Human Immunodeficiency Virus-1 Tat suppresses gp120 specific T-cell response in IL-10 dependent manner
3.1 Introduction

The clinical manifestations observed in HIV-1 infected patients are primarily due to the virus induced immuno-suppression that cannot be explained solely by the direct lytic effect of the virus on infected CD4+ T lymphocytes. One of the major features of HIV-1 infection is that the virus has direct and indirect pathogenic effects on both mature CD4+ T cells and on their progenitor cells (McCune, 2001). HIV-1 can infect target cells and remain integrated in the form of a latent provirus (McCune, 1995). In addition to latency, the virus uses other strategies involving viral and cellular factors to evade the immune system (Fauci, 1996). Despite extensive effort, no effective HIV vaccine has emerged till date (Berkley and Koff, 2007; Tonks, 2007). Increasing evidences indicate that the host’s natural immunity has major but usually insufficient role in limiting HIV-1 infection. CD8+ cytotoxic T cells (CTLs) appear to be the major mediator of viral control, as demonstrated by the dramatic increase in viremia in primate model after depletion of CD8+ T cell (Schmitz et al, 1999; Jin, et al, 1999).

The intramuscular injection of naked plasmid DNA induces long lived humoral and cellular immune responses both in experimental system and in human and protective immunity in animal challenge model (Donnelly et al, 2005), although multiple immunizations with DNA is generally required (Wang et al, 1998). T cell responses can be induced to different HIV-1 proteins by immunization with genes encoding viral proteins (Wahren, et al, 1995; Calarota et al, 1998; Wang et al, 1998). HIV-1 Tat protein has been known to have multiple regulatory roles including replication of the virus and modulation of cytokine expression in the infected and bystander cells. Tat protein has long been implicated as an important factor in the manifestation of immune dysfunction in many HIV-1 infected individuals before substantial loss of CD4+ T cells (Rosenberg, et al, 1997; Viscidi, et al, 1989). In fact, a number of reports have unequivocally established that Tat possesses a unique biological activity that alters the function of monocytes, dendritic cells, CD4+ and CD8+ T cells in vivo (Lafrenie et al, 1996; Rosenberg et al, 2000).

Recent reports suggest that deregulation of cytokine production contributes to the attenuated functioning of the immune system during the course of HIV-1 infection. Due to its important role in virus life cycle and relatively well conserved sequence in various
isolates, Tat has been used as an immunogen both alone or as a part of multi-component vaccines. Although results from several studies strongly indicate Tat as a potential vaccine candidate, some studies show an immunosuppressive role of Tat, particularly against the co-immunogens, in the host (Cohen et al, 1999; Agwale. et al, 2002). In addition, studies from patients have indicated increase in IL-10 production from infected cells (Clerici et al, 1994) and Tat has been implied in such IL-10 induction (Li et al, 2005; Gee et al, 2007). IL-10 is known to inhibit a broad spectrum of cellular immune response. It suppresses the function of APCs and T cells by inhibiting cytokine production, co-stimulation, MHC class II expression and chemokine secretion.

In the present study we have used a bicistronic vector expressing both gp120 and Tat along with vectors expressing gp120 or Tat alone for DNA immunization in mice and the studied immune response of Tat towards gp120 when it is co-expressed. We also demonstrate that Tat modulates antigen specific CD8+ T cell responses by regulating CD4+ T helper cell function, which are central players in the development of functional cytotoxic CD8+ T cell. Furthermore, the immunosuppressive activity of Tat is not observed in IL-10 deficient mice. Our results thus suggest that IL-10 induced by Tat could alter antigen specific CD8+ T cell response and may play a role in immune dysregulation observed in HIV-1 infection.

3.2 Materials and Methods

3.2.1 Mice and immunization
C57BL/6 mice (6-8 week old) and IL-10 deficient mice on C57BL/6 background were obtained from Jackson Lab (Bar Harbor, ME, USA) and were maintained in Experimental Animal Facility of National Center of Cell Science, Pune. Mice were injected intramuscularly (i.m.) in the quadriceps muscle using 26 gauge needle with 3 doses of 100 µg plasmid encoding either viral protein Tat or gp120 alone or with the bicistronic vector containing gp120 & Tat on day 0, 15 and 30. The spleen was taken out 10 days after the last immunization and the cells were used for T cell proliferation and CTL assay. GST-Tat protein was used for immunization in WT and IL-10−/− mice along with plasmid encoding only gp120 in one group of mice. Ovalbumin (Sigma, USA) was
used to immunize WT mice as a non viral protein along with plasmid encoding Tat or GST–Tat protein. 30 µg of GST-Tat and 50 µg of OVA was injected subcutaneously (s.c.) with CFA in first injection and subsequent injections were given with IFA. 2 ng of rIL-10 and 100 µg anti IL-10 was injected (i.p.) in mice was injected in IL-10 deficient and WT mice respectively. The experiment accorded with the committee for the purpose of control and supervision of experiments on animal approved protocols.

