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IL-10 Inhibits HIV-1 Replication and Is Induced by Tat

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SUMMARY: Interleukin 10 (IL-10) is produced by T_H2 lymphocytes and regulates both lymphoid and myeloid cells. In the present study we demonstrate that IL-10 is expressed and produced spontaneously in the peripheral blood mononuclear cells (PBMCs) of all HIV-1 infected individuals tested, 3 of 19 cases of HIV-negative lymphoma and none of five healthy controls. IL-10 mRNA was detectable in both monocytes/macrophages and T lymphocytes isolated from PBMCs of HIV infected patients. We have also shown that infection of promonocytic (U937) and T (H9) cell lines with HIV stimulates IL-10 secretion. Furthermore, a T cell line (H9) stably transfected with a HIV tat expression-vector secreted higher levels of IL-10. We have also demonstrated that rhIL-10 inhibited HIV-1 replication in infected monocytes and PBMCs in a dose dependent manner. IL-10 may thus participate in long latency between HIV-1 infection and development of AIDS. © 1994 Academic Press, Inc.

HIV-1 infection is associated with polyclonal hypergammaglobulinemia (1) enhanced spontaneous production of immunoglobulins by B lymphocytes (2), high grade B cell lymphoma, and defective T-cell mediated immune response (3). Peripheral blood mononuclear cells (PBMCs) from HIV-1 infected individuals show both reduced levels of interferon γ (IFN-γ), IL-2 and elevated levels of IL-4 and IL-6, an imbalance favoring enhanced expression of T_H2 type cytokines (4-6). IL-10 is another member of the T_H2 type of cytokines which inhibits T_H1 (IFN-γ) production, inhibits T cell proliferation and induces B cells to secrete high amounts of IgG, IgM, and IgA (7-8).

The mechanisms responsible for conversion for HIV latency to HIV replication are unclear, although cytokine activation signals are implicated in this process. Several cytokines like IL-1, TNFα, TNFβ, IL-6 and granulocyte macrophage colony stimulating factor (GM-

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CSF) have all been shown to induce the expression of HIV-1 in infected T cells and promonocytic cell lines (9-11).

In the present study, we have demonstrated that IL-10 is expressed and produced spontaneously in the PBMCs of all HIV-infected patients at the mRNA level detected by PCR and at the protein level shown by ELISA. Moreover, HIV infected U937 and H9 cell lines secrete higher levels of IL-10. Further rhIL-10 inhibits HIV-1 replication. IL-10 thus may play a role in the long latency between HIV-1 infection and disease manifestation.

