



Syphilis Infection Differentially Regulates the Phenotype and Function of $\gamma\delta$ T Cells in HIV-1-Infected Patients Depends on the HIV-1 Disease Stage

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A rapidly escalating outbreak of syphilis infection has been affected men who have sex with men, particularly those with HIV-1 infection. $\gamma\delta$ T cells are unconventional immune cells with two main subsets, V δ 1 T cells and V δ 2 T cells, which possess a combination of innate and adaptive immune features allowing them against HIV-1. However, whether syphilis infection affects the phenotype and function of $\gamma\delta$ T cells in HIV-1-infected patients remains unclear, especially in acute HIV-1 infection (AHI). In this study, we enrolled 57 HIV-1-infected patients (24 with HIV-1 infection only and 33 coinfecting with syphilis) from an acute HIV-1-infected cohort in Beijing (PRIMO). A comprehensive analysis of $\gamma\delta$ T-cell phenotype and function was performed by flow cytometry. We found syphilis coinfection could reverse the imbalance of V δ 1/V δ 2 ratio in AHI. Syphilis infection results in decreased $\gamma\delta$ T-cell activation in AHI, but increased $\gamma\delta$ T-cell activation in chronic HIV-1 infection (CHI). Moreover, patients with CHI had larger numbers of IL-17-producing $\gamma\delta$ T cells than those with AHI, regardless of syphilis status. Thus, syphilis affected the $\gamma\delta$ T-cell immune response differently in patients depending on the stages of HIV-1 disease. In addition, the percentage of IL-17-producing $\gamma\delta$ T cells was positively correlated with the percentage of neutrophils. These results suggest that the $\gamma\delta$ T-cell/IL-17/neutrophil axis is involved in HIV-1 pathogenesis and disease progression. Taken together, our observations provide new insight into the roles of $\gamma\delta$ T cells in immunopathogenesis of syphilis and HIV-1 coinfection, particularly during AHI, and our findings may be helpful for the prevention of syphilis and other sexually transmitted infections and highlight the great significance on the remedy of patients coinfecting with HIV-1.

Keywords: syphilis, acute/chronic HIV-1 infection, $\gamma\delta$ T cells, innate immune response, IL-17

INTRODUCTION

Syphilis is a chronic bacterial infection caused by the sexually transmitted pathogen, *Treponema pallidum*. The incidence of syphilis has increased sharply in recent years in men who have sex with men (MSM), especially those with HIV-1 infection (1–7). Syphilis worsens disease progression in HIV-1-infected patients, as demonstrated by CD4⁺ T-cell depletion and increased plasma HIV-1 RNA levels (8–10). Syphilis may also increase the risk of HIV-1 infection and transmission in MSM population (11, 12). Meanwhile, HIV-1 infection may effect on the presentation, disease progression, and efficacy of treatment on syphilis (13). The host immune system plays a key role in controlling HIV-1 disease progression through host restriction factors (TRIMs, APOBEC3G, SAMHD1), cytokines (IL-6, IFN- γ , TNF- α), and chemokines (MIP-1 α , MIP-1 β , RANTES) (14–18). Cells producing IL-17 and IFN- γ have been implicated in inflammation and central nervous system damages (19, 20). However, the cellular immune responses elicited by HIV-1 and syphilis coinfection have not been well studied.

$\gamma\delta$ T cells account for only a small proportion (1–10%) of T lymphocytes, but are nevertheless a critical component of the innate immune system and play important roles in the first line (21). Human $\gamma\delta$ T cells have been defined into two main subsets, V δ ₁ T cells and V δ ₂ T cells. V δ ₁ $\gamma\delta$ T cells are located predominantly in the mucosae, where they recognize stress-induced molecules (such as ULBPs and MICA/B), and play a protective role. By contrast, V δ ₂ $\gamma\delta$ T cells recognize phosphoantigens (such as IPP). They account for most of the circulating $\gamma\delta$ T cells in the bloodstream and are directed against tumors and infectious diseases. In the peripheral blood, V δ ₁ T cells, also known as regulatory $\gamma\delta$ T cells, play an immunosuppressive role, through Foxp3 expression or the secretion of cytokines, such as TGF- β and IL-10 (22). On the contrary, V δ ₂ T cells mainly served as a control of antiviral immunity; cytotoxic cells kill tumor cells or pathogen-infected cells by secreting cytokines, perforin, granzyme B, or through Fas-FasL pathway; antigen presentation; or B helper T-cell function (21, 23, 24). HIV-1 infection strongly depletes the numbers of V δ ₂ T cells and induces V δ ₂ T-cell anergy, resulting in weaker responses to IPP stimulation (25). In our previous study, we found that $\gamma\delta$ T cells were over-activated in HIV-1 infection and were associated with HIV-1 disease progression (26). IL-17-producing $\gamma\delta$ T cells increased in HIV-1-infected patients with fast disease progression and were involved in HIV-1 pathogenesis (26). However, to our knowledge, there are few studies describing the antisyphilitic role of $\gamma\delta$ T cells in HIV-1 infection. In addition, increasing IFN- γ /IL-17-producing CD8⁺ T cells were considered as a compensation in HIV⁺ individuals, but they were not sufficient to eliminate the spirochete (27). Therefore, we propose that $\gamma\delta$ T cells involved in cell-mediated antisyphilitic response in HIV-1-infected patients. However, how the phenotype and function of $\gamma\delta$ T cells in HIV-1-infected patients with syphilis coinfection change remains unclear, especially in acute HIV-1 infection (AHI).

In the current study, we performed a comprehensive analysis of $\gamma\delta$ T-cell phenotype and function in patients coinfecting with syphilis and HIV-1. We found that syphilis affected the phenotype

and function of $\gamma\delta$ T cells differently in HIV-1-infected patients, depending on the stages of HIV-1 disease. Our observations provide new insight into the roles of $\gamma\delta$ T cells in immunopathogenesis of syphilis and HIV-1 coinfection. These findings suggest syphilis monitoring, follow-up, and treatment in MSM or HIV-1-positive individuals should be enhanced.

MATERIALS AND METHODS

Study Subjects

Study subjects used in this study were enrolled from the Beijing PRIMO Clinical Cohort, a prospective study cohort of HIV-1-negative MSM to identify AHI at Beijing You'an Hospital, Beijing, China, since October 2006 (28). After enrollment, they were screened for plasma HIV-1 antibody, HIV-1 RNA level, clinical signs of acute infection, and syphilis status. Then they were followed up every 2 months, and HIV-1 antibody and syphilis were detected at each visit. AHI was defined as a negative or indeterminate HIV-1 antibody status but a positive HIV RNA (29). Once acute HIV-1-infected cases were identified, patients were followed up at 1, 2, 4, 8, and 12 weeks, and then every 3 months and syphilis status was monitored at each visit (Figure 1). Blood samples were collected, and peripheral blood mononuclear cells (PBMCs) and plasma were isolated and cryopreserved. Estimated date of infection was defined as the mid-point between the last HIV-1 antibody negative test and the first HIV-1 antibody positive test, or as 14 days prior to a positive RNA PCR assay on the same day as a negative HIV Enzyme Immunoassay. Patients whose infection time was longer than 180 days were defined as chronic HIV-1 infection (CHI). 26 AHI and 31 CHI patients were recruited randomly. The estimated infection date of AHI and CHI patients were 62 ± 33 and 565 ± 307 days, respectively. Early HIV-1 infection can be depicted as six discrete stages proposed by Fiebig et al. (30, 31). Stage I–II: HIV-1 RNA positive, ELISA negative with 3 patients; stage III–IV: HIV-1 RNA positive, ELISA positive, and Western blot negative or indeterminate with 4 patients; stage V–VI: HIV-1 RNA positive, ELISA positive, Western blot without/with p31 band with 19 patients. Syphilis was diagnosed on the basis of a compatible history, and the results of rapid plasma reagin (RPR) test (Shanghai Kehua Company, China) and *T. pallidum* particle agglutination assay (TPPA) (Fujirebio Diagnostics, Inc., Japan) (32). Based on the RPR and TPPA results, AHI and CHI patients were divided into HIV⁺RPR⁺ group ($n = 15$, $n = 18$) and HIV⁺RPR⁻ group ($n = 11$, $n = 13$), respectively. All AHI and CHI patients were ART-naïve treated. 6 patients in acute HIV⁺RPR⁺ group and 4 patients in chronic HIV⁺RPR⁺ group received benzathine benzylpenicillin treatment for syphilis before their enrollment in this study. Patients with opportunistic infections or coinfections with tuberculosis, hepatitis B virus, or hepatitis C virus were excluded. Twenty age-matched male MSM HIV-1-negative controls (HC) were included as controls. The characteristics of all subjects are described in the Table 1.