3.2.2 Purification of GST-Tat protein
GST-Tat was purified from *E.coli* BL21-DE3 transformed with expression vector GST-Tat 86R TK as reported (Rhim et al, 1994) with minor modification. The bacterial culture was grown at 37 °C to 0.4 OD and induced with 0.1 mM isopropyl β-D thiogalatoside and further grown to 0.6 OD. The bacterial pellet after centrifugation of culture was lysed with in 50 mM TrisHCl pH 8.0, 0.5 mM DTT 1X protease inhibitor cocktail, 0.5 % NP40 and 120 mM NaCl. The lysate was incubated with glutathione –sepharose beads (Amersham, USA) to allow binding of GST-Tat protein. GST-Tat from glutathione –sepharose beads was eluted with 10 mM reduced gluthione, 5 mM DTT and 50 mM Tris HCl. The GST-Tat was dialyzed with Polyethylene glycol (PEG). SDS PAGE was used to confirm the purity of GST-Tat protein.

3.2.3 ELISA Assay for gp120
Sera were collected from immunized mice after 10 days of last immunization. Direct ELISA was used to measure antibody response against gp120. Briefly, ELISA plate (Costar, USA) was coated overnight at 4 °C with 50 µl of 5 µg/ml gp120 protein in PBS obtained from Dr. Ian M Jones, University of Reading U.K. (Morikawa et al, 1990; Wang et al, 1995). Following wash with PBS containing 0.05 % Tween-20, the wells were blocked for 2 h with 5 % BSA (Amersham, USA) and 0.05 % Tween-20 in PBS. Sera were diluted in 5 % BSA/ 0.05 % Tween-20 and added to ELISA wells. Following incubation at 37 °C, the plate was washed five times and incubated with 1:500 dilutions of peroxidase conjugated rabbit anti-mouse secondary antibody (KPL, USA). After washing the titer of serum antibody was checked by development of color with ABTS
substrate (Roche Biochemicals, USA). The reaction was stopped with 0.33 N HCl and analyzed at 405 nm on ELISA reader.

3.2.4 ELISA for Isotype analysis
In order to analyze the isotype profile of gp120 specific antibody response, 96-well Costar plates were coated with 5 µg/ml of gp120 protein in bicarbonate buffer pH 9.6 for 2 h at 37 °C. The plates were blocked with 5 % BSA in PBS overnight at 4 °C. After blocking, plates were washed 3 times and the sera from pCDNA, pCgp120 and pIRESgp120-Tat immunized mice were added at various dilutions and kept for 2 h at 37 °C. Plates were washed 5 times with wash buffer and bound antibodies were detected using biotin-conjugated goat anti-mouse IgM, IgG1 and IgG2a (BD pharmingen, USA). This was followed by incubation with HRP-streptavidin and development of color by ABTS substrate.

3.2.5 Preparation of murine splenocytes for CTL assay
Ten days after the last immunization mice were sacrificed and their spleens were aseptically removed. A single cell suspension was prepared by crushing the spleen with frosted end slides. RBCs were removed by treating the spleen cells with Geys solution (Barbara B Mishell and Stanely M shiigi 1980) for 5 min at 4 °C following two washes in RPMI1640.

3.2.6 T cell purification
RBC depleted cells were incubated in nylon wool (Robbins Scientific, USA) column for 1 h at 37 °C in 5 % CO2 at sterile condition. Cells were eluted and spun down at 1200 rpm at 4 °C. Resulting cells were directly subjected to CD8+ and CD4+ T cell enrichment system (Stem cell technology, USA), which contains rat serum and CD4+ or CD8+ enrichment cocktail. Cells were incubated with rat serum for 30 min on ice and then CD4+ or CD8+ T enrichment cocktail was added and kept for 15 min on ice. Cells were then washed with PBS containing 1 % FCS. Pellet was incubated with M-280 Dynal beads for 45 min at constant mixing at 4 °C. After incubation cells were kept on magnet (Dynal, USA) for separation. Unwanted cells bind to the magnet, while desired cells
come out in supernatant. Supernatant was spun down at 1200 rpm at 4 °C and pellet contained purified CD8⁺ or CD4⁺ T cell.

3.2.7 CTL assay by JAM TEST
The CTL assay was performed following the method developed by Matzinger (Matzinger P, 1991). Naïve C57BL/6 Splenocytes were incubated with 10 µM of HIV gp120 peptides HXB2 334-349 KENWTDLQRVSKKL, 320-324 SIRIGPGQTFYYATGE, 102-116 NQMHDVISLWDQSL and HIV-1 Tat peptide 16-35 SQPKTAACTNCYCKKFHCQ and 31-50 CFHCQVCFITKALGISYGRK (Sigma, USA) for in vitro stimulation The incubation was for 2 h at 37 °C and then the cells were irradiated in gamma chamber. 2x10⁶ splenocytes from immunized mice were stimulated with 1x10⁶ peptide pulsed irradiated normal syngenic splenocytes in 24-well tissue culture plate (Nunc, USA). All cultures were incubated in RPMI 1640 supplemented with 10 % heat inactivated FCS and 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen, USA). After 5 days, dead cells were removed by Histopaque (Sigma, USA). The viable T cells were counted by trypan blue exclusion method. These effector cells were used in Cytotoxic T Lymphocyte (CTL) assay. EL-4 cell line was pulsed with pool of gp120 or Tat peptide for 2 h at 37 °C and 5 % CO₂. The target cells EL-4 were pulsed over night with 1 µCi thymidine ³H (BRIT India) at 37 °C in sterile condition. After two washes the radiolabeled target cells were plated in complete RPMI 1640 at a concentration of 2.5x10⁴ cells /100 μl. The effector cells were plated in various ratios with the target cells in a total volume of 100 μl in triplicate in to the wells of a 96-well U bottom tissue culture plate (Greiner, Germany). After 3 h of incubation at 37 °C, cells were harvested in cell harvester (Packard, USA) and counts were analyzed on Top count microplate counter (Perkin Elmer, USA).

