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Potent immune responses of Ag-specific V γ 2V δ 2+ T cells and CD8+ T cells associated with latent stage of *Mycobacterium tuberculosis* coinfection in HIV-1-infected humans

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Abstract

Objective—To investigate immune responses of peptide-specific CD4+ and CD8+ T cells, and nonpeptide-specific V γ 2V δ 2+ T cells during clinical quiescence of latent *Mycobacterium tuberculosis* coinfection in HIV-1-infected humans.

Methods—One hundred HIV-1-infected individuals who had HIV infection only [HIV +tuberculosis–(TB–)], latent *Mycobacterium tuberculosis* coinfection (HIV +LTB), LTB), or active tuberculosis (HIV +TB) were recruited to measure mycobacterium purified protein derivative (PPD)-specific IFN γ + CD4+ and CD8+ T cells, and phospho-antigen HMBPP-specific IFN γ + V γ 2V δ 2+ T cells using enzyme-linked immunospot and intracellular cytokine staining assays.

Results—Both HIV +TB and HIV +LTB groups had low levels of PPD-specific IFN γ + CD4+ T cells regardless of CD4+ peripheral blood lymphocytes counts. However, numbers of PPD-specific IFN γ +CD8+ T cells in the HIV +LTB group were significantly greater than those in the HIV +TB group. Surprisingly, numbers of phosphoantigen hydroxy-3-methyl-but-2-enyl pyrophosphate-specific IFN γ + V γ 2V δ 2+ T cells in the HIV +LTB group were much greater than those in the HIV +TB group ($P < 0.001$). This difference was present in the subgroups of HIV +LTB whatever the levels of CD4+ T-cell counts more than 200/ μ l or less than 200/ μ l. Numbers of hydroxy-3-methyl-but-2-enyl pyrophosphate-specific IFN γ + V γ 2V δ 2+ T cells were even five times greater than those of PPD-specific IFN γ + CD8 T cells within the HIV +LTB group.

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The role of each of the authors in the study: L.S.: designing the study; setting up the experimental systems; analyzing the data; drawing the figures and finishing the article.

W.Z.: designing the study; organizing HIV positive people for screening; revising the manuscript; S.Z.: working out the questionnaires; performing PBL isolation, whole blood staining, ICS; drawing the figures; analyzing the data; C.Y.C.: performing flow cytometric and statistical analysis; W.J.: organizing HIV-positive people for screening; collecting medical history of the enrolled subjects; Y.X.: organizing HIV-positive people for screening; collecting blood samples; C.M.: ordering reagents and supplies, performing PBL isolation, T-SPOT.TB and ELISPOT assays; X.W.: designing the study; revising the manuscript and Z.W.C.: designing the study and revising the article.

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Conclusion—Potent immune responses of hydroxy-3-methyl-but-2-enyl pyrophosphate-specific IFN γ + V γ 2V δ 2+ T cells and PPD-specific IFN γ + CD8+ T cells were detected in HIV + LTB persons but not HIV + TB patients. The robust immune responses of V γ 2V δ 2+ and CD8+ T effector cells were associated with the latent stage of *Mycobacterium tuberculosis* coinfection in HIV-1-infected humans.

Keywords

HIV/AIDS; latent TB; T-cell-based enzyme-linked immunospot assay for tuberculosis; tuberculosis; V γ 2V δ 2+ T cells

Introduction

Tuberculosis (TB), HIV/AIDS and malaria remain three leading causes of infectious morbidity and mortality worldwide. HIV/AIDS clearly increases the pandemics and mortality of TB. The WHO estimates that about 8% of the 8.8 million new TB cases and 229 000 of 1.7 million TB deaths were probably HIV-infected in 2003. Clinical studies have demonstrated that purified protein derivative (PPD)+ persons not infected with HIV-1 have approximately 10% lifetime risk of developing active tuberculosis, whereas coinfection with *Mycobacterium tuberculosis* and HIV-1 is associated with a 5–15% yearly risk of active TB [1]. It is important to elucidate immune mechanisms underlying the increased susceptibility to primary *M. tuberculosis* infection and reactivation tuberculosis in HIV-infected individuals.