MATERIALS AND METHODS

Antibodies: The monoclonal antibodies used for the phenotyping of the monocytes, B cells and T cell preparation from PBMCs were purchased from the following manufacturers, monoclonal antihuman CD3, CD4, CD8, CD19 and CD20 were purchased from Sigma Chemical Company, St. Louis, MO. FITC conjugated anti CD19, CD14, and CD3 were purchased from Ortho Pharmaceutical Diagnostic Systems, Raritan, N.J. Bio- Magnetic Goat Anti-Mouse IgG was purchased by Advanced Magnetics, Inc. Cambridge, MA.
Cell Lines: The H9 cell line stably transfected with the HIV-1 tat gene (H-9 tat) was the kind gift of Dr. George N. Pavlakis (National Cancer Institute, Frederick, M.D.). H-9, H-9 tat and U937 cell lines were maintained in complete medium containing RPMI 1640, 2mM L-glutamine, 100U/ml penicillin, 100 μg/ml streptomycin and 10% heat inactivated fetal calf serum (Gibco BRL, MD). cDNA probe for human IL-10 was provided by Dr. Kevin Moore (DNAX, Palo Alto, CA). Oligonucleotides were synthesized at the Norris Comprehensive Cancer Center Core Laboratories.
Blood Samples: Heparinized peripheral blood was obtained from 39 HIV-1 infected patients, 19 HIV-1 negative malignant lymphoma and 7 HIV-1 negative healthy heterosexual control from the Los Angeles County USC Medical Center Hospital. PBMCs were separated from whole blood within 3-4 hour of blood collection by Ficoll-hypaque (Pharmacia fine chemical Piscataway, NJ) density gradient centrifugation ($d=1.077$ g/cm^3). Interface cells were washed twice with Hanks balanced salt solution (HBSS) and resuspended (10^6 cells/ml) in RPMI 1640. Cell viability as tested by trypan blue dye exclusion was always >95%.
Purifications of Monocytes, T and B lymphocytes: PBMCs were plated at a denisty of 1 x 10^6 cells/ml in RPMI 1640 supplemented with $10^{\%}$ (v/v) heat inactivated fetal calf serum (Gibco) in plastic petri dishes (Falcon, Lincoln Park, NJ) for 1 hr at 37°C. Adherent cell-depleted peripheral blood subpopulation contained <2% monocytes when analyzed by indirect immunofluorescence using the CD14 monoclonal antibody (MoAb). Non-adherent cells were sorted by positive selection using MoAbs bound to magnetic beads (CD2, CD4 and CD8 for T lymphocytes and CD19 for B lymphocytes). Each fraction was over 95% pure as confirmed by indirect immunofluorescence using FITC conjugated anti CD3 for T cells and CD20 for B cells.
RT-PCR for IL-10: Total RNA was extracted from cell pellets by guanidinium thiocyanate (12) (RNAzol,Tel-Test, Inc. TX) cDNA was prepared by reverse transcription of 1.0-2.0 μg total RNA using random hexamer as a primer in a total volume of 20 μl, using the protocol recommended by the manufacturer. (Superscript, Gibco BRL, Gaithersburg, MD). Five μl of cDNA reaction was amplified with 2.5U of Taq Polymerase (Perkin Elmer) and 50 pmol of each of the 5' and 3' primers, dNTP (2.5 mM) and $MgCl_2$ (1.5 mm) in a total volume of 50 μl. The samples were amplified for 40 cycles. Each cycle consisted of denaturing at 94°C for 60 sec, primer annealing at 60°C for 60 sec and extending at 72°C for 2 min. To ensure that specific amplification of hIL-10 was achieved, we used two primers that do not amplify DNA from a plasmid template containing the $BCRF_1$ open reading frame from EBV

which is homologous to hIL-10 (13). Primer 1 consisted of the protein coding region: nucleotides 323 to 349 (sense strand) 5' CTG-AGA-ACC-AAG-ACC-CAG-ACA-TCA-AGG; primer 2 corresponding to nucleotides 648-674 from the 3' untranslated region (antisense strand) 5' CAA-TAA-GGT-TTC-TCA-AGG-GGC-TGG-GTC. Similarly, PCR reactions using RNA as templates in place of cDNA failed to show any product demonstrating the absence of genomic DNA contamination (data not shown). A positive reaction yields 350 base pair amplification product which was visualized on a 1.5% agarose gel and subsequently confirmed with hybridization to an internal oligodeoxynucleotide probe and labeled with [γ-^{32}P] ATP comprised of nucleotides 381 to 422. RT-PCR of housekeeping β-actin mRNA was performed on all samples using the oligonucleotide 5'primer (sense strand) GTG-GGG-CGC-CCC-AGG-CAC-CA and 3'primer (antisense strand) CTC-CTT-AAT-GTC-ACG-CAC-GAT-TTC to demonstrate the integrity of mRNA and cDNA. Samples were amplified for 30 cycles. Amplified product of size 548 base pair was visualized in 1.5% agarose gel.

Short Term Culture of Whole PMBCs: PBMCs were separated from freshly collected heparinized whole blood, by ficoll-hypaque density centrifugation. PMBCs were washed three times in HBSS and cultured in RPMI 1640 supplemented with 2mm glutamine, 10% FCS, 100 U/ml of penicillin and 100 μg/ml of streptomycin. Supernatants were collected after 48 hours, centrifuged to remove cell debris. IL-10 levels were measured using the protocol recommended by the manufacturer (Biosource International, Camarillo, CA).

