figs. 16 and 17). Addition of IL-6 to the PHA-stimulated $CD4^+$ and $CD8^+$ T cells had no effect on IL-10 expression (Figs. 16 and 17). Furthermore, the effect of IL-6 and IL-12 on IL-10 expression by both $CD4^+$ and $CD8^+$ T cells was additive (Fig 17). IL-1α, IL-1β, TNF-α, GM-CSF and M-CSF did not influence IL-10 expression by unstimulated or PHA-stimulated $CD4^+$ and $CD8^+$ T cells (no detectable amount of IL-10 ELISA).

A

cDNA Dilution 1/256 1/128 1/64 1/32 1/16 1/8 1/4 1/2

β Actin



IL-10

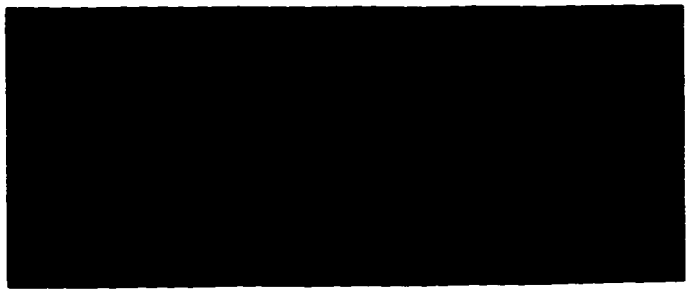


Figure 14. Semi-quantitative RT-PCR analysis of IL-10 mRNA. Total RNA (1μg) isolated from PHA-stimulated PBMC was reverse transcribed. Equal volumes of various dilutions of cDNA (1:2-1:256) were amplified for IL-10 and β-actin. The amplified PCR products were resolved by ethidium bromide-stained 2% agarose gel electrophoresis. Note: the ratio of IL-10 to β-actin for various dilutions remained constant (1.17 ± 0.1, mean ± SEM). The specific bands for IL-10 and β-actin obtained at dilutions of 1:256 were not detectable.

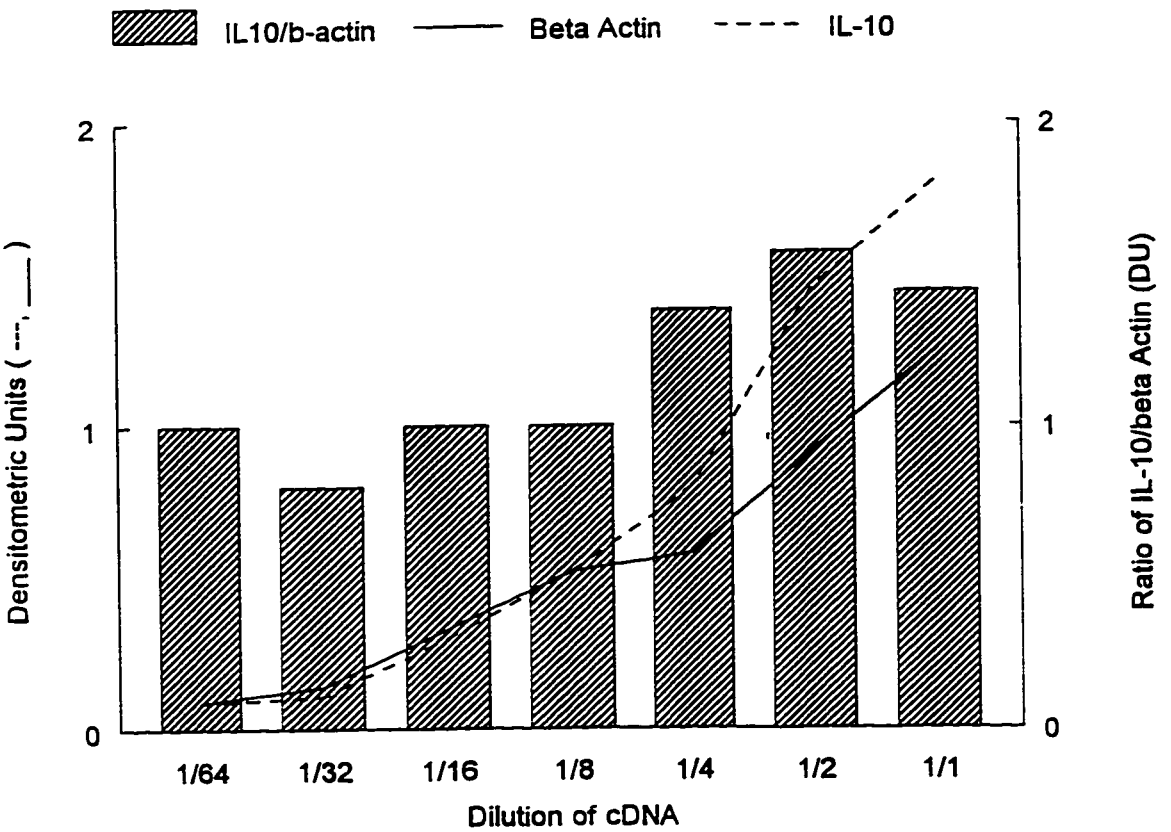
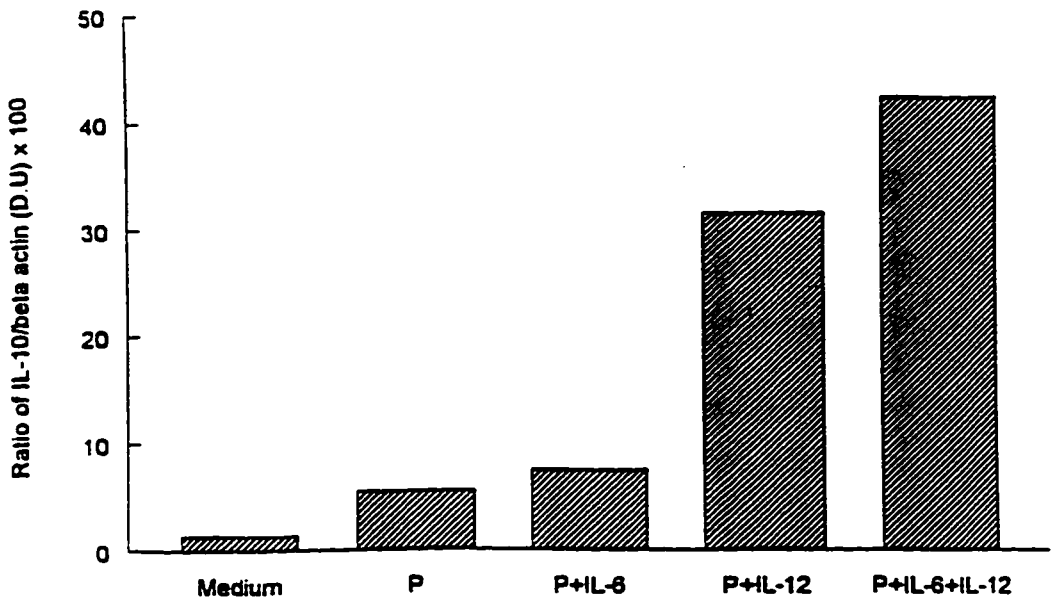

IL10/b-actin
Beta Actin
IL-10
Densitometric Units (---, ___)
Ratio of IL-10/beta Actin (DU)
2
1
0
1/64
1/32
1/16
1/8
1/4
1/2
1/1
Dilution of cDNA

Figure 15. Semi-quantitative RT-PCR analysis of IL-10 mRNA. Total RNA (1μg) isolated from PHA-stimulated PBMC was reverse transcribed. The specific bands for IL-10 and β-actin were quantified using densitometric analysis by assigning arbitrary units. The values for IL-10 and β-actin and the ratio of IL-10 to β-actin were plotted . The specific bands for IL-10 and β-actin obtained at dilutions of 1:256 were not detectable.



Stimulation of CD4+ T cells

Figure 16. Effect of IL-6 and IL-12 on IL-10 expression following stimulation of $CD4^+$ T cells with PHA. $CD4^+$ T cells were stimulated with PHA in the presence or absence of IL-6 (50 ng/ml), IL-12 (200 units/ml), or IL-6 (50 ng/ml) + IL-12 (200 units/ml), for 24 hours. The cells were harvested for RNA isolation and quantification of IL-10 by semi-quantitative RT-PCR as described in the Materials and Methods. Equal volumes of cDNA (5μl) were amplified for IL-10 and β-actin. The signals obtained were subjected to densitometric analysis and assigned arbitrary units. IL-10 expression was normalized with respect to β-actin and expressed as a ratio of values for IL-10 and β-actin. Fig 16 shows results from one representative experiment of three, each performed for $CD4^+$ T cells. P, PHA.

