CCL5-Secreting Virtual Memory CD8+ T Cells Inversely Associate With Viral Reservoir Size in HIV-1–Infected Individuals on Antiretroviral Therapy

Wei Hu1,2†, Yan-Jun Li3†, Cheng Zhen2†, You-Yuan Wang1,2, Hui-Huang Huang2, Jun Zou3, Yan-Qing Zheng3, Gui-Chan Huang3, Si-Run Meng3, Jie-Hua Jin3, Jing Li2, Ming-Ju Zhou2, Yu-Long Fu2, Peng Zhang2, Xiao-Yu Li2, Tao Yang2, Xiu-Wen Wang2, Xiu-Han Yang2, Jin-Wen Song2, Xing Fan3, Yan-Mei Jiao2, Ruo-Nan Xu2, Ji-Yuan Zhang2, Chun-Bao Zou2, Jin-Hong Yuan2, Lei Huang2, Ya-Qin Qin3, Feng-Yao Wu3, Ming Shi2, Fu-Sheng Wang1,2,3* and Chao Zhang2,3*

1 Medical School of Chinese People’s Liberation Army (PLA), Beijing, China, 2 Department of Infectious Diseases, The Fifth Medical Center of Chinese PLA General Hospital, National Clinical Research Center for Infectious Diseases, Beijing, China, 3 Guangxi Acquired Immune Deficiency Syndrome (AIDS) Clinical Treatment Centre, The Fourth People’s Hospital of Nanning, Nanning, China

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Recent studies highlighted that CD8+ T cells are necessary for restraining reservoir in HIV-1-infected individuals who undergo antiretroviral therapy (ART), whereas the underlying cellular and molecular mechanisms remain largely unknown. Here, we enrolled 60 virologically suppressed HIV-1-infected individuals, to assess the correlations of the effector molecules and phenotypic subsets of CD8+ T cells with HIV-1 DNA and cell-associated unspliced RNA (CA usRNA). We found that the levels of HIV-1 DNA and usRNA correlated positively with the percentage of CCL4+CCL5- CD8+ central memory (TCM) cells while negatively with CCL4-CCL5+ CD8+ terminally differentiated effector memory cells (TEMRA). Moreover, a virtual memory CD8+ T cell (TVM) subset was enriched in CCL4-CCL5+ TEMRA cells and phenotypically distinctive from CCL4+ TCM subset, supported by single-cell RNA-Seq data. Specifically, TVM cells showed superior cytotoxicity potentially driven by T-bet and RUNX3, while CCL4+ TCM subset displayed a suppressive phenotype dominated by JUNB and CREM. In viral inhibition assays, TVM cells inhibited HIV-1 reactivation more effectively than non-TVM CD8+ T cells, which was dependent on CCL5 secretion. Our study highlights CCL5-secreting TVM cells subset as a potential determinant of HIV-1 reservoir size. This might be helpful to design CD8+ T cell-based therapeutic strategies for cure of the disease.
INTRODUCTION

Established antiretroviral therapy (ART) efficiently suppresses HIV-1 replication and de novo infection arising in people living with HIV-1 (PLWH). The implementation of a “treat-all” policy has led to infection with HIV-1 becoming a manageable chronic health condition (1–3). However, lifelong ART is mandatory to maintain viral suppression because latent HIV-1 reservoirs are major obstacles to curing HIV-1 (4).

Several clinical parameters are associated with the size of HIV-1 reservoir, such as pre-ART CD4 cell count (5), the CD4/CD8 ratio (5) and HIV-1 viral load (6, 7), residual viral replication during ART (6), and the duration of ART (6, 7). Moreover, the immune system, especially CD8+ T cells, plays an essential role in constraining HIV-1 reservoirs and limiting HIV-1 rebound during ART (8, 9). Defining evidence has been generated from SIV-infected macaques with viral suppression under ART (10). Plasma viremia develops in all animals when CD8+ T cells are depleted, and this can be controlled by repopulation with CD8+ T cells (11, 11). In PLWH who receive early ART, a preserved CD8+ T-cell effector function is associated with a smaller reservoir (12, 13). Thus, CD8+ T cells are important in limiting the HIV-1 reservoir in PLWH and might be harnessed to induce therapeutic antiviral effects (14). However, the exact functional subset and mechanism of action are not well understood.

