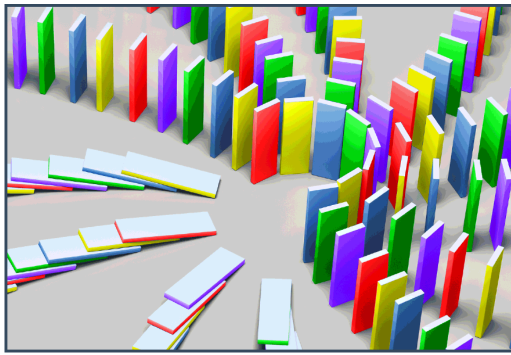
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# *Mycobacterium tuberculosis* Recall Antigens Suppress HIV-1 Replication in Anergic Donor Cells via CD8$^+$ T Cell Expansion and Increased IL-10 Levels[1]

**Shahin Ranjbar,* Nary Ly,† Sok Thim,‡ Jean-Marc Reynes,† and Anne E. Goldfeld[2]***

***Mycobacterium tuberculosis* (MTb) is the leading cause of death in the setting of AIDS. MTb enhances the pathogenicity and accelerates the course of HIV disease and, furthermore, infection with HIV-1 increases the risk of reactivation or reinfection with MTb. In this study, we show that host-specific recall responses to one pathogen, MTb, has a direct effect upon the regulation of a second pathogen, HIV-1. Using cells from immunocompetent former tuberculosis (TB) patients who displayed either a persistently positive (responsive) or negative (anergic), delayed-type hypersensitivity (DTH) reaction to intradermal injection of purified protein derivative (PPD), we investigated the effect of recall Ags to MTb upon the replication of HIV-1 primary isolates in vitro. We show that HIV-1 replication of a T cell-tropic isolate was significantly impaired in MTb-stimulated PBMC from PPD-anergic donors. Furthermore, these donors displayed a significant increase in CD8$^+$ T cells and IL-10 levels and lower levels of IL-2 and TNF-$\alpha$ relative to PPD-responsive donors in response to PPD stimulation. Strikingly, CD8$^+$ T cell depletion and blocking of IL-10 significantly increased HIV-1 replication in these PPD-anergic donors, indicating that an immunosuppressive response to MTb recall Ags inhibits HIV-1 replication in PPD-anergic individuals. Therefore, immunotherapeutic approaches aimed at recapitulating Ag-specific MTb anergy in vivo could result in novel and effective approaches to inhibit HIV-1 disease progression in MTb/HIV-1 coinfection.** *The Journal of Immunology,* **2004, 172: 1953–1959.**

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Tuberculosis (TB)[3] and AIDS are the largest causes of infectious disease death globally (1). In countries with a high incidence of TB and AIDS, reactivation of latent TB disease associated with HIV-1-induced immunosuppression is the major cause of TB disease in coinfected individuals (2). In addition, a proportion of TB cases are also due to infection with a second MTb strain (3) in patients who previously achieved a chemotherapeutic TB cure. Notably, TB disease accelerates the course of HIV-1 disease progression (4, 5) and is the largest single cause of death in the setting of AIDS (6). Since HIV-1 plasma viral load strongly predicts progression to AIDS and death (7, 8), host-specific immune responses to TB reactivation or reinfection that influence HIV-1 replication could thus be expected to impact survival of coinfected patients.

To investigate the impact of host-specific recall responses to *Mycobacterium tuberculosis* (MTb) Ags upon HIV-1 replication, we established an ex vivo HIV-1 infection model using cells from a unique cohort of immunocompetent HIV-negative former Cambodian pulmonary TB patients. These individuals, all of whom achieved a chemotherapeutic and microbiological cure of TB, do not manifest a delayed-type hypersensitivity (DTH) skin response to intradermal injection of purified protein derivative (PPD), but do manifest a response to the injection of other Ags such as mumps or *Candida* (9, 10). Concordant with these in vivo results, T cells from PPD-anergic donors display impaired responses to PPD but not to other Ags in vitro. By contrast, T cells from former TB patients with a positive skin reaction to PPD (PPD-responsive donors) in vivo respond equally to PPD and other Ags in vitro (9, 10). HIV-1 infection of cells from these PPD-anergic and PPD-responsive donors thus afforded us the unique opportunity of investigating Ag-specific recall responses to MTb upon replication of HIV-1 primary isolates.

Strikingly, we demonstrate that MTb Ag stimulation of PPD-anergic donor cells results in an immunosuppressive state resulting in a significant impairment of T-tropic HIV-1 replication. We show that there is a disproportionate expansion of CD8$^+$ T cells and increased IL-10 levels in anergic donor PBMC upon MTb Ag challenge and that CD8$^+$ T cell depletion and blocking IL-10 in vitro results in a significant restoration of HIV-1 replication. Thus, an immunosuppressive state induced by a host-specific recall response to MTb Ags can control the level of HIV-1 replication in certain individuals. These results provide new insight into immune responses to MTb infection and suggest that host-specific responses to MTb have a significant impact upon HIV-1 disease pathogenesis in the coinfected host.