HIV infected U937 and H9 cell lines: U937 a promonocytic cell line and H9 a T cell line, were maintained in RPMI 1640 containing 10% FCS (heat inactivated), 2mM L-glutamine, penicillin and streptomycin. U937 cells were plated at a density of 1 x 10^6 cells/ml and infected with HIV-$1_{ba\text{-}L}$ with 50 TCID/ml for 24 hours. Controls were treated identically except no HIV-1 was added. The cells were washed to remove free virus and fresh media was added; medium was changed every three to four days. Supernatants from the cell cultures were collected, centrifuged to remove cell debris and stored at -70°c until tested for hIL-10 protein levels and the reverse transcriptase (RT) activity. H9 cells were infected with HIV-1_{111b} as above. Twenty one days after infection equal number of uninfected H9 and infected H9 were plated at a density of 1 x 10^6 cells/ml. Supernatants were collected after 48 hr and analyzed for RT activity (14) and hIL-10 levels.

RT Activity Assay: 5μl of culture supernatants was added to 25$\mu\ell$ of a mixture containing poly (A) oligo (dT) (Pharmacia Fine Chemicals, Piscataway, NJ) $MgCl_2$ and [^{32}p] - labeled deoxythymidine 5'-triphosphate (dTTP) (Amersham, Arlington Heights, IL) and incubated for 2 h at 37°C (14). 10μl of the mixture was spotted onto DE 81 paper, air-dried, washed five times in 2XSSC buffer and two additional times in 95% ethanol. The paper was then dried cut and counted on a scintillation counter. Each experiments were done in duplicate.

Northern Blot: H9 and H9 tat cells were seeded at a density of 1x10^6 cells/ml in 10 ml of culture medium, with PHA (1μg/ml) and PMA (0.1 ng/ml). Cells were harvested after 6 hr and 24 hr (data not shown). Total RNA was extracted by guanidinium thiocyanate (RNAzol, Tel-Test Inc., Friendswood, TX) (12). Twenty μg of total cellular RNA were electrophoresed through a 1% agarose/formaldehyde denaturing gel and transferred to a nylon membrane. The nucleic acid was bound to the membrane by ultraviolet crosslinking (Stratagene, La Jolla, CA). The membrane was prehybridized in 10 ml of Quikhyb solution containing 100 μg salmon sperm DNA (Stratagene) at 68°C for 30 min. The plasmid DNA encoding hIL-10 was digested with BglII and HindIII to generate at 760 bp fragment which was purified and used as a probe. After prehybridization the filter was hybridized with ^{32}P-labeled human IL-10 cDNA for 2 hr at 68°C. The membrane was washed twice for 15 min at room temperature with 2xSSC/0.1% SDS followed by a single wash at 60°C for 30 min with 0.1xSSC/0.1% SDS. After the membrane was exposed overnight to autoradiography film, was stripped of the labeled probe and reprobed with ^{32}P-labeled GAPDH cDNA (15).

Effect of IL-10 on HIV-1 replication: Heparinized peripheral blood was obtained from healthy donors. PBMCs were separated from whole blood by ficoll-hypaque density gradient centrifugation. Monocytes were purified on petri dishes coated with human Ab- serum as

described earlier and seeded in 24 well plates at a density of $0.5x10^6$ cell/well. Twenty-four hours after infection with HIV-1 JR-FL (50 $TCID_{50}$), the cells were washed extensively to remove input virus and were treated with 0, 1, 5, and 10ng/ml of rhIL-10 (R & D system, MN.) PBMCs were stimulated with PHA (1μg/ml), and infected with HIV-1_{IIIb}. Input virus was washed out after 24 hours and cells were treated with 0, 1, 10, 50, 100 ng/ml of rhIL-10. Supernatants were collected on day 5 and P_{24} antigen levels were measured (16). A chronically infected T cell line (ACH-2) was also studied for IL-10 effect on HIV-1 replication. Equal number of cells ($0.5x10^6$) were treated with rhIL-10 at doses of 0, 1, 5, 10, 50 and 100 ng/ml. Supernatants were collected on day 5 and HIV-P_{24} levels were measured.

