HIV-1 Tat Suppresses gp120-Specific T Cell Response in IL-10-Dependent Manner

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HIV-1 Tat Suppresses gp120-Specific T Cell Response in IL-10-Dependent Manner

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A large number of multicomponent vaccine candidates are currently in clinical evaluation, many of which also include the HIV-1 Tat protein, an important regulatory protein of the virus. However, whether Tat, a known immune effectormolecule with a well-conserved sequence among different HIV subtypes, affects the immune response to a coimmunogen is not well understood. In this study, using a bicistronic vector expressing both gp120 and Tat, we have analyzed the role of Tat in elicitation of the gp120-specific immune response. The T cell responses to gp120 were greatly diminished in mice coimmunized with Tat as compared with mice immunized with gp120 alone. This immunosuppressive activity of Tat was not confined to viral Ag only because it also suppressed the immune response of unrelated Ag. Analysis of the cytokine profile suggests that Tat induces IL-10 and since IL-10 has been demonstrated to have appreciable T cell inhibitory activity, it is plausible that IL-10 could be responsible for Tat-mediated immunosuppression. Finally, the immunosuppressive effect of Tat was not observed in IL-10-deficient mice, confirming the role of IL-10 in Tat-mediated immunosuppression. Thus, our results demonstrate for the first time that the immunosuppressive effect of Tat is mediated through IL-10 and suggests that Tat-induced IL-10-mediated immune suppression seems to cripple immune surveillance during HIV-1 infection. The Journal of Immunology, 2008, 180: 79–88.

The clinical manifestations observed in HIV-1-infected patients are primarily due to the virus-induced immunosuppression 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 (1). HIV-1 can infect target cells and remain integrated in the form of a latent provirus (2). In addition to latency, the virus uses other strategies involving viral and cellular factors to evade the immune system (3). Despite extensive effort, no effective HIV vaccine has emerged to date (4, 5). Increasing evidences indicate that the host’s natural immunity has a major but usually insufficient role in limiting HIV-1 infection. CD8+ CTLs appear to be the major mediator of viral control, as demonstrated by the dramatic increase in viremia in a primate model after depletion of CD8+ T cells (6, 7).

The i.m. injection of naked plasmid DNA induces long-lived humoral and cellular immune responses both in an experimental system and in human and protective immunity in an animal challenge model (8), although multiple immunizations with DNA are generally required (9). T cell responses can be induced to different HIV-1 proteins by immunization with genes encoding viral proteins (10–13). 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 (14, 15). 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 (16, 17).

Recent reports suggest that dysregulation 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 multicomponent 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 coimmunogens, in the host (18, 19). In addition, studies from patients have indicated an increase in IL-10 production from infected cells (20) and Tat has been implicated in such IL-10 induction (21, 22). IL-10 is known to inhibit a broad spectrum of cellular immune responses. It suppresses the function of APCs and T cells by inhibiting cytokine production, costimulation, MHC class II expression, and chemokine secretion.

In this report, 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 results show that Tat diminishes the cellular immune response toward gp120 when it is coexpressed. We also demonstrate that Tat modulates Ag-specific CD8+ T cell responses by regulating CD4+ Th 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 Ag-specific CD8+ T cell responses and may play a role in immune dysregulation observed in HIV-1 infection.
Materials and Methods

Construction of HIV-1 gp120 and Tat-expressing bicistronic vector

The gp120 sequence was amplified from a subtype C Indian isolate IN301904 (National Institutes of Health AIDS Research and Reagent Program) (23) by PCR and was cloned first in multiple cloning site A of the pRES vector (BD Clontech). The subtype B Tat sequence was subcloned in the multiple cloning site B of pRES taken from the pcDNA-Tat vector previously reported from this laboratory (24). The subtype C gp120 sequence was also cloned in pCDNA3.1 (Invitrogen Life Technologies). The cloning of gp120 and Tat in the pRES and pcDNA vectors was confirmed by restriction digestion and DNA sequencing.