% Percent lysis = S-E/S x100
Where, E=experimentally retained DNA in presence of Effectors (in cpm)
S=Retained DNA in the absence of Effectors.

3.2.8 T cell proliferation Assay
The ³H TdR uptake assay was used to measure the proliferation of splenocytes after antigenic stimulation. Splenocytes from immunized mice were resuspended at a
concentration of $2 \times 10^5$ cells/200μl in RPMI 1640 containing 10 % FCS and antibiotics. gp120 and Tat peptides were added at final concentration of 10 μg/ml. After 60 h, 1 μCi of $^3$H thymidine TdR (BRIT, India) was added in each well and incubated for 12 h at 37 °C in 5 % CO$_2$. The cells were harvested on glass fiber filter paper using Packard cell harvester and the thymidine uptake was counted in Top count microplate counter (Perkin Elmer, USA).

### 3.2.9 Cytokine ELISA

Cytokine levels in culture supernatants were detected by standard sandwich ELISA for cytokines as described in manufacturers manual (BD Pharmingen, USA). In brief, ELISA plates were coated with 100 μl of anti mouse cytokine monoclonal antibody in 0.1 M NaHPO$_4$ (pH 9.0) overnight at 4 °C. Plates were washed three times with wash buffer (PBS with 0.05 % Tween-20) and blocked with 200 μl of blocking (PBS with 1 % BSA, 0.05 % Tween-20, and 0.05 % NaN$_3$) for 1 h at room temperature. Plates were then washed three times and 100 μl of murine recombinant cytokine standard (BD pharmingen, USA) or culture supernant in binding buffer (1 % BSA and 0.05 % Tween-20 in PBS) was added and incubated overnight at 4 °C. After overnight incubation plates were washed and 100 μl of biotin-conjugated anti-mouse cytokine monoclonal antibody was added for 1h at room temperature. Then the plate was washed and 100 μl of peroxidase–conjugated streptavidin was added and incubated for 45 min at room temperature. Plates were then washed six times and 100 μl of tetramethyl benzidine substrate (TMB) was added to each well and color was allowed to develop for 30 min at room temperature before stopping the reaction with 100 μl of 1N H$_2$SO$_4$ in double distilled H$_2$O. Absorbance at 450 nm was measured using an automated microplate absorbance reader (Bio-Tek Instrument, USA).

### 3.2.10 RT-PCR

Total RNA was isolated from macrophage-T cell co-culture using Trizol (Invitrogen, USA) according to manufacturers instructions. 5 μg RNA was used for first strand cDNA synthesis. The cDNA was then used as template for PCR amplification of mouse IFNγ and IL-10 using gene specific primers. The primers used for PCR are IFNγ forward 5’-
AAC GCT ACA CAC TGC ATC TTG G-3’ and Reverse 5’-CTC ATG AAT GCA TCC TTT TTC G-3’; IL-10 forward 5’ GAA AGA CAA TAA CTG CAC C -3’ and Reverse 5’-CAT TTC CGA TAA GGC TTG G-3’and β-actin forward 5’-GTG GGC CGC TCT AGG CAC CA-3’ and Reverse 5’-TGG CC TTA GGG TTC AGG GGG-3’ Each sample was amplified for mouse β-actin to ensure equal input.

### 3.2.11 Statistical Analysis

Each individual experiment was repeated at least three times. The error bars represent the mean±SD of triplicate cultures in vitro. For in vivo experiment, error bars represent the mean±SD, which is minimum of four mice per group. Statistical analysis of the experimental data was conducted by student t test with the levels of significance defined as p<0.05.

### 3.3 Results

#### 3.3.1 Tat modulates gp120 specific humoral response

Serum was collected from immunized mice and ELISA was performed to assess gp120 specific antibody response. Antibody response was well observed in serum of pCgp120 and pIRESgp120-Tat immunized mice as compared to pCDNA immunized mice (Fig 3.1A). We then analyzed the isotype profile of gp120 specific immune response in presence and absence of Tat. Less IgM was observed in pCDNA, pCgp120, pIRESgp120-Tat immunize mice (Fig 3.1B), however, higher IgG2a response was observed in pCgp120 immunized mice as compared to pIRESgp120-Tat immunized mice (p<0.05). Tat co-immunization reduced gp120 mediated IgG2a (Fig 3.1C) response but enhanced IgG1 (p<0.05) (Fig 3.1D), which are known to be controlled by IFNγ and IL-4, respectively. These findings suggest that Tat enhances Th2 responses leading to IL-4 and IL-10 mediated suppression of Th1 response and reduced gp120 mediated IgG2a response.
Fig 3.1. gp120 specific humoral response and Tat induced modulation of isotypes in co-immunized mice. The sera from pCDNA, pCgp120 and pIRESgp120-Tat immunized wild type mice were assayed for gp120 reactive antibody response followed by isotype analysis (IgM, IgG2a, IgG1) on gp120 protein coated ELISA plate. A. Analysis of gp120 antibody response; B. gp120 specific IgM profile; C. gp120 specific IgG2a profile; D. gp120 specific IgG1 profile.