RESULTS

PBMCs from HIV-1 infected individuals spontaneously expressed hIL-10 mRNA:

Spontaneous expression of IL-10 was examined by RT-PCR analysis of PBMCs from HIV-1 infected individuals (Table 1). RT-PCR analysis was performed using primers specific for hIL-10 which does not amplify transcripts of BCRF-1 (13). For each sample, RT-PCR amplification of β-actin mRNA, a ubiquitously expressed gene, served as a control for the integrity of mRNA. Total PBMCs of all 27 HIV-infected patients analyzed by RT-PCR were positive for IL-10 mRNA expression. In contrast, PBMCs from only three of 19 cases of HIV-1 negative, B cell malignant lymphoma cases and none of five healthy controls spontaneously expressed IL-10 mRNA (Table 1 and Figure 1).

Table 1

IL-10 expression by RT-PCR

	HIV+ (N=39)	HIV- (N=19)	Controls (N=7)
PBMCS	27/27	3/19	0/5
Monocytes	9/10	ND	0/2
T cells	6/12	ND	0/2
B cells	1/12	ND	0/2

IL-10 mRNA expression by RT-PCR was examined on the whole PBMCs or monocytes, B and T cells isolated from PBMCs of HIV-1 infection, and healthy control were also used for IL-10 detection. Not done=ND.

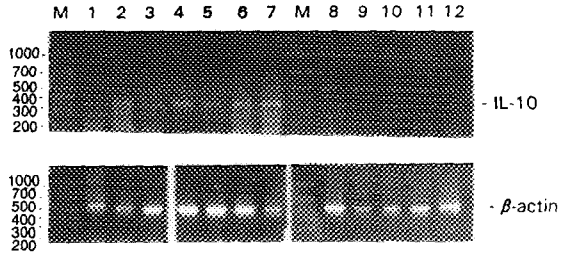

Figure 1. hIL-10 expression by RT-PCR from PBMCs of HIV seropositive cases and healthy donors. PBMCs were isolated from whole blood and total RNA was extracted from freshly isolated PBMCs. 1-2 μg of RNA was used for cDNA synthesis. RT-PCR was performed for hIL-10 and β-actin as described in Materials and Methods. M represents size markers (Research Genetics, AL) lanes 1-7 represent seven HIV-1 infected cases and lanes 8-12 represent five healthy donors.

IL-10 can be induced in T cells, B cells and monocytes (2,3). We thus examined IL-10 expression in monocytes/ macrophages, T cells and B cells, (T and B cells). Cells were allowed to adhere to the plastic for 1 hr. Non-adherent (A-) cell populations were positively selected for T cells (CD_2^+ A^-) or B cells (CD_{20}^+ A^-) (>95% pure population). RT-PCR analysis on PBMCs, purified for each cell type showed IL-10 expression in 9/10 monocytes, 6/12 T cells and 1/12 B cells. In contrast, no expression of IL-10 was detected in either cell population from two healthy seronegative controls. Integrity of mRNA was confirmed by the amplification of β-actin in all samples included in the analyses (Table 1).

Supernatants of PBMCs from HIV-1 infected constitutively secretes hIL-10 protein: We examined IL-10 protein secretion by PBMCs from patients with HIV-1 infection. Freshly collected PBMCs were cultured in RPMI 1640 containing 10% FCS (heat inactivated), without any mitogen for a period of 48 hr. The supernatants were analyzed for IL-10 by ELISA. Elevated levels of IL-10 were observed in 17 of 21 (81%) of HIV-1 infected patients ranging from 17 to >300 pg/ml (figure 2). CD_4 levels or clinical diagnosis did not predict for IL-10 levels. We also examined IL-10 protein secretion by PBMCs from five healthy donors which served as controls. The levels of IL-10 from supernatant was found to be below detectable level in all (Figure 2).