The polyfunctional anti-HIV-1 effects exerted by CD8+ T cells are characterized by the production of cytokytic granules such as granzyme B (GZMB) and perforin (PRF), the secretion of effector cytokines such as interferon-γ (IFNγ), tumor necrosis factor alpha (TNFα), and interleukin (IL)-2 (12, 13), as well as production of β chemokines such as chemokine (C-C motif) ligand 3 (CCL3, also known as [aka] MIP-1α), CCL4 (aka MIP-1β) and CCL5 (aka RANTES) (15). Although those molecules are often used to evaluate the anti-HIV-1 functionality of CD8+ T cells, different cytokine-producing CD8+ T-cell subsets might be heterogeneous. For example, PLWH with poor immune reconstitution showed increased frequency of CCL4+-CD8+ T cells (16). In another Uganda women ART cohort, it was reported that the TNFα+ CD8+ T-cell frequency positively correlates with HIV-1 reservoir size (7).

In some cases, CD8+ T cells can be activated without cognate antigen stimulation (17). Such innate-like, bystander effect was also observed in HIV-1 infection. For instance, it was reported that major histocompatibility complex (MHC) matching is not necessary for CD8+ T cells to kill HIV-1-infected cells during primary infection (18, 19). Furthermore, bystander CD8+ T cells play an important role in maintaining the suppression of viremia in individuals undergoing ART (18, 20–25). Of which, we previously showed that a virtual memory T (TVM) cells subset, defined by CD44hi CD122hi CD49dlo in mice and CD45RA+ killer Ig-like receptors (KIR)+ and/or CD94 natural killer group 2 member A (NKG2A)+ in humans (17, 26, 27), senses and inhibits the reactivation of HIV-1 reservoir through KIR (28). Thus, TVM cells might represent a bystander CD8+ T-cell subset of interest in terms of constraining the size of HIV-1 reservoirs (29). The clinical relevance and functionality of this subset warrants further investigation.

The present study aimed to determine the effector mechanisms of CD8+ T cells, particularly TVM cells, and their potential impacts on HIV-1 reservoir. We found CCL4-CCL5+ terminally differentiated effector memory cells (TEMRA) negatively correlated with HIV-1 reservoir. TVM cells were enriched in CCL4-CCL5+ TEMRA cells and efficiently suppressed HIV-1 in vitro in a CCL5 dependent manner. Our findings highlight the role of CCL5-secreting TVM cells in constraining HIV-1 reservoirs and the potential of utilizing this mechanism to shrink HIV-1 reservoirs in future studies.

MATERIALS AND METHODS

Study Participants

We enrolled PLWH from the Fourth People’s Hospital of Nanning, Nanning, China. The inclusion criteria comprised: age 18–65 years, confirmed HIV-1 positive, successful ART for > 2 years, undetectable blood HIV-1 RNA < 20 copies/mL (below the limits of detection, undetectable, ND) for at least 6 to 12 months, and a CD4 cell count > 250/μL within 7 days of clinical sample collection. Key exclusion criteria comprised co-infection with hepatitis B or C viruses, pregnancy, illicit intravenous drug use, and common comorbidities, including seizure disorder, and liver and kidney-related diseases. Table 1 summarizes the characteristics of the participants. Individuals included in ex vivo functional assays were additionally recruited virologically suppressed HIV-1–infected individuals with the same inclusion criteria.