3.3.2 Induction of gp120 specific cell mediated immune response

Groups of C57Bl/6 mice (n=4) were immunized with 100 μg of one of the following vectors, pCDNA, pCgp120, pCTat, pCgp120+pCTat, pCgp120+GST-Tat and pIRESgp120-Tat intramuscularly on 0, 15 and 30 days. The mice were sacrificed after 10 days of last immunization. Spleens were isolated from mice and splenocytes were isolated for analyzing cellular immune response. Cytotoxic T lymphocyte assay for gp120 and Tat was performed with splenocytes isolated from all groups of mice as described in materials and methods. gp120 specific CTL response observed in mice immunized with pCgp120 alone was reduced in pIRESgp120-Tat immunized mice.
(p<0.05) as shown in (Fig 3.2A). However, Tat specific CTL activity did not show significant change in mice immunized with pCTat or pIRESgp120-Tat as compared to the disparity observed in gp120 specific CTL response (Fig 3.2B). The effect of Tat on gp120 specific T cell response was due to DNA immunization with bicistronic vector or the effect was universal irrespective of the nature of Tat immunization. Similar results on gp120 specific CTL response were obtained with mice immunized with pCgp120 along with either pCTat vector or GST-Tat. The above data clearly indicate that Tat induced suppression does not take place at the level of co-expression of genes but at a later stage, when the immune effectors are activated.

Fig 3.2. Tat suppresses T cell responses in mice immunized with bicistronic vector pIRESgp120-Tat. Splenocytes from pCDNA, pCgp120, pCTat, pIRESgp120-Tat, Cgp120+pCTat and pCgp120+GST-Tat injected mice were plated 2x10^6 per well in 24 well plate with gp120 or Tat peptide pulsed 1x10^6 irradiated naïve splenocytes. After 5 days of culture viable cells were harvested and plated against ^3H thymidine-incorporated gp120 or Tat peptide pulsed EL-4 cells and tested for their cytolytic activity in standard 3½ h in JAM test. The E:T ratio used are shown in the figure. Each data point is the mean of triplicate samples. A. gp120 specific CTL response in pCDNA, pCgp120, pIRESgp120-Tat pCgp120+pCTat and pCgp120+GST-Tat immunized mice. B. Tat specific CTL response in pCDNA, pCTat, pCgp120+pCTat pCgp120+pCTat, pCgp120+GST-Tat, GST-Tat and pIRESgp120-Tat immunized mice.
3.3.3 Tat suppresses proliferation in mice immunized with pIRESgp120-Tat

Splenocytes isolated from different immunized mice mentioned above were also used for T cell proliferation using Tat and gp120 peptides. As expected, no gp120 and Tat specific response was detectable in mice immunized with pCDNA. Proliferation was readily observed in the mice immunized with pCgp120 or pCTat, when in vitro stimulated with gp120 or Tat peptide (Fig 3.3C and 3.3D). Strikingly, the proliferative responses from mice immunized with bicistronic pIRESgp120-Tat were diminished when cells were stimulated with gp120 peptide (p<0.05) as compared to splenocytes stimulated with Tat peptide (Fig 3.3A and 3.3B). This observation again suggests that Tat has an immunosuppressive effect on gp120 specific T cell response. Similar results were also obtained in mice co-immunized with pCgp120 along with pCTat or GST-Tat and pCTat vector. Thus the results obtained from both CTL and proliferation assay indicate that Tat has a suppressive effect on immune response towards gp120.

Fig 3.3. Tat suppresses T cell responses in mice immunized with bicistronic vector pIRESgp120-Tat

Also Splenocytes from injected mice were plated 2x10^5 cells per well in 96 well plates and was pulsed with 10 µg of gp120 or Tat peptides or without antigen (medium). Proliferation was assessed by ^3^H thymidine-incorporated assay. The results represent three individual experiments and error bar represent the mean±SD of a given group. A. gp120 specific proliferation in splenocytes from mice immunized with different vectors mentioned above. B. Tat specific proliferation in splenocytes from mice immunized with different vectors mentioned above.
3.3.4 Tat also suppress Ovalbumin induced immune response

In order to test whether the immunosuppressive effect of Tat on viral envelope protein also holds true for other antigens, we have used ovalbumin (OVA) antigen for co-immunization with Tat. There are several reports, which have shown that ovalbumin is immunogenic when immunized with CFA (Ke et al, 1995). Groups of mice (n=4) were immunized with OVA alone or OVA with GST-Tat or OVA with pCTat injected subcutaneously with complete Freunds adjuvant (CFA) on first injection and subsequent injections were given with incomplete Freund’s adjuvant (IFA) at 0, 15 and 30 days.

![Graphs showing immune response to OVA and Tat](image)

**Fig 3.4.** Tat also suppresses immune response to Ovalbumin (OVA) antigen. Mice were immunized with OVA, OVA + pCTat and OVA + GST-Tat as detailed in materials and methods. Splenocytes isolated from immunized mice were used for both CTL and proliferation assay as described in materials and methods. 