U937 a promonocytic and H9, a T cell lines secrete high amounts of hIL-10 after HIV infection: To examine whether HIV-1 infection induces IL-10 protein, U937 a promonocytic leukemia cell line which does not express IL-10 was infected with HIV-$1_{ba\text{-}L}$ (17) virus and the supernatants were examined for IL-10 secretion every 2 to 4 days for a period of 21 days. IL-10 was not detectable in the supernatants of uninfected cells in any of the samples examined during 21 day period. In contrast, HIV-$1_{ba\text{-}L}$ infected U937 showed a progressive increase of IL-10 levels in the supernatants beginning 6 days post infection: the HIV-1 titer

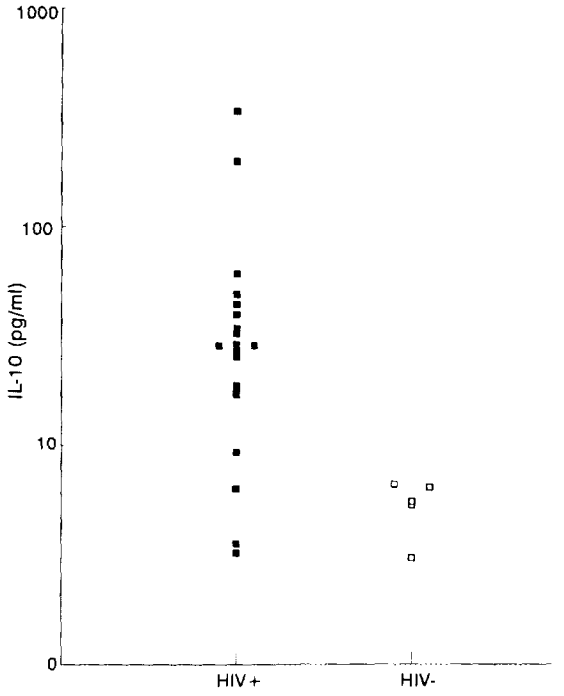

Figure 2. IL-10 secretion by PBMCs from patients with HIV-1 infection and healthy donors. Supernatant collected after 48 hours were tested for IL-10 by ELISA.

represented by RT levels (14), correlated with IL-10 protein level (Figure 3A). We also examined the effect of HIV-1 infection in a chronically infected T-cell line (H9), infected with HIV-1_{IIIb}. H9, a clone of HUT78, which is derived from the peripheral blood of a

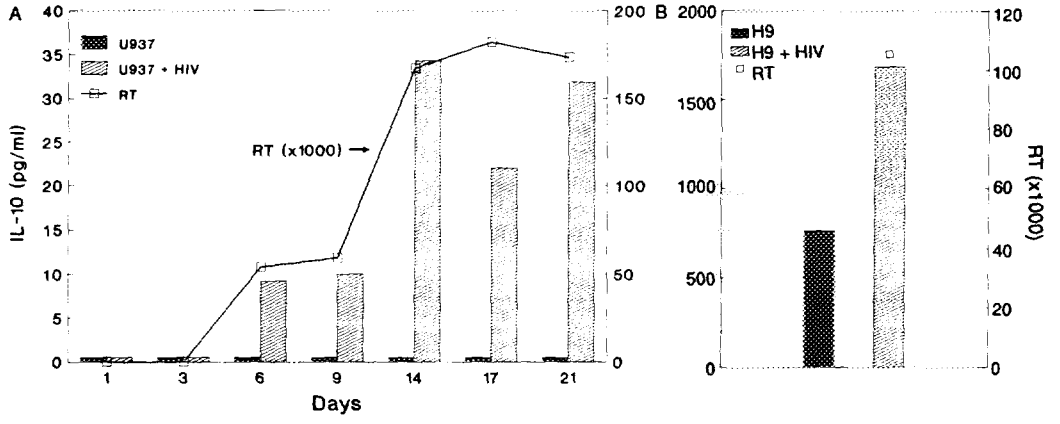

Figure 3. hIL-10 expression in promonocytic (U937) (A) or T cell (H9) (B) lines after HIV-1 infection. U937 and H9 cell lines were uninfected (■) or infected (▨) with HIV-$1_{ba\text{-}L}$ and HIV-I_{IIIb}, respectively, as described in Materials and Methods. The supernatants were collected at various time interval and then tested for IL-10 levels and RT assay (⊟).