Flow Cytometry

Peripheral blood samples were collected upon enrollment, and peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque density gradient centrifugation. Sorted PBMCs were cryopreserved in liquid nitrogen. Before phenotypic staining (detection of GZMB, GNLY and PRF) or functional

| TABLE 1 | Characteristics of study population (n = 60). |
|----------|---------------------------------------------|
| Characteristic | Value |
| Age (y) | 46 (26–56) |
| Gender (female/male) | 22/38 |
| Pre-ART | |
| CD4 cell count (cells/μL) | 185.5 (5–934) |
| CD8 cell count (cells/μL) | 591.5 (50–2650) |
| CD4/CD8 ratio | 0.23 (0.01–1.23) |
| Time on ART (y) | 6.6 (2.5–14.9) |
| ART regimen—no. | |
| 2 NRTIs + 1 NNRTIs | 51 (85%) |
| 2 NRTIs + 1 PIs | 8 (13%) |
| 1 NRTIs + 1 PIs | 1 (2%) |
| At enrolment | |
| Plasma HIV RNA | ND |
| CD4 cell count (cells/μL) | 504.5 (283–1378) |
| CD8 cell count (cells/μL) | 743 (377–1846) |
| CD4/CD8 ratio | 0.70 (0.24–1.87) |

Data are shown as medians with ranges (min to max) or no. with percentages. ART, Antiretroviral therapy; ND, undetectable; NRTIs, nucleoside reverse transcriptase inhibitors; NNRTIs, non-nucleoside reverse transcriptase inhibitors; PIs, protease inhibitors.
stimation (detection of CCL3, CCL4, CCL5, IL-2, IFNγ and TNFα), cryopreserved PBMCs were thawed and incubated in RPMI 1640 containing 10% fetal bovine serum (FBS) for 2 h at 37°C and 5% CO2. Then cells were stained with monoclonal antibody (mAb) for surface markers at 4°C for 30 min. For intracellular staining, the samples were fixed and permeabilized for intracellular staining with the indicated antibodies at 4°C for 30 min using the Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific Inc., Walhama, MA). The cells were fixed in 2% formaldehyde and examined by flow cytometry on a BD Canto II flow cytometer (BD Biosciences, San Diego, CA) within 24 h. Flow cytometry data were analyzed using FlowJo software (version 10.5.3, BD Biosciences). Aberrant events were removed using the FlowAI plugin for t-distributed stochastic neighbor embedding (t-SNE) analysis (30). The DownSample plugin was then used to randomly down-sample CD8+ T cells into 3,000 cells, which were then concatenated into a single file. This file was visualized using the t-SNE plugin with CD45RA, CD27, CCL3, CCL4, and CCL5 markers under the following conditions: Iterations 300, Perplexity 30, Eta 7% of cell number, and Theta 0.5. The subsets were then separately gated and plotted onto a t-SNE map.

The following fluorochrome-conjugated anti-human mAbs or reagents were used: APC/Fire™ 750 anti-CD3 (SK7), PerCP anti-CD8 (SK1), BV510 anti-CD45RA (HI100), PE-Cy7 anti-CD27 (O323), BV421 anti-PRF (dG9), APC anti-IL-2 (MQ1-17H12), FITC anti-IFNγ (4S.B3), and BV421 anti-TNFα (MAb11) (BioLegend, San Diego, CA); PerCP anti-CD4 (SK3), AF647 anti-GZMB (GB11), AF488 anti-GLNL (RB1), and BV421 anti-CCL5 (2D5) (BD Biosciences); PE anti-NKG2A (REA110), PE anti-KIR2D (DX27), and PE anti-KIR3DL1 (DX9) (Miltenyi Biotec, Auburn, CA); FITC anti-CCL3 (CR3M) and AF647 anti-CCL4 (FL34Z3L) (Thermo Fisher Scientific Inc.); FITC anti-Gag P24 (kc57) (Beckman Coulter Brea, CA); and Live/dead dye (Thermo Fisher Scientific Inc.).