## Materials and Methods

### *Donors*

PBMC were obtained from 12 healthy individuals with a history of pulmonary TB with either a positive intradermal skin reaction (>10 mm) to PPD (5TU; Adventis Pasteur, Swiftwater, PA) (PPD$^+$; six individuals) or negative (no skin reaction, anergic) to PPD (six individuals) during and following successful TB chemotherapy. The patients were recruited from a cohort of consenting volunteers identified by the Cambodia Health Committee in the Svay Rieng Province, Cambodia, as previously described (9, 10). The diagnosis of clinical TB was made on the basis of medical history,

*Center for Blood Research and Department of Medicine, Harvard Medical School, Boston, MA 02115; †Unité de Virologie, Institut Pasteur du Cambodge, Phnom Penh, Cambodia; and ‡Cambodian Health Committee, Phnom Penh, Cambodia

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[2] Address correspondence and reprint requests to Dr. Anne.E. Goldfeld, Center for Blood Research, Harvard Medical School, 800 Huntington Avenue, Boston, MA 02115. E-mail address:goldfeld@cbr.med.harvard.edu

[3] Abbreviations used in this paper: TB, tuberculosis; MTb, *Mycobacterium tuberculosis*; PPD, purified protein derivative; DTH, delayed-type hypersensitivity; $TCID_{50}$, 50% tissue culture-effective dose; MFI, mean fluorescence intensity.

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physical examination, and the detection of acid-fast bacilli in sputum by light microscopy. All 12 had completed TB therapy according to the protocol of the Cambodian National TB program: isoniazid/rifampin/pyrazinamide/ethambutol for 2 mo followed by ethambutol and isoniazid for 6 additional months. All patients were tested for clearance of acid-fast bacilli from their sputum at 2 and 6 mo after beginning anti-TB therapy and at the end of therapy. All PPD-anergic as well as PPD-responsive donors displayed a positive skin reaction to another intradermal Ag such as *Candida* or mumps and all 12 were HIV-1 and HIV-2 negative as determined by the ELISA assay as previously described (10). Whole heparinized blood was collected and kept at room temperature and processed <24 h after collection. The experiments were repeated on sets of 12 individuals (6 anergic and 6 PPD-responsive) three different times over a 2-year period except for the $CD8^+$ T cell depletion experiment, which was performed twice.

### *Viruses*

The viruses used in this study were HIV-$1_{KR25}$ (T-tropic) and HIV-$1_{98IN22}$ (M-tropic). HIV-1 $_{KR25}$ was isolated from a 28-year-old Cambodian male with advanced AIDS (S.R. and A.E.G., unpublished data) according to standard techniques (11). HIV-$1_{98IN22}$ was obtained from the Centralized Facility for AIDS Reagents, National Institute for Biological Standard and Control, United Kingdom and was isolated from a 27-year old male from India without evidence of immunocompromise (12). We determined the secondary receptor usage of HIV-$1_{KR25}$ and HIV-$1_{98IN22}$ by incubating them with a set of U87.CD4 cell lines expressing CCR1-CCR3, CCR5, or CXCR4 receptor (13) and found that HIV-$1_{KR25}$ utilizes only CXCR4 and is thus T-tropic (X4), whereas HIV-$1_{98IN22}$ utilizes only CCR5 as its secondary receptor and is thus M-tropic (R5; data not shown).

### *Cell culture and PPD stimulation*

PBMC were isolated by centrifugation on Ficoll-Hypaque gradients as previously described (10), $2 \times 10^6$ PBMC were cultured in 24-well plates in 1.5 ml of RPMI 1640 medium supplemented with 10% FCS containing 20 U/ml penicillin and 20 $\mu$g/ml streptomycin and were mock stimulated or stimulated with 10 $\mu$g/ml PPD as indicated. Separate cultures were set up for each time point and the cells were not washed and the medium was not changed for the duration of the experiment. At days 3, 8, and 14 poststimulation, culture supernatants were collected and IL-10, IL-2, IFN-$\gamma$, and TNF-$\alpha$ levels of cultures were measured using OptEIA ELISA kits (BD PharMingen, San Diego, CA).

### *HIV-1 infection*

The cells were set up as above and were mock stimulated or stimulated with PPD (10 $\mu$g/ml) or PHA (3.5 $\mu$g/ml) and IL-2 (20 IU/ml) and were incubated at 37°C for 2 days before being infected with 10 tissue culture-effective dose (TCID) 50%/ml HIV-$1_{KR25}$ or 50 $TCID_{50}$/ml HIV-$1_{98IN22}$. The cultures were further incubated at 37°C in 5% $CO_2$ and 95% humidity for 1, 6, 12, and 17 days after HIV-1 infection, after which supernatants were collected and IL-10, IL-2, IFN-$\gamma$, and TNF-$\alpha$ levels of cultures were measured as described above. The HIV-1 p24 levels were determined in infected cultures by measuring p24 Ag (picograms per milliliter) in the supernatants using a p24 ELISA kit (NEN, Boston, MA). The medium of the cultures that were terminated at days 1, 6, and 12 postinfection were not changed for the duration of the experiment; however, cultures terminated at day 17 were fed with fresh patient PBMC on day 11 postinfection to maintain viability of the cultures.

### *$CD8^+$ T cell depletion*

$CD8^+$ T cells were depleted from PBMC by positive selection using magnetic beads coated with an anti-CD8 mAb (Dynal Biotech, Great Neck, NY). The purity of the cells was ~96% as judged by flow cytometry (data not shown). $CD8^+$ T cells depleted or bulk PBMC were stimulated with 10 $\mu$g/ml PPD for 2 days before infection with 10 $TCID_{50}$ HIV-$1_{KR25}$. Supernatants were collected at day 3 postinfection and virus replication was determined by measuring HIV-1 p24 Ag in the culture supernatants.

### *Neutralization of IL-10 and TNF-$\alpha$*

mAbs to IL-10 (22 $\mu$g/ml; R&D Systems, Minneapolis, MN) or TNF-$\alpha$ (2.5 $\mu$g/ml; R&D Systems) were added at the time of PPD stimulation of PBMC cultures (time zero) and cells were infected with HIV-$1_{KR25}$ or HIV-$1_{98IN22}$ 2 days later as described above.