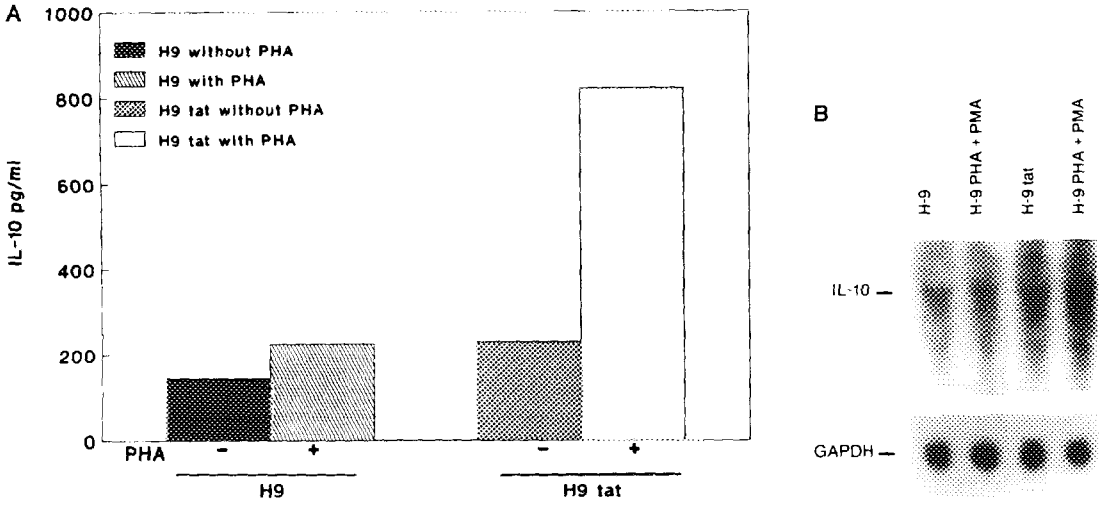

Figure 4. IL-10 expression in H9 and H9 stably transfected with HIV-1 tat gene. Supernatant(A) of H9 and H9 tat with (▨ □) or without PHA (■,▨) were collected after 48 hours and analyzed for hIL-10. The data represents the average from two independent experiments. RNAs (B) were isolated from H9 and H9 tat with or without PHA (1μg/ml) and PMA (0.1ng/ml). The northern blot was probed with ^{32}P labeled IL-10 and GAPDH cDNAs.

patient with mycosis fungoides, (14) constitutively expresses high levels of IL-10. HIV-1 infection leads to an over 2-fold increase in the level of IL-10 in the supernatant (Fig 3B).

H9 stably transfected with HIV-1 tat gene secrete high levels of hIL-10 protein and mRNA as compared to H9 cells: To examine the effect of tat on IL-10 regulation, supernatants from the T cell line H9, and H9 stably expressing tat (H9 tat) were assayed for IL-10 levels. H9 tat cell line expressed nearly twice the level of IL-10 when compared to H9 cells. After Phytohemagglutinin (PHA) stimulation, cells expressing 'tat' exhibited a nearly 4-fold increase in the amount of IL-10 compared to similarly treated cells not expressing HIV-tat (Figure 4A).

H9 and H9 tat cells with or without stimulation by PHA and PMA (phorbol 12 myristate 13 acetate) were also studied for the expression of IL-10 specific mRNA at various time points. IL-10 mRNA basal expression is higher in H9 tat compared to H9 (Figure 4B). Furthermore, IL-10 mRNA is induced after 6 hr with PHA and PMA treatment in H9 tat but no significant effect was observed in H9 cells.