A. OVA specific CTL response in mice immunized with OVA alone or with Tat. B. Tat specific CTL response in mice immunized with Tat. C. T cell proliferation assay with splenocytes isolated from mice immunized with OVA alone or along with Tat.
The mice were sacrificed 10 days after the last immunization. Spleens were isolated from immunized mice and splenocytes were used to analyze OVA specific CTL and proliferation response. As shown in Fig 3.4A and 3.4C, both OVA specific CTL and proliferation response was impaired in presence of Tat (p<0.05); however, Tat specific CTL response was unaffected in presence of OVA (Fig 3.4B).

3.3.5 IL-10 produced by the splenocytes mediate the suppressor function

The decreased T cell proliferation in response to gp120 peptides in mice immunized with pIRESgp120–Tat as compared with pCgp120 immunized mice could be explained by two possible mechanisms. First, the suppression of T cell proliferation could be due to Tat since it is known to play important role in T cell apoptosis (Zhang et al, 2001; Kim et al, 2003). The second possibility could be active suppression of the host protective T cell response due to production of the counteractive disease promoting cytokines such as IL-10 and IL-4. Therefore, we examined the kinetics of production of these cytokine along with proliferation of T cells in response to gp120 peptides. Groups of mice (n=4) were immunized with 100 μg of pCDNA, pCgp120, pCTat and pIRESgp120-Tat intramuscularly at 0, 15 and 30 days. The mice were sacrificed after 10 days of last immunization. Proliferation assays were done with splenocytes isolated from immunized mice and supernatants were collected for cytokine ELISA at different time points at 24, 48 and 72 h. The proliferation profile of splenocytes isolated from pCgp120 and pIRESgp120-Tat mice suggests that Tat reduces time dependent proliferation of cells in response to gp120 peptides from pIRESgp120-Tat mice (Fig 3.5A) as compared to pCgp120 mice (p<0.05). However, Tat does not seem to induce apoptosis since proliferative response were well observed in splenocytes incubated with Tat in all the three groups of mice (Fig 3.5B). IL-2 and IL-4 levels were maintained during the time course (Graziosi et al, 1996); however IFNγ and IL-10 exhibited reciprocal patterns in gp120 stimulated cells of pIRESgp120-Tat mice. pCgp120 injected mice show elevated levels of IFNγ (p<0.05) (Fig 3.5C) whereas IFNγ production was decreased in pCTat (p<0.001) (Fig 3.5D) and pIRESgp120-Tat (p<0.05) (Fig 3.5E). IL-10 production increased in Tat immunized mice either alone or in pIRESgp120-Tat (p<0.05) (Fig 3.5F).
This pattern of cytokines suggest that Tat induces IL-10 and since IL-10 has been demonstrated to have appreciable T cell inhibitory activity, particularly on Th1 cells in both human and mice (Fiorentino et al, 1989; Fiorentino et al, 1991), it is plausible that IL-10 could be responsible for Tat mediated immunosuppression.

![Graphs showing cytokine profiles and cell proliferation](image.png)

**Fig 3.5.** Tat suppresses the immune response toward gp120 in co-immunized mice by modulating cytokine expression. Splenocytes isolated from pCDNA, pCgp120, pCTat and pIRESgp120-Tat immunized mice were plated in 96 well plates and stimulated with gp120 or Tat peptide as described in materials and methods. Tritiated Thymidine was added at 12, 36 and 60 h for time kinetics proliferation assay. **A.** Cell proliferation at different time points of splenocytes stimulated with gp120. **B.** Cell proliferation at different time points of splenocytes stimulated with Tat. **C.** Secretion of IL-2, IL-4, IL-10 and IFNγ cytokines by splenocytes of pCgp120 immunized mice in vitro stimulated with gp120 peptide as assessed by ELISA of the culture
supernatant collected at 24, 48 and 72 h in the experiment described above. D. Secretion of IL-2, IL-4, IL-10 and IFNγ cytokines by splenocytes of pCTat immunized mice with Tat peptide stimulated as assessed by ELISA in the experiment described above. E. Secretion of IL-2, IL-4, IL-10 and IFNγ cytokines by splenocytes of pIRESgp120-Tat immunized mice gp120 peptide stimulated as assessed by ELISA in the experiment described above. F. Secretion of IL-2, IL-4, IL-10 and IFNγ cytokines by splenocytes of pIRESgp120-Tat immunized mice in vitro stimulated with Tat peptide as assessed by ELISA in the experiment described above.