### *Cell proliferation*

The proliferation of total PBMC from PPD-anergic and PPD-responsive donors was assessed by culturing $2 \times 10^5$ cells/well in a final volume of 200 $\mu$l of medium containing 10 $\mu$g/ml PPD or 3.5 $\mu$g/ml PHA and 20 IU/ml IL-2. Cultures were incubated for 3 and 5 days at 37°C and pulsed with 1 $\mu$Ci of [$^3$H]thymidine for the final 20 h. [$^3$H]Thymidine incorporation was expressed in cpm.

### *Flow cytometry*

The percentage of $CD3^+CD4^+$, $CD3^+CD8^+$ T cells and $CD4^+CXCR4^+$ cells was determined by flow cytometry using conjugated mAbs CD3-FITC, CD4-Cy5, CD8-PE, and CXCR4-PE (R&D Systems). Triple staining was performed by incubation of $2 \times 10^5$ cells with the conjugated Abs in HBSS with 2% FCS for 20 min at 4°C. Cells were then washed in HBSS and resuspended in FACS buffer containing 2.7% formaldehyde for flow cytofluorometric analysis. Nonspecific staining was controlled for by incubation of cells with appropriately labeled mouse IgG1 Abs (R&D Systems). Data were acquired and analyzed with CellQuest software (BD Biosciences, Mountain View, CA).

### *Statistical analysis*

Results are expressed as a mean ± SEM. The Mann-Whitney $U$ test was used to analyze statistical differences between two groups. A value of $p < 0.05$ was taken as statistically significant.

## Results

### *Cytokine profiles of PPD-anergic and PPD-responsive donors are distinct and are not influenced by HIV-1 infection*

We isolated PBMC from six HIV-negative immunocompetent persistently PPD-responsive and six PPD-anergic former pulmonary TB patients who had achieved chemotherapeutic cure of TB. Long-term follow-up of the anergic individuals after successful treatment revealed that lack of DTH to PPD was not a transient phenomenon associated with active pulmonary TB, but was an Ag-specific and persistent finding in the anergic individuals (9, 10).

Cells were mock stimulated or stimulated with PPD and were either uninfected or infected with HIV-1 two days after PPD stimulation and the levels of IL-10, IL-2, IFN-$\gamma$, and TNF-$\alpha$ were measured (Fig. 1). By 3 days after PPD stimulation, there was a significant increase in the production of all four cytokines in both PPD-anergic and -responsive cultures. However, although after PPD stimulation, IL-2, IFN-$\gamma$, and TNF-$\alpha$ levels were higher in PPD-responsive cultures, by contrast, IL-10 levels were higher in PPD-anergic cultures at all time points (Fig. 1*A*).

We next sought to determine whether the different cytokine milieu elicited by PPD stimulation in anergic vs PPD-responsive PBMC was influenced by HIV-1 infection or viral tropism. A subset of PPD-anergic or PPD-responsive donor PBMC were thus infected with a T lymphocyte (T)-tropic HIV-$1_{KR25}$ (Fig. 1*B*) or a macrophage (M)-tropic HIV-$1_{98IN22}$ (Fig. 1*C*) primary isolate 2 days after PPD or mock stimulation, and cytokine levels were measured 1, 6, and 12 days after infection and thus 3, 8, and 14 days after PPD stimulation.

Without PPD prestimulation, there were no detectable changes in the cytokine profiles of HIV-1-infected vs uninfected cells (Fig. 1). Whereas HIV-1 infection of PPD-stimulated cells further increased the levels of all four cytokines as compared with HIV-1-uninfected PPD-stimulated cultures, the pattern of cytokine induction in PPD-anergic and PPD-responsive cells remained unchanged (Fig. 1). Thus, the cytokine profile of PPD-stimulated cells is distinct in PPD-anergic and PPD-responsive PBMC and is not changed by HIV-1 infection.

### *T-tropic HIV-1 replication is significantly lower in the PPD-stimulated PBMC from PPD-anergic compared with PPD-responsive donors*

We next measured p24 levels in the infected culture supernatants to determine whether the different cytokine milieus of PPD-anergic and -responsive PBMC affected HIV-1 replication. Strikingly, infection of PPD-stimulated cells with T-tropic HIV-$1_{KR25}$ resulted