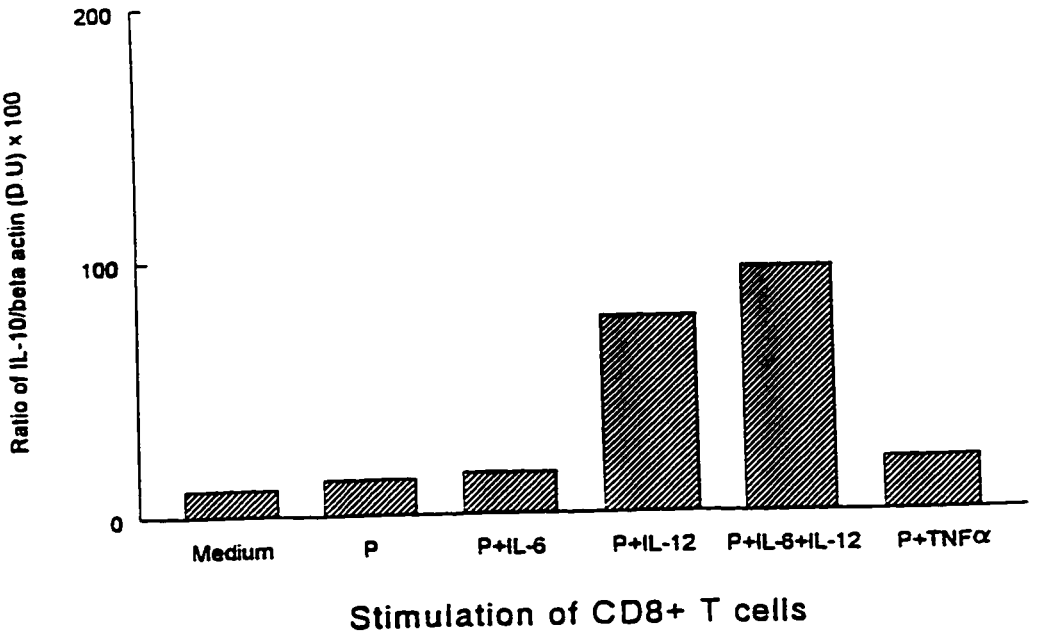

200
100
0
Ratio of IL-10/beta actin (D U) x 100
Medium
P
P+IL-6
P+IL-12
P+IL-6+IL-12
P+TNFα
Stimulation of CD8+ T cells

Figure 17. Effect of IL-6 and IL-12 on IL-10 expression following stimulation of $CD8^+$ T cells with PHA. $CD8^+$ T cells were stimulated with PHA in the presence or absence of IL-6 (50 ng/ml), IL-12 (200 units/ml), IL-6 (50 ng/ml) + IL-12 (200 units/ml), and TNF-α (100 units/ml) for 24 hours. The cells were harvested for RNA isolation and quantification of IL-10 by semi-quantitative RT-PCR as described in the Materials and Methods. Equal volumes of cDNA (5μl) were amplified for IL-10 and β-actin. The signals obtained were subjected to densitometric analysis and assigned arbitrary units. IL-10 expression was normalized with respect to β-actin and expressed as a ratio of values for IL-10 and β-actin. Fig 17 shows results from one representative experiment of three each performed for $CD8^+$ T cells. P, PHA.

13. Measurement of IL-10 expression by $CD4^+$ and $CD8^+$ T cells by ELISA

In order to confirm the results obtained in RT-PCR assays (section 11.) IL-10 was also detected at the protein level. $CD4^+$ and $CD8^+$ T cells were isolated by anti-CD4 antibody and anti-CD8 antibody-coated magnetic beads, respectively. Following stimulation with PHA alone, $CD4^+$ and $CD8^+$ cells produced minimal levels of IL-10 as determined by ELISA (Figs. 18 and 19). Addition of IL-6 and IL-12 independently to PHA-stimulated $CD4^+$ T cells enhanced IL-10 production by two and three-fold, respectively, compared to unstimulated $CD4^+$ T cells and $CD4^+$ T cells stimulated with PHA alone (Fig 18). PHA-stimulated $CD4^+$ T cells cultured in the presence of both IL-6 and IL-12 produced five fold higher amounts of IL-10 compared to $CD4^+$ T cells stimulated with PHA alone (Fig 18). Furthermore, the level of IL-10 produced by PHA-stimulated $CD4^+$ T cells cultured in the presence of both IL-6 and IL-12 was comparable to the levels of IL-10 produced in the presence of monocyte-conditioned medium. Similar results were obtained for $CD8^+$ T cells stimulated with PHA in the presence of IL-6 and/or IL-12.

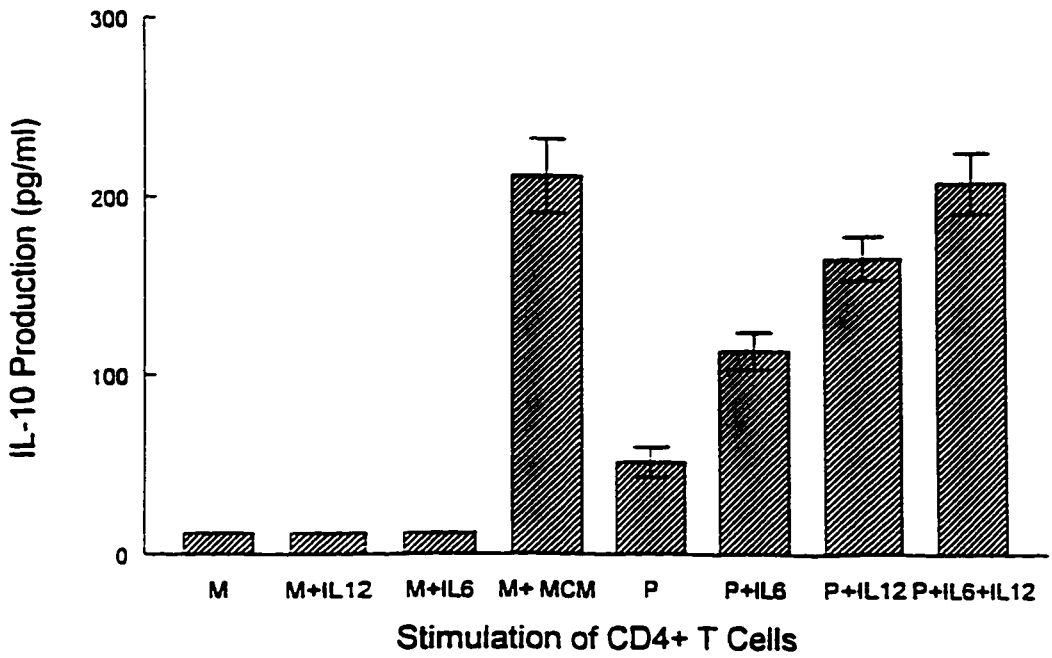

300
200
100
0
IL-10 Production (pg/ml)
M
M+IL12
M+IL6
M+ MCM
P
P+IL6
P+IL12
P+IL6+IL12
Stimulation of CD4+ T Cells

Figure 18. Effect of IL-6 and IL-12 on IL-10 expression following stimulation of $CD4^+$ T cells with PHA. $CD4^+$ T cells were stimulated with PHA in the presence or absence of IL-6 (50 ng/ml), IL-12 (200 units/ml), IL-6 (50 ng/ml) + IL-12 (200 units/ml). The supernatants harvested after 48 hours of stimulation were analyzed for IL-10 production by ELISA as described in the Materials and methods. The results are expressed as mean ± S.E.M. of IL-10 production in triplicate wells. IL-10 production from one representative experiment of the three performed for $CD4^+$ T cells, is presented. P, PHA; MCM, monocyte conditioned medium.

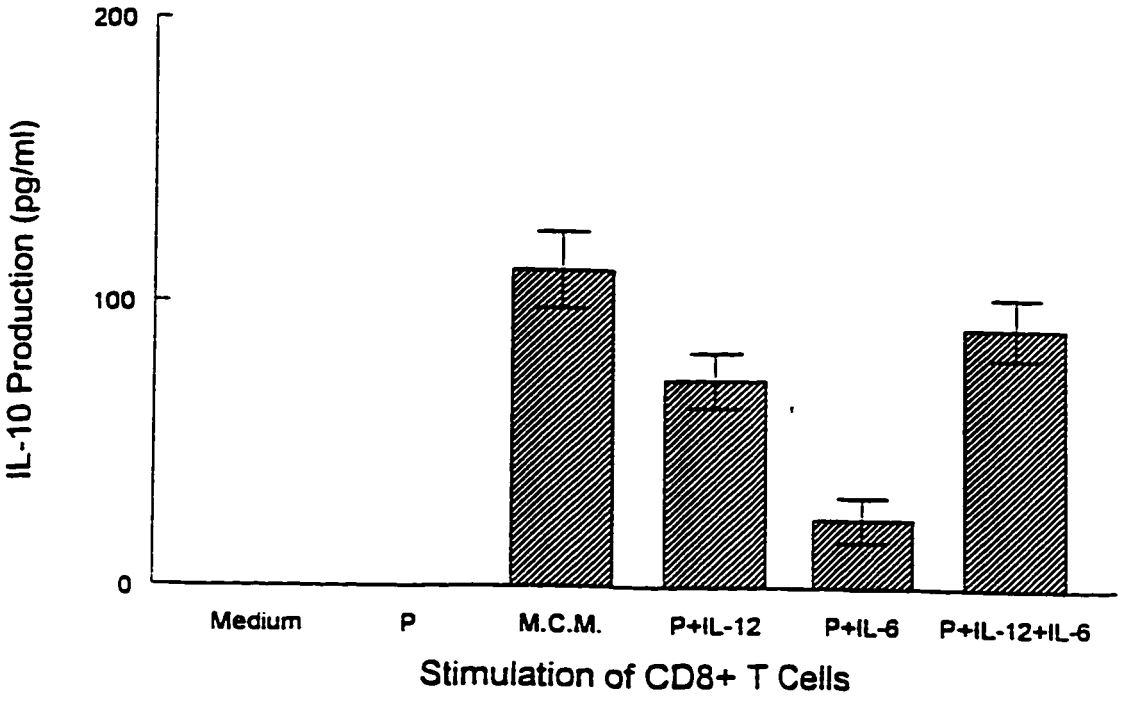

200
100
0
IL-10 Production (pg/ml)
Medium
P
M.C.M.
P+IL-12
P+IL-6
P+IL-12+IL-6
Stimulation of CD8+ T Cells