Tat possesses a unique biological activity that it alters the function of macrophage, monocytes, dendritic cells, CD4+ T cell and CD8+ T cells in vivo. Several studies have also shown that extra-cellular Tat mediated cytokine deregulation may play role in destruction of uninfected bystander cells. In order to identify the source of IL-10 induced by Tat observed in the previous experiment, we have isolated T cells from naïve C57BL/6 mice and co-cultured them with peritoneal macrophages isolated from IL-10 deficient mice and stimulated with GST and GST-Tat. Inversely, T cells isolated from naïve IL-10 deficient mice were co-cultured with peritoneal macrophages from WT mice and stimulated with GST and GST-Tat. IFNγ and IL-10 secretion was analyzed by ELISA in the culture supernatant and the RNA from the co-cultured cells were used for RT-PCR for IFNγ and IL-10. The up regulation of IL-10 indicates that Tat induces IL-10 from both T cells and macrophages (p<0.05) (Fig 3.6A). However, T cells seem to secrete more IL-10 as compared to macrophages, a profile confirmed by RT-PCR (Fig 3.6B). These results indicate that Tat induces IL-10 secretion from both T cells and macrophages.
Fig 3.6. Tat induces IL-10 from naïve T cell and macrophages. T cells isolated from naïve IL-10 deficient mice were co-cultured with peritoneal macrophage from WT mice and stimulated with GST and GST-Tat. In same experiment T cells were isolated from naïve C57BL/6 mice and co-cultured with peritoneal macrophages isolated from IL-10 deficient mice and stimulated with GST and GST-Tat for 12 h. After incubation, culture supernatants were used for cytokine ELISA and cells were used for preparation of RNA. A. Analysis of IFNγ and IL-10 in culture supernatants by ELISA in co-cultures indicated in the figure. B. RT-PCR analysis for IFNγ and IL-10 gene. Lane 1, marker; Lane 2, medium; Lane 3, IL-10-/- T cell + WT macrophage +GST; Lane 4, IL-10-/- T cell + WT macrophage + GST-Tat; Lane 5, medium; Lane 6, WT T cell+IL-10-/- macrophage + GST; Lane 7, WT T cell+ IL-10-/-macrophage + GST-Tat.

3.3.6 gp120 specific antibody response in IL-10-/- mice in presence of Tat
In order to investigate the impact of IL-10 on observed Tat mediated immunosuppression, we have immunized IL-10-/- mice with three vectors as done in the case of wild type mice. Serum was collected after 10 days of last immunization and isotype
profile of gp120 specific antibody was analyzed. The results indicate that IgM was minimal in pCDNA, pCgp120 and pIRESgp120-Tat immunized mice (Fig 3.7A), but IgG2a was more than IgG1 in both pCgp120 and pIRESgp120-Tat immunized mice (Fig 3.7B and 3.7C). This observation was in contrast to WT mice, where IgG1 was higher in pIRESgp120-Tat immunized mice but IgG2a was more in pCgp120 mice (Fig 3.1C and 3.1D). The absence of switch from IgG2a to IgG1 in presence of Tat in IL-10 deficient mice indicates that IL-10 plays a role in Tat induced changes in isotype switching of co-immunized gp120 antigen.

Fig 3.7. gp120 specific antibody response in IL-10−/− mice. The sera from pCDNA, pCgp120 and pIRESgp120-Tat immunized IL-10 deficient mice were assayed for gp120 reactive antibody response by isotype analysis (IgM, IgG2a, IgG1) on gp120 protein coated ELISA plate. A. gp120 specific IgM profile; B. gp120 specific IgG2a profile; C. gp120 specific IgG1 profile.
3.3.7 IL-10 deficiency augments T cell response in pIRESgp120-Tat immunized mice

IL-10 is known to be an anti-inflammatory cytokine responsible for the suppression of the immune response, thus preventing the development of chronic inflammation. The function of IL-10 is to inhibit specific cytokine produced by T helper 1 (Th1) and natural killer cells as well as macrophage. In order to further analyze the role of IL-10 in Tat mediated immunosuppression, we have immunized groups of IL-10−/− mice (n=4) were immunized with 100μg of pCDNA, pCgp120, pCTat, pCgp120+pCTat, pCgp120+GST-Tat and pIRESgp120-Tat i.m. at 0, 15 and 30 days and were sacrificed after 10 days of last immunization. Anti-gp120 specific CTL assays were performed with splenocytes isolated from immunized mice. A strong gp120 specific CD8+ T cell response was observed in pIRESgp120-Tat injected IL-10−/− mice (Fig 3.8A) as compared to wild type mice described in Fig 3.2A, where anti-gp120 CTL response were diminished in presence of Tat, suggesting that Tat mediates its effect through IL-10. Tat specific CTL response remains unchanged in IL-10−/− mice (Fig 3.8B). Proliferation assays were also performed with immunized IL-10−/− mice but no immunosuppression was observed in pIRESgp120-Tat immunized IL-10−/− mice (Fig 3.8C and 3.8D). Similar results were also observed when Tat was immunized either as protein or independent vector. The collective data obtained from CTL and proliferation assay in IL-10−/− mice immunized with pIRESgp120-Tat show that immunosuppressive effect of Tat is mediated through IL-10, which plays a disease-exacerbative role by suppressing the host protective T cells and inhibiting IFNγ production.
Fig 3.8. Immunosuppressive activity of Tat on gp120 immune response is abrogated in IL-10 deficient mice. IL-10-/- mice were immunized with pCDNA, pCgp120, pCTat, pCgp120+pCTat, pCgp120+GST-Tat, GST-Tat and pIRES-gp120-Tat. Splenocytes were isolated from immunized mice and plated with gp120 or Tat peptide pulsed 1x10^6 irradiated naïve splenocytes. After 5 days of culture viable cells were harvested and plated against ^3^H thymidine-incorporated gp120 or Tat peptide pulsed EL-4 cells and tested for their cytolytic activity in standard 3½ h JAM test. The E:T ratio was as shown in the figures. Each data point is the mean of triplicate samples. The results represent three individual experiments and error bar represent the mean±SD of a given group. Also Splenocytes from injected mice were plated 2x10^5 cells per well in 96 well plates and was pulsed with 10 μg of gp120 or Tat peptides or without antigen (medium). Proliferation was assessed by ^3^H thymidine-incorporation assay. The results represent three individual experiments and error bar represent the mean±SD of a given group. A. gp120 specific CTL response in IL-10-/- mice immunized with pIRESgp120-Tat. B. Tat specific CTL response in IL-10-/- mice immunized with pIRESgp120-Tat. C. gp120 specific proliferation assay in IL-10-/- mice immunized with pIRESgp120-Tat. D. Tat specific proliferation assay in IL-10-/- mice immunized with pIRESgp120-Tat.
3.3.8 CD4⁺T cell secreted IL-10 reduces the anti-gp120 CTL response in Tat co-immunized mice