Protein expression from the bicistronic vector

The expression of gp120 and Tat from pRESgp120-Tat was tested by transient transfection of the 293T cell line. 293T cells were transfected with 1 μg of pEGFPN1 along with 5 μg of pcDNA, pcPgp120, pcPgp120 plus pCTat, pcCTat, and pcRESgp120-Tat using the calcium phosphate precipitation method. After 48 h, cells were lysed by cell lysis buffer and analyzed for gp120 and Tat expression by ELISA using gp120 and Tat Ab. The transfection efficiency was normalized by quantitation of enhanced GFP expression using microfluorometry (25).

Protein expression in mice

To test the expression of gp120 and Tat in vivo, mice were immunized i.m. with pcDNA, pcCTat, pcPgp120, pcPgp120 plus pCTat, and pcRESgp120-Tat. Quadriceps muscles from immunized mice were excised 72 h after plasmid DNA immunizations. Muscles were then homogenized with no. 10 Medicen homogenizers (Wheaton) in 1 ml of PBS (pH 7.2) containing 0.05% Tween 20. Muscle homogenates were incubated on ice for 30 min, cell debris was removed by centrifugation, and supernatant was used to analyze for the presence of gp120 and Tat by ELISA.

ELISA for gp120 and Tat expression

An ELISA plate was coated overnight at 4°C with 50 μl of either transfected cell lysates containing enhanced GFP units or 50 μl of muscle lysates containing 200-μg proteins from different experiments described above. Following three washes with PBS containing 0.05% Tween 20, the wells were blocked for 2 h with 5% BSA (Amersham Biosciences) and 0.05% Tween 20 in PBS. Polyclonal Ab against Tat (26) or polyclonal Ab against gp120 (Santa Cruz Biotechnology) was diluted 1/500 in blocking 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 Ab (KPL). After washing, the titer of serum Ab was checked by development of color with the ABTS substrate (Roche Biochemicals). The reaction was stopped with 0.33 N HCl and analyzed at 405 nm on an ELISA reader.

ELISA for isotype analysis

To analyze the isotype profile of the gp120-specific Ab response, 96-well Costar plates were coated with 5 μg/ml 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 three times and the sera from pCDNA-, pcPgp120-, and pRESgp120-Tat-immunized mice were added at various dilutions and kept for 2 h at 37°C. Plates were washed five times with wash buffer and bound Abs were detected using biotin-conjugated goat anti-mouse IgM, IgG1, and IgG2a (BD Pharman- gen). This was followed by incubation with HRP-streptavidin and development of color using the ABTS substrate.

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 Gey’s solution (29) for 5 min at 4°C following two washes in RPMI 1640.

T-cell proliferation assay

RBC-depleted cells were incubated in a nylon wool (Robbins Scientific) column for 1½ h at 37°C in 5% CO2 in sterile condition. Cells were eluted and spun down at 1200 rpm at 4°C. Resulting cells were directly subjected to the CD8+ and CD4+ T cell enrichment system (StemCell Technologies), which contains rat serum and CD4+ or CD8+ enrichment mixtures. Cells were then incubated with 1 ml of serum for 30 min on ice and then CD4+ or CD8+ T enrichment mixture was added and kept for 15 min on ice. Cells were then washed with PBS containing 1% FCS. The pellet was incubated with M-280 Dynal beads for 45 min with constant mixing at 4°C. After incubation, cells were kept on a magnet (Dynal) for separation. Unwanted cells bound to the magnet, whereas desired cells came out in the supernatant. Supernatant was spun down at 1200 rpm at 4°C and the pellet contained purified CD8+ or CD4+ T cells.