**Functional Assays for CD8+ T Cells**

We stimulated PBMCs with IL-15 (50 ng/mL) for 48 h and IL-2 (500 IU/mL), anti-CD3 (1 ng/mL), anti-CD28 (1 ng/mL), and anti-CD49d (1 ng/mL) for 6 h to detect intracellular CCL3, CCL4, and CCL5 (31), and with IL-12 (20 ng/mL), IL-15 (10 ng/mL), and IL-18 (20 ng/mL) for 48 h to detect intracellular IL-2, IFNγ and TNFα (26). GolgiPlug was added to the medium 6 h before harvest to detect intracellular cytokines. Recombinant human IL-12 and IL-18 were purchased from PeproTech (Rocky Hill, NJ). Recombinant human IL-2 and IL-15, anti-human CD3 (clone OKT3), anti-human CD28 (clone CD28.2), and anti-human CD49d (clone 9F10) antibodies were purchased from BioLegend.

**Detection of HIV-1 DNA and CA usRNA**

Total cellular HIV-1 DNA and RNA were extracted using Qiagen QIAsymphony DNA Mini Kits (Qiagen, Valencia, CA) and HiPure Total RNA Plus Mini Kits (Magen, Guangzhou, China), respectively. We quantified HIV-1 DNA and CA usRNA using fluorescence-based real-time SUPBIO HIV-1 Quantitative Detection Kits (SUPBIO, Guangzhou, China). The quantitation range was 5–10 ×10^6 copies/10^6 PBMCs.

**Viral Inhibition Assay**

Latency-reversal CD8+ T cells were co-cultured with killer cells to analyze their suppressive capability as described with minor modifications (32–34). Briefly, CD8+ T and CD56+ NK cells were isolated using anti-CD8 and anti-CD56 microbeads (Miltenyi Biotec, respectively). CD8+ T cells were maintained in RPMI 1640 medium containing 10% FBS and 500 IU/mL IL-2 before coculture. The remaining CD8− and CD56− depleted cells were mainly CD4+ T cells with >90% purity, and were cultured in RPMI 1640 medium containing 10% FBS, 50 ng/ml PMA, 1 μM ionomycin, and 500 IU/mL IL-2 for 48 h. Latency-reversal CD8+ T cells were cultured alone or co-cultured with CD8+ T cells at a 1:3 ratio in RPMI 1640 medium containing 10% FBS and 500 IU/mL IL-2 for 24 h. Viral inhibition was determined as a decrease in P24+ % CD8+ T cells and as % inhibition.

The inhibition abilities of TVM and non-TVM were compared as follows. The CD8+ T cells selected using microbeads were further sorted into TVM and TVM−depleted (non-TVM CD8+ T cells) subsets using a FACS Aria II cell sorter (BD Bioscience). The sorted subsets were separately cultured for 2 days, then co-cultured with latency-reversal CD8− and CD56−depleted cells to calculate viral suppression rates as described above. To examine the inhibition mechanisms of TVM cells, 5 μg/mL CCL5 blockade antibody (αCCL5, clone 21445, R&D Systems, Minneapolis, MN) and 5 μg/mL αCCL3 (clone W16009B, BioLegend) were separately added to the co-culture system.