Figure 19. Effect of IL-6 and IL-12 on IL-10 expression following stimulation of $CD8^+$ T cells with PHA. $CD8^+$ T cells were stimulated with PHA in the presence or absence of IL-6 (50 ng/ml), IL-12 (200 units/ml), IL-6 (50 ng/ml) + IL-12 (200 units/ml). The supernatants harvested after 48 hours of stimulation were analyzed for IL-10 production by ELISA as described in the Materials and Methods. The results are expressed as mean ± S.EM.. of IL-10 production in triplicate wells. IL-10 production from one representative experiment of the three performed for $CD8^+$ T cells, is depicted. P, PHA; MCM, monocyte conditioned medium.

Addition of IL-12 to $CD8^+$ T cells stimulated with PHA induced significantly ($p<0.002$) higher levels of IL-10 compared to PHA-stimulated $CD8^+$ T cells alone (76.98 versus 13.97 pg/ml; Fig 19). IL-6 induced IL-10 expression in PHA-stimulated $CD8^+$ T cells, but the enhancement was not as dramatic. Furthermore, consistent with the results obtained by semi-quantitative RT-PCR analysis, IL-6 and IL-12 together exhibited an additive effect on IL-10 production by PHA stimulated $CD8^+$ T cells. IL-1α, IL-1β, TNF-α, GM-CSF and M-CSF did not influence IL-10 production by unstimulated or PHA stimulated $CD4^+$ and $CD8^+$ T cells (IL-10 induced by these stimuli plus PHA was comparable with that of PHA alone, 32 ± 17).

14. Kinetics of IL-10 production by PHA activated T cells following stimulation with IL-6 and IL-12

To determine the relative effectiveness of IL-6 and IL-12 in IL-10 induction in T cells, expression of IL-10 was analyzed at sequential time-points after stimulation of T cells with PHA in the presence of IL-6 and IL-12. Since PHA-activated $CD4^+$ and $CD8^+$ T cells exhibited IL-10 induction in the presence of both IL-6 and IL-12, purified $CD4^+$ and $CD8^+$ T cells were mixed in order to obtain sufficient numbers of cells from the same donor to perform both RT-PCR analysis and ELISA for IL-10. Semiquantitative RT-PCR analysis revealed that IL-12 induced more than a two-fold increase in IL-10 mRNA expression compared to stimulation with PHA alone at 12 hours post-stimulation (Fig. 20). Similarly, IL-6 enhanced the expression of IL-10 by two-fold compared to

stimulation with PHA alone. However, this enhanced expression was relatively delayed and observed at 24 hours post-stimulation (Fig. 20). Analysis of IL-10 production by ELISA showed that enhanced IL-10 production was detectable at 24 hours post-stimulation with both IL-6 and IL-12, and this increase was significantly higher after 36 hours of stimulation (Fig 21). Taken together, these results suggest that IL-12 and IL-6 induce IL-10 production by PHA activated $CD4^+$ and $CD8^+$ T cells, and IL-12 induces the expression of mRNA for IL-10 earlier compared to IL-6.

15. TNF-α induces IL-10 production by monocytes

Wanidworanun and Strober have shown that TNF-α is the predominant inducer of IL-10 expression by human monocytes (Wanidworanun and Strober, 1993). In order to evaluate factors affecting IL-10 production by monocytes, purified monocytes isolated by anti-CD14 coated immunobeads were cultured under varying conditions for 24 hrs to quantify IL-10 mRNA by semiquantitative RT-PCR. The supernatants harvested after 48 hrs of culture were assayed for IL-10 production by ELISA. Unstimulated monocytes expressed marginal levels of IL-10 as determined by both RT-PCR analysis [ratio of IL-10/β-actin densitometric units (D.U.), 1.45] and ELISA (15-20 pg/ml). IL-10 was measured after culturing monocytes with various cytokines. IL-1α, IL-4, IL-6, IL-12, GM-CSF and M-CSF did not induce IL-10 production either by unstimulated or LPS stimulated monocytes (no detectable amounts of IL-10 by ELISA). However, TNF-α induced IL-10 production as determined by ELISA (Table

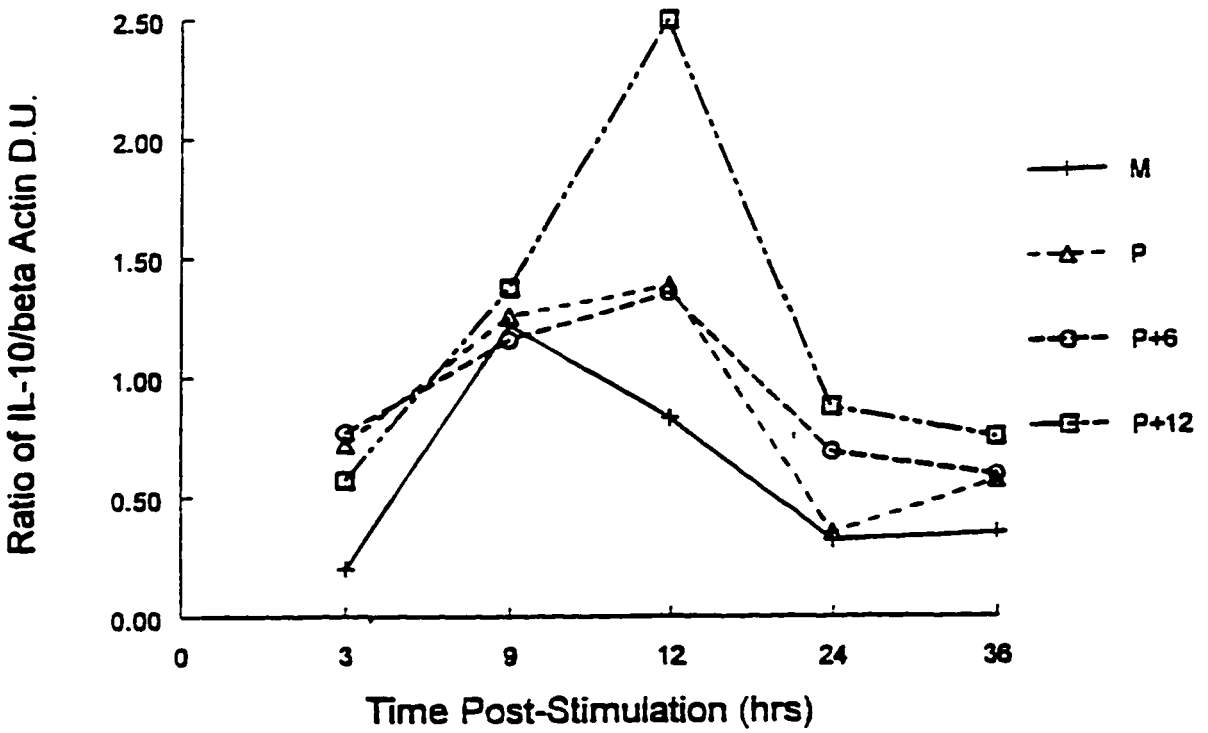

Ratio of IL-10/beta Actin D.U.
2.50
2.00
1.50
1.00
0.50
0.00
0
3
9
12
24
36
Time Post-Stimulation (hrs)
M
P
P+6
P+12

Figure 20. Kinetics of IL-10 production by PHA-activated T cells following stimulation with IL-6 and IL-12 by semiquantitative RT-PCR analysis. Purified $CD4^+$ T cells and $CD8^+$ T cells were mixed in order to obtain sufficient numbers of cells from the same individual to perform both RT-PCR analysis and ELISA for IL-10. T cells were stimulated with PHA in the presence or absence of IL-6 (50 ng/ml) and IL-12 (200 units/ml; 170 units = 1 ng/ml). The cells and supernatants were harvested at different time points (3, 6, 12, 24 and 36 hours) for analysis of IL-10 by RT-PCR and ELISA respectively. The semiquantitative RT-PCR analysis was performed as described in Materials and methods. This figure shows results from one representative experiment of two performed. M, medium; P, PHA; P+6, PHA + IL-6; P+12, PHA + IL-12.