Immune components for maintaining clinical quiescence of latent *M. tuberculosis* infection remain poorly characterized in both healthy individuals and HIV-infected humans [2]. Murine CD4+ T cells do not appear to have a role in controlling reactivation of latent infection despite the fact that they are absolutely required for immunity against the acutely active or persistent *M. tuberculosis* infection in mice [3–5]. Human CD4 T cells may play a role in immunity against reactivation of latent *M. tuberculosis* infection, as HIV-induced immune suppression and decline of CD4 T-cell counts are associated with increased susceptibility to *M. tuberculosis* infection and reactivation tuberculosis. The role of human CD8+ T cells in immunity against reactivation tuberculosis has not been demonstrated although mycobacterium-specific histocompatibility complex (MHC) class I-restricted CD8+ T cells can be detected in humans [6–8]. Surprisingly, the depletion of murine CD8+ T cells induces reactivation of latent *M. tuberculosis* infection in mice, although there is a limited role of these cells in controlling active *M. tuberculosis* infection [9]. Circulating V γ 2V δ 2+ T cells exist only in primates and, in humans, constitute 60–95% of total blood $\gamma\delta$ T cells. Although immune responses of V γ 2V δ 2+ T cells in patients with chronic tuberculosis appear to be suppressed [10–12], it has been debated as to whether the depression of V γ 2V δ 2+ T-cell response in TB is caused by the infection or allows the infection to progress (for review see [13]). As HIV does not infect small laboratory animals, studies of T-cell immune responses in *M. tuberculosis* coinfection of HIV can only be undertaken in humans and nonhuman primates.

We have recently undertaken extensive studies of potential roles of antigen-specific $\alpha\beta$ CD8 T cells and V γ 2V δ 2+ T cells in AIDS virus infection, *M. tuberculosis* infection and mycobacterium-AIDS virus coinfection in nonhuman primates [6,13–15]. Particularly, we have shown that a loss of mycobacterium-specific T-cell responses can result in reactivation of AIDS-related tuberculosis-like disease in SIV/mycobacterium-coinfected monkeys [16]. In addition, macaque V γ 2V δ 2+ T cells share with their human counterparts the capability to recognize mycobacterial phosphoantigen (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP), and that HMBPP can stimulate prolonged antimicrobial and cytotoxic responses of systemic and pulmonary V γ 2V δ 2+ T cells [17–19]. While HMBPP-specific V γ 2V δ 2+ T cells can undergo extraordinary transendothelial migration and interstitial localization in tissues

during *M. tuberculosis* infection, the ability of these cell to rapidly expand is associated with the immunity against acutely fatal tuberculosis in juvenile rhesus monkeys [14,20]. These findings therefore prompted us to investigate mycobacterium-specific V γ 2V δ 2+ T cells, CD4+ and CD8+ T cells as well as their association with maintenance of latent stage of *M. tuberculosis* coinfection in HIV-1-infected humans.

Materials and methods

Patients and controls

One hundred and ten individuals were recruited in this study, among which 100 were HIV-1-infected individuals and 10 were uninfected healthy controls. The HIV-1-infected individuals were recruited from Yunnan province, the HIV and TB pandemic area in China. HIV-1 infection was confirmed by clinical records, routine serum tests [enzyme linked immunosorbent assay (ELISA) confirmation] and CD4T-cell counts. All individuals had a history of newborn Bacille Calmette–Guerin (BCG) vaccination.

Criteria for determining HIV + TB, HIV + LTb, HIV + TB–, and HIV – TB– individuals