**scRNA-seq Analysis**

The raw scRNA-seq data of CD8+ T cells purified from PBMCs of 3 ART-treated individuals (clinical characteristics were presented in Supplementary Figure 6A) were downloaded from the Genome Sequence Archive of the Beijing Institute of Genomics Data Center, Chinese Academy of Sciences [http://bigd.big.ac.cn/gsa-human, accession code HRA000190, uploaded by our group (35)]. The downloaded data were processed with Cell ranger (v.6.0.1) pipeline against GRCh38 human reference genome to generate gene expression matrices. Subsequently, the Cell control process was performed with the Seurat package (v.4.0.5) in R (v.4.1.0). Briefly, the genes expressed in ≥5 cells were kept for every sample; cells that met these following criteria were retained: (1) > 500 genes; (2) ≥1000 but < 20000 unique molecular identifiers (UMIs); (3) <10% UMIs derived from the mitochondrial genome (Supplementary Figure 6B). Scrublet package (v.0.2.3) in Python (v.3.9.7) was applied to each dataset to remove potential doublets with the expected doublet rate of 6%. All the cells predicted as “doublets” were filtered out. The NormalizeData function in Seurat was applied to normalize the inhibition abilities of TVM and non-TVM were compared as follows. The CD8+ T cells selected using microbeads were further sorted into TVM and TVM−depleted (non-TVM CD8+ T cells) subsets using a FACS Aria II cell sorter (BD Bioscience). The sorted subsets were separately cultured for 2 days, then co-cultured with latency-reversal CD8− and CD56−depleted cells to calculate viral suppression rates as described above. To examine the inhibition mechanisms of TVM cells, 5 μg/mL CCL5 blockade antibody (αCCL5, clone 21445, R&D Systems, Minneapolis, MN) and 5 μg/mL αCCL3 (clone W16009B, BioLegend) were separately added to the co-culture system.

Three scRNA-seq datasets (each for one person) were assembled with Seurat package into an integrated dataset to eliminate the batch effect. In detail, anchors among the three datasets were identified with the FindIntegrationAnchors function, and then were imported into the IntegrateData.
function to generate a corrected and integrated Seurat object. Consequently, uniform manifold approximation and projection (UMAP) of all cells was performed by the RunUMAP function in Seurat to reduce the dimension and visualize the clusters. All these clusters were manually annotated according to the expression of selected markers (36).

The differentially expressed genes (DEGs) were calculated with the FindMarkers function using Wilcoxon Rank Sum test and p-values were adjusted through Bonferroni correction. For gene ontology (GO) analysis and transcription factor analysis, DEGs with log2 fold change (log2FC) of more than 0.3 and adjusted p-values of less than 0.05 were selected. In volcano plots, threshold of log2FC was set to 0.5 and threshold for adjusted p-values was 0.05. GO analysis were carried out with ClusterProfiler package (v.4.0.5) in R.

To reconstruct the gene regulatory networks, transcriptional factor (TF) analysis and clustering were performed with SCENIC package (v.1.2.4) in R and Arboreto package (v.0.1.6) in Python (37). Briefly, the normalized and quality-controlled single-cell RNA-seq expression matrix was used as input, while both 500 bp upstream of the transcription start site (TSS) and ±10kb around the TSS were explored for TF binding motifs. SCENIC identify potential targets for each TF based on co-expression matrix and GRNBoost2 algorithm in Arboreto package was applied to filter out unqualified genes to form co-expression modules. Potential direct-binding targets (regulons) based on DNA-motif analysis was selected and the network activity in each individual cell was calculated with AUCell. The cell-regulon activity matrix was visualized as heatmap and the gene regulatory networks was drawn with Cytoscape (v.3.9.0).

Statistical Analysis
Data were statistically analyzed using GraphPad Prism version 8.0.1 (GraphPad Software Inc., San Diego, CA, USA). Data are shown as medians with ranges (min to max). For two group comparison, Mann Whitney tests were used for unpaired data and Wilcoxon matched-pairs signed rank tests for paired data. For three group comparison, an overall primary analysis was performed (Kruskal-Wallis tests for unpaired data and Friedman tests for paired data). If the results were statistically significant, comparisons between two group were further performed. Correlations were evaluated using nonparametric Spearman correlation tests. Values were considered statistically significant at p < 0.05. Due to current cohort size and the exploratory nature of the study, Bonferroni corrections were employed to adjust the threshold of p value in multiple comparison analysis.