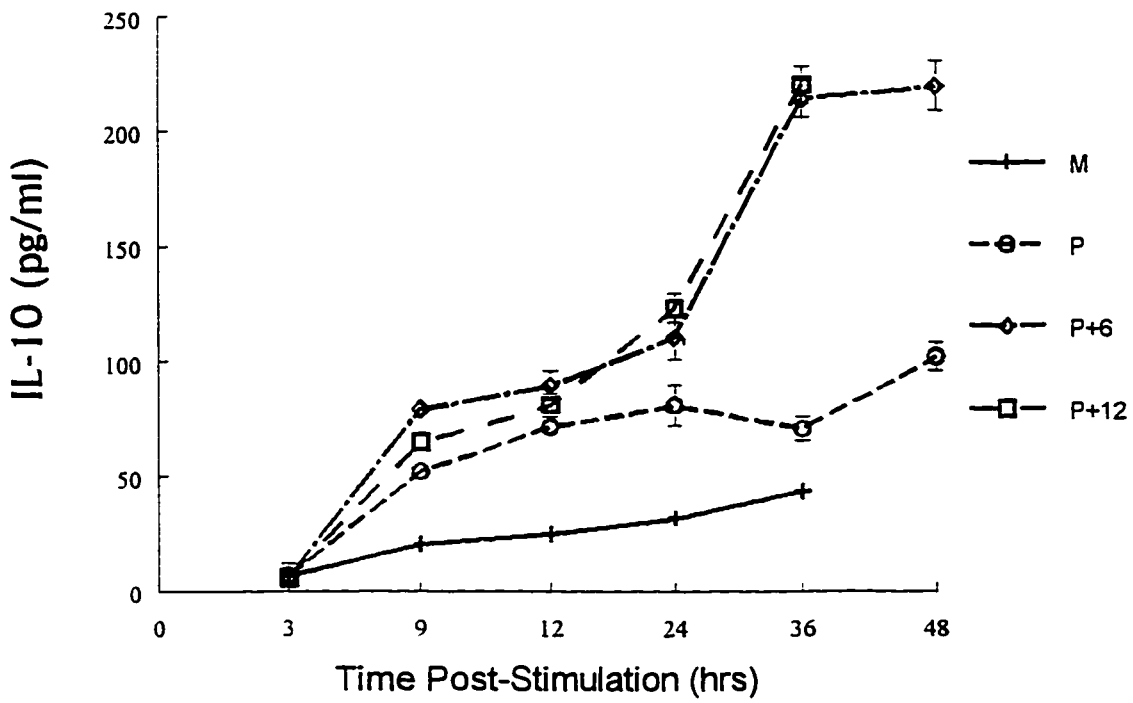

250
200
150
100
50
0
IL-10 (pg/ml)
0
3
9
12
24
36
48
Time Post-Stimulation (hrs)
M
P
P+6
P+12

Figure 21. Kinetics of IL-10 production by PHA activated T cells following stimulation with IL-6 and IL-12 by ELISA. Purified $CD4^+$ T cells and $CD8^+$ T cells were mixed in order to obtain sufficient numbers of cells from the same individual to perform both RT-PCR analysis and ELISA for IL-10, as it was described in the Fig. 20. This figure shows results from one representative experiment of two performed. M, medium; P, PHA; P+6, PHA + IL-6; P+12, PHA + IL-12.

9). Similar results were obtained by semiquantitative RT-PCR analysis (ratio of IL-10/β-actin D.U. 10.73 and 17.75 following stimulation of monocytes with 10 and 20 units/ml of TNF-α, respectively). Stimulation of monocytes with LPS enhanced IL-10 expression and this level was further enhanced by culturing monocytes with TNF-α (Table 9).

16. Preliminarily studies on the effect of IL-12 on IL-10 production by PBMC from HIV^+ persons

I have shown that IL-12 enhances IL-10 production by T cells from HIV^- persons. In the next series of experiments, the changes induced in IL-10 production by T cells from HIV^+ individuals were evaluated. PBMC from HIV^+ subjects were stimulated with recall antigens or PHA in the presence or absence of IL-12. IL-10 was measured 2 and 7 days after addition of IL-12 to whole PBMC and to the monocyte-depleted PBMC from HIV^+ individuals and the IL-10 levels were compared with IL-12 untreated PBMC. PBMC from twenty seven HIV^+ and five HIV^- individuals were studied for the *in vitro* effect of IL-12 on IL-10 production. PBMC were cocultured with recall antigens or PHA with or without IL-12. Since IL-10 production (in monocytes) is influenced by TNF-α, in order to be able to relate IL-10 production to IL-12, therefore, TNF-α also needed to be measured. IL-10 and TNF-α were measured after 48 hours (PHA) and 7 days (recall antigens).

IL-12 was added to PBMC from HIV^+ and HIV^- individuals and IL-10 and TNF-α were measured at different time points. Upon IL-12 treatment, IL-10 was increased in 16 out

Table 9. Effect of tumor necrosis factor (TNF)-α on IL-10 production by resting and lipopolysaccharide stimulated monocytes.

Stimulant	IL-10 Production (pg/ml)		
	Exp-1	Exp-2	Exp. 3
Medium	15.5 ± 3.1	17 ± 2.18	19 ± 3.25
Medium + TNF-a	143.1 ± 16.19	110 ± 15.65	74 ± 10.25
LPS	890.6 ± 62.34		
LPS + TNF-a	1462 ± 77.25		
IL-1α	17.1 ± 5.5		
IL-6	17.0 ± 2.8		
IL-12	16.8 ± 3.33		
IL-4	15.8 ± 5.2		
GM-CSF	18.11± 2.6		
M-CSF	17.0 ± 3.7		

Monocytes isolated by positive selection using anti-CD14 conjugated immunobeads were cultured with either TNF-α (100 units/ml), LPS (1 μg/ml) or LPS (1 μg/ml) in the presence of TNF-α (100 units/ml) for 48 hours. The supernatants were analyzed for IL-10 production by ELISA. Monocytes stimulated with IL-6 (50 ng/ml), IL-12 (200 U/ml), IL-1α (4 ng/ml), IL-4 (2ng/ml), GM-CSF (5ng/ml), and M-CSF (10ng/ml) produced levels of IL-10 similar to unstimulated monocytes. Results are expressed as mean ± SEM of IL-10 production in triplicate wells.

of 27 HIV-infected patients. IL-12 was most effective in the IL-10 induction when it was used with PHA. TNF-α was elevated in PBMC from all (27/27) HIV^+ individuals as well as all HIV^- controls (5/5). When IL-12 was used with PHA, IL-10 production reached its peak after 48 hours. TNF-α was enhanced in the absence of PHA and reached its peak in 48 hours.

Preliminary results showed that in 8 out of 12 HIV^+ patients (regardless of status of IL-10 production) IL-12 addition could increase IL-10 expression (Table 10). Whether there is a correlation between the ability of PBMC and in particular T cells to produce IL-10 and the HIV disease progression remains to be revealed.

Table 10. Effect of IL-12 on IL-10 production of PBMC from HIV^+ individuals.

	Media	IL-12	PHA	PHA+IL-12
HIV^+ (n = 8)	52±8	81±14	121±25	215±22

Effect of IL-12 on IL-10 production of PBMC from HIV^+ individuals. PBMC from HIV^+ (n = 8) individuals were cultured in the presence of media alone, PHA (1:50), and PHA plus IL-12 (170 IU/ml). IL-10 was measured by ELISA after 48 hours. Results are shown as means ± SEM.

Chapter IV. Discussion

IV. 1. Analysis of Th1 and Th2 cytokines in HIV infection

HIV infection of $CD4^+$ cells and monocytes initiates a series of complex events in the immune system of the host that ultimately results in depletion of $CD4^+$ T cells, infection with opportunistic infections and AIDS. Dysregulation of production of cytokines may contribute to HIV immunopathogenesis. It also has been suggested that progression to AIDS is associated with an alteration in T helper cytokines, from Th1 to Th2 which may be responsible in part for undermining the effectiveness of the protective host immune responses. The first objective of this study, hence, was to evaluate Th1 and Th2 type cytokine production by unstimulated resting PBMC and mitogen-activated PBMC of HIV^- individuals.

My results show that unstimulated PBMC from HIV^+ patients, particularly those with <400 $CD4^+$ T cells/mm^3, expressed significantly higher levels of IL-10 than did PBMC from HIV- controls. These results also show that IFN-γ expression is reduced significantly in unstimulated PBMC from HIV^+ patients with <400 $CD4^+$ T cells/mm^3. Since the levels of IFN-γ in the supernatants of unstimulated PBMC were not detectable by ELISA, RT-PCR was employed for detection of IFN-γ mRNA. This is the first report showing enhanced expression of IL-10 and downregulation of IFN-γ in unstimulated PBMC from HIV^+ individuals. In this study, however, the levels of expression of other cytokines was not measured in unstimulated PBMC. RT-PCR has some limitations, these

results, however, are consistant in 30 HIV patients and 16 HIV^- controls. Predominance of IL-10 and reduced IFN-γ, in unstimulated PBMC of HIV^+ individuals, may contribute to unresponsiveness observed in these patients.