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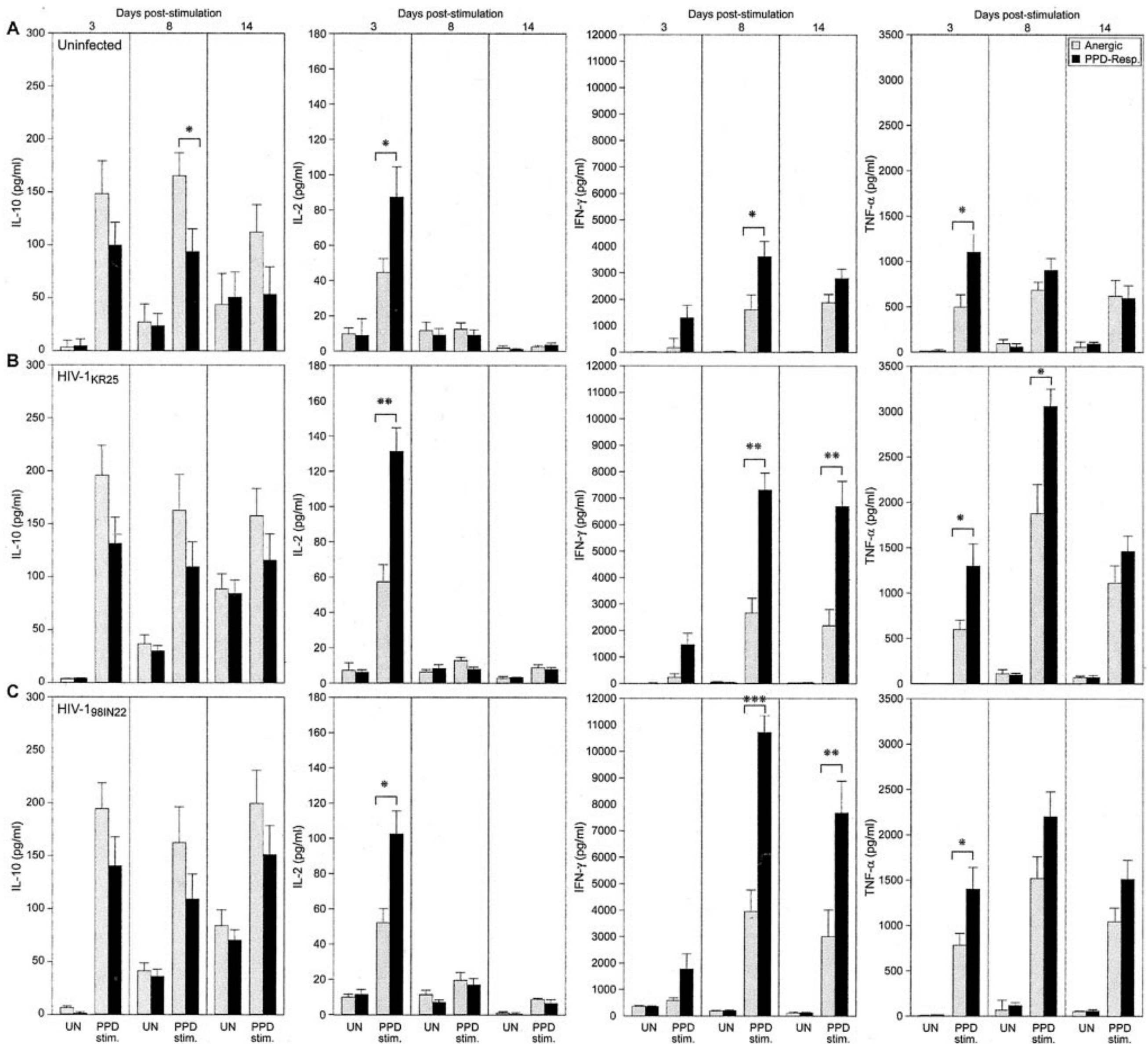


**FIGURE 1.** Cytokine profiles of PPD-anergic and PPD-responsive donors are distinct. PBMC from PPD-anergic (□) and PPD- responsive (■) donors were cultured at day 0 and either mock stimulated (UN) or PPD-stimulated (PPD stim) for the times indicated. Levels of IL-10, IL-2, IFN-γ, and TNF-α were determined in HIV-1-uninfected (*A*), T-tropic HIV-$1_{KR25}$-infected (*B*), or M-tropic HIV-$1_{98IN22}$-infected cultures (*C*). The cells were infected with virus at day 2 after PPD stimulation and the cytokines were measured at days 3, 8, and 14 poststimulation. Separate cultures were set up for each time point and the cells were not washed and the medium was not changed for the duration of the experiment. Results are expressed as a mean ± SEM and are representative of three separate experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.

in dramatically lower virus replication in PPD-anergic compared with PPD-responsive cultures (Fig. 2*A*). In PPD-stimulated and HIV-$1_{KR25}$-infected anergic cultures, p24 levels were ~5-fold lower ($p < 0.001$) at day 6 and 3-fold lower ($p < 0.005$) at day 12 postinfection as compared with PPD-responsive cultures, whereas no difference was observed in p24 levels of both subgroups stimulated with the positive control PHA/IL-2 (Fig. 2*A*). By contrast, the p24 levels in the PPD-responsive cultures were similar whether stimulated with PPD or PHA/IL-2 at all time points. Notably, infection with the M-tropic HIV-$1_{98IN22}$ after stimulation with PPD or with PHA/IL-2 resulted in very low levels of virus replication and there was no difference in the p24 levels between PPD-anergic and -responsive donors up to 17 days postinfection (Fig. 2*B*).

*Blocking of IL-10 significantly increases HIV-1 replication in anergic donor cells and blocking of TNF-α significantly decreases HIV-1 replication in PPD-responsive donor cells*

Given the higher levels of IL-10 in anergic PBMC compared with IL-10 levels in PPD-responsive PBMC and given the ability of IL-10 to inhibit Ag-specific responses in general (14–16), we next tested the effect of neutralizing IL-10 upon HIV-$1_{KR25}$ p24 levels. Using an anti-IL-10 mAb at the time of PPD stimulation, p24 levels were significantly increased in PPD-anergic HIV-$1_{KR25}$-infected cultures at day 6 ($p < 0.05$) and day 12 ($p < 0.01$) postinfection. By contrast, blocking IL-10 only minimally increased p24 levels in PPD-responsive cultures at all time points. Thus, blocking IL-10 relieved the inhibition of HIV-$1_{KR25}$ replication in PPD-anergic cells.