CTL assay by just another method (JAM) test

The CTL assay was performed following the method developed by Matzinger (30). Naive C57BL/6 splenocytes were incubated with either 10 μM HIV gp120 peptides HXB2 335–349 KENWTDLTQRVSKKL, 320–322 SIRIGPGQTFYYATGE, 102–116 NQMHEDVISLWDQSL or Tat pep- tides 16–35 SQPKTAACTNCYCKKCFHCQ and 31–50 CFHCQVCFIT KALGSYGRK (Sigma-Aldrich) for in vitro stimulation. The incubation was for 2 h at 37°C and then the cells were irradiated in a gamma chamber. In brief, 2 × 10^6 splenocytes from immunized mice were stimulated with 1 × 10^6 peptide-pulsed irradiated normal syngeneic splenocytes in 24-well tissue culture plates (Nunc). 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 Life Technologies). After 5 days, dead cells were removed by Histopaque (Sigma-Aldrich). The viable T cells were counted using the trypan blue exclusion method. These effector cells were used in the CTL assay. The EL-4 cell line was pulsed with a pool of either gp120 or Tat peptides, mentioned above, for 2 h at 37°C and 5% CO2. The EL-4 target cells were pulsed overnight with 1 μCi of [3H]Tdr (BRIT) at 37°C in sterile conditions. After two washes, the radiolabeled target cells were plated in complete RPMI 1640 at a concentration of 2.5 × 10^3 cells/100 μl. The effector cells were plated in various ratios with the target cells in a total volume of 100 μl in triplicate into the wells of a 96-well U-bottom tissue culture plate. After 3 h of incubation at 37°C, cells were harvested in a Packard cell harvester and counts were analyzed on a Top count microplate counter (PerkinElmer). The following formula was used: percent lysis = S - E/S X 100, where E = experimentally retained DNA in the presence of effectors (in cpm) and S = retained DNA in the absence of effectors.

T cell proliferation assay

The [3H]Tdr uptake assay was used to measure the proliferation of splenocytes after antigen 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 a final concentration of 10μg/ml. After 60 h, 1μCi of $[^3H]$Tdr (BRIT) was added in each well and incubated for 12 h at 37°C in 5% CO₂. The cells were harvested on glass fiber filter paper using a Packard cell harvester and the thymidine uptake was counted in a Top count microplate counter (PerkinElmer).

Cytokine ELISA

Cytokine levels in culture supernatants were detected by standard sandwich ELISA for cytokines as described in the manufacturer’s manual (BD Pharmingen). In brief, ELISA plates were coated with 100μl of anti-mouse cytokine mAb in 0.1 M NaH₂PO₄ (pH 9.0) overnight at 4°C. Plates were washed three times with wash buffer (PBS with 0.05% Tween 20) and blocked with 200μl of blocking buffer (PBS with 1% BSA, 0.05% Tween 20, and 0.05% NaN₃) for 1 h at room temperature. Plates were then washed three times and 100μl of murine recombinant cytokine standard (BD Pharmingen) or culture supernatant 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 100μl of biotin-conjugated anti-mouse cytokine mAb was added for 1 h at room temperature. Then the plate was washed and 100μl of peroxidase-conjugated streptavidin was added and incubated for 45 min at room temperature. Plates were then washed six times and 100μl of tetrathymolbenzidine substrate was added to each well and color was allowed to develop for 30 min at room temperature before stopping the reaction with 100μl of 1 N H₂SO₄ in ddH₂O. Absorbance at 450 nm was measured using an automated microplate absorbance reader (Bio-Tek Instruments).

RT-PCR

Total RNA was isolated from a macrophage-T cell coculture using TRIzol (Invitrogen Life Technologies) according to the manufacturer’s instructions. Five micrograms of RNA was used for first-strand cDNA synthesis. The cDNA was then used as template for PCR amplification of mouse IFN-γ and IL-10 using gene-specific primers. The primers used for PCR are IFN-γ forward 5'-AAC GCT AAC TGC ATC TTG G-3' and reverse 5'-GAA AGA CAT ACC TGC CAT C-3'; and IL-10 forward 5'-GAA AGA CAA TAA CTG CAC C-3' and reverse 5'-CAT TCG TTC GGT CTT TGG C-3'; and β-actin forward 5'-GTC GCC CGC TCT AGG CAT CAC CA-3' and reverse 5'-TTG CGG TTC AGG GGG-3'. Each sample was amplified for mouse β-actin to ensure equal input.