As BCG vaccination is still routine clinical practice in China, we used T-cell-based enzyme-linked immunospot assay for tuberculosis (T-SPOT.TB assay) to distinguish BCG vaccination from *M. tuberculosis* infection. The individuals were divided into four groups based on T-SPOT.TB assay and extensive clinical evaluation of tuberculosis including TB contact history. T-SPOT TB kit (Oxford Immunote Ltd., Oxford, UK), a novel commercial enzyme-linked immunospot (ELISPOT) assay to detect IFN- γ release induced by *M. tuberculosis* ESAT-6 and CFP-10, was employed to identify *M. tuberculosis* infection including latent and active *M. tuberculosis* infection. The test result of T-SPOT TB assay was considered positive if either or both of Panel A (containing peptide antigens derived from ESAT-6) or Panel B (containing peptide antigens derived from CFP-10) had six or more spots than the negative control, and this number was at least two times greater than the number of spots in the negative controls. The combined T-SPOT TB assay and extensive clinical evaluation resulted in the classification of four groups of human individuals: HIV + TB, HIV + LTb, HIV + TB–, and HIV – TB– (healthy controls). The patients and groups were described in Table 1. Thirty-two patients (22 men and 10 women, 37.0 ± 8.6 years of age, range 26–65 years) were defined as having active TB (HIV + TB) by the presence of recent clinical symptoms of TB, positive culture of *M. tuberculosis*, smear test for acid-fast bacilli (AFB) from sputum, or both and abnormal chest radiograph. Large numbers of these newly diagnosed TB patients had been treated with antiretrovirals for months and routine anti-TB drugs for less than 2 months when they were recruited for studies (Table 1). Antiretrovirals can improve immune responses of V γ 2V δ 2T cells and $\alpha\beta$ T cells; anti-TB therapy appears to increase IFN- γ /granulysin production effector function of V γ 2V δ 2T cells but decrease their expansion capacity [12,21]. Forty-six (24 men and 22 women, 33.9 ± 6.7 years of age, range 24–45 years) were diagnosed with latent TB (HIV + LTb) by positive T-SPOT TB assay and no evidence of active tuberculosis, without clinical manifestations of pulmonary and extrathoracic tuberculosis, and abnormal chest radiograph. Twenty-two HIV-1-infected individuals (nine men and 13 women, 33.7 ± 5.8 years of age, range 22–44 years) were defined as no *M. tuberculosis* coinfection group (HIV + TB –) based on negative T-SPOT assay and the absence of any clinical evidence of tuberculosis. Given the possibility that HIV infection might reduce sensitivity of T-SPOT.TB test, the reduced sensitivity might lead to potential misclassification of some of the patients in the HIV + TB– group. However, this speculative misclassification if ever would have little impact on the main findings seen in comparison between LTb and TB+ groups. Ten individuals (three men and seven women, 31.9 ± 14.2 years of age, range 24–70 years) were healthy control who had negative results of T SPOT and anti-HIV antibodies assays.

Peripheral blood lymphocytes isolation

Blood samples collected freshly from all groups of patients were handled, and analyzed by phenotyping, ELISPOT and intracellular cytokine staining (ICS) at the biocontainment laboratory of Honghe No.1 People's Hospital in Yunnan, China. Peripheral blood lymphocytes (PBLs) were isolated from ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood of the individuals by density gradient sedimentation using Lympholyte-H (Cedarlane Laboratories Ltd, Ontario, Canada).

Immunofluorescent staining and flow cytometric analysis

For cell-surface staining, 100 μ l EDTA blood was treated with red blood cell (RBC) lysing buffer and washed twice with 5% fetal bovine serum (FBS)-phosphate-buffered saline (PBS) before staining. PBLs were stained with up to four Abs (conjugated to FITC, PE, allophycocyanin, pacific blue, and PE-Cy5) for at least 10 min at room temperature. After staining, cells were fixed with 2% formaldehyde-PBS prior to analysis on a BD FACSAria flow cytometer (BD Bioscience, San Diego, California, USA). Lymphocytes were gated based on forward-scatters and side-scatters; at least 20 000-gated events were analyzed using WinMDI 2.9 Software. Absolute cell numbers were calculated based on flow cytometry data and complete blood counts. The following mouse antihuman mAbs were used: V γ 2 (7A5), V δ 2 (15D), CD3 (SP34, SP34-2), CD8 (RPA-T8), CD28 (CD28.2), CD49d (9F10), IFN γ (4S.B3), (BD Pharmingen, San Diego, California, USA); CD4 (OKT4) (eBioscience, San Diego, California, USA); CD8 (DK25) (Dako, Glostrup, Denmark). The secondary Ab PE-conjugated goat antimouse IgG (Beckman Coulter, Marseille, France) was used for indirect staining.

T-SPOT.TB assay

The T-SPOT.TB assay was performed according to the manufacturer's instructions. The spots were read using the ELISPOT plate reader (AID-Gmb-H, Germany). The results were double checked by other laboratory workers and, if deemed necessary, corrected by manual counting. The laboratory technicians were blinded to the subject identifiers. The positive results were as described earlier.