This study and all relevant experiments have been approved by the Beijing You'an Hospital Research Ethics Committee and written informed consent was obtained from each participant

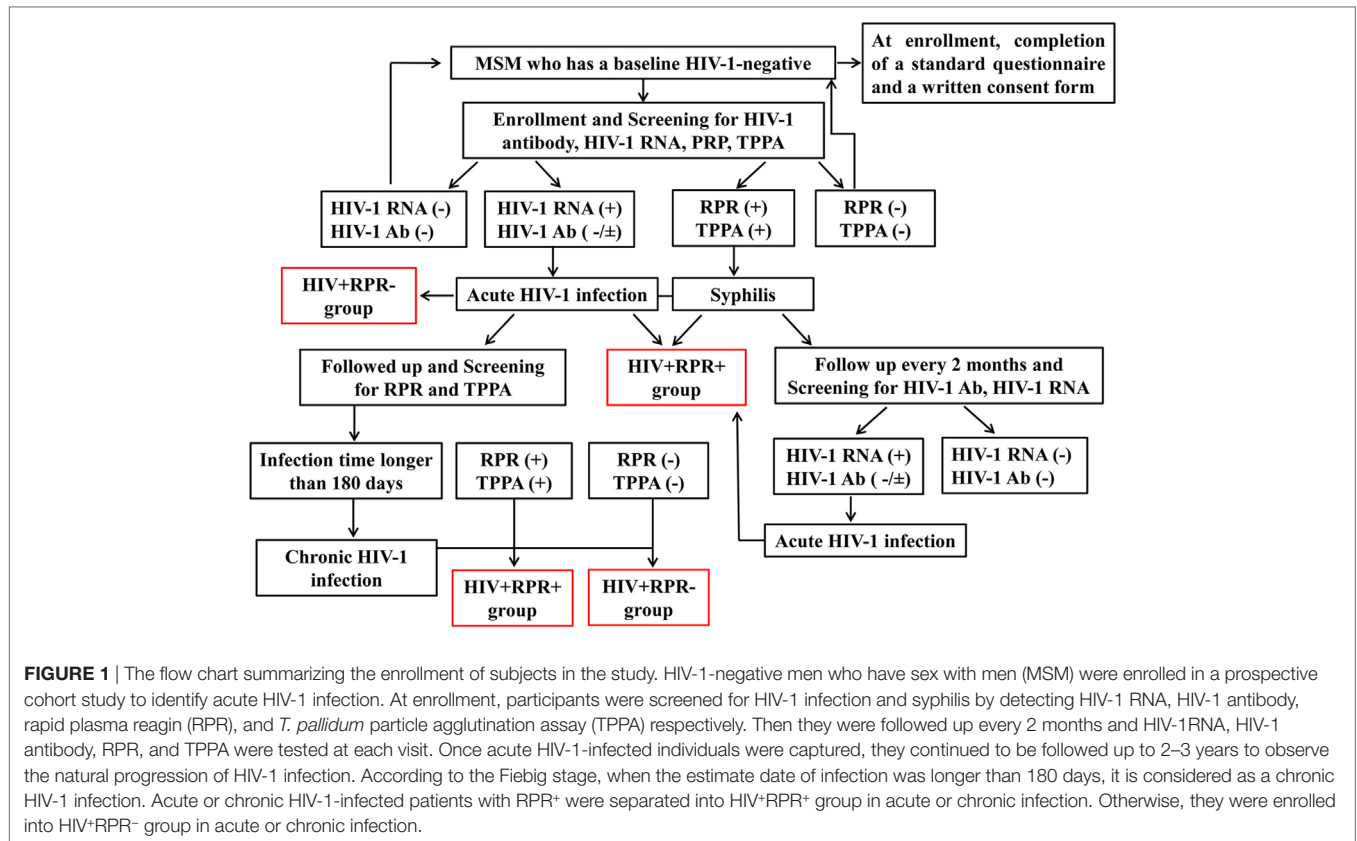


TABLE 1 | Basic characteristics of all participants enrolled in this study.

Characteristics	Healthy control	Acute HIV-1 infection		P-value	Chronic HIV-1 infection		P-value
		RPR ⁺	RPR ⁻		RPR ⁺	RPR ⁻	
Cases	20	15	11		18	13	
Chinese Han population (%)	100	100	100		100	100	
Age (years)	29.5 ± 7.7	31.5 ± 6.8	30.3 ± 7.5	0.678	34.6 ± 7.6	33.6 ± 8.8	0.703
Infection time (day)	NA	58.6 ± 23.8	66.6 ± 43.8	0.917	500.9 ± 249.4	655.4 ± 364.1	0.483
CD3 ⁺ T-cell count (cells/μl)	1751 ± 467	1622 ± 499	1545 ± 537	0.958	1801 ± 846	1629 ± 394	0.857
CD4 ⁺ T-cell count (cells/μl)	865 ± 300**	423 ± 150	438 ± 144	0.815	415 ± 118	521 ± 143	0.097
CD8 ⁺ T-cell count (cells/μl)	824 ± 291	1173 ± 503	1024 ± 396	0.716	1072 ± 558	1070 ± 397	0.561
CD4:CD8 ratio	1.13 ± 0.55**	0.38 ± 0.22	0.49 ± 0.23	0.203	0.37 ± 0.27	0.56 ± 0.32	0.063
Plasma viral load (log ₁₀ copies/ml)	NA	4.45 ± 0.74	4.66 ± 1.15	0.978	4.27 ± 0.88	3.86 ± 0.69	0.267

Data were presented as mean ± SD.

**P < 0.001, compared with HIV-1-infected patients.

P values were determined by Mann-Whitney test.

NA, not applicable; RPR, rapid plasma reagin.

according to the Declaration of Helsinki. All the participants provided written informed consent for their information, and clinical samples were stored and used for research. At enrollment, all subjects provided baseline demographic, clinical, and epidemiological information by completing a standardized questionnaire. The methods were carried out in accordance with approved guidelines and regulations.

Whole-Blood Analysis

Venous blood was collected into tubes containing EDTA as an anticoagulant. Whole blood cells analysis was performed on

Uritest-3000 Fully Automated Hematology Analyzer (URIT medical electronic company, Shenzhen, China). Absolute counts of CD3⁺, CD4⁺, and CD8⁺ T lymphocytes were obtained as previously described (26).

Viral Load Testing

Plasma HIV-1 viral load (copies/ml) were quantified using the COBAS AmpliPrep/COBAS TaqMan 48 Analyser (Roche Diagnostic, Branchburg, NJ, USA), or by real-time PCR (Abbott, Des Plaines, IL, USA), with a detection limit of 40 copies/ml of plasma.

Serological Assays

HIV-1 infection status was determined by screening with a HIV-1/2 antigen/antibody combo enzyme immunoassay (Beijing Wantai Biological Medical Company, Beijing, China). Positive specimens were further conformed by using Western blot for HIV-1/2 (HIV Blot 2.0 MP Diagnostics, Singapore). The diagnosis of syphilis was performed with the RPR test (Shanghai Kehua Company, China) and TPPA (Fujirebio Diagnostics, Inc., Japan) (32). A seropositive result for TPPA was defined as the presence of a past or current syphilis infection, while a seropositive result for both TPPA and RPR was diagnosed as a current syphilis infection.