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 experiments, error bars represent the mean ± SD, which is the minimum of four mice per group. Statistical analysis of the experimental data was performed using Student’s t test, with the levels of significance defined as $p < 0.05$.

Results

Cloning and expression of gp120 and Tat from a bicistronic vector

To analyze the immune response against HIV-1 gp120, when co-immunized with the viral regulatory protein Tat, we constructed a bicistronic mammalian expression vector. The envelope gene gp120 of a subtype C Indian isolate was cloned along with subtype B Tat under the regulation of a CMV promoter in an internal ribosome entry site-containing expression vector and was named as pIRESGp120-Tat. Cloning of gp120 and Tat was confirmed by restriction enzyme analysis and DNA sequencing. Expression of gp120 and Tat by pCgp120, pCTat, and pIRESGp120-Tat was confirmed using transient transfection of 293T cells. ELISA was performed with the transfected cell lysate to analyze gp120 expression (Fig. 1A). The expression of gp120 was clearly observed in pCgp120-, pIRESGp120-Tat-, and pCgp120 plus pCTat-transfected cell lysates as compared with pCDNA (p = 0.0001), and there was no significant difference between the expression levels in pCgp120- and pIRESGp120-Tat-transfected cells (p = 0.587) or between pCgp120 and pCgp120 plus pCTat (p = 0.797). We have also performed ELISA for Tat expression in pCTat-, pIRESGp120-Tat-, and pCgp120 plus pCTat-transfected cell lysates (Fig. 1B). Again, Tat expression by pIRESGp120-Tat- and pCgp120 plus pCTat-transfected cell lysates was similar to that of pCTat-transfected cell lysates (p = 0.131 and 0.068, respectively). In vivo expression in the muscle of immunized mice with pCDNA, pCgp120, pCTat, pIRESGp120-Tat, and pCgp120 plus pCTat was analyzed by ELISA using the muscle lysate for both gp120 and Tat. As shown in Fig. 1, C and D, the muscle lysates show expression of both gp120 and Tat as compared with pCDNA-immunized mice (p = 0.001). The expression levels of gp120 from pIRESGp120-Tat and pCgp120 plus pCTat was similar to those of pCgp120 (p = 0.154 and 0.654, respectively). The expression level of Tat was also similar in pIRESGp120-Tat and pCgp120 plus pCTat as compared with pCTat (p = 0.087 and 0.248, respectively).
Tat modulates gp120-specific humoral response

Serum was collected from immunized mice and ELISA was performed to assess gp120-specific Ab response. Ab response was well observed in the serum of pCgp120- and pIRESgp120-Tat-immunized mice as compared with pCDNA-immunized mice (p = 0.0007; Fig. 2A). We then analyzed the isotype profile of the gp120-specific immune response in the presence and absence of Tat. Less IgM was observed in pCDNA-, pCgp120-, pIRESgp120-Tat-immunized mice (Fig. 2B); however, a higher IgG2a response was observed in pCgp120-immunized mice as compared with pIRESgp120-Tat-immunized mice (p = 0.004). Tat coimmunization reduced the gp120-mediated IgG2a (Fig. 2C) response but enhanced IgG1 (p = 0.001; Fig. 2D), which are known to be controlled by IFN-γ and IL-4, respectively. These findings suggest that Tat may enhance Th2 responses, leading to IL-4- and IL-10-mediated suppression of the Th1 response and reduced gp120-mediated IgG2a response.

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 plus pCTat, and pIRESgp120-Tat i.m. on 0, 15, and 30 days. The mice were sacrificed 10 days after the last immunization. Splenocytes were isolated from mice and splenocytes were isolated for analyzing cellular immune responses. The CTL assay for gp120 and Tat was performed with splenocytes isolated from different groups of mice as described in Materials and Methods. The gp120-specific CTL response observed in mice immunized with pCgp120 alone was reduced in pIRESgp120-Tat-immunized mice (p = 0.010) as shown in (Fig. 3A). However, Tat-specific CTL activity did not

FIGURE 2. gp120-specific humoral response and Tat-induced modulation of isotypes in coimmunized mice. The sera from pCDNA-, pCgp120-, and pIRESgp120-Tat-immunized WT mice were assayed for the gp120-reactive Ab response followed by isotype analysis (IgM, IgG2a, IgG1) on gp120 protein-coated ELISA plates. A, Analysis of gp120 Ab response; B, gp120-specific IgM profile; C, gp120-specific IgG2a profile; and D, gp120-specific IgG1 profile.