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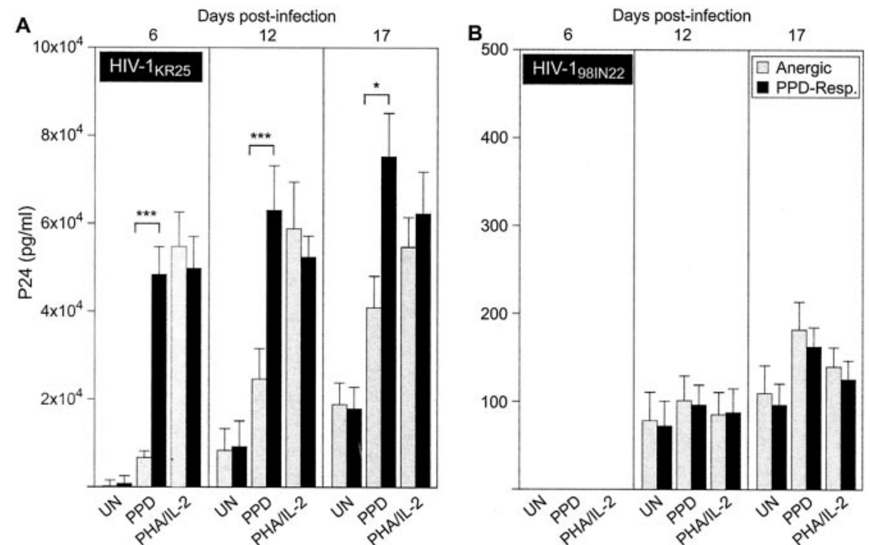


**FIGURE 2.** T-tropic HIV-1, but not M-tropic HIV-1, replication is significantly impaired in PPD-anergic as compared with PPD-responsive donor cells. PBMC from PPD-anergic (□ and PPD-responsive (■) donors were unstimulated (UN) or stimulated with PPD or PHA/IL-2 for 2 days before infection with T-tropic HIV-$1_{KR25}$ (*A*) or M-tropic HIV-$1_{98IN22}$ (*B*). p24 levels were determined at days 6, 12, and 17 postinfection. The medium of the cultures that were terminated at days 6 and 12 was not changed for the duration of the experiment. However, cultures terminated at day 17 were fed with fresh patient PBMC on day 11 postinfection to maintain viability of the cultures. Results are expressed as a mean ± SEM and are representative of three separate experiments.*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$. Please note the different scales used for measuring p24 levels in cell infected with T-tropic HIV-$1_{KR25}$ (*A*) or M-tropic HIV-$1_{98IN22}$ (*B*).

Since TNF-$\alpha$ levels were higher in PPD-responsive cultures, and given that TNF-$\alpha$ enhances HIV-1 replication (17), we next tested the effect of blocking TNF-$\alpha$ upon p24 levels in both groups of donor cells. When PPD-responsive cultures were treated with an anti-TNF-$\alpha$ mAb at the time of PPD stimulation, p24 levels of HIV-$1_{KR25}$-infected cultures were significantly decreased at day 6 ($p < 0.005$) and day 17 ($p < 0.01$) postinfection, whereas p24 levels were minimally reduced in the PPD-anergic cultures at all time points after infection (Fig. 3*A*). By contrast, there was no observable effect of blocking TNF-$\alpha$ or IL-10 on p24 levels in cultures infected with the M-tropic HIV-$1_{98IN22}$ (Fig. 3*B*). This could be due to very low levels of HIV-$1_{98IN22}$ replication within the time points studied.

Thus, the immunosuppressive cytokine milieu (with relatively higher IL-10 levels) elicited by MTb recall Ags in PPD-anergic cultures compared with the immunostimulatory milieu elicited in PPD-responsive cultures (with relatively higher TNF-$\alpha$ and IL-2 levels) had a significant impact upon the replication of a HIV-1 T-tropic primary isolate. Furthermore, blocking TNF-$\alpha$ or IL-10 did not influence the replication of the M-tropic HIV-1 primary isolate in the period of time studied.

### *CXCR4 receptor expression and cellular proliferation in PPD-stimulated PPD-anergic and PPD-responsive PBMC are equivalent*

Several reports have demonstrated an important role for $CD4^+$ T cell activation and CXCR4 receptor expression in the ability of T- tropic HIV-1 to establish a productive infection (18–20). Since cytokines can influence the expression of CXCR4, we next examined the percentage of $CD4^+$ cells expressing CXCR4 and the intensity (mean fluorescence intensity (MFI)) of this receptor in PPD-anergic and -responsive PBMC stimulated with PPD. While following PPD stimulation for 3 days, the percentage of $CD4^+$ cells expressing CXCR4 was slightly higher in PPD-responsive cells and the MFI of CXCR4 was marginally higher in anergic cells, PPD stimulation significantly increased the CXCR4 intensity in both groups of donor $CD4^+$ cells (Fig. 4). Therefore, differences in CXCR4 receptor expression and intensity could not be responsible for the significant difference in HIV-$1_{KR25}$ p24 levels observed between PPD-anergic and -responsive PBMC stimulated with PPD.

We next stimulated bulk PBMC from anergic and PPD-responsive PBMC with PPD or PHA/IL-2 for 3 and 5 days and assessed