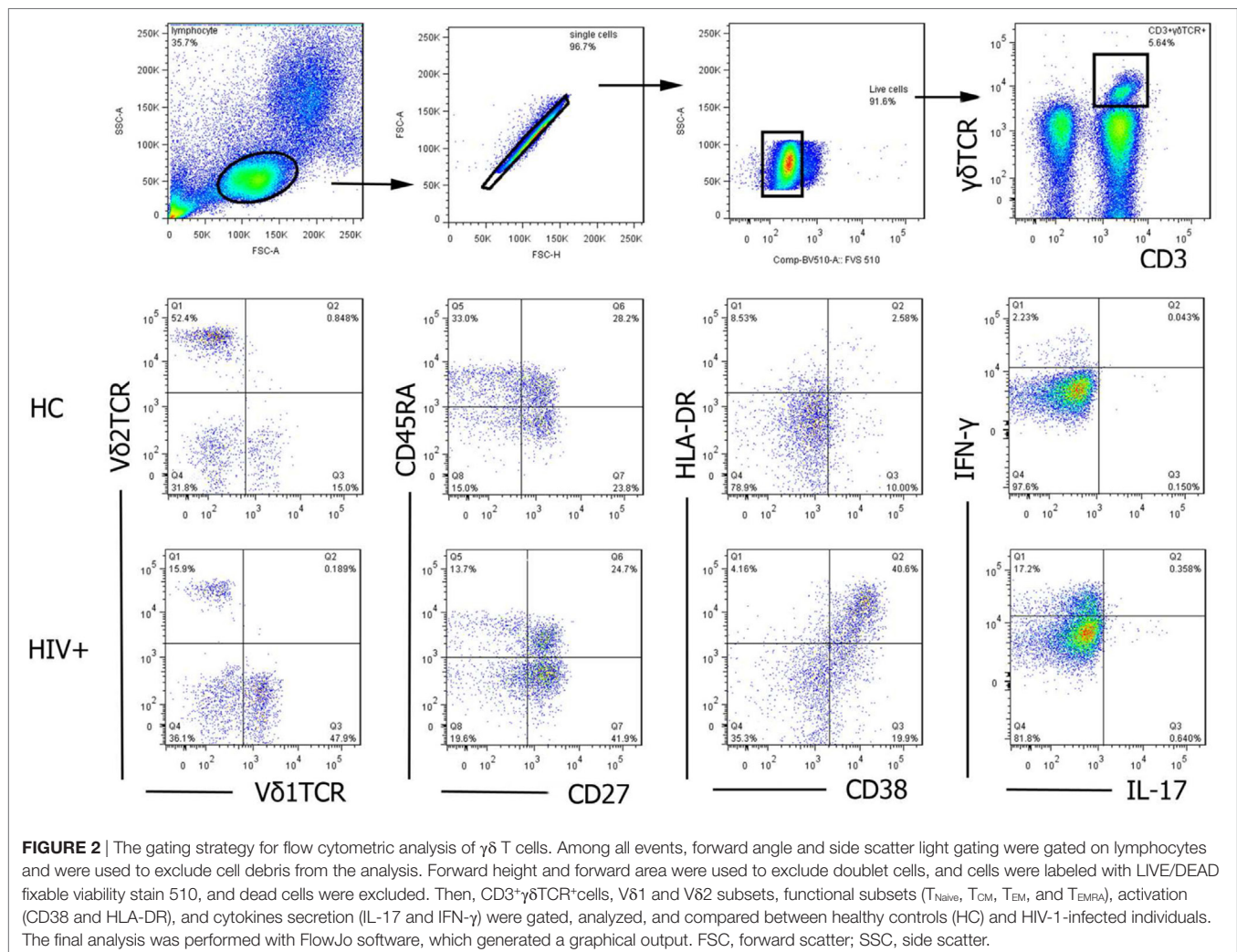
Antibodies

Phycoerythrin (PE)-conjugated anti-human CD38 (HIT2) monoclonal antibody (mAb), allophycocyanin (APC)-conjugated anti-human HLA-DR (L243) mAb, phycoerythrin-cyanine 7 (PE-cy7)-conjugated anti-human CD3 (HIT3a) mAb, peridinin-chlorophyll protein complex cyanine 5.5 (PerCP-cy5.5)-conjugated anti-human CD27 (O323) mAb, and APC-conjugated anti-human CD45RA (HI100) mAb were purchased from Biolegend (San Diego, CA, USA). Fluorescein

isothiocyanate (FITC)-conjugated anti-human pan TCR $\gamma\delta$ mAb, PE-conjugated anti-human pan TCR $\gamma\delta$ (IMMU510) mAb and FITC-conjugated anti-human pan V δ_2 TCR mAb (IMMU389) were purchased from Immunotech (Beckman Coulter, Fullerton, France). FITC-conjugated anti-human pan V δ_1 TCR (TS8.2) mAb was purchased from Pierce (Rockford, IL, USA). PE-conjugated IL-17A (SCPL1362) mAb and APC-conjugated interferon- γ (IFN- γ) (B27) mAb were purchased from BD Pharmingen (San Diego, CA, USA). The isotype control mAbs were purchased from the corresponding companies.

Flow Cytometry

For surface staining, PBMCs were isolated from HC and HIV-1-infected patients with or without syphilis infection. Cells were washed with 1% bovine serum albumin in PBS and were labeled with LIVE/DEAD fixable viability stain 510 (BD Biosciences, San Jose, CA, USA), and dead cells were excluded. Then, cells labeled with specific surface antibodies: gating strategy on CD3 $^+$ $\gamma\delta$ TCR $^+$ cells, V δ_1 and V δ_2 T cells, or $\gamma\delta$ T $_{Naive}$ (CD27 $^+$ CD45RA $^+$), $\gamma\delta$ T $_{CM}$ (CD27 $^+$ CD45RA $^-$), $\gamma\delta$ T $_{EM}$ (CD27 $^-$ CD45RA $^-$), and $\gamma\delta$ T $_{EMRA}$ (CD27 $^-$ CD45RA $^+$) were displayed (Figure 2). Cytometer setup



and tracking calibration particles were used to ensure that fluorescence intensity measurement was consistent in all experiments. Flow cytometry Comp-Beads kits (BD Biosciences, San Jose, CA, USA) were used for compensation. Forward angle and side scatter light gating were gated on lymphocytes and were used to exclude cell debris from the analysis. Forward height and forward area were used to exclude doublet cells. Cells were performed with a FACScan flow cytometer, as previously described (33, 34). The final analysis was performed with FlowJo software (version 7.6.2; Tree Star Inc., Ashland, OR, USA), which generated a graphical output. The strategies for the analysis of flow cytometry data are detailed in **Figure 2**.

Intracellular Staining

Peripheral blood mononuclear cells were incubated with 50 ng/ml phorbol 12-myristate-13-acetate (PMA), 1 μ g/ml ionomycin, and 10 μ g/ml brefeldinA (Sigma, St Louis, MO, USA) at 37°C for 6 h. Cells were collected, washed, and labeled with specific surface antibodies. Then, cells were washed, fixed, permeabilized, and incubated with IL-17 and IFN- γ mAbs. Cells were performed with a FACScan flow cytometer. Cells were first gated on 510 negative cells (live cells), then gated on CD3⁺ $\gamma\delta$ TCR⁺ cells, the expression of IL-17 or IFN- γ was analyzed (**Figure 2**). Data were analyzed by FlowJo software, as described above.