FIGURE 3. Tat suppresses T cell responses in mice co-immunized with gp120. Splenocytes from pCDNA-, pCgp120-, pCTat-, pCgp120 + pCTat-, and pIRESgp120-Tat-injected mice were plated 2 × 10^5/well in 24-well plates with gp120 or Tat peptide-pulsed 1 × 10^5 irradiated naive splenocytes. After 5 days of culture, viable cells were harvested and plated against [3H]Tdr-incorporated gp120 or Tat peptide-pulsed EL-4 cells and tested for their cytolytic activity in a standard 3 h in JAM test. The E/T ratios used are shown. Each data point is the mean of triplicate samples. Also, splenocytes from injected mice were plated 2 × 10^5 cells/well in 96-well plates and was pulsed with 10 μg of gp120 or Tat peptides or without Ag (medium). Proliferation was assessed using a [3H]thymidine incorporation assay. The results represent three individual experiments and error bars represent the mean ± SD of a given group. A, gp120-specific CTL response in pCDNA-, pCgp120-, pCgp120 + pCTat-, and pIRESgp120-Tat-immunized mice. B, Tat-specific CTL response in pCDNA-, pCTat-, pCgp120 + pCTat-, and pIRESgp120-Tat-immunized mice. C, gp120-specific proliferation in splenocytes from mice immunized with different vectors mentioned above. D, Tat-specific proliferation in splenocytes from mice immunized with different vectors mentioned above.
alone or with Tat.

C mice immunized with pCgp120 along with pCTat (Fig. 3A). We then examined whether the effect of Tat on the gp120-specific T cell response was due to immunization with bicistronic vector or the effect was universal irrespective of the nature of Tat immunization. Similar results on the gp120-specific CTL response were also obtained with mice immunized with pCgp120 alone along with pCTat (Fig. 3A). The above data clearly indicate that Tat-induced suppression does not take place at the level of coexpression of genes but at a later stage, when the immune effectors are activated.

**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 the gp120 or Tat peptide (Fig. 3, C and D). Strikingly, the proliferative responses from mice immunized with bicistronic pIRESgp120-Tat were diminished when cells were stimulated with the gp120 peptide ($p = 0.015$) as compared with splenocytes stimulated with the Tat peptide (Fig. 3, C and D). This observation again suggests that Tat has an immunosuppressive effect on the gp120-specific T cell response. Similar results were also obtained in mice coimmunized with the pCgp120 plus pCTat vector (Fig. 3C). Thus, the results obtained from both the CTL and proliferation assay indicate that Tat has a suppressive effect on the immune response toward gp120.

**Tat also suppresses OVA-induced immune response**

To test whether the immunosuppressive effect of Tat on viral envelope protein also holds true for other Ags, we have used OVA Ag for coimmunization with Tat. There are several reports that envelope protein also holds true for other Ags, we have used OVA Ag for coimmunization with Tat. There are several reports that

show a significant change in mice immunized with pCTat or pIRESgp120-Tat as compared with the disparity observed in the gp120-specific CTL response (Fig. 3B). We then examined whether the effect of Tat on the gp120-specific T cell response was due to immunization with bicistronic vector or the effect was universal irrespective of the nature of Tat immunization. Similar results on the gp120-specific CTL response were also obtained with mice immunized with pCgp120 alone along with pCTat (Fig. 3A). The above data clearly indicate that Tat-induced suppression does not take place at the level of coexpression of genes but at a later stage, when the immune effectors are activated.