The CD8⁺ T cell responses are dependent on concurrent help from CD4⁺ T cells (Wodarz et al, 2001; Castellino et al, 2006). Tat is known to be secreted by infected cells and can act on other cells including macrophages (Badou et al, 2000) and T cells irrespective of whether they are infected or not. Failure of CD4⁺ T cells can disrupt the ability of CD8⁺ T cells to become effective CTLs and CD4⁺ T cell response in HIV infection has long been known to be poor. So we investigated the role of CD4⁺ T cells in Tat mediated suppression of gp120 immune response. We have immunized WT and IL-10 deficient mice with pCgp120 and pIRESgp120-Tat and isolated CD4⁺ T cells and co-cultured them with CD8⁺ T cells isolated from pCgp120 immunized wild type and IL-10 deficient mice. gp120 peptide pulsed irradiated macrophages from naïve wild type mice were used as antigen presenting cells in the co-culture. The co-culture was kept for 6 days in sterile condition and gp120 specific CTL assay were performed there after. CD8⁺ T cells from pCgp120 immunized wild type mice co-cultured with CD4⁺ T cells from pIRESgp120-Tat immunized mice show suppressed CTL response (p<0.05) as compared to co-culture containing CD4⁺ T cells from pCgp120 immunized wild type mice (Fig 3.9A). CD8⁺ T cell from pCgp120 immunized IL-10 deficient mice, when co-culture with CD4⁺ T cell isolated from and IL-10 deficient pIRESgp120-Tat have shown up regulation as compared to wild type pIRESgp120-Tat (Fig 3.9B) which indicate that IL-10 is possible reason for Tat mediated immunosuppression. Further more the Tat mediated suppressor activity is regained in the CD4⁺ T cells isolated from IL-10 deficient mice co-immunized with pIRESgp120-Tat and recombinant IL-10. These results indicate that suppressive activity of Tat is mediated through CD4⁺ T cells expressing IL-10 (Barcellini et al, 1994; Klein et al, 1997).
Fig 3.9. CD4⁺ T cells from Tat co-immunized mice are responsible for IL-10 mediated suppressor function and co-administration of rIL-10 restores the suppressive function in IL-10⁻/⁻ mice. A. CD8⁺ T cells isolated from B6 wild type (B6-WT) mice immunized with pCgp120 were co-cultured with CD4⁺ T cells isolated from different immunized B6 wild type and IL-10⁻/⁻ mice as shown in the figure in 1:2 ratio in a six well plate. gp120 peptide pulsed peritoneal macrophage was used as APC in the co-culture. After 5 days of culture cells were harvested and plated against [³H] thymidine-incorporated gp120 pulsed EL-4 cells and tested for their cytolytic activity in standard 3½ h JAM test. The E:T ratio was as shown in the figure. Each data point is the mean of triplicate samples. The results represent three individual experiments and error bar represents the mean±SD of a given group. B. CD8⁺ T cells isolated from IL-10⁻/⁻ mice immunized with pCgp120 were co-cultured with CD4⁺ T cells isolated from different immunized B6 wild type and IL-10⁻/⁻ mice as shown in the fig in 1:2 ratio in a six well plate. gp120 peptide pulsed peritoneal macrophage was used as APC in the co-culture. After 5 days of culture viable CD8⁺ T cells were harvested and plated against [³H] thymidine-incorporated gp120 pulsed EL-4 cells and tested for their cytolytic activity in standard 3½ h JAM test. The E:T ratio was as shown in the figure. Each data point is the mean of triplicate sample. The results represent three individual experiments and error bar represents the mean±SD of a given group.

We have further investigated the role of neutralizing antibody against IL-10. We have immunized WT with pCgp120, pIRESgp120-Tat and pIRESgp120-Tat+antiIL-10 and isolated CD4⁺ T cells and co-cultured with CD8⁺ T cells isolated from pCgp120 immunized mice. We have performed gp120 specific CTL assay. The gp120 specific CTL response is impaired in pIRESgp120-Tat, However administration of anti IL-10
rescues the immunosuppressive effect observed with pIRESgp120-Tat. This observation clearly demonstrates the role of Tat induced IL-10 in suppression of immune response.