Purified protein derivative and hydroxy-3-methyl-but-2-enyl pyrophosphate-based enzyme-linked immunospot assay

The ELISPOT assay using PPD and HMBPP was done using our established protocol [16–18,20]. In these experiments, 96-well nitrocellulose-backed plates were coated with 10 μ g/ml antihuman IFN γ (B27) by overnight incubation at room temperature; 2×10^5 cells were plated in triplicate in each well with or without PPD (25 μ g/ml), or HMBPP (40 ng/ml) or media alone. PPD were purchased from Mycos Research (Loveland, Colorado, USA), and were shown to carry large and small peptide proteins without detectable phosphoantigen activity (data not shown). HMBPP was synthesized using a previously described procedure [22], which was scaled-up to a final yield of 10 g. The purity determined by ion chromatography was more than 98%. Eighteen hours after culture, the plates were washed and incubated for 2 h at room temperature with 500 ng of the biotinylated anti-IFN γ . Spots were developed by adding horseradish peroxidase (HRP)-streptavidin and freshly prepared substrate. The numbers of spot-forming cells in response to the mycobacterial antigens were calculated and expressed by spot-forming cells per 10^6 PBL.

Intracellular cytokine staining

This was done using the standard protocol as we recently described [17,18]. For the assay of ICS, 10^6 PBL plus costimulatory mAbs CD28 (1 μ g/ml) and CD49d (1 μ g/ml) were incubated with PPD (25 μ g/ml), HMBPP (40 ng/ml) or media alone in 200 μ l final volume for 1 h at 37°

C, 5% CO₂ followed by an additional 5 h incubation in the presence of brefeldin A (GolgiPlug; BD Bioscience). After staining cell-surface CD3/CD4/CD8/V γ 2 for at least 15 min at room temperature, cells were permeabilized for 45 min (Cytofix/cytoperm; BD Bioscience) at 4°C and stained another 45 min for IFN γ at room temperature before resuspending in 2% formaldehyde-PBS. As only TCR bearing both V γ 2 and V δ 2 elements can recognize HMBPP [23], the V γ 2 T cells that produce IFN γ in response to HMBPP are interpreted as V γ 2V δ 2 T cells.

Statistical analysis

Statistical analysis was done using Student's *t*-test and ANOVA as previously described [18, 20].

Results

HIV + LTb individuals appeared to have relatively conserved numbers of CD4+ T cells, CD8+ T cells and V γ 2V δ 2+ T cells compared with HIV + TB patients

As an initial effort to explore an association between effector T cells and latent stage of *M. tuberculosis* coinfection in HIV-infected humans, we examined the numbers of circulating CD4+ T cells, CD8+ T cells and V γ 2V δ 2 T cells between HIV + LTb group and HIV + TB group. A group infected with HIV-1 only (HIV + TB-) and a normal healthy group without any evidence of infections also served as controls (Table 1). Although the mean percentage numbers and absolute numbers of CD4+ T cells in all three HIV-infected groups were significantly lower than the healthy control group ($P < 0.05$), the HIV + TB group had lower CD4+ T-cell counts than HIV + LTb group [$P < 0.001$ (Fig. 1a, b)]. Interestingly, mean absolute numbers of CD8+ T cells in the HIV + LTb group were significantly higher than those in the HIV + TB group ($P < 0.001$), despite the lack of differences in mean percentage numbers of CD8+ T cells between these two groups (Fig. 1c). Furthermore, both percentage and absolute numbers of V γ 2V δ 2+ T cells in the HIV + LTb group were significantly higher than those in the HIV + TB group ($P < 0.01$), although both the groups had significantly lower numbers of these $\gamma\delta$ T cells than the healthy control group [$P < 0.05$, (Fig. 1d)]. However, percentage and absolute numbers of V γ 2V δ 2+ T cells in HIV + TB was much lower than those in HIV + LTb group and other two groups [$P < 0.05$ for percentages, $P < 0.01$ for absolute numbers, (Fig. 1d)]. Thus, the phenotyping data from our well defined groups indicate that HIV + LTb individuals appear to have relatively conserved numbers of CD4+ T cells, CD8+ T cells and V γ 2V δ 2+ T cells compared with HIV + TB patients, although both groups exhibited lower numbers of CD4+ T cells and V γ 2V δ 2+ T cells than the normal healthy controls.