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isolated from IL-10-deficient mice and stimulated with GST and GST-Tat. Inversely, T cells isolated from naive IL-10-deficient mice were cocultured with peritoneal macrophages from WT mice and stimulated with GST and GST-Tat. IFN-γ and IL-10 secretion was analyzed by ELISA in the culture supernatant and the RNA from the cocultured cells were used for RT-PCR of IFN-γ and IL-10. The up-regulation of IL-10 indicates that Tat induces IL-10 from both T cells and macrophages. However, T cells seem to secrete more IL-10 as compared with macrophages, a profile confirmed by RT-PCR. These results indicate that Tat induces IL-10 secretion from both T cells and macrophages.

gp120-specific Ab response in IL-10−/− mice in the presence of Tat

To investigate the impact of IL-10 on observed Tat-mediated immunosuppression, we have immunized IL-10−/− mice with pCDNA, pCgp120, and pRESgp120-Tat vectors as done in the case of WT mice. Serum was collected 10 days after the last immunization and the isotype profile of gp120-specific Ab was analyzed. The results indicate that IgM was minimal in pCDNA-, pCgp120-, and pRESgp120-Tat-immunized mice, but the presence of IgG2a was clearly more evident than IgG1 in both pCgp120- and pRESgp120-Tat-immunized mice.
There was no significant difference in the IgG2a response in pCgp120- and pIRESgp120-Tat-immunized mice ($p$ = 0.486). This observation was in contrast to WT mice, where IgG1 was higher in pIRESgp120-Tat-immunized mice but IgG2a was higher in pCgp120 mice (Fig. 2, C and D). The absence of switch from IgG2a to IgG1 in the presence of Tat in IL-10-deficient mice indicates that IL-10 possibly plays a role in Tat-induced changes in isotype switching of coimmunized gp120 Ag.

**IL-10 deficiency augments T cell response in pIRESgp120-Tat-immunized mice**

To further analyze the role of IL-10 in Tat-mediated immunosuppression, we have immunized WT and IL-10−/− mice. Groups of mice ($n$ = 4) were immunized with 100 μg of pCDNA, pCgp120, pCTat, and pIRESgp120-Tat i.m. at 0, 15, and 30 days and were sacrificed 10 days after the last immunization. gp120-specific CTL assays were performed with splenocytes isolated from immunized mice. A strong gp120-specific CD8+ T cell response was observed in both pCgp120- and pIRESgp120-Tat-injected IL-10−/− mice (Fig. 8A, right panel) as compared with WT mice, where the gp120-specific CTL responses were diminished in the presence of Tat ($p$ = 0.008; Fig. 8A, left panel), suggesting that Tat mediates its effect through IL-10. The Tat-specific CTL response remains unchanged in both IL-10−/− and WT mice (Fig. 8B). Proliferation assays were also performed with immunized WT and IL-10−/− mice but no immunosuppression was observed in pIRESgp120-Tat-immunized IL-10−/− mice upon gp120 stimulation as observed in WT mice ($p$ = 0.018; Fig. 8C). There was no difference...
lytic activity in a standard 3.5-h JAM test. The E:T ratio is as shown. Each data point is the mean of triplicate samples. The results represent three individual experiments and the error bars represent the mean ± SD of a given group.

FIGURE 9. CD4+ T cells from Tat-coimmunized mice are responsible for IL-10-mediated suppressor function and coadministration of rIL-10 restores the suppressive function in IL-10−/− mice. A, CD8+ T cells isolated from B6 WT mice immunized with pCgp120 were cocultured with CD4+ T cells isolated from different immunized B6 WT and IL-10−/− mice as shown by the 1:2 ratio in a 6-well plate. gp120 peptide-pulsed peritoneal macrophages were used as APCs in the coculture. After 5 days of culture, cells were harvested and plated against [3H]Thymidine (H11002)-incorporated gp120-pulsed EL-4 cells and tested for their cytolytic activity in a standard 3.5-h JAM test. The E:T ratio is as shown. Each data point is the mean of triplicate samples. The results represent three individual experiments and the error bars represent the mean ± SD of a given group. B, CD8+ T cells isolated from IL-10−/− mice immunized with pCgp120 were cocultured with CD4+ T cells isolated from different immunized B6 WT and IL-10−/− mice as shown by the 1:2 ratio in a 6-well plate. gp120 peptide-pulsed peritoneal macrophages were used as APCs in the coculture. After 5 days of culture, viable CD8+ T cells were harvested and plated against [3H]Thymidine (H11002)-incorporated gp120-pulsed EL-4 cells and tested for their cytolytic activity in a standard 3.5-h JAM test. The E:T ratio is as shown. Each data point is the mean of triplicate samples. The results represent three individual experiments and the error bars represent the mean ± SD of a given group.