![Graph showing lysis percentage against effector to target ratio for pCgp120, pIRESgp120-Tat, and pIRESgp120-Tat+anti IL-10](image)

**Fig 3.10.** CD4⁺ T cells from Tat co-immunized mice are responsible for IL-10 mediated suppressor function and co-administration of anti IL-10 rescues the suppression observed with Tat. CD8⁺ T cells isolated from B6 wild type (B6-WT) mice immunized with pCgp120 were co-cultured with CD4⁺ T cells isolated from pCgp120, pIRESgp120-Tat and pIRESgp120-Tat+antiIL-10 immunized B6 wild type. gp120 peptide pulsed peritoneal macrophage was used as APC in the co-culture. After 5 days of culture cells were harvested and plated against ³H thymidine-incorporated gp120 pulsed EL-4 cells and tested for their cytolytic activity in standard 3½ h JAM test. The E:T ratio was as shown in the figure. Each data point is the mean of triplicate samples. The results represent three individual experiments and error bar represents the mean±SD of a given group.

### 3.4 Discussion

Tat, the HIV-1 transactivator of transcription protein is the most important regulator of the virus, as it is essential for virus replication. It is a relatively conserved protein among all the viral isolates and status of antibodies against Tat protein has been shown to be directly proportional to the progression of the disease in several clinical studies (Ensoli et al, 2001). The hypothesis that Tat plays an important role in the immunopathogenesis of HIV-1 has led to several studies using Tat as a candidate protein for HIV vaccine as both...
individual and multi-component vaccine candidates (Ensoli et al, 2006). Several studies indicate that antibodies to Tat play a host protective role in HIV-1 infection and thus have been also considered as a target for the development of novel AIDS therapy (Goldstein, 1996). Although Tat has been found to be a successful candidate, few reports imply that Tat acts as an immunosuppressor for the co-immunized viral antigens (Agwale et al, 2002). The mechanism by which Tat mediates its immunosuppression remains to be elucidated. In order to identify unambiguously the role of Tat in immune response of a co-immunized antigen, we have constructed a bicistronic vector expressing Tat and gp120 from CMV promoter with an internal ribosome entry site and have used intramuscular DNA immunization in mice. Isotype analysis of the elicited antibody indicates that Tat induces a switch from IgG2a to IgG1 in mouse co-immunized with gp120 and Tat. Our results also show that Tat suppresses T cell responses against co-immunized gp120 antigen. This immunosuppresion by Tat was observed not only in mice immunized with bicistronic vector but also where gp120 and Tat has been immunized separately either as DNA or protein. The immunosuppressive activity of Tat was not confined to viral antigen but it also suppressed the immune response of unrelated antigen, ovalbumin. Furthermore, cytokine analysis of stimulated splenocytes showed induction of IL-10 in Tat immunized mice as compared to IFNγ in gp120 immunized mice. Finally, Tat mediated induction of IL-10 was confirmed by stimulating a co-culture of naïve T cells (WT or IL-10−/−) and macrophages (WT or IL-10−/−) with GST or GST-Tat protein. Thus our finding that Tat induces production of IL-10, a cytokine with known immunosuppressive activity (Moore et al 1991), could be a crucial player in immune response to the virus.

Our result clearly shows that Tat modulates antigen specific CD8+ T cell response towards gp120. There could be two mechanisms of down regulation of immune response by Tat. Firstly, Tat probably impairs IFNγ responsiveness of T cell resulting in suppression of IFNγ mediated cytotoxic killing although it has been demonstrated that in chronic HIV-1 subjects develop a subset of HIV-1 specific CD8+ T cell that express IFNγ but lack cytotoxic effector function (Appay et al, 2000; Shankar et al, 2000). Secondly, Tat seems to exploit the hosts IL-10 dependent auto regulatory or a feed back servo–mechanism that prevents excessive inflammation mediated host tissue pathology. It has
been shown that Tat induces IL-10 production in infected PBMCs both in vitro and also in infected patients (Blazevic et al, 1996). This phenomenon seems to be not only restricted to HIV-1 as certain other viruses like cytomegalovirus and pox viruses skew the immune response similarly (Fleming et al, 1997; Spencer et al, 2002). In order to look at the role of IL-10 in Tat mediated immunosuppression, we have immunized IL-10−/− mice with bicistronic vector and found that immunosuppressive effect of Tat was abrogated suggesting that Tat uses host’s immune regulation pathway for benefit of virus. IL-10 is known to inhibit a broad spectrum of cellular immune response. It suppresses the function of APCs and T cells by inhibiting cytokine production, co-stimulation, MHC class II expression and chemokine secretion. Moreover, CD4+ and CD8+ T cells have been shown to express high level of IL-10 in HIV-1 infected individuals (Graziosi et al, 1994; Zanussi et al, 1996). In addition, higher frequency of IL-10 producing CD4+ cells in HIV-1 infected individual with progressive disease or active HIV replication has been reported as compared to infected individuals in the latent phase of disease. Furthermore, we have tried to dissect the role of CD4+ and CD8+ T cells in Tat mediated suppression of gp120 immune response, the results of which indicate that CD4+ T cells from wild type mice mediate the suppression through IL-10 as CD4+ cells from IL-10 deficient mice failed to show immunosuppression. This clearly demonstrates for that Tat suppresses T cell response by inducing IL-10. The degree to which Tat modulates CD8+ T cell response probably depends upon shift in CD4+ T cell cytokine secretion in immune inductive site.
3.5 References