RESULTS
Characteristics of Study Population
The present study included 60 participants with chronic HIV-1 infection that had sustained virological suppression during ART. They had been under ART for > 2 years (median, 6.6 years). Table 1 summarizes the characteristics of the study population. The median age was 46 years, 37% were female, the median pre-ART and present CD4 cell counts were 185.5 and 504.5/μL, respectively. Figure 1A shows the design of current study. The median numbers of HIV-1 DNA and HIV-1 CA usRNA copies were 217 and 468/10^6 cells, respectively. We analyzed CD4+ and CD8+ T-cell subsets based on CD45RA and CD27 expression and effector molecule expression in CD8+ T cells and phenotypic subsets. Pre-ART CD4 cell counts and the pre-ART CD4/CD8 ratio were negatively associated with HIV-1 DNA (r = -0.2844, p = 0.0263 and r = -0.3012, p = 0.0183, respectively; Supplementary Figure 1). There correlations became insignificant after Bonferroni correction (p_adj set to 0.0063). Although not statistically significant, the increase in CD4 cell count tended to positively correlate with HIV-1 DNA (r = 0.2411, p = 0.0613; Supplementary Figure 1). ART duration was insignificantly correlated with HIV-1 DNA and immune subset parameters (Supplementary Figures 1, 8). Associations between the analyzed clinical parameters and HIV-1 CA usRNA were not identified.

CD8+ TEMRA Percentage Negatively Correlates With the HIV-1 Reservoir in ART Individuals
Based on CD45RA and CD27 expression, CD4+ and CD8+ T cells were classified as CD45RA-CD27- effector memory (TEM), CD45RA+CD27- TEMRA, CD45RA-CD27+ central memory (TCM), and CD45RA+CD27+ naive (TN) populations (Supplementary Figure 2A shows gating strategy) (38). Statistical trends in associations were found between the CD4+ TCM percentage and HIV-1 DNA (r = 0.239, p = 0.0636) and CA usRNA (r = 0.2382, p = 0.064), and between the CD4+ TN percentage and HIV-1 DNA (r = -0.2450, p = 0.0570) (Figure 1B). No other CD4+ sub-population percentages were associated with HIV-1 DNA or CA usRNA. Percentage of CD8+ TCM positively correlated with HIV-1 DNA (r = 0.4911, p < 0.0001) and CA usRNA (r = 0.4925, p < 0.0001), whereas CD8+ TEMRA percentage correlated negatively with HIV-1 DNA (r = -0.4706, p = 0.0001) and CA usRNA (r = -0.3527, p = 0.0057) (Figure 1B and Supplementary Figure 3). These correlations remained statistically significant after Bonferroni corrections (p_adj set to 0.0063). The CD8+ TEM or TN percentages were not associated with HIV-1 DNA or CA usRNA.

CCL5 Expression in CD8+ T Cells Negatively Correlates With HIV-1 Reservoir Size
CD8+ T cells are important in restraining HIV-1 rebound and reservoir size (10, 28, 39). We further analyzed effector molecule expression in CD8+ T cells (Supplementary Figure 2B for gating strategy) and their correlations with HIV-1 DNA and CA usRNA. The percentages of CCL5+, IL-2+, PRF+, and GZMB+ CD8+ T cells were negatively associated with HIV-1 DNA (CCL5+ CD8+ T cells, r = -0.4036, p = 0.0080; IL-2+ CD8+ T cells, r = -0.4317, p = 0.0017; PRF+ CD8+ T cells, r = -0.3307, p = 0.0098; GZMB+ CD8+ T cells, r = -0.2739, p = 0.0342) and CA usRNA (CCL5+ CD8+ T cells, r = -0.3712, p < 0.0001; IL-2+ CD8+ T cells, r = -0.2716, p < 0.0001; PRF+ CD8+ T cells, r = -0.4315, p = 0.0006; GZMB+ CD8+ T cells, r = -0.3061, p = 0.0174) (Figure 1C and Supplementary Figure 3).
After Bonferroni correction, there were no significant correlation between the above effector molecule expressed CD8+ T cells and HIV-1 DNA, with exception for IL-2+ CD8+ T cells, no significant correlation between GZMB+ CD8+ T cells and HIV-1 CA usRNA, but still significant correlations between IL-2+, CCL5+ and PRF+ CD8+ T cells and HIV-1 CA usRNA (p_adj set to 0.0056). In agreement with the crucial role of PRF in the killing effects of GZMB, percentages of GZMB+ polyfunctional CD8+ T cells co-expressing PRF correlated negatively with HIV-1 DNA and CA usRNA (Supplementary Figure 4). Among the effector CD8+ T-cell percentages, the TNFα+ (r = -0.3366, p = 0.0168, insignificant after Bonferroni correction), and IFNγ+ (r = -0.4394, p = 0.0014, remains significant after Bonferroni correction) were negatively associated with HIV-1 CA usRNA (Figure 1C), whereas the CCL4+ CD8+ T-cell percentage was positively associated with HIV-1 CA usRNA (r = 0.5206, p = 0.0003, remains significant after Bonferroni correction; Figure 1C).