The appreciable mycobacterium-specific IFN γ + CD8+ T-cell responses were detectable during the latent stage of *M. tuberculosis* coinfection in HIV-1-infected humans

We then sought to compare the capacity of $\alpha\beta$ T cells to recognize *M. tuberculosis* antigens and to exert effector function of IFN γ production between the HIV + LTb and HIV + TB groups. Given that PPD has been repeatedly used as multiple protein peptide antigens for measuring peptide-specific immune responses of both CD4+ and CD8+ T cells [14,17,18], we used PPD as specific stimuli in ELISPOT and intracellular ICS assays. ELISPOT data indicated that mean numbers of PPD-specific IFN γ + cells in the HIV + LTb group were significantly higher than those in the HIV + TB group and the other two control groups: HIV + TB- and healthy control [$P < 0.05$, (Fig. 2a)]. Surprisingly, ICS data showed that regardless of their status of CD4+ T-cell counts, both the HIV + LTb and HIV + TB groups exhibited low levels of PPD-specific IFN γ + CD4+ T cells, suggesting that HIV-1 infection affected IFN γ immune responses of PPD-specific CD4+ T cells (Fig. 3a). In contrast, the HIV + LTb group exhibited an increase in numbers of PPD-specific IFN γ + CD8+ T cells compared with the HIV + TB group [$P < 0.05$, (Fig. 3b, c)]. The fact that some of HIV + TB- or healthy individuals exhibited

good numbers of PPD-specific T cells might represent ongoing memory responses of BCG vaccination. Therefore, these results demonstrated that whereas both HIV + TB and HIV + LTB groups had low levels of PPD-specific CD4+ T-cell immune responses, appreciable IFN γ responses of mycobacterium-specific CD8+ T cells were detectable during the latent stage of *M. tuberculosis* coinfection in HIV-1-infected humans.

Potent immune responses of hydroxy-3-methyl-but-2-enyl pyrophosphate-specific IFN γ + V γ 2V δ 2+ T cells were associated with the latent stage of *M. tuberculosis* coinfection in HIV-1-infected humans

As HMBPP is the sole *M. tuberculosis* phosphoantigen recognized by V γ 2V δ 2+ T cells [14, 17,18], we evaluated the numbers of HMBPP-specific IFN γ + V γ 2V δ 2+ T cells between the HIV + LTB and HIV + TB groups. Surprisingly, HIV + LTB individuals developed potent HMBPP-specific IFN γ + V γ 2V δ 2+ T-cell responses. ELISPOT data indicated that the absolute numbers of HMBPP-specific IFN γ + V γ 2V δ 2+ T cells in the HIV + LTB group were five times greater than those in the HIV + TB group [$P < 0.01$, (Fig. 2b)]. Even within the HIV + LTB group for comparing $\alpha\beta$ and $\gamma\delta$ T cells, HMBPP-specific IFN- γ producing V γ 2V δ 2+ T cells were five times greater than PPD-specific IFN γ + CD8+ T cells (Fig. 2a, b). Consistently, ICS data showed that the mean numbers of HMBPP-specific IFN γ + V γ 2V δ 2+ T cells in the HIV + LTB group were significantly higher than those in the HIV + TB group [$P < 0.001$, (Fig. 3de,)]. Importantly, the numbers of HMBPP-specific IFN γ + V γ 2V δ 2+ T cells in the HIV + LTB group were even greater than those detected in the two control groups: HIV + TB- and healthy control groups not infected with *M. tuberculosis* [$P < 0.05$, (Fig. 3c,e)].

Given that both HIV + LTB and HIV + TB groups had detectable but low PPD-specific IFN γ + CD4 T cells, it is important to evaluate antigen-specific T-cell responses between the subgroups whose CD4 T-cell counts were categorized. We, therefore, subgrouped HIV + LTB and HIV + TB patients based on their CD4 PBL counts more than 200/ μ l and less than 200/ μ l, and then compared magnitudes of HMBPP-specific V γ 2V δ 2 T-cell responses as well as PPD-specific CD4 and CD8 T-cell responses between the CD4 T-cell-matched subgroups. The rationale for using the number 200 CD4 T cells/ μ l as cutoff for sub-grouping was that CD4 PBL counts 200/ μ l were defined as AIDS in HIV-infected humans. Interestingly, both 'high' and 'low' CD4-PBL-count subgroups of HIV + LTB persons exhibited statistically greater numbers of HMBPP-specific IFN γ +V γ 2V δ 2 T cells than those matched subgroups of HIV + TB patients (Fig. 4a, b). Only the 'high' CD4-PBL-count subgroup of HIV + LTB persons exhibited statistically greater numbers of PPD-specific IFN γ +CD8 T cells than the matched subgroup of HIV + TB patients (Fig. 4a, b).