Statistical Analysis

Data are expressed as mean \pm SD. Statistical analysis was performed with GraphPad Prism software version 5.03 (GraphPad Software, San Diego, CA, USA). Statistical significance *P* values for differences between groups were assessed by Mann-Whitney tests and one-way ANOVA test. The statistical dependence between variables was assessed by performing Spearman's rank correlation analysis. All tests were two-tailed, and values of *P* < 0.05 were considered significant.

RESULTS

Characteristics of Participants

26 AHI, 31 CHI patients, and 20 HIV-1-negative HC were enrolled in this study (**Table 1**). Both AHI and CHI patients were further divided into two groups (HIV⁺RPR⁺ or HIV⁺RPR⁻) based on the syphilis status. The information of all the subjects is presented in **Table 1**. The age, sex, and the nationality of the HIV-1-infected patients and HC are matched. The estimated date of infection and viral load of HC are unavailable. The absolute number of CD4⁺ T cells and CD4/CD8 ratio in AHI and CHI patients are much lower than that in HC (*P* < 0.001). The absolute number of CD3⁺ and CD8⁺ T cells between HIV-1-infected patients and HC are not significant different (*P* > 0.05, **Table 1**).

Syphilis Infection Has Differential Effects on the Frequencies of $\gamma\delta$ T-Cell Subsets in Patients with AHI

First, we investigated the effect of syphilis on the frequencies of total $\gamma\delta$, V δ ₁, and V δ ₂ T-cell in HIV-1-infected patients. The frequencies of total $\gamma\delta$, V δ ₁, and V δ ₂ T cells among three groups: HC

(*n* = 20), HIV⁺RPR⁻ (*n* = 24), and HIV⁺RPR⁺ (*n* = 33) patients were compared. As shown in **Figure 3A**, the frequencies of total $\gamma\delta$ T cells were not significantly different among the three groups. The frequencies of V δ ₁ T cells was significantly higher in the HIV⁺RPR⁻ group than in HC, but no significant difference was found compared with HIV⁺RPR⁺ group. The frequencies of V δ ₂ T cells was significantly lower in both HIV⁺RPR⁻ and HIV⁺RPR⁺ groups compared with HC. However, we found no significant difference in V δ ₂ T-cell frequency between HIV⁺RPR⁻ and HIV⁺RPR⁺ groups (**Figures 3B,C**). Based on the above results, we assumed that HIV-1 infection status might affect the frequencies of total $\gamma\delta$, V δ ₁, and V δ ₂ T cells. Therefore, we split the HIV⁺RPR⁻ and HIV⁺RPR⁺ groups into subgroups of patients with 26 AHI and 31 CHI patients. The frequencies of total $\gamma\delta$, V δ ₁, and V δ ₂ T cells were compared between the HIV⁺RPR⁻ and HIV⁺RPR⁺ subgroups with AHI and CHI. We found no significant difference of the frequencies of total $\gamma\delta$ T cells between HIV⁺RPR⁻ and HIV⁺RPR⁺ groups for either acute or CHI (**Figure 3D**). The frequencies of V δ ₁ T cells were significantly lower in the HIV⁺RPR⁻ group than in the HIV⁺RPR⁺ group for AHI patients. By contrast, in CHI, no significant difference of the frequencies of V δ ₁ T cells was found between the HIV⁺RPR⁺ and HIV⁺RPR⁻ groups. Moreover, in the HIV⁺RPR⁺ group, the frequencies of V δ ₁ T cells were significantly higher in CHI patients than in AHI patients (**Figure 3E**). Conversely, in AHI patients, the frequencies of V δ ₂ T cells were significantly higher in the HIV⁺RPR⁺ group than in the HIV⁺RPR⁻ group. However, no significant difference of the frequencies of V δ ₂ T cells between HIV⁺RPR⁻ and HIV⁺RPR⁺ groups was observed in CHI patients. Moreover, for HIV⁺RPR⁺ group, the frequencies of V δ ₂ T cells in AHI patients were significantly higher than that in CHI patients (**Figure 3F**). Taken together, these results suggest that syphilis has differential effects on the frequencies of V δ ₁ and V δ ₂ T cells in patients with HIV-1 infection, depending on that this infection is acute or chronic.

Syphilis Affects $\gamma\delta$ T-Cell Differentiation in Patients with AHI

Memory CD4⁺ T-cell depletion is a major cause of HIV-1 pathogenesis and disease progression (35, 36). Like CD4⁺ T cells, $\gamma\delta$ T cells can be split into naïve and memory T-cell subsets on the basis of their surface expression of CD45RA and CD27 (37). Herein, we found that the frequencies of naïve (CD27⁺CD45RA⁺, T_{Naïve}) $\gamma\delta$ T cells in HIV⁺RPR⁺ group in AHI patients were significantly increased compared with HC, and HIV⁺RPR⁻ patients with both acute and CHI. The frequencies of T_{Naïve} $\gamma\delta$ T cells in HIV⁺RPR⁺ group in CHI patients were significantly higher compared with HC, but no significant differences were shown compared with the other 3 groups (**Figure 4A**). In AHI patients, the frequencies of central memory (CD27⁺CD45RA⁻, T_{CM}) $\gamma\delta$ T cells in the HIV⁺RPR⁻ group were significantly lower than that in HC and HIV⁺RPR⁺ group. In CHI patients, the frequencies of T_{CM} $\gamma\delta$ T cells were significantly higher than that in HIV⁺RPR⁻ group in AHI patients (**Figure 4B**). Moreover, the frequencies of effector memory (CD27⁻CD45RA⁻, T_{EM}) $\gamma\delta$ T cells in the HIV⁺RPR⁻ and HIV⁺RPR⁺ groups in both AHI and CHI patients were significantly lower than that in HC. However, there were no significant differences of the frequencies of T_{EM} $\gamma\delta$ T cells