Mitogenic stimulation of PBMC from HIV^+ individuals revealed varying patterns of cytokine production. I have observed two groups of HIV^+ individuals based on levels of IL-10 produced by PHA stimulated PBMC. I have shown, decreased production of IL-10 in a subset of HIV^+ patients (low IL-10 producers), whereas, in another subset of HIV^+ individuals its production was found to be comparable with that of HIV^- controls (normal IL-10 producers), although in unstimulated PBMC of HIV patients IL-10 was higher than that of normals. IL-10 production in HIV^+ patients after PHA and anti-CD3 stimulation was not higher than HIV^- controls. These results are in disagreement with an earlier report showing enhanced levels of IL-10 and IL-4 production by PBMC following PHA stimulation (Barcellini et al. 1994; Clerici and Shearer, 1993). These results also do not completely support the hypothesis of a switch from Th1 to Th2 type responses in which IL-10 was predicted to be elevated during disease progression. However, lower levels of IL-4 and IL-10 have also been reported (Graziosi et al. 1994).

Various groups of investigators have reported differing levels of IL-10 production by HIV^+ PBMC following stimulation with PHA (Barcellini, 1994; Emilie, 1994). Different stimulation protocols, stage of disease, concurrent infections, and administration of antiviral medications may have contributed to the observed variability.

The reason for enhanced IL-10 production in unstimulated and low IL-10 production after PHA stimulation (in case of low IL-10 producers) is not clear. HIV

infection may results in a complex dysregulation of immunoregulatory cytokines. It is possible that, after HIV infection, T cells gradually lose the ability to produce IL-10, while IL-10 production by monocytes is increased. Recently, we have shown that in HIV-infected individuals IL-10 production by T cells is defective (Daftarian et al. 1997; Kumar et al. 1997b). We (Kumar et al. 1997; Daftarian et al. 1997) and others (Akridge, 1994; Yoo et al. 1996) have shown that IL-10 production by monocytes in HIV^+ individuals is enhanced. Stimulation with other mitogens PWM (figure 10) and LPS (Kumar et al. 1997b) also resulted in IL-10 production in PBMC of HIV^+ patients comparable to the levels produced in HIV^- controls. The extent of defect in monocytes may determine their response to PHA for IL-10 production. Such defect may be reflected in an altered production of cytokines in HIV infection. The mechanism by which IL-10 production following HIV infection is differentially regulated in T cells and monocytes may be attributed to the altered production of immunoregulatory cytokines. We and others have shown that in HIV-infection, IL-12 induces IL-10 production by T cells whereas TNF-α induces IL-10 production by monocytes (Gerosa et al. 1996; Daftarian et al. 1996a; Daftarian et al. 1996b). IL-12 synthesis is downregulated (Chehimi et al. 1994; Daftarian et al. 1995; Than et al. 1997; Harrison et al. 1997) and TNF-α production is enhanced in HIVinfection (Navikas et al. 1995; Fauci 1996), that may acount for impaired IL-10 production by T cells and the ability of monocytes to synthesize IL-10. Alternatively, HIV antigens tat, nef, or gp120 may regulate IL-10 production differentially in T cells and monocytes.

IL-4 production was comparable to controls in HIV^+ individuals >400 $CD4^+$ T cells/mm^3, but was decreased in HIV^+ individuals with a $CD4^+$ count of <400 cells/mm^3.

Lower levels of IL-4 were detected at 24, 48 and 72 hours after mitogenic stimulation, thus arguing against the possibility of differential kinetics of cytokine production. This may be due to decreased numbers of $CD4^+$ cells rather than a dysregulation. Alternatively, lower levels of IL-4 in HIV^+ individuals with <400 $CD4^+$ T cells may be due to reduced number of Th2 cells, as Th2/Th0 cells have been shown to be preferentially susceptible to HIV infection and apoptosis and cell death (Del Prete et al. 1995; Clerici and Shearer, 1994). Nevertheless, IL-4 in HIV^+ patients was not higher than HIV^- controls, another finding that was not in agreement a switch from Th1 to Th2 in HIV disease. These results are in agreement with with low levels of IL-4 in HIV-infected individuals shown by Graziosi et al. (Graziosi et al. 1994).

On the other hand, IL-12 production following PMA stimulation of PBMC was found to be significantly decreased. In this study, I have measured p40 production by ELISA. It has recently been shown that, in order to evaluate production of active form of IL-12, both p70 and p40 have to be measured. High levels of p40 may inhibit IL-12 production so a high p40 needs to be compared with p70. However, my results show dramatic decrease in p40 in HIV^+ patients. Defective production of IL-12 has been repeatedly reported by others as well (Harrison and Levitz 1997). It is generally believed that IL-2 production is reduced in HIV infected individuals even before the depletion of $CD4^+$ T cell is observed. Despite impaired production of IL-2 and IL-12 in HIV infection, the ability of total or positively selected $CD4^+$ PBMC to produce IFN-γ was not impaired. Normal levels of IFN-γ was produced after PHA stimulation of PBMC and $CD4^+$ T cells from HIV^+ individuals. This observation is not consistant with the original hypothesis of a

switch from Th1 to Th2, in which IFN-γ would be decreased during disease progression. However, taken together, these observations also suggest that Th1 cytokine production, with an exception of IFN-γ, is generally decreased.

The effect of HIV infection on IFN-γ production is not clear. Both low and high levels of IFN-γ production by PBMC from HIV^+ individuals have been reported (Meyaard et al. 1994; McGowan et al. 1994; Maggi et al. 1994; Romagnani et al. 1994). Here, it has been shown that IFN-γ production following mitogenic stimulation of PBMC was comparable in total PBMC as well as in positively selected $CD4^+$ cells of HIV^+ and normal individuals. The kinetics of IFN-γ production by PHA stimulated PBMC, however, revealed differences between normal and HIV^+ individuals. Peak levels of IFN-γ were detected on day 2 by PBMC from normal controls as compared to day 3 in PBMC from HIV^+ individuals. High levels of IFN-γ in serum and in the supernatants of PBMC of HIV^+ individuals have been reported (Emilie et al. 1994; Navikas et al. 1994). It has been suggested that these high levels of IFN-γ are produced by $CD8^+$ T cells (Emilie et al. 1994). To determine the ability of $CD4^+$ cells to produce IFN-γ, I have analyzed IFN-γ production following stimulation of positively selected $CD4^+$ cells, which includes $CD4^+$ T cells and monocytes. IFN-γ production by $CD4^+$ cells of HIV^+ and normal individuals was comparable, which again suggested that ability to produce IFN-γ was not impaired in $CD4^+$ T cells of HIV^+ individuals.

The disease status of the two groups of HIV^+ individuals with respect to $CD4^+$ T cell number and stability of IL-10 phenotype (low and normal IL-10 producers) is not clear. The present study does not address the issue of effect of virus load in low and

normal IL-10 producers. There was a trend of higher $CD4^+$ T-cell counts in low IL-10 producers than in normal IL-10 producers (305 ± 273 per mm^3 versus 217 ± 101 per mm^3; $p = 0.045$) (Daftarian et al. 1995a). Association of $CD4^+$ T cell number with IL-10 levels needs confirmation in larger populations and in different stages of the disease. The significance of this observation is not clear, but since low IL-10 producers showed proliferative responses to FLU and HIV p25 antigens while normal IL-10 producers did not show such responses, these results suggest an association of IL-10 production with unresponsiveness to recall antigens as was shown by others (Sher et al. 1992, Clereci et al. 1993). Studies are in progress to investigate such an association, including the significance of IL-10 production with respect to clinical manifestations.

The question of which cell types produce IL-10 in HIV infection was further investigated. Human IL-10 is produced by monocytes, EBV-transformed B cells and both Th1 and Th2 type cells (Banchereau, 1995). Among T cells, IL-10 is primarily produced by the Th2 subset (Banchereau, 1995; Sosroseno et al. 1994). I have studied the cell type producing IL-10 by PHA-stimulated-PBMC-depleted of T cells or monocytes. IL-10 PHA stimulated HIV^+ PBMC, upon T cell depletion, IL-10 production was unchanged; while following T cell depletion IL-10 production was reduced in PBMC from HIV^- healthy adults. On the other hand, monocyte depletion could abrogate IL-10 production in PBMC from both HIV^+ and HIV^- individuals. These results suggest that in HIV infection, IL-10 is produced by non-T, non-B cells. Thus, in HIV^+ PBMC, IL-10 is mainly produced by monocytes, in contrast to HIV^- PBMC, in which IL-10 is produced by both T cells and monocytes. When stimulated with LPS and PWM, PBMC from HIV^+ individuals produce

IL-10 levels similar to those of normal controls, in addition T cells in these patients are defective in IL-10 production, suggesting that IL-10 production in monocytes is enhanced in these patients. In fact, elevation of IL-10 upon infection of monocytes has been well documented (Akridge, 1994; Daftarian et al. 1997; Kumar et al. 1997). Enhanced IL-10 and decreased IL-12 in monocytes of HIV^+ individuals may be a reflection of a defect in monocytes in HIV disease.