These results therefore demonstrated that potent immune responses of HMBPP-specific IFN γ + V γ 2V δ 2+ T cells together with PPD-specific $\alpha\beta$ CD8+ T cells were associated with the latent stage of *M. tuberculosis* coinfection in HIV-1-infected humans.

Discussion

In this study, we combined the T-SPOT.TB assay and clinical evaluation to define latent and active *M. tuberculosis* coinfections in HIV-1-infected humans in China, one of 22 high-TB-burden countries in the world [4,24]. As the T-SPOT.TB is a *M. tuberculosis*-specific, ESAT6-/CFP10-based ELISPOT assay, its capability to detect *M. tuberculosis* infection is usually not affected by moderate HIV infection [25–27]. Thus, the combined use of T-SPOT.TB assay and clinical evaluation allowed us to define three groups of HIV-infected individuals as no TB, latent TB and active TB. The data derived from three defined groups of HIV-infected humans appeared to be more convincing than those derived from earlier studies using PPD skin test for distinguishing BCG vaccination from latent *M. tuberculosis* infection. This is particularly important for the population studies in China, a country where newborns are vaccinated with

BCG. The finding that some of HIV + TB⁻ or healthy individuals showed significant numbers of PPD-specific IFN γ + cells in ELISPOT or PPD-specific IFN γ + CD8 T cells might be explained by BCG-elicited immune responses rather than latent TB, as they displayed negative T-SPOT.TB results without any evidence of clinical TB. The detectable responses of V γ 2V δ 2+ T cells after HMBPP stimulation in some of these individuals without *M. tuberculosis* infection might also be due to the innate-like capacity of these cells or the BCG vaccination background.

Both HIV + LTB and HIV + TB groups of HIV-1-infected individuals had low levels of PPD-specific CD4+ T-cell responses compared with PPD-specific CD8+ T-cell responses. Such low levels of PPD-specific CD4+ T-cell responses were not related to their CD4+ T-cell counts in the blood. This result did not appear to support the notion that potent IFN γ immune responses of mycobacterium-specific CD4+ T cells were required for the immunity against reactivation tuberculosis in HIV-1-infected humans. However, the finding does suggest that low levels of antigen-specific CD4+ T cell responses may be efficient enough to facilitate *M. tuberculosis*-specific CD8+ and V γ 2V δ 2+ T-cell responses, and therefore contribute to the cell-mediated immunity against reactivation tuberculosis in HIV-1-infected humans with latent *M. tuberculosis* coinfection. In-depth long-term follow-up studies are needed to define the precise role of mycobacterium-specific CD4+ T cells in the immunity against reactivation tuberculosis in HIV-1-infected humans.

In HIV negative children, numbers of V γ 2V δ 2+ IFN γ + T effector cells are reduced in active TB but restorable under anti-TB therapy [11,12]. Here in HIV+ patients, despite low levels of PPD-specific CD4+ T cells, potent IFN γ immune responses of HMBPP phosphoantigen-specific V γ 2V δ 2+ T cells and PPD peptide-specific CD8+ T cells were detected in the latent stage of *M. tuberculosis* coinfection of HIV + LTB persons compared with the active TB stage of HIV + TB patients. The potent T-cell responses detected in the HIV + LTB group were unlikely attributed to immune improvement after successful antiretroviral treatment [21], as only three of 46 HIV + LTB patients received antiretrovirals whereas 23 of 32 HIV + TB patients were treated with antiretrovirals (Table 1). Furthermore, our finding was actually seen in the subgroups of HIV + LTB persons whose CD4 T-cell counts were greater or lower than 200/ μ l. Such findings suggest that these two T-cell populations driven by both peptide and nonpeptide antigens may play a role in the immunity against reactivation tuberculosis. In fact, it has been shown in mice that CD8+ T cells contribute to immunity against reactivation of latent *M. tuberculosis* infection [9]. It is important to note that CD8+ and V γ 2V δ 2+ T cells that were able to produce IFN γ in recognition of PPD or HMBPP *in vitro* accounts for more than 2% mean numbers of CD3 T cells or more than 600/10⁶ peripheral blood mononuclear cells (PBMC) mean absolute numbers in the circulation of HIV + LTB patients. Such a large number of antigen-specific IFN γ +T cells may represent an enriched source of T-helper and antimicrobial responses in HIV-infected patients with latent *M. tuberculosis* coinfection. In fact, IFN γ is important cytokine mounting Th1-like immune responses, and has been shown to be critical for controlling TB in murine TB model.