in the Tat-stimulated proliferation response in WT or IL-10-deficient mice. The collective data obtained from the CTL and proliferation assay in IL-10−/− mice immunized with pIRESgp120-Tat show that the immunosuppressive effect of Tat is mediated through IL-10, which plays a disease-exacerbating role by suppressing the host-protective T cells and inhibiting IFN-γ production.

CD4+ T cell-secreted IL-10 reduces the gp120-specific CTL response in Tat-coimmunized mice

The CD8+ T cell responses are dependent on concurrent help from CD4+ T cells (37, 38). Tat is known to be secreted by infected cells and can act on other cells, including macrophages (39) 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 the CD4+ T cell response in HIV infection has long been known to be poor. In addition, there are studies which suggest that CD8+ T cells have two subsets, Tc1 and Tc2. These subsets display a cytokine profile that resembles the Th1 and Th2 subsets (40). Therefore, to check whether IL-10 secreted possibly by such a Tc2 subset might be involved in the observed immunosuppression, we have immunized WT and IL-10-deficient mice with pCgp120 and pIRESgp120-Tat and isolated CD4+ T cells and cocultured them with CD8+ T cells isolated from pCgp120-immunized WT or IL-10-deficient mice. gp120 peptide-pulsed irradiated macrophages from naive WT mice were used as APCs in the coculture. The coculture was kept for 5 days in sterile conditions and the gp120-specific CTL assay was performed thereafter. CD8+ T cells from pCgp120-immunized WT mice cocultured with CD4+ T cells from pIRESgp120-Tat-immunized WT mice show a suppressed CTL response (p = 0.001) as compared with a coculture containing CD4+ T cells from pCgp120-immunized WT mice (Fig. 9A). Tat-mediated suppressor activity was not observed in CD4+ T cells isolated from pIRESgp120-Tat-immunized IL-10−/− mice as compared with the pCgp120-immunized IL-10−/− mice (p = 0.581). Tat-mediated suppression is regained in the CD4+ T cells isolated from IL-10-deficient mice coimmunized with pIRESgp120-Tat and rIL-10 (Fig. 9A). Similar results were also obtained with CD8+ T cells from pCgp120-immunized IL-10-deficient mice (Fig. 9B), indicating thereby that IL-10 secreted from CD8+ T cells may not be involved in the Tat-mediated immunosuppression observed here. All of these results indicate that suppressive activity of Tat is mediated through CD4+ T cells expressing IL-10 (41, 42). Our results also indicate that IL-10 secreted from CD8+ T cells, if any, does not play a significant role in the observed immunosuppression.