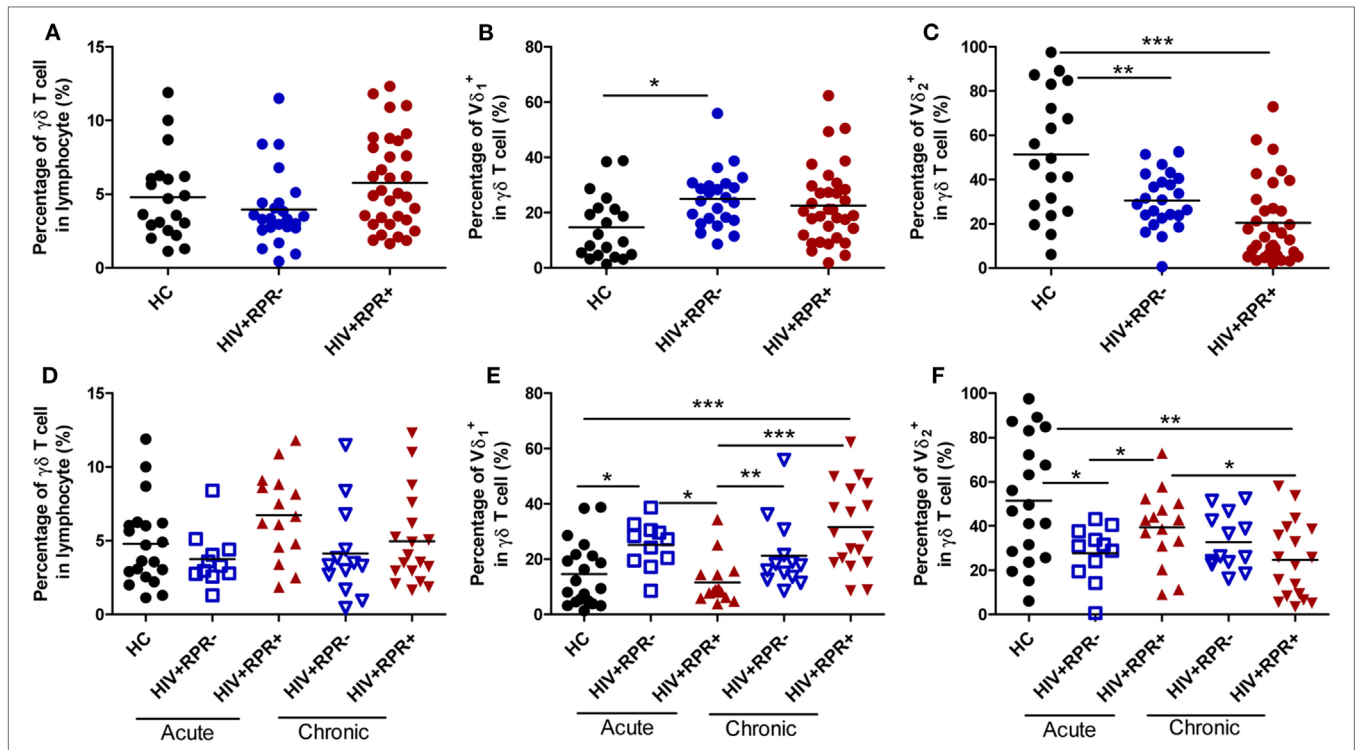


FIGURE 3 | Comparison of the frequencies of $\gamma\delta$ T cells, $V\delta_1$ and $V\delta_2$ T cells among healthy controls, HIV-1-infected and HIV-1/syphilis coinfecting patients. Based on the results of rapid plasma reagin (RPR), HIV-1-infected patients were divided into HIV+RPR⁻ and HIV+RPR⁺ groups. The percentages of $\gamma\delta$ T cells (A), $V\delta_1$ T cells (B), and $V\delta_2$ T cells (C) were compared among HC (●), HIV+RPR⁻ (●), and HIV+RPR⁺ (●) groups. Next, HIV+RPR⁻ and HIV+RPR⁺ groups were subdivided according to the acute or chronic nature of their HIV-1 infection. The percentages of $\gamma\delta$ T cells (D), $V\delta_1$ T cells (E), and $V\delta_2$ T cells (F) were compared among HIV+RPR⁻ (□) and HIV+RPR⁺ (▲) groups in acute HIV-1 infection and HIV+RPR⁻ (▽) and HIV+RPR⁺ (▼) groups in chronic HIV-1 infection. The significance of differences was determined by calculating *P* values in Mann–Whitney tests and one-way ANOVA test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. HC, healthy controls; HIV+RPR⁺, patients coinfecting with HIV-1 and syphilis; HIV+RPR⁻, patients infected with HIV-1 without syphilis.

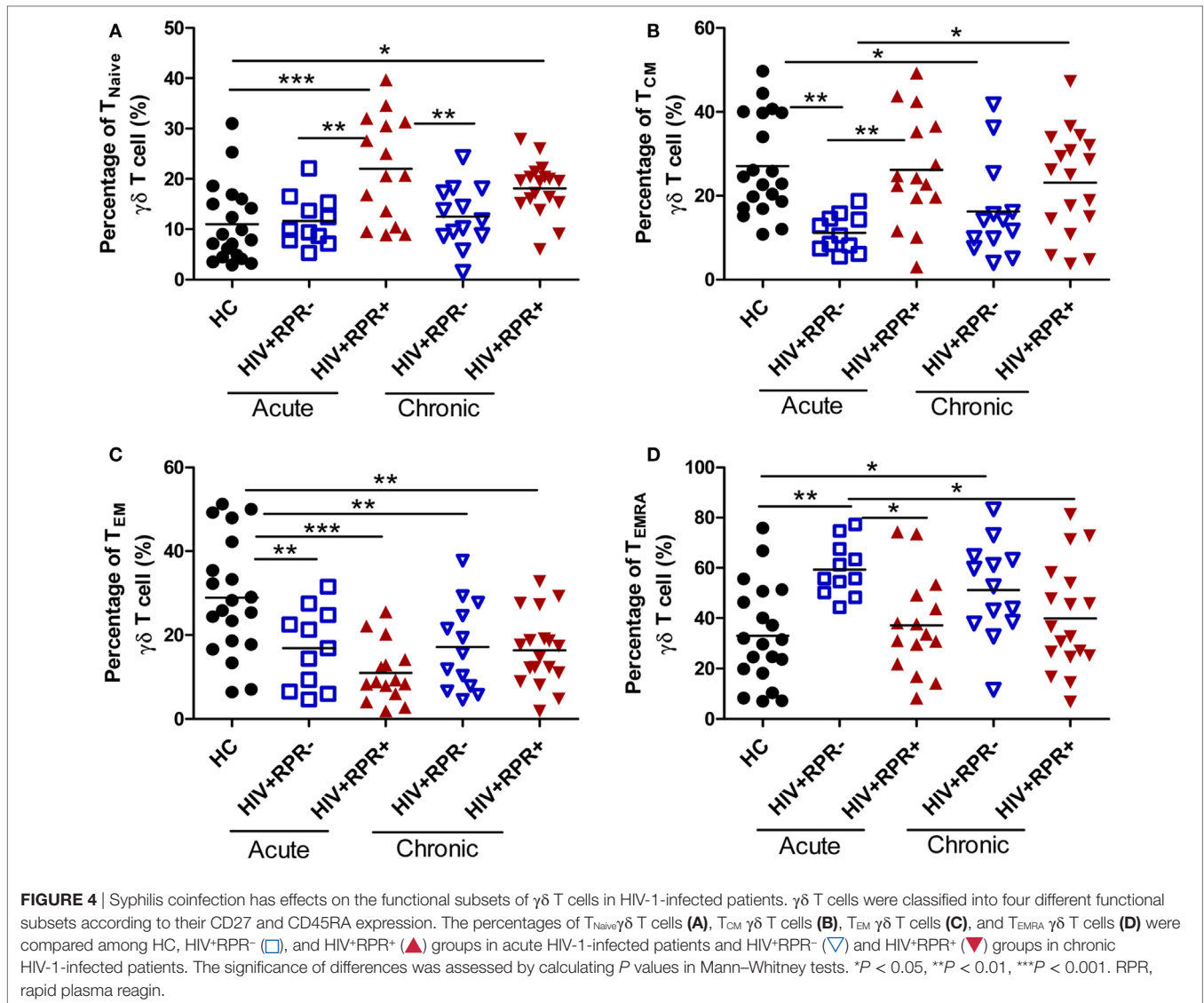
between the HIV+RPR⁻ and HIV+RPR⁺ groups in both AHI and CHI patients (Figure 4C). Finally, we found the frequencies of terminally differentiated (CD27⁻CD45RA⁺, T_{EMRA}) $\gamma\delta$ T cells in HIV+RPR⁻ group in AHI patients were significantly higher than that in HC and HIV+RPR⁺ groups in both AHI and CHI patients. There was no significant difference between the HIV+RPR⁻ and HIV+RPR⁺ groups in CHI patients (Figure 4D).

Different $\gamma\delta$ T-cell subsets exhibit different effector functions. CD27⁻ V γ_9 V δ_2 T cells are considered as the major producer of IL-17 (38, 39) and neutrophils can regulate IL-17 production by $\gamma\delta$ T cells (40–42). Therefore, we analyzed the relationships between the frequencies of T_{Naive}, T_{CM}, T_{EM}, and T_{EMRA} $\gamma\delta$ T cells and the frequencies of neutrophils (Figure S1 in Supplementary Material). We found that the frequencies of T_{EM} $\gamma\delta$ T cells were positively correlated with the frequencies of neutrophils. However, no relationships were observed between the frequencies of T_{Naive}, T_{CM}, and T_{EMRA} $\gamma\delta$ T cells and the frequencies of neutrophils (Figure S1 in Supplementary Material).