Unfractionated PBMC which contain other cells in addition to $CD4^+$ Th1/Th2 cells, may not be the ideal source for analysis of Th1/Th2 cytokines. However, the cytokine profile of mitogen stimulated PBMC indicates the preferential activation of Th1/Th2 type cells that may be helpful in determining the prognosis of disease. The question of Th1/Th2 type cytokines in HIV infections has recently been addressed by several investigators (Romagnani et al. 1994a; Clerici and Shearer, 1994a; Del Prete et al. 1995a; Maggi et al. 1994a; Romagnani et al. 1994a; Saville et al. 1994a; Romagnani et al. 1994a; Rook et al. 1993a). Results from some other laboratories have not supported the hypothesis of Th2 type cytokine predominance in HIV infections (Romagnani, 1995).

These results suggest that production of Th1 cytokines except IFN-γ is markedly decreased. However, production of IL-4 and IL-10 in HIV^+ individuals with >400 $CD4^+$ T cells remain comparable with that of HIV^- individuals. With respect to the Th1/Th2 hypothesis, at least in patients with >400 $CD4^+$ T cells and upon stimulation, production of IL-4, IFN-γ and overall production of IL-10 are comparable with that of normal controls, while levels of IL-12 are reduced. These results are in agreement with the exhaustive studies using T cell lines, PBMC, or lymph nodes from HIV^+ individuals

reported by Fauci et al and Romagnani et al. in which they have suggested a possible tendency towards what is known as Th0 profile (Romagnani et al. 1994; Romagnani et al. 1995; Fauci et al. 1994; Fauci et al. 1996; Fauci et al. 1997).

IV. 2. The role of IL-10 and IL-12 in HIV immunopathogenesis

The cellular basis for the gradual immunodeficiency in HIV infection is not well understood. The sequential loss of proliferative responses to recall antigens, alloantigens and lectins observed with disease progression may be attributed to perturbation of cytokine cross-regulation (Romagnani, 1995; Fishman and Perelson, 1994; Clerici and Shearer, 1993; Sher et al. 1992; Clerici et al. 1989). Various observations suggest that dysfunction of T helper responses may occur with disease progression. Immunosuppression induced by HIV proteins such as gp120, nef and tat proteins has been attributed to interference with signal transduction and cytokine production (Sharma et al. 1995; Banchereau, 1995). Non-responsiveness to antigens in the settings of HIV infection may result from impaired IL-2 production and has shown to be reversed by its exogenous administration (Romagnani, 1995; Fishman and Perelson, 1994; Emilie et al. 1994; Clerici and Shearer, 1993; Sher et al. 1992). Selective loss of IL-2 production is further correlated with the loss of IFN-γ production and a corresponding increase in IL-4 production (Del Prete et al. 1995; Clerici and Shearer, 1994; Romagnani et al. 1994; Emilie et al. 1994) suggesting upregulation of Th2 type and downregulation of Th1 type responses. In addition, Th2 type responses such as B cell hyperplasia, enhanced levels of

IgG and IgE in serum and B cell lymphomas are frequently observed in HIV infection (Del Prete et al. 1995).

Immunoregulatory cytokines play an important role in regulation of HIV replication in $CD4^+$ T cells as well as in cells of monocyte/macrophage system (Weissman et al. 1995b; Gruber et al. 1995b; Foli et al. 1995b; Emilie et al. 1994b). HIV induced cytokines such as TNF-α, IL-6 and GM-CSF trigger HIV replication whereas transforming growth factor-β, IFN-α and IFN-β have been shown to suppress virus replication (Molina et al. 1990; Than et al. 1994; Barcellini et al. 1994; Romagnani et al. 1994a; Gruber et al. 1995; Takeshita et al. 1995). IFN-γ exerts dichotomous effects on HIV replication depending on experimental conditions. It directly enhanced viral replication in the U1 promonocytic cell line but prevented replication following stimulation of cells with PMA (Emilie et al. 1994). Spontaneous production of IL-10 by unstimulated PBMC from HIV^+ individuals may also contribute to the defective antigen/recall response in HIV infection due to its downregulatory effects on IFN-γ, IL-2 and IL-12 production, major histocompatibility class II molecule and B7-1 expression and antigen presentation (Go et al. 1990; Dallman et al. 1993; Chehimi et al. 1994; Doherty, 1995).

Since IL-10 and IL-12 mediate humoral immune responses and CMIR, respectively, HIV infection may disrupt the balance between IL-10 and IL-12 production resulting in loss of immune competence. Therefore, as a second objective, the role of IL-10 and IL-12 in responses to recall antigens was studied.. I analysed IL-10 and IL-12 production by mitogen-stimulated PBMC from HIV^+ individuals and correlated their levels with proliferative responses to the recall antigens. Low IL-10 producers had PBMC that

proliferated in response to recall antigens, and demonstrated enhanced recall antigen-induced proliferation upon addition of anti-IL-10 antibodies and/or IL-12. IL-10 producers, conversely, had PBMC that failed to proliferate to recall antigens, and did not demonstrate increased proliferation following addition of anti-IL-10 and/or IL-12. Similar results were later reported by others (Landay et al. 1996).

IL-10 has been reported to be upregulated in B cell lymphomas from HIV^+ patients, but was attributed to EBV transformation (Banchereau, 1995; Sosroseno et al. 1994). The patients in the present study exhibited neither B cell lymphomas nor infectious mononucleosis. However, the possibility that IL-10 expression could be due to the presence of opportunistic infections can not be ruled out. It has been shown that patients with high levels of EBV replication had an increased risk of rapid HIV disease progression (Diaz-Mitoma et al. 1990). It is not known whether there is a direct correlation between high EBV load and IL-10 expression in HIV infection, but overexpression of IL-10 in this setting may contribute to the observed B cell hyperplasia and hypergammaglobulinemia owing to its activity as a B cell growth factor (Go et al. 1990; Benjamin et al. 1992; Emilie et al. 1992; Benjamin et al. 1994).

These results show that monocytes/macrophages from HIV infected individuals are the predominant source of IL-10. There is evidence to suggest loss of production of IL-2, a Th1 type cytokine, by PBMC of HIV^+ individuals (Fishman and Perelson, 1994; Romagnani et al. 1994) which may contribute to the development of immune unresponsiveness. This may eventually result in the loss of innate immunity and cellular

immune responses. Spontaneous production of IL-10 in HIV^+ raises the question of the effect of IL-10 on *in vivo* HIV immunopathogenesis as well as replication and virus load.

Generation and maintenance of a type 1 response might be of great importance in control or protection of certain infectious diseases such as HIV infection. IL-12 has been shown to stimulate differentiation of uncommitted (Th0) cells into Th1 cells (Romagnani, 1995). These cells secrete IL-2 and IFN-γ and thereby promote generation of type 1 response while inhibiting type 2 response (Romagnani, 1995; Hyjek et al. 1995; Paganin et al. 1995; Trinchieri et al. 1992). In this study I have demonstrated an association of abnormal patterns of IL-10 and IL-12 secretion by HIV^+ PBMC with alterations in proliferative responses to recall antigens. PBMC from HIV^+ individuals producing low levels of IL-10 proliferated in response to HIV p25 antigen, and their proliferation could be enhanced by the addition of anti-IL-10 antibodies and/or IL-12. Conversely, PBMC from HIV^+ IL-10 producers failed to proliferate in response to HIV p25. These results are in agreement with an earlier report suggesting IL-10 production in sera and by PHA-stimulated HIV^+ PBMC is correlated with disease progression (Ameglio et al. 1994). Since a sequential loss of responsiveness to recall antigens, alloantigens and mitogens has also been correlated with disease progression (Clerici et al. 1989), low IL-10 producing individuals in our study may be in an early stage of HIV infection and may progress to a more advanced stage of disease characterized by higher IL-10 production and unresponsiveness to recall antigens. The present cross-sectional study of asymptomatic HIV^+ individuals does not address the question of stability of IL-10 producing phenotype

over time, or its association with clinical or virological markers of disease progression. Longitudinal studies are currently underway to address these issues.

IL-12 is a potent inducer of Th1 type responses which eventually may induce CMIR in both human and mice (Doherty, 1995). IL-12 might be a potential candidate for generation of protective responses in any disease in which a strong cellular response is desired. Stimulation of HIV^+ PBMC with either PWM, PHA or anti-CD3 antibodies did not induce IL-12 production above that produced by unstimulated PBMC. These results are in agreement with other observation of impaired IL-12 production by HIV^+ PBMC (Chehimi et al. 1994). My results do not distinguish between a direct inhibitory effect of HIV on IL-12 production and an indirect effect through counter-regulatory cytokines such as IL-10 and TGF-β (Germann and Rude, 1995). Addition of exogenous IL-10 *in vitro* inhibited IL-12 production by PBMC indicating a possible role for IL-10 in altered IL-12 production, at least in HIV^+ IL-10 producers.