Discussion

Tat, the HIV-1 transactivator of transcription protein, is the most important regulator of the virus, because it is essential for virus replication. It is a relatively conserved protein among all of the viral isolates, and the status of Abs against Tat protein has been shown to be directly proportional to the progression of the disease in several clinical studies (43). 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 multicomponent vaccine candidates (44). Several studies indicate that Abs to Tat play a host-protective role in HIV infection and thus have been also considered as a target for the development of novel AIDS therapy (45). Although Tat has been found to be a successful candidate, few reports imply that Tat acts as an immunosuppressor for the coimmunized viral Ags (18, 19). The mechanism by which Tat mediates its immunosuppression remains to be elucidated. To identify unambiguously the role of Tat in the immune response of a coimmunized Ag, we have constructed a bicistronic vector expressing Tat and gp120 from CMV promoter with an internal ribosome entry site and have used i.m. DNA immunization in mice. Isootype analysis of the elicited Ab indicates that Tat induces a switch from IgG2a to IgG1 in mouse immunized with gp120 and Tat. Our results also show that Tat suppresses the T cell responses against coimmunized gp120 Ag. This immunosuppression by Tat was observed not only in mice immunized with the bicistronic vector but also where gp120 and Tat have been immunized separately. The immunosuppressive activity of Tat was not confined to viral Ag but it also suppressed the immune response of unrelated Ag, OVA. Furthermore, cytokine analysis of stimulated splenocytes showed induction of IL-10 in Tat-immunized mice as compared with IFN-γ in gp120-immunized mice. Finally, Tat-mediated induction of IL-10 was confirmed by stimulating a coculture of naive T cells (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 (46), could be a crucial player in the immune response to the virus. Tat-mediated immunosuppression has also been observed by a few other groups (18, 19), but we did not observe any suppression of the Ab response as reported by Cohen et al. (19) due to the presence of Tat. However, we have observed the Ab switch from IgG2a to IgG1 in the presence of Tat. Such reciprocity between the IgG1 and IgG2a isotype has been explained previously through IFN-γ (47) and could be happening here due to Tat-mediated induction of IL-10, which is known to inhibit the production and function of IFN-γ. Our results are apparently in contrast to another report which suggests that Tat coimmunization with gag leads to enhancement of the cellular immune response (48). This mixed outcome probably reflects species difference, route of immunization, difference in concentration or type of Tat immunogen, and nature of coimmunized Ag. Studies have also suggested that Tat in its native form binds to and is taken up by dendritic cells, in which Tat induces activation and maturation into myeloid dendritic cells (49). This increases the up-regulation of MHC class I and costimulatory molecules CD80, CD86, and LFA on the surface. Tat contains a cationic domain which may play a role in up-regulation of epitope-MHC class I complexes on the cell surface (50). However, in the present study, the down-regulation of the immune response toward coimmunized Ag is due to up-regulation of IL-10 (immunosuppressive cytokine) and down-regulation of IFN-γ, leading to an overall suppression of the cellular immune response. Recently, a study has shown that Tat induces IL-10 in the U937 cell line through Ets and sp1 transcription factors (51).

Our result clearly shows that Tat modulates the Ag-specific CD8+ T cell response toward gp120. There could be two mechanisms of down-regulation of the immune response by Tat. First, Tat probably impairs IFN-γ responsiveness of T cells, resulting in suppression of IFN-γ-mediated cytotoxic killing, although it has been demonstrated that chronic HIV-1 subjects develop a subset of HIV-1-specific CD8+ T cells that express IFN-γ but lack cytotoxic effector function (52, 53). Second, Tat seems to exploit the host’s IL-10-dependent autoregulatory or a feedback 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 (54). This phenomenon seems to be not only restricted to HIV-1 since certain other viruses like CMV and pox viruses skew the immune response similarly (55, 56). To look at the role of IL-10 in Tat-mediated immunosuppression, we have immunized IL-10−/− mice with bicistronic vector and found that the immunosuppressive effect of Tat was abrogated, suggesting that Tat uses the host’s immune regulation pathway for benefit of virus. IL-10 is known to inhibit a broad spectrum of the cellular immune response. It suppresses the function of APCs and T cells by inhibiting cytokine production, costimulation, 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 (57, 58). In addition, higher frequency of IL-10-producing CD4+ cells in HIV-1-infected individuals with progressive disease or active HIV replication has been reported as compared with 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 the gp120 immune response, the results of which indicate that CD4+ T cells from WT mice mediate the suppression through IL-10 as CD4+ T cells from IL-10-deficient mice failed to show immunosuppression. In summary, data presented in the present report clearly demonstrate for the first time that Tat suppresses the T cell response by inducing IL-10. The degree to which Tat modulates the CD8+ T cell response depends upon a shift in CD4+ T cell cytokine secretion in the immune inductive site.

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Disclosures
The authors have no financial conflict of interest.

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