IL-10 inhibits HIV-1 replication: Effect of IL-10 was studied on PBMCs of healthy donor or freshly isolated monocytes infected with HIV-1. In a dose dependent manner IL-10 inhibited HIV-1 replication. Monocytes were more sensitive and showed over 90% inhibition at doses of 10 ng/ml. PBMCs however were less sensitive and showed IC_{50} of

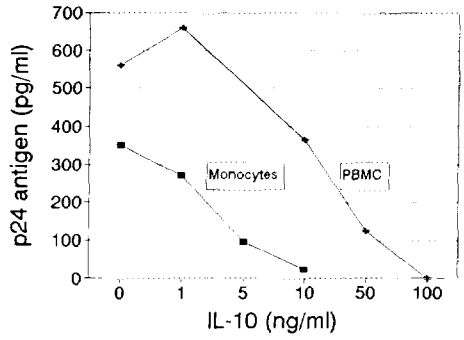

Figure 5. IL-10 inhibits HIV replication. Monocytes and PBMCs were infected with HIV-1 JR-FL and HIV-1_{111b}, respectively, supernatants were analyzed for P_{24} as described in Materials and Methods.

about 25 ng/ml and over 90% inhibition at 100 ng/ml of rhIL-10 (Figure 5). The sensitivity of chronically infected T cell line, ACH-2 (infected with HIV-1_{111b}) was similar to PBMCs with IC_{50} around 25 ng/ml (data not shown). IL-10 did not have any effect on the viability or replication of any cell type utilized in these experiments (data not shown).

Discussion: The results presented in this report demonstrated that IL-10 is expressed spontaneously in the PBMCs of HIV infected patients. In contrast no IL-10 was detected in PBMCs of HIV seronegative cases and healthy controls. We have further shown that the monocytes/macrophage and T cells and B cell fraction from the PBMCs of HIV infected patients express IL-10. We therefore, analyzed IL-10 levels from HIV-1 infected cases to determine if there were any differences among HIV disease status (KS, NHL, others) and CD_4 count. No differences were observed in IL-10 levels among the groups. Furthermore, no significant correlation was seen between CD_4 counts and IL-10 levels. Studies of large cohorts are currently in progress, to determine if any trends can be observed.

Our studies show that HIV plays a direct role in the induction of IL-10 in HIV infected patients. IL-10 is induced in both promonocytic (U937) and T cell (H-9) lineage after acute and chronic infection with different strains of HIV-1.

The HIV-1 tat protein can transregulate cellular genes, including several which encode cytokines such as IL-6 (18), TNF (19) and IL-4R (20). Our finding that H-9 tat cells secreted 4-fold high levels of IL-10 after PMA treatment in comparison to similarly-treated H-9 cells is evidence that tat may be involved in regulating IL-10 expression during HIV-1 infection. Several studies have shown that tat regulation of cellular and HIV-1 gene expression involves cooperation with or activation of host transcription factors, including NF-kB (21-23). We are currently investigating in detail how HIV regulates IL-10.

These findings demonstrate that HIV-1 can induce IL-10, another T_H2 type cytokine both in-vitro and in-vivo. It would thus be tempting to suggest that IL-10 could contribute to the depressed cytotoxic T-cell and enhance B cell responses seen in patients with HIV-1 infection. Inhibitory effect of IL-10 in HIV-1 replication however is dramatic. Particularly marked sensitivity of HIV-1 in monocytes, which is the main reservoir in-vivo, and effect of IL-10 on chronically infected T cells argues for beneficial effects of IL-10. IL-10 may in fact participate in the long latency of HIV, as evident from our data that IL-10 is induced in PBMCs of patients with HIV-1 regardless of the CD4 count. The effect of IL-10 on T_H1 function and thus cellular immune function is well known and thus the net effect of IL-10 in HIV-1 is far from clear.

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