The potent IFN γ responses of HMBPP-specific V γ 2V δ 2+ T cells in HIV + LTB patients are somehow consistent with mycobacterial phosphoantigen-mediated immune responses and effector function of V γ 2V δ 2+ T cells in nonhuman primates. We have demonstrated that a single-dose HMBPP treatment plus IL-2 can induce prolonged cytotoxic and IFN γ responses of V γ 2V δ 2+ T cells in the systemic and pulmonary compartments [18]. The ability of V γ 2V δ 2+ T cells to rapidly expand and migrate to lungs after pulmonary *M. tuberculosis* infection correlates with BCG vaccine-induced immunity against acutely fatal tuberculosis in juvenile rhesus monkeys [14,17,20]. It is worth mentioning that our data obtained from peripheral blood may not adequately reflect the pulmonary immune response, as V γ 2V δ 2+ T cells can migrate to and accumulate in lungs despite a lack of apparent responses in the blood.

during *M. tuberculosis* infection [20]. Further studies are needed to elucidate antigen-specific T-effector cells in lungs in *M. tuberculosis* coinfection of HIV-infected humans. As V γ 2V δ 2 + T cells and CD8+ T cells have cytotoxic potentials, they may exert killing effector function through TCR recognition of peptides or phosphoantigen on the cell-surface of latently *M. tuberculosis*-infected macrophages or dendritic cells. Such cytotoxic activities might keep latent *M. tuberculosis* coinfection on check and prevent from reactivation tuberculosis in HIV-1-infected individuals. Thus, V γ 2V δ 2+ T cells and CD8 T cells might emerge as appreciable anti-TB effector T cells in HIV-infected patients whose CD4 T-cell counts are low. Our findings therefore suggest that V γ 2V δ 2+ T cells and CD8 T cells may be potential targets for TB immunotherapeutic and vaccine development.

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Fig. 1. Both HIV + LTB and HIV + TB groups had lower mean numbers of CD4+ T cells and Vγ2Vδ2+ T cells than the healthy control, but the HIV and latent tuberculosis group had relatively reserved numbers of these cells compared with the HIV and tuberculosis group

The percentage and absolute numbers of CD3+ T cells (a), CD4+ T cells (b), CD8+ T cells (c) and Vγ2Vδ2+ T cells (d) in PBLs of all the four groups. The horizontal lines represent the means of T cell subsets of each group. The percentage and absolute numbers of CD4+ T cells in the HIV + TB group were lower than those in other groups ($P < 0.01$). The numbers of Vγ2Vδ2+ T cells in the HIV + TB group were significantly lower than those in three groups ($P < 0.05$). HC, healthy control; LTB, latent tuberculosis; PBLs, peripheral blood lymphocytes, TB, tuberculosis.

Fig. 2. Enzyme-linked immunospot data show that the HIV + LTB group developed stronger immune responses of purified protein derivative-specific IFN γ + cells and hydroxy-3-methyl-but-2-enyl pyrophosphate-specific IFN γ + V γ 2V δ 2+ T cells than the HIV + TB group

Freshly isolated PBL from patients and controls were stimulated overnight with PPD (a) or HMBPP (b) and measured for numbers of IFN γ + cells by ELISPOT. The horizontal lines are the means of SFCs of each group. Numbers of PPD-specific IFN γ + cells in the HIV + LTB group were greater than those of other three groups ($P < 0.05$). Numbers of HMBPP-specific IFN γ + V γ 2V δ 2+ T cells in the HIV + LTB were much greater than those of the HIV + TB group and other two groups ($P < 0.01$). HMBPP, hydroxy-3-methyl-but-2-enyl pyrophosphate;

LTB, latent tuberculosis; PBL, peripheral blood lymphocyte; PPD, purified protein derivative; SFCs, spot forming cells.