**CCL4+ CCL5+ CD8+ TEMRA Percentage Negatively Correlates With the HIV-1 Reservoir in ART Individuals**

Both CCL4 and CCL5 were reported as HIV-1-suppressive chemokines (15). A potential explanation for the opposite correlations between the HIV-1 reservoir and CCL4 and CCL5 producing CD8+ T cells is that these producing cells were distinctive subsets. To test the hypothesis and characterize their differences, we analyzed associations between CCL4 and CCL5 producing polyfunctional CD8+ T cells co-expressing PRF correlated negatively with HIV-1 DNA and CA usRNA (Supplementary Figure 4). Among the effector CD8+ T-cell percentages, the TNFα+ (r = -0.3366, p = 0.0168, insignificant after Bonferroni correction), and IFNγ+ (r = -0.4394, p = 0.0014, remains significant after Bonferroni correction) were negatively associated with HIV-1 DNA and CA usRNA (Figure 1C), whereas the CCL4+ CD8+ T-cell percentage was positively associated with HIV-1 CA usRNA (r = 0.5206, p = 0.0003, remains significant after Bonferroni correction; Figure 1C).

**TVM Cells Are Enriched in CCL4- CCL5+ CD8+ TEMRA Cells**

We previously identified a restraining role of the TVM subset in the HIV-1 reservoir (28) and here examined the connections of the TVM subset with the HIV-1 reservoir and the potential effector mechanisms (Supplementary Figure 2A shows gating strategy for TVM cell; Supplementary Figure 2C shows gating strategy for effector molecules of TVM cells). Phenotypically, TVM cells were mainly allocated to the CD8+ TEMRA subpopulation (p < 0.0001 vs. CD45RA+ and CD8+ TN; Figures 3A, B). Furthermore, compared with total TEMRA, TVM cells were enriched more in CCL4-CCL5+ TEMRA (p = 0.0034; Figure 3C). Percentage of TVM+ TEMRA in CD8+ T cells negatively correlated with HIV-1 DNA (r = -0.4324, p = 0.0006) and CA usRNA (r = -0.3328, p = 0.0094) (Figure 3D), whereas that of non-TVM TEMRA didn’t significantly correlate
with HIV-1 DNA and CA usRNA (Figure 3E). These data support the notion that TVM cells are the major functional anti-HIV-1 subset in CD8+ TEMRA cells.

**TVM Cells Are Transcriptionally Distinctive From CCL4+ TCM Cells**

To further characterize the transcriptional profiling of TVM cells, we analyzed the scRNA-seq datasets of purified CD8+ T cells from 3 ART-treated individuals (Supplementary Figure 6A). After normalization and batch correction, a total of 12033 CD8+ T cells were obtained. UMAP was applied to the transcriptional expression data, and identified 9 CD8+ T-cell clusters (Figures 4A, B; Supplementary Table