The mechanism through which HIV^+ IL-10 producers lose their ability to respond to recall antigens, even in the presence of anti-IL-10 antibodies, is unclear. The relevant T cell subsets in this group of individuals may have become tolerized through long-term exposure to IL-10. I have shown a high spontaneous production of IL-10 by unstimulated HIV^+ PBMC (Diaz-Mitoma et al. 1995). IL-10 has been shown to induce tolerance through inhibition of Th1 type cytokines (Fishman and Perelson, 1994; Dallman et al. 1993), possibly through altered expression of co-stimulatory molecules such as MHC class II molecules and/or B7/BB1 (Dallman et al. 1993; Doherty, 1995; Creery et al. 1996). IL-10 has also been shown to induce immune tolerance *in vivo* if administered during the

induction phase of the immune response (Dallman et al. 1993; Boussiotis et al. 1994). The reasons underlying the failure of IL-12 to enhance proliferation in the IL-10 producing subset of HIV^+ individuals is also not clear. Their PBMC may have lost IL-12 receptors or developed defects in IL-12 receptor-induced cytokines or signalling molecules. It is possible that the differences seen among IL-10 producers and low IL-10 producers in their responses to recall antigens might be a reflection of $CD4^+$ numbers. However, it is unlikely since neutralizing antibodies against IL-10 increases these responses for IL-10 low producers. Lack of such improvement among IL-10 producers upon treatment with neutralizing antibodies against IL-10, may be due to the fact that T cells in these patients are in a state of anergy or unresponsiveness.

A number of mechanisms may be operative in the loss of IL-10 production by the low IL-10 producing subset of patients. IL-10 producing Th2 type cells that support viral replication (Del Prete et al. 1995; Clerici and Shearer, 1994) may have been preferentially lost. Alternatively, HIV^+ individuals may exhibit a deficiency of IL-10 inductive cytokines. IL-10 production in monocytes is regulated by TNF-α (Wanidworanun and Strober, 1993), whereas little is known about the regulation of IL-10 production by T cells. Altered production of TNF-α, IL-1 or other cytokines may thus have affected the levels of IL-10 production by HIV^+ PBMC.

Cytokines secreted by monocytes/macrophages following exposure to antigens/pathogens may play a vital role in driving Th0 cells to develop into cells with distinct Th1 and Th2 phenotypes (Romagnani, 1995; Germann and Rude, 1995). IL-12 and IFN-α have been shown to induce the development of Th1-type cells, while IL-1 and

IL-10 induce the development of Th2-type cells (Romagnani, 1995; Germann and Rude, 1995). I and others have shown the loss of IL-12 and IFN-α production in monocytes from HIV infected patients (Chehimi et al. 1994; Daftarian et al. 1995). My results indicate that in HIV^+ PBMC, IL-10 is mainly produced by monocytes, in contrast to HIV^- PBMC in which IL-10 is produced by both T cells and monocytes. IL-12 production was significantly reduced in both subsets of HIV^+ individuals, irrespective of their IL-10 synthesis which may be through direct or indirect mechanisms (Daftarian et al. 1995; Diaz-Mitoma et al. 1995).

IV. 3. Regulation of IL-10 production in T cells and monocytes

Altered production of IL-10 by T cells and monocytes suggest that they may be regulated by different immunoregulatory cytokines. Little is known about the regulation of IL-10 synthesis in T cells. To better understand abnormal mechanisms of IL-10 regulation in HIV infection individuals, I, next, studied the regulation of IL-10 in T cells from HIV seronegative individuals.

IL-10 occupies a central role in the development of immune responses and inflammatory reactions. It limits the development of Th1 type responses by inhibiting the production of IL-12 and IFN-γ, and also acts as an anti-inflammatory cytokine by inhibiting the production of cytokines such as IL-6 and TNF-α. Furthermore, it also plays an important role in the development of immune unresponsiveness by regulating the expression of co-stimulatory molecules HLA class II molecules, CD-28 and B7. The regulatory cytokines that limit the

development of immune responses and inflammatory reactions by inducing IL-10 production are not known. I found that human IL-10 production is differentially regulated by T cells and cells of monocytes/macrophages lineage (Daftarian et al. 1996). Resting T cells as well as PHA activated T cells are unable to produce detectable levels of IL-10 in the absence of monocytes. IL-6 and IL-12 independently enhance IL-10 production by PHA stimulated $CD4^+$ and $CD8^+$ T cells but have no effect on IL-10 production either by unstimulated or activated monocytes. Conversely, TNF-α enhances IL-10 production by unstimulated as well as activated monocytes but does not affect IL-10 production by $CD4^+$ and $CD8^+$ T cells.

To precisely define the cytokines capable of inducing IL-10 by T cells, I used highly purified populations of $CD4^+$ and $CD8^+$ T cells (>98%). To discount the possibility of cytokines produced by the contaminating monocytes/macrophages, purified $CD4^+$ T cells were treated with L-LME to deplete $CD4^+$ monocytes. However, these experiments do not rule out the possibility of cytokines produced in trace amounts by contaminating non T cells that may act either independently or in concert with other cytokines to influence IL-10 production. It is possible that IL-6 and IL-12 induce IL-10 production by T cells indirectly by affecting other cytokines. However, addition of IL-1 α, IL-1β, IFN-α, TNF-α, IL-4, GM-CSF and M-CSF to either resting T cells or PHA activated $CD4^+$ and $CD8^+$ T cells did not enhance IL-10 production. The above cytokines in different combinations may have an effect on IL-10 expression but it is unlikely as addition of neutralizing anti-IL-6 and anti-IL-12 antibodies to the monocyte conditioned medium abrogated its ability to enhance IL-10 production. Furthermore, induction of IL-10 in activated T cells by IL-12 was determined by two methods, highly sensitive semiquantitative RT-PCR analysis and ELISA which were consistent to each

other. Discrepancies in the induction of IL-10 expression in both $CD4^+$ and $CD8^+$ T cells by IL-6 as determined by RT-PCR and ELISA may be attributed to the differences in the kinetics of IL-10 expression as the cells and supernatants were harvested at different times following stimulation.

The molecular mechanisms by which IL-6 and IL-12 induce upregulation of IL-10 production in $CD4^+$ and $CD8^+$ T cells but not in monocytes/macrophages are not clear. This may be attributed to the relative lack of functional high affinity IL-6 and IL-12 receptors on cells of monocyte lineage. IL-6 and IL-12 are produced by activated monocytes/macrophages but mediate their effects on T cells, and indirectly on monocytes/macrophages via IFN-γ. The possibility that IL-6 and IL-12 may induce IL-10 production by interacting with a common IL-6 receptor (IL-6R) cannot be ruled out. The functional IL-12 molecule consists of heterodimeric molecules p35 and p40. IL-12 p40 shares extensive amino-acid homology to the human IL-6R and IL-6 has homology to the p35 chain of IL-12 heterodimer. The p35-p40 complex of IL-12 may thus act in a fashion akin to IL-6/soluble IL-6R and may induce signals via the gp130 receptor subunit of the IL-6 receptor complex. Furthermore, cellular IL-12R might be functionally equivalent to gp130 of the IL-6 receptor complex. Although there is no direct evidence to support this hypothesis, the additive effect of IL-6 and IL-12 on IL-10 production suggest a common regulatory pathway.

These results strongly suggest that IL-6 and IL-12 exert their effects on activated T cells and TNF-α on monocytes/macrophages by enhancing IL-10 mRNA transcription and translation. Furthermore, maximal increase in IL-10 production was observed only after 48 hrs of stimulation. The mechanism by which IL-6 and IL-12 enhance RNA transcription in T cells

and TNF-α in monocytes is not understood. The IL-10 gene in man and mouse is preceded by 5' upstream regulatory sequences that contain possible AP-1 and NF-kB binding sites. Whether IL-6 and IL-12 enhance IL-10 transcription via binding to these regulatory DNA binding sequences is not known. Molecular studies in this regard are needed to answer this question. The fact that anti-TNF-α neutralizing antibodies did not completely inhibit IL-10 production in monocytes suggest that, perhaps, in addition to induction of TNF-α, LPS induces IL-10 expression by monocytes by unknown cytokine(s) or a combination of cytokines.

These results on the induction of IL-10 by IL-12 are surprising in view of recent reports which suggested that IL-12 inhibits production of Th2 type cytokines such as IL-4, IL-5 and IL-10. In infections characterized by protective Th2 type cytokines responses, administration of IL-12 downregulated IL-4 producing Th2 type cells. *In vivo,* administration of IL-12 in mice infected with *Schistisoma mansoni,* inhibited IL-10 secretion in addition to IL-4 and IL-5. Conversely, in infections characterized by protective Th1 type responses, administration of IL-12 resulted in expansion of IFN-γ producing Th1 type T cells. Thus, one could expect that addition of IL-12 to the PHA activated T cells could result in downregulation of IL-10 production. However, addition of IL-12 to *in vitro* cultures of PHA activated purified $CD4^+$ and $CD8^+$ T cells enhanced IL-10 production. Furthermore, these results also suggest that IL-10 upregulation may be attributed to the direct effect of IL-12 on activated T cells. This finding is also in agreement with the observations made *in vivo* in which administration of IL-12 in normal mice and in mice treated with goat anti-mouse IgD (a common stimuli) antibodies enhanced the production of IFN-γ and IL-10 but suppressed IL-3 and IL-4 gene

expression. The reasons for the variable effect of IL-12 on IL-10 production under *in vivo* conditions are not clear but may depend upon the state of T cell activation and/or immunizing agents.