Fig. 3. Intracellular cytokine staining showed that potent immune responses of purified protein derivative-specific CD8+ IFN γ + T cells and hydroxy-3-methyl-but-2-enyl pyrophosphate-specific IFN γ + V γ 2V δ 2+ T cells were detected during the latent stage of *M. tuberculosis* coinfection in the HIV + LTB group

PBL from patients and controls were stimulated for 6 h with PPD or HMBPP, and measured for numbers of IFN- γ + T cell by ICS. Shown are PPD-specific CD4+ T cells (a), PPD-specific IFN γ + CD8+ T cells (c), and HMBPP-specific IFN γ + V γ 2V δ 2+ T cells (e) in PBL of all the four groups. The horizontal lines represent the means of cell numbers for each group.

Representative CD3-gated histograms of PPD-specific CD8 T cells (b) and HMBPP-specific IFN γ + V γ 2V δ 2+ T cells (d) were shown for the HIV + LTB and HIV + TB groups, respectively. Numbers of PPD-specific CD4+ T cells were similar between all four groups ($P > 0.05$). The numbers of PPD-specific IFN γ + CD8+ T cells in the HIV + LTB group were greater than those in the HIV + TB group ($P < 0.05$); the numbers of HMBPP-specific IFN γ + V γ 2V δ 2+ T cells

in the HIV + LTB group were much greater than those in the HIV + TB group ($P < 0.001$). The numbers in the HIV + LTB group were even higher than those in the HC and HIV + TB – groups ($P < 0.05$). HC, healthy control; HMBPP, hydroxy-3-methyl-but-2-enyl pyrophosphate; ICS, intracellular cytokine staining; LTB, latent tuberculosis; PBL, peripheral blood lymphocyte; PPD, purified protein derivative; TB, tuberculosis.

Fig. 4. HIV and latent tuberculosis persons whose CD4 T cell counts more than 200/ μ l (a) and less than 200/ μ l (b) had higher numbers of hydroxy-3-methyl-but-2-enyl pyrophosphate-specific IFN γ + V γ 2V δ 2+ T cells than HIV and tuberculosis patients with the matched CD4 T-cell counts. Forty-six HIV + LTB persons and 32 HIV + TB patients were divided into two subgroups, respectively, based on CD4 T-cell counts more than 200/ μ l and less than 200/ μ l. The CD4 count-matched subgroups between HIV + LTB and HIV + TB TB groups ($n = 41$ versus 10; and $n = 5$ versus 22) were compared for numbers of PPD-specific IFN γ + CD4 or CD8 T cells as well as HMBPP-specific IFN γ + V γ 2V δ 2 T cells. Shown are mean numbers with SEM bars. ** $P < 0.001$; * $P < 0.05$. HMBPP, hydroxy-3-methyl-but-2-enyl pyrophosphate; LTB, latent tuberculosis; PPD, purified protein derivative; TB, tuberculosis.

Table 1

Patient and control groups.

Group	Number of subjects	Description	^a HARRT treatment	^a Anti-TB treatment
HIV + TB−	22	HIV-1-infection only: no evidence of <i>M. tuberculosis</i> coinfection	3 were treated for mean 10 months (8–12 months)	No
HIV + LTB	46	HIV-1-infection and latent <i>M. tuberculosis</i> coinfection: no symptoms of TB infection; normal chest radiograph; negative AFB smear and/or <i>M. tuberculosis</i> culture	3 were treated for mean 19.5 months (15–24 months)	No
HIV + TB	32	HIV-1-infection and active TB: recent clinical symptoms of TB; abnormal chest radiograph; positive AFB smear and/or <i>M. tuberculosis</i> culture	23 were treated for mean 3.4 months (0.3–18 months)	26 were treated for <2 months
HC	10	Healthy control: no evidence of HIV and <i>M. tuberculosis</i> infections	No	No

^aDenotes months treated with antiretrovirals or anti-TB drugs at the time when patients were recruited for studies. AFB, acid-fast bacilli; HC, healthy control; TB, tuberculosis.