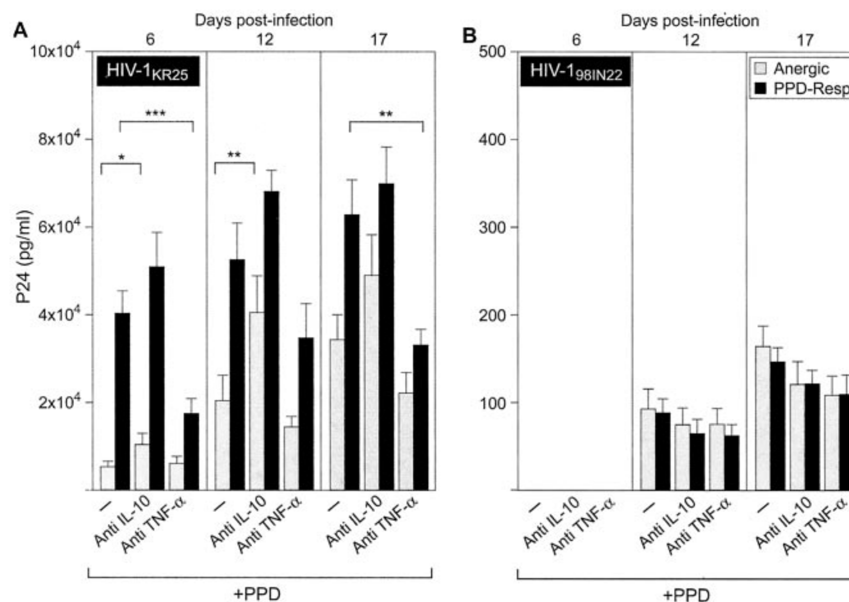


**FIGURE 3.** Blocking IL-10 and TNF-$\alpha$ have opposite effects upon T-tropic HIV-1 replication. PBMC from PPD-anergic (□) and PPD-responsive (■) donors were stimulated with PPD in the presence or absence of anti-IL-10 or anti-TNF-$\alpha$ mAbs as indicated for 2 days before being infected with HIV-$1_{KR25}$ (*A*) or HIV-$1_{98IN22}$ (*B*). The p24 levels were measured at days 6, 12, and 17 after virus infection. Results are expressed as a mean ± SEM and are representative of three separate experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.

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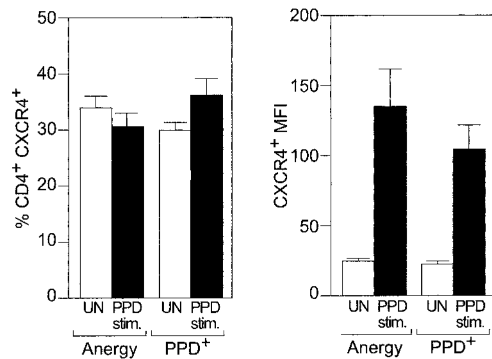


**FIGURE 4.** CXCR4 expression is similar in PPD-anergic and -responsive PBMC. PPD-anergic and -responsive PBMC were freshly isolated (□) or were stimulated with PPD (■) for 3 days and the percentage of $CD4^+CXCR4^+$ cells was determined. Data are represented as the difference between specifically stained cells and nonspecifically stained cells obtained by setting a quadrant marker for nonspecific staining in dot plots. Data on MFI are represented as the difference between MFI of specifically stained cells and nonspecifically stained cells obtained by setting a quadrant marker for nonspecific staining in dot plots. Results are expressed as a mean ± SEM and are representative of three separate experiments.

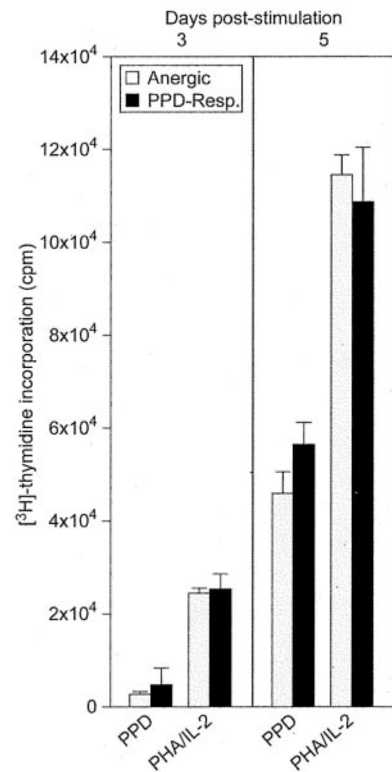


**FIGURE 5.** Cellular proliferation of PPD-anergic and -responsive bulk PBMC is equivalent. The proliferation of total PBMC from PPD-anergic (□) and PPD-responsive (■) donors was measured by $[^3H]$thymidine incorporation (expressed in cpm) in response to stimulation with PPD or PHA/IL-2 for 3 and 5 days as indicated. Results are expressed as a mean ± SEM and are representative of three separate experiments.

cellular proliferation by (21) thymidine incorporation. In response to PPD stimulation, proliferation was slightly higher in the PPD-responsive cells as compared with anergic cells, whereas there was no difference in the two donor subgroups stimulated with PHA/IL-2 (Fig. 5). Intriguingly, PHA/IL-2 stimulation resulted in equivalent T-tropic virus p24 levels in PPD-anergic and -responsive cultures (Figs. 2 and 5). However, although proliferation was equivalent in PPD-stimulated anergic and PPD-responsive cultures, p24 levels were significantly different in the two donor groups (Figs. 2 and 5). Thus, proliferation of bulk PBMC stimulated with MTb recall Ag is not concordant with T-tropic HIV-1 replication in anergic and PPD-responsive PBMC.

### *PPD-anergic $CD8^+$ T lymphocytes are significantly increased in response to PPD stimulation*

Previous studies have shown that both $CD4^+$ and $CD8^+$ T cells play an active role in the immune response to MTb Ags (22, 23). Although PPD presentation by autologous APC to $CD4^+$ T cells results in significantly lower $CD4^+$ T cell proliferation in PPD-anergic cells as compared with PPD-responsive cells (9), the effect of PPD stimulation upon $CD8^+$ T cell expansion in this system was unknown. As shown in Fig. 6, no difference in the CD4:CD8 ratio was observed in resting PPD-anergic and -responsive donor cells. Strikingly however, after 3 days of PPD stimulation, there was a significant increase in the percentage of $CD8^+$ T cells (from ~20 to 33%, $p < 0.01$) and there was a decrease in the $CD4^+$ T cells (from ~39 to 25%), resulting in a dramatic decrease in the CD4:CD8 ratio of PPD-anergic PBMC (from 1.92 to 0.77; Fig. 6). By contrast, PPD stimulation of PPD-responsive PBMC resulted in nonsignificant increases in both $CD8^+$ T cells (from 18 to 26%) and $CD4^+$ T cells (from 30 to 33%) and the CD4:CD8 ratio remained above one (1.23; Fig. 6). The percentage of CD4CD8 double-positive cells was also increased in both subgroups to a similar level. Thus, following MTb recall Ag stimulation, there is a disproportional expansion of $CD8^+$ T cells and a lack of $CD4^+$ T cell proliferation in PPD-anergic compared with PPD-responsive PBMC from hosts previously exposed to MTb.