IL-12 is a required cytokine for the Th1 developmental pathway and is produced as one of the first host responses to infection. IL-12 production by monocytes/macrophages has been shown to be mediated by the endogenously produced TNF-α which is produced very early following activation (Germann and Rude, 1995). TNF-α also induces IL-10 production by resting and activated monocytes/macrophages as shown by others and I (Germann and Rude, 1995; Daftarian et al, 1996). Activated monocytes/macrophages also constitute the principal source of IL-6 that is a potent regulator of T cell activation, proliferation, differentiation and B cell differentiation and antibody production (Akira et al. 1993). Taken together, these studies suggest that activated monocytes/macrophages produce IL-6 and IL-12 that induce IL-10 production by T cells, and TNF-α that enhance IL-10 production by monocytes/macrophages. IL-10, in turn, has been shown to negatively regulate IL-6, IL-12, TNF-α and its own production (de Waal Malefyt et al. 1991; Germann et al. 1995). Thus, activated monocytes/macrophages may produce a cascade of immunoregulatory cytokines such as IL-1, IL-6, IL-12, TNF-α and IL-10 which seem to act in an autoregulatory fashion in the development of an appropriate immune response. Dysregulation of any of these cytokines may skew the immune response towards either cell mediated or humoral responses. Understanding the molecular mechanisms of regulation of these cytokines may help in the induction of desired cytokine profile in response to antigens/pathogens. This approach may be

of potential therapeutic significance in the design of vaccines or immune reconstitution in immunosuppressive states.

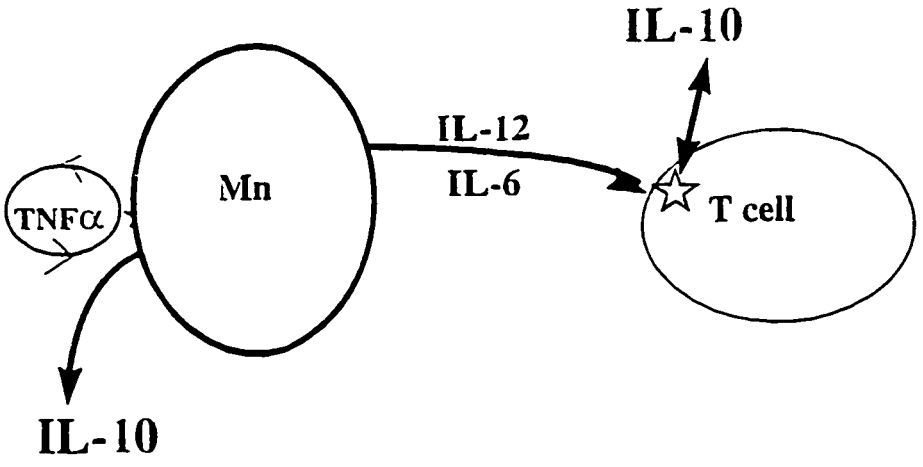
V. Conclusion

The role of T helper subsets and cytokine patterns in HIV infection has recently been the focus of various researchers. Different groups of investigators have suggested that progression to AIDS is associated with an alteration in T helper cell phenotype, from a Th1 to Th2, the first hypothesis of my Ph.D thesis. I have analyzed the expression of Th1 (IFN-γ and IL-12) and Th2 (IL-4 and IL-10) cytokines by PBMC from HIV^+ and normal controls. In mitogen stimulated PBMC, my results suggest that production of IL-12 and not that of IFN-γ is markedly decreased. However, production of IL-4 and IL-10 in HIV^+ individuals with >400 $CD4^+$ T cells remains comparable with that of HIV^- individuals. IL-2, another major Th1 cytokine, has been shown to be impaired in HIV-infected individuals by a number of investigators. Thus, in patients with >400 $CD4^+$ T cells, overall production of IL-4, IFN-γ, and IL-10 is comparable with that of HIV^- controls, while levels of IL-12 and IL-2 are reduced. These results are in agreement with the exhaustive studies using T cell lines, PBMC, or lymph node cells from HIV^+ individuals as reported by Fauci et al and Romagnani et al. and do not support a switch from Th1 to Th2 phenotype. These authors have suggested a possible tendency towards Th0 profile. In HIV^+ individuals with <400 $CD4^+$ T cell, IL-4 was reduced. IL-4 is produced only by T cells and its defect in patients with $CD4^+$ T cell counts of <400 may be due to the preferential depletion of $CD4^+$ T cells and especially selective depletion of IL-4 producing T cells (Th2 type cells) by HIV.

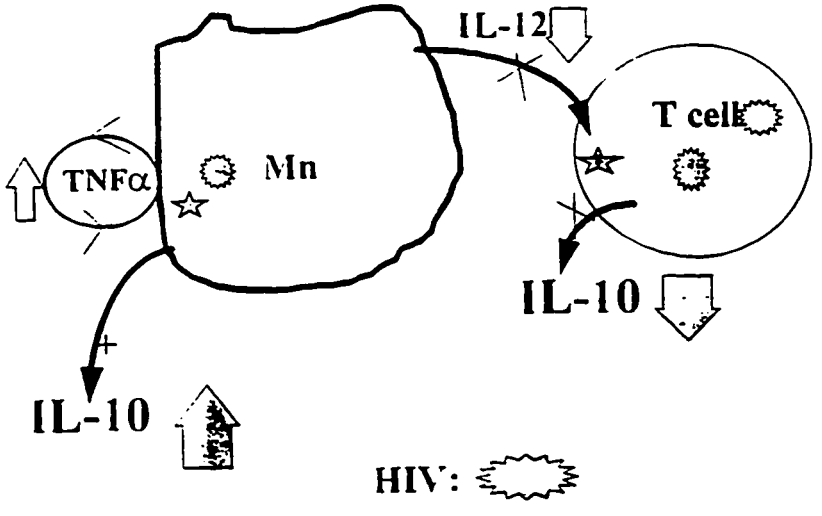
Studying the impact of altered expression of IL-10 on immunoresponsiveness in HIV-infected individuals constituted my second objective. IL-10 has immunosuppressive effects and downregulates Th1 through different mechanism such as inhibition of IL-2 and IL-12 production. My results indicate that in PBMC from HIV^+ individuals, IL-10 is mainly produced by monocytes, in contrast to PBMC from HIV^- individuals in which IL-10 is produced by both T cells and monocytes. Based on the ability of their PBMC to produce IL-10 after PHA stimulation, I stratified HIV-infected individuals into low and normal IL-10 producers. The levels of IL-10 was correlated with proliferative responses to antigens such as p25 and FLU. In these studies, a correlation between low IL-10 production with preserved proliferative responses and IL-10 production with loss of proliferative responses to recall antigens were observed. Later studies revealed that a defect in IL-10 production was observed only in T cells, whereas monocytes retained the ability to produce IL-10. The overall production of IL-10 by PBMC stimulated with PWM and LPS was comparable with that of HIV^- controls. Further experiments confirmed these results in HIV^+ T cell and monocyte subsets at the single cell level by intracellular staining and by flowcytometry analysis (Kumar et al. 1997b; Daftarian et al. 1997).

Finally, I studied the regulation of IL-10 production in T cells and monocytes. The molecular mechanism underlying the production of IL-10 are not clear. IL-10 production by monocytes has been shown to be regulated by TNF-α. In this study, I have shown that monocytes are required for IL-10 production by normal activated T cells. Monokines responsible for IL-10 regulation in T cells were further investigated (Model 1). IL-6 and IL-12

Regulation of IL-10 production in T cell and monocytes



Regulation of IL-10 production in T cell and monocytes; effect of HIV infection



Model 1. The regulation of IL-10 production in T cells and monocytes of HIV^- (top) and HIV^+(bottom) individuals. Mn, monocytes; T, T cells.

independently enhance IL-10 production by PHA stimulated $CD4^+$ and $CD8^+$ T cells but have no effect on IL-10 production either by unstimulated or activated monocytes. TNF-α was shown to enhances IL-10 production by unstimulated as well as activated monocytes but does not affect IL-10 production by $CD4^+$ and $CD8^+$ T cells. The fact that, IL-12 synthesis is downregulated and TNF-α production is enhanced in HIV infection, may account for impaired IL-10 production by T cells and retained IL-10 production in monocytes. I have shown that IL-12 enhances IL-10 production in PBMC from HIV^+ individuals. This finding is consistant with a recent report from Gerosa (Gerosa et al. 1996).

Taken together my results suggest that upon HIV infection T cells gradually lose the ability to produce IL-10 while IL-10 production by monocytes is gradually enhanced (Model 1 top). It is possible that the IL-10 deficit in a subset of patients may be due to decreased IL-12 production by monocytes as addition of IL-12 enhanced IL-10 production in some patients (Model 1 bottom). The ability to produce IL-10 by T cells or monocytes may provide a useful parameter for immune disease progression in HIV infection. High levels of IL-10 produced in monocytes of HIV^+ individuals may be an indicator of disease progression. Further studies are needed to clarify these issues. My observation that IL-12 induces IL-10 in T cells may have applications beyond the scope of this work such as in inflammation and autoimmunity research.

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