Syphilis Coinfection Effects on $\gamma\delta$ T-Cell Activation following Different Stages of HIV-1 Infection

We have shown that $\gamma\delta$ T cells were over-activated in HIV-1-infected patients, and associated with disease progression

(26, 43). Here, we investigated the effects of syphilis on $\gamma\delta$ T-cell activation by comparing $\gamma\delta$ T-cell activation among HC, the HIV+RPR⁺ and HIV+RPR⁻ groups in both AHI and CHI patients. Consistent with our previous results, we found that the frequencies of CD38⁺ $\gamma\delta$ T cells were significantly higher in both the HIV+RPR⁺ and HIV+RPR⁻ groups in AHI and CHI patients, compared with HC. In AHI patients, the frequencies of CD38⁺ $\gamma\delta$ T cells in HIV+RPR⁺ group were significantly lower than that in HIV+RPR⁻ group (Figure 5A). In addition, we found that, in AHI patients, the frequencies of HLA-DR⁺ $\gamma\delta$ T cells and CD38⁺HLA-DR⁺ $\gamma\delta$ T cells were significantly lower in the HIV+RPR⁺ group than in the HIV+RPR⁻ group (Figures 5B,C). By contrast, in CHI patients, the frequencies of HLA-DR⁺ $\gamma\delta$ T cells and CD38⁺HLA-DR⁺ $\gamma\delta$ T cells were significantly higher in the HIV+RPR⁺ group than that in the HIV+RPR⁻ group (Figures 5B,C). Moreover, the frequencies of CD38⁺, HLA-DR⁺, and CD38⁺HLA-DR⁺ $\gamma\delta$ T cells in CHI patients, both HIV+RPR⁺ and HIV+RPR⁻ groups, were significantly higher than that in HIV+RPR⁺ group in AHI patients (Figures 5A–C). In a previous study, it was reported that neutrophils inhibited $\gamma\delta$ T-cell activation (44), we therefore analyzed the relationship between $\gamma\delta$ T-cell activation and the percentage of neutrophils. We found that there were no correlations between the frequencies of CD38⁺, HLA-DR⁺, and CD38⁺HLA-DR⁺ $\gamma\delta$ T cells and the percentages of neutrophils



(Figure S2 in Supplementary Material). Thus, HIV-1 infection appears to lead to $\gamma\delta$ T-cell over-activation. However, syphilis can differentially affect $\gamma\delta$ T-cell activation status, depending on the acute or chronic nature of the HIV-1 infection.

IL-17-Producing $\gamma\delta$ T Cells Are Positively Correlated with the Percentage of Neutrophils in HIV-1-Infected Patients

Previous studies have shown that IL-17 levels in the peripheral blood or central nervous system (CFS) increase in patients with asymptomatic neurosyphilis and secondary syphilis, possibly as part of the immune response to *T. palladium* infection (19, 20, 27, 45). $\gamma\delta$ T cells are a major source of IL-17, which is involved in inflammation and immune response (46, 47). Therefore, we assessed and compared the frequencies of IL-17-producing and IFN- γ -producing $\gamma\delta$ T cells in HC, and in the HIV+RPR⁺ and HIV+RPR⁻ patients with acute and CHI. We

found that the frequencies of IL-17-producing $\gamma\delta$ T cells were significantly higher in CHI patients than in HC, particularly for the HIV+RPR⁺ group (Figure 6A). Surprisingly, we found a significant difference in the frequencies of IL-17-producing $\gamma\delta$ T cells between the HIV+RPR⁺ and HIV+RPR⁻ groups was observed in CHI patients, but not in AHI patients. Moreover, the frequencies of IL-17-producing $\gamma\delta$ T cells in HIV+RPR⁺ group in CHI patients were significantly higher than that in both HIV+RPR⁺ and HIV+RPR⁻ groups in AHI patients (Figure 6A). In addition, the frequency of IFN- γ -producing $\gamma\delta$ T cells was significantly lower in the HIV+RPR⁺ group in CHI patients than that in HC. The frequencies of IFN- γ -producing $\gamma\delta$ T cells in HIV+RPR⁺ group in AHI patients were significantly higher than that in HIV+RPR⁺ group in CHI patients. However, there was no significant difference in the frequency of IFN- γ -producing $\gamma\delta$ T cells between the HIV+RPR⁺ and HIV+RPR⁻ groups in both AHI and CHI patients (Figure 6B). Thus, both IL-17 and IFN- γ may be involved in $\gamma\delta$ T-cell-mediated immune