### *MTb-activated $CD8^+$ T cells significantly suppress HIV-$1_{KR25}$ levels in PPD-anergic PBMC*

Given that activated $CD8^+$ T cells are known to suppress HIV (24–26), we next investigated the effect of depleting $CD8^+$ T cells from PPD-anergic and PPD-responsive bulk PBMC upon HIV-$1_{KR25}$ replication. Bulk PBMC and $CD8^+$ T cell-depleted PBMC from both donor subgroups were PPD stimulated for 2 days before being infected with HIV-$1_{KR25}$ and p24 levels were then measured 3 days following infection. Depletion of $CD8^+$ T cells significantly increased p24 levels in PPD-anergic (~2.5-fold, $p < 0.01$) cells, whereas there was only a nonsignificant increase in p24 levels in PPD-responsive cultures (~1.7-fold, $p > 0.1$; Fig. 7). Taken together, these results demonstrate that MTb Ag-stimulated $CD8^+$ T cells differentially suppress HIV-1 expression in PPD-anergic donor PBMC.

## Discussion

We have shown that an immunosuppressive and reversible host response to MTb recall Ags resulting in increased IL-10 levels and $CD8^+$ T cell expansion significantly suppresses HIV-$1_{KR25}$ replication in cells from PPD-anergic donors in vitro. These results thus demonstrate that PPD-anergic individuals have an advantage in controlling HIV-1 viral load if re-challenged with MTb Ags.

Even using an optimal anti-TB chemotherapeutic regimen, it is estimated that ~5–10% of patients with apparent cure, relapse with TB disease (27). Thus, host-specific responses to TB reactivation or reinfection that result in higher viral loads can be expected to promote HIV-1 disease progression, which in turn causes further depletion of $CD4^+$ cells, resulting in MTb disease progression (2). Consistent with these observations, isoniazid prophylaxis

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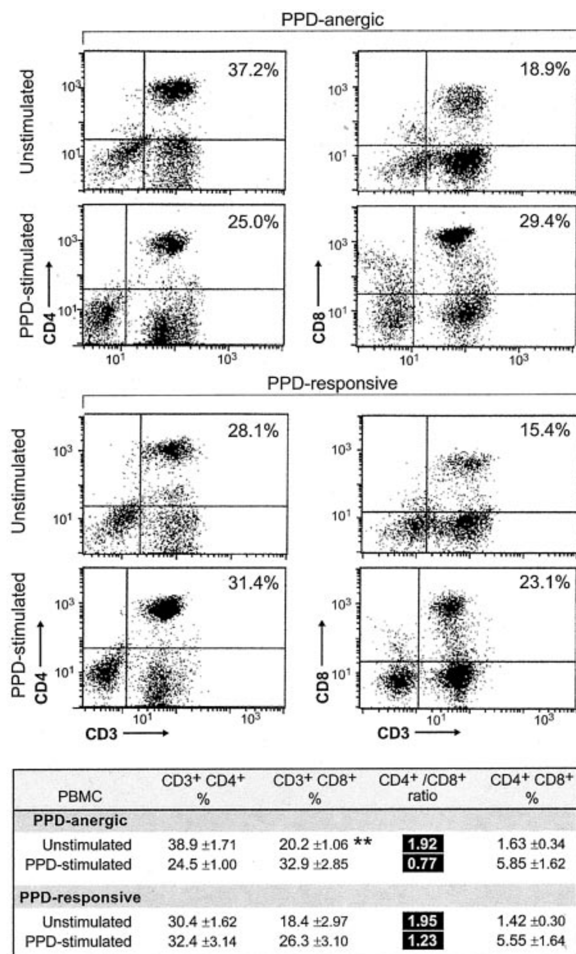


| PBMC | CD3+ CD4+ % | CD3+ CD8+ % | CD4+ /CD8+ ratio | CD4+ CD8+ % |
|---|---|---|---|---|
| **PPD-anergic** | | | | |
| Unstimulated | 38.9 ±1.71 | 20.2 ±1.06 ** | 1.92 | 1.63 ±0.34 |
| PPD-stimulated | 24.5 ±1.00 | 32.9 ±2.85 | 0.77 | 5.85 ±1.62 |
| **PPD-responsive** | | | | |
| Unstimulated | 30.4 ±1.62 | 18.4 ±2.97 | 1.95 | 1.42 ±0.30 |
| PPD-stimulated | 32.4 ±3.14 | 26.3 ±3.10 | 1.23 | 5.55 ±1.64 |

**FIGURE 6.** $CD8^+$ T lymphocytes differentially expand in anergic PBMC in response to PPD stimulation. The percentage of $CD3^+CD4^+$, $CD3^+CD8^+$ T cells was determined by flow cytometry. Data are represented as the difference between specifically stained cells and nonspecifically stained cells obtained by setting a quadrant marker for nonspecific staining in dot plots. The dot plot is representative of PBMC freshly isolated or PPD stimulated for 3 days from six PPD-anergic and six PPD-responsive donors. Results are expressed as a mean ± SEM and are representative of three separate experiments. **, $p < 0.01$.

in HIV-infected individuals who are latently infected with TB has been reported to delay the onset of HIV-1-related illnesses and to prolong survival in the coinfected host (5).