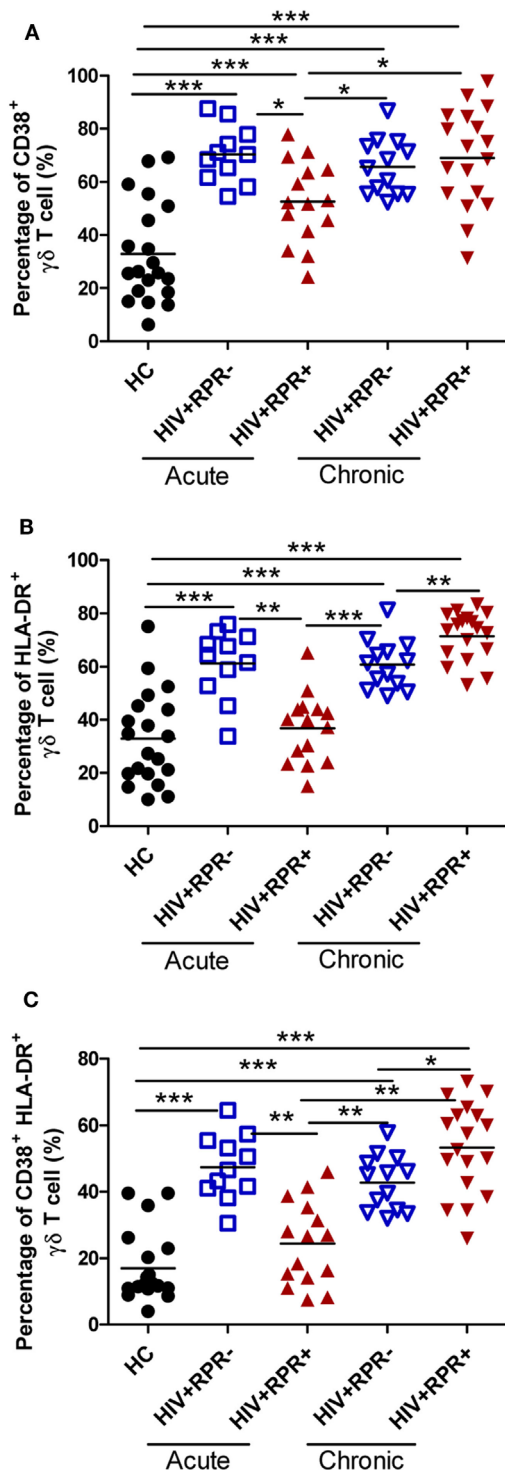


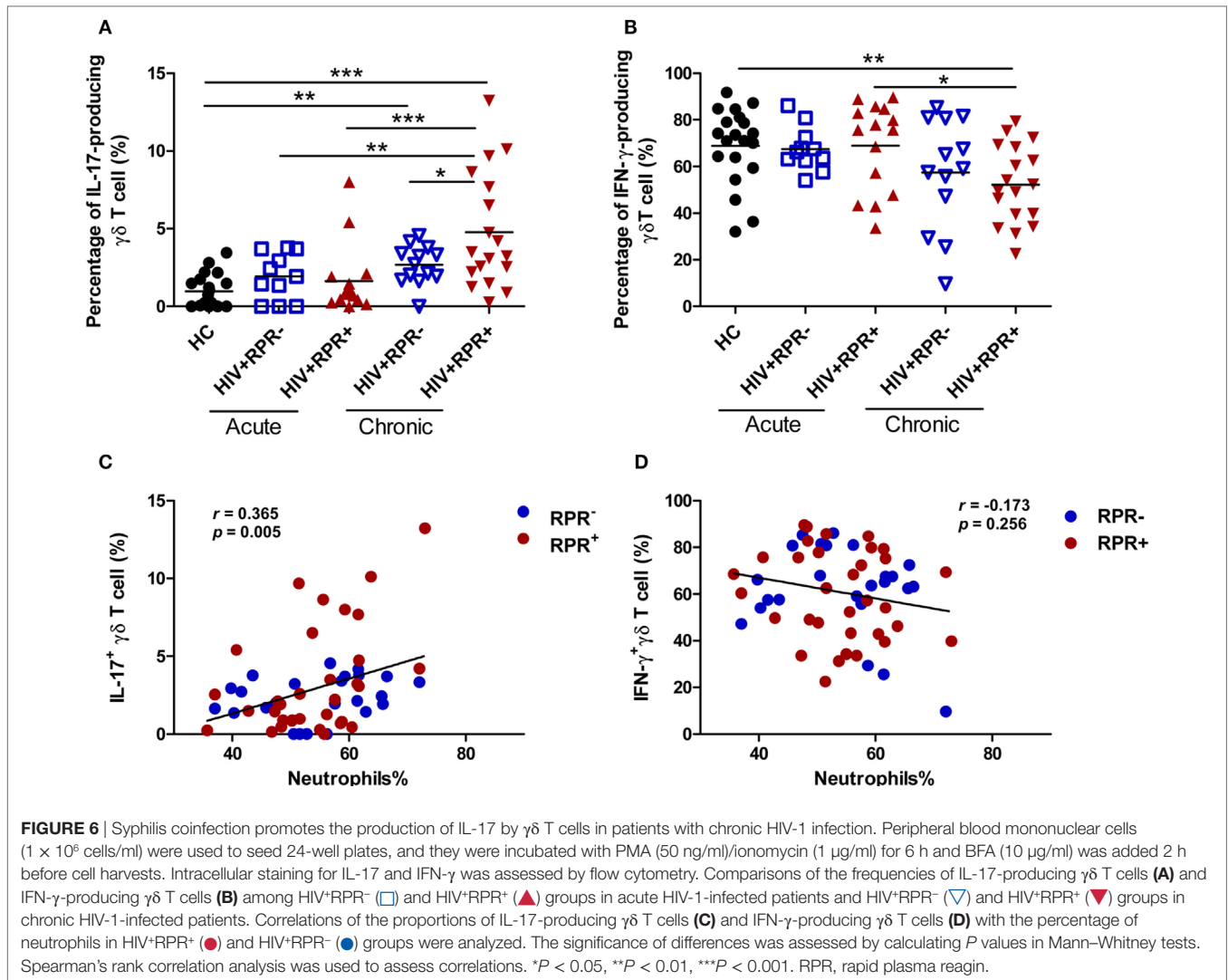
FIGURE 5 | Syphilis coinfection affects differently $\gamma\delta$ T-cell activation in patients with acute and chronic HIV-1 infection. $\gamma\delta$ T-cell activation was assessed by evaluating the expression of CD38 and HLA-DR. The frequencies of CD38⁺ $\gamma\delta$ T cells (A), HLA-DR⁺ $\gamma\delta$ T cells (B), and CD38⁺HLA-DR⁺ $\gamma\delta$ T cells (C) were compared among HC, HIV+RPR⁻ (□), and HIV+RPR⁺ (▲) groups in acute HIV-1-infected patients and HIV+RPR⁻ (▽) and HIV+RPR⁺ (▼) groups in chronic HIV-1-infected patients. The significance of differences was determined by calculating *P* values in Mann–Whitney tests. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. RPR, rapid plasma reagin.

response to syphilis, which seems to depend on HIV-1 disease stage. Recent studies have shown that neutrophils can suppress $\gamma\delta$ T-cell activation, proliferation, and IFN- γ production (44, 48). However, Coffelt et al. reported that neutrophils promoted IL-17 production by $\gamma\delta$ T cells, leading to tumor metastasis (49). We therefore hypothesized that the differences in $\gamma\delta$ T-cell functions in AHI and CHI patients might be associated with neutrophils. We analyzed the relationships between IL-17- or IFN- γ -producing $\gamma\delta$ T cells and the percentage of neutrophils. We found that the frequencies of IL-17-producing $\gamma\delta$ T cells were positively correlated with the percentage of neutrophils (Figure 6C). However, no correlation was found between the frequencies of IFN- γ -producing $\gamma\delta$ T cells and the percentage of neutrophils (Figure 6D). Taken together, these results suggest that syphilis may lead to the recruitment of neutrophils to local sites, where they promote the production of IL-17 by $\gamma\delta$ T cells, leading to inflammation, immune activation, and an acceleration of HIV-1 disease progression.

DISCUSSION

HIV-1 and syphilis, two sexually transmitted diseases, show rapidly increasing incidences of coinfection in MSM population (2, 11). $\gamma\delta$ T cells, a critical component of the host immune system, play an important role in controlling HIV-1 infection. However, the effects of syphilis on $\gamma\delta$ T-cell phenotype and function remain unclear. Herein, we examined and compared the phenotype and function of $\gamma\delta$ T cells in the HIV⁺RPR⁺ and HIV⁺RPR⁻ groups of patients with AHI and CHI separately. Consistent with previous studies (26, 50), we found that HIV-1 infection disrupted the balance of V δ ₁ and V δ ₂ T cells, increased the frequency of V δ ₁ T cells, and decreased the frequency of V δ ₂ T cells (Figures 3B,C). Interestingly, we found that syphilis corrected the imbalance of V δ ₁ and V δ ₂ T cells in patients with AHI, by decreasing the proportion of V δ ₁ T cells and increasing that of V δ ₂ T cells (Figures 3E,F). However, this effect was not observed in patients with CHI. The mechanisms by which syphilis affects the proportions of V δ ₁ and V δ ₂ T cells in patients with AHI are unknown, but may involve an induction of V δ ₁ T-cell proliferation by *T. pallidum* antigens early in infection (51). In this study, we found that syphilis coinfection among HIV-1-infected patients did not affect the frequency of total $\gamma\delta$ T cells; this may be due to the mutual compensation of V δ ₁ and V δ ₂ T cells. The frequencies of V δ ₂ T cells in healthy controls are diffusion, which appeared to be donor dependent. V δ ₁ and V δ ₂ T cells exhibit different features in location site, cytokine secretion, and cytotoxicity (24, 46, 52, 53). Therefore, it would be more helpful to understand the mechanisms of how syphilis differentially regulates the phenotype and function of $\gamma\delta$ T cells by analyzing the phenotypes and functions of V δ ₁ and V δ ₂ T cells separately in the further studies.

$\gamma\delta$ T cells can be classified into T_{Naive}, T_{CM}, T_{EM}, and T_{EMRA} four functional subsets. T_{Naive} and T_{CM} $\gamma\delta$ T cells are located in lymph nodes and short of immediate effector function, whereas T_{EM} and T_{EMRA} $\gamma\delta$ T cells prefer to locate in inflammatory sites and show immediate effector functions (54). We found that the frequencies of T_{Naive} and T_{CM} $\gamma\delta$ T cells were significantly higher,



but the frequencies of T_{EM} and T_{EMRA} $\gamma\delta$ T cells were significantly lower in HIV+RPR⁺ group than those in HIV+RPR⁻ group in patients with acute HIV-1-infected patients (Figure 4). These results suggest that *T. pallidum* may trigger $\gamma\delta$ T-cell proliferation and differentiation, in a manner dependent on the HIV-1 stage in coinfecting patients, and that the changes of the frequencies of T_{Naive} , T_{CM} , T_{EM} , and T_{EMRA} $\gamma\delta$ T cells may be associated with $\gamma\delta$ T-cell activation (26). Furthermore, we observed a positive correlation between the frequencies of T_{EM} $\gamma\delta$ T cells and the percentage of neutrophils in AHI. This may indicate that mutual interactions between immune cells may also have some effects on $\gamma\delta$ T-cell differentiation.