Intriguingly, our data also indicate that MTb recall Ags differentially induce viral replication of a T-tropic, but not an M-tropic, HIV-1 primary isolate up to 17 days postinfection. Consistent with other studies (28, 29) using primary M-tropic HIV-1 isolates from patients in an early stage of HIV-1 infection, we have observed that replication of the M-tropic HIV-$1_{98IN22}$ (50 $TCID_{50}$) was significantly less (more than a 1000-fold less) than what was observed using the T-tropic primary isolate HIV-$1_{KR25}$ (10 $TCID_{50}$). We note that both of the isolates used in this study had only three short passages within normal PBMC cultures. Our data demonstrate PPD stimulation of PBMC from PPD-responsive donors does not differentially activate M-tropic replication as it does when the T-tropic virus is used within the early time points studied. Given the low levels of HIV-$1_{98IN22}$ replication we observed, it is not surprising that we were unable to detect any effect of blocking IL-10 or TNF-$\alpha$ in these cultures even stimulated with PPD or PHA and IL-2.

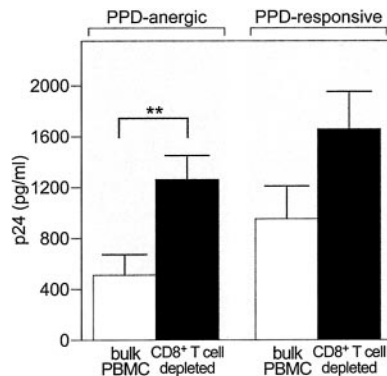


**FIGURE 7.** $CD8^+$ T cells differentially suppress HIV-1 replication in PPD-anergic PBMC. $CD8^+$ T cells were depleted from PBMC by positive selection using magnetic beads coated with an anti-CD8 mAb (Dynal Biotech). The purity of the cells was ~96% as judged by flow cytometry (data not shown). p24 levels were measured 3 days after HIV-$1_{KR25}$ infection of PPD-prestimulated, PPD-anergic, and PPD-responsive bulk PBMC (□ or $CD8^+$-depleted PBMC (■). Results are expressed as a mean ± SEM and are representative of two separate experiments. **, $p < 0.01$.

In the case of the T cell-tropic HIV-1 infection, TNF-$\alpha$ is significantly increased by PPD stimulation of PBMC from PPD-responsive donors and its neutralization results in a significant decrease of HIV-1 replication. By contrast neutralization of IL-10 has a nonsignificant impact upon HIV-1 replication in the PPD-responsive PBMC. Thus, PPD-stimulated TNF-$\alpha$ is directly linked to an increase in HIV-1 replication in PBMC from PPD-responsive donors. Furthermore, IL-2 and IFN-$\gamma$ are differentially increased in PPD-responsive donors and may also contribute to the observed increase in viral replication of the T-tropic isolate. Given that HIV-1 viral load is directly linked to HIV-1 and AIDS disease progression (30), these data provide a direct demonstration that the state of Ag-specific activation of the immune system by MTb and potentially the stage of HIV-1 infection as reflected by the tropism of the infecting viral strain has a direct impact upon HIV-1 progression in the setting of rechallenge with MTb Ags.

Notably, CXCR4-using T-tropic viruses, which infect $CD4^+$ T cells, predominate comparatively late in HIV-1 infection (31, 32). Thus, MTb Ag-specific $CD4^+$ T cell activation that occurs later in HIV-1 infection, when T cell-tropic viruses predominate, may be particularly destructive. Furthermore, our data support the concept that MTb infection, reinfection, or reactivation may in fact promote HIV-1 disease through the creation of a cytokine and cellular milieu that favors the transition of M-tropic to T-tropic viral strains and the rapid expansion of T-tropic viruses in the PPD-responsive host.

Since more than one-third of the global population is infected with MTb, mostly in the regions with high a incidence of HIV-1, the majority of individuals infected with HIV-1 are PPD responsive before the occurrence of HIV-1-associated immunosuppression (33). TNF-$\alpha$ has a clear role in increasing HIV-1 replication via its engagement of the signaling pathway, resulting in activation of the transcription factor NF-$\kappa$B, which binds to the HIV-1 long terminal repeat and thus drives HIV-1 transcription (34, 35). Thus, our demonstration of the differential increase of TNF-$\alpha$ and HIV-1 replication in PPD-stimulated PPD-responsive cultures underscores the importance of chemotherapy for TB as early as possible in the HIV-1/MTb-coinfected host.

$CD8^+$ T cells control HIV-1 levels by inhibiting viral infection and replication via the production of soluble antiviral factors, including $CD8^+$ antiviral factor, which is capable of inhibiting both M- and T- tropic HIV-1 replication (24, 26, 36) and by their ability

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to directly kill infected cells (37, 38). Previously we have shown that MTb Ag-specific anergic patients have a constitutive increase in $CD4^+$ IL-10-producing T cells and increased levels of soluble IL-10 in PPD-stimulated PBMC supernatants (9, 10). Given that IL-10 is a chemotactic factor for $CD8^+$ T lymphocytes and enhances both the precursor frequency and activity of $CD8^+$ T lymphocytes (39), the increased levels of IL-10 in PPD-anergic individuals may in fact predispose them to the observed differential increase in $CD8^+$ T cell expansion and subsequent suppression of HIV-1 replication in response to MTb recall Ags.

In the case of individuals with persistent MTb Ag-specific anergy after successful Tb treatment, MTb recall challenge causes an expansion of $CD8^+$ T cells and increased IL-10 production. Thus a host-specific environment that is unfavorable to T-tropic HIV-1 replication is created. Finally, the identification of a subset of individuals whose cells have an innate advantage in controlling HIV-1 viral load in response to MTb recall challenge suggests that immunotherapeutic approaches aimed at recapitulating Ag-specific MTb anergy in vivo could result in novel treatment strategies of the MTb/HIV-1-coinfected host.

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