Chronic immune activation is a key hallmark of HIV-1 infection and pathogenesis (55). We found here that $\gamma\delta$ T cells are activated in all patients with both AHI and CHI, regardless of syphilis (Figure 5). However, syphilis infection regulates $\gamma\delta$ T-cell activation status following the different stage of HIV-1 infection, that is, the frequencies of CD38⁺, HLA-DR⁺, and CD38⁺HLA-DR⁺ $\gamma\delta$ T cells were decreased in patients with AHI but they were increased in patients with CHI (Figure 5).

These differential effects may be mediated by the spirochetal lipoproteins, abundant membrane proteins inducing a strong immune response. Moreover, these lipoproteins, which enable the spirochetes to adhere to the host, determine the interaction between the spirochetes and the host environment and immune system (56). It has been suggested that spirochetal membrane lipoproteins bear pathogen-associated molecular patterns that bind to pattern recognition receptors such as Toll-like receptors (TLRs). Spirochetal lipoproteins (*T. pallidum* lipoproteins) are therefore pro-inflammatory. They may activate monocytes by binding to CD14, or macrophages through a TLR-dependent pathway, leading to the production of pro-inflammatory cytokines (57–60), which drive inflammation and induce $\gamma\delta$ T-cell activation in patients with syphilis. The fact that syphilis differentially modulates $\gamma\delta$ T-cell activation may be ascribed to the different host immune environments exhibited in patients with AHI and CHI. Moreover, interactions among immune cells may affect $\gamma\delta$ T-cell activation (61). However, we did not observe any correlation between $\gamma\delta$ T-cell activation and neutrophils in AHI. The precise mechanisms by which syphilis differentially

modulates $\gamma\delta$ T-cell activation are not fully understood and deserve further investigation.

IL-17, an inflammatory cytokine, has two principal effects: host protection and immunopathogenesis, with high levels leading to cancer progression and autoimmune diseases (62). Plasma IL-17 concentration increases in secondary syphilis, possibly as part of the immune response (20, 45). Syphilis also affects the liver, where it may cause syphilitic hepatitis or hepatic inflammatory tumors in HIV-1-positive MSM (63). However, the role of IL-17 and IL-17-producing $\gamma\delta$ T cells in HIV-1-infected patients with syphilis remains unclear. In this study, we found that patients with CHI, particularly for the HIV⁺RPR⁺ group have an increase in the proportion of IL-17-producing $\gamma\delta$ T cells, which was positively correlated with the percentage of neutrophils (Figures 6A,C). Our findings suggest that the immune response to *T. pallidum* promotes the production of IL-17 by $\gamma\delta$ T cells in patients with CHI, leading to the recruitment of neutrophils to the inflammatory site, and to mediate the immune response (44, 63, 64). We previously showed that the frequency of IL-17-producing $\gamma\delta$ T cells was correlated with $\gamma\delta$ T-cell activation (26). We also show here that change of activated $\gamma\delta$ T cells has a similar trend with the frequency of IL-17-producing $\gamma\delta$ T cells in all patients (Figures 5 and 6). Together, these findings suggest that syphilis induces the production of IL-17 by $\gamma\delta$ T cells, thereby promoting neutrophil-mediated immunity, triggering $\gamma\delta$ T-cell immune activation, and accelerating HIV-1 disease progression. Neutrophils have recently been recognized as a source of IL-17 (40). However, the effects of neutrophils on $\gamma\delta$ T cells remain unclear. Further studies on the $\gamma\delta$ T-cell/IL-17/neutrophil axis are therefore required to elucidate the mechanisms of $\gamma\delta$ T-cell action in HIV-1 pathogenesis and HIV-1 disease progression.

In summary, syphilis infection decreases immune activation and the secretion of inflammatory cytokines in AHI. To our knowledge, it's the first time to reveal the role of $\gamma\delta$ T cells in HIV-1 and syphilis coinfection. However, it remains unclear why and how syphilis differentially regulates the $\gamma\delta$ T-cell immune response at different stages of HIV-1 infection. The different effects of syphilis on $\gamma\delta$ T cells may relate to the different host immune environments in AHI and CHI and the natural features of $\gamma\delta$ T cells which respond in the early stages of infection. Moreover, younger age other than CD4⁺ T-cell, nadir CD4⁺ T-cell, or CD8⁺ T-cell counts may be one of the key factors that affect the phenotype and function of $\gamma\delta$ T cells (65). Therefore, syphilis should be followed up, monitored, and treated in the MSM population and in patients with AHI. In addition, there are still some limitations in this study. First, the sample size is small that may cause experimental deviation; second, we did not take into consideration other pathogens in this study, such as human cytomegalovirus and *Candida albicans* infection, which also have some effects on $\gamma\delta$ T cells (66–69), although the medians of CD4 T-cell count among HIV-1-infected patients more than 400 cells/ μ l (Table 1). Therefore, larger samples and more detailed studies in further investigation are required to address these issues in depth. Our observations provide new insight into the roles of $\gamma\delta$ T cells in immunopathogenesis of

syphilis and HIV coinfection, particularly during AHI. Our findings may be helpful for the prevention of syphilis and other sexually transmitted infections, which is just the dedication in this research and highlights the great significance on the remedy of patients coinfecting with HIV.

ETHICS STATEMENT

This study and all relevant experiments have been approved by the Beijing You'an Hospital Research Ethics Committee and written informed consent was obtained from each participant according to the declaration of Helsinki. All the participants provided written informed consent for their information, and clinical samples were stored and used for research. At enrollment, all subjects provided baseline demographic, clinical and epidemiological information by completing a standardized questionnaire. The methods were carried out in accordance with approved guidelines and regulations.

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AUTHOR CONTRIBUTIONS

ZL, HW, BS, and TZ conceived and designed the experiments; QZ, AS, YG, JY, and XH collected the sample information, contributed to reagents and materials; ZL, XL, ZH, ZWL, AS and DM performed the experiments and analyzed the data; and ZL, BS, WJ, HW, and TZ wrote the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://journal.frontiersin.org/article/10.3389/fimmu.2017.00991/full#supplementary-material>.

FIGURE S1 | Correlations between the frequencies of T_{Naive} , T_{CM} , T_{EM} , and $T_{\text{EMRA}}\gamma\delta$ T cells and neutrophils. $\gamma\delta$ T cells can be divided into four functional subsets according to the expression of CD27 and CD45RA. There were no correlations between the frequencies of T_{Naive} (A), T_{CM} (B), T_{EM} (C), and T_{EMRA} (D) $\gamma\delta$ T cells and the percentage of neutrophils in all HIV-1-infected patients with RPR⁻ and RPR⁺. The frequencies of T_{EM} $\gamma\delta$ T cells were positively correlated with the percentage of neutrophils in acute and all HIV-1-infected patients with RPR⁻ (●) and RPR⁺ (●). Correlations were calculated by using Spearman's rank correlation. $P < 0.05$ was considered to be statistically significant. RPR, rapid plasma reagin.

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FIGURE S2 | Correlation between $\gamma\delta$ T-cell activation and neutrophils. Two immune activation markers CD38 and HLA-DR were used to evaluate and compare $\gamma\delta$ T-cell activation status in both HC and HIV-1-infected patients with RPR⁻ and RPR⁺. The relationship between $\gamma\delta$ T-cell activation and neutrophils was analyzed. There were no correlations between the frequencies of CD38⁺ (A), HLA-DR⁺ (B), and CD38⁺HLA-DR⁺ (C) $\gamma\delta$ T cells and the percentage of neutrophils in all HIV-1-infected patients with RPR⁻ (●) and RPR⁺ (●). Correlations were calculated by using Spearman's rank correlation. $P < 0.05$ was considered to be statistically significant. RPR, rapid plasma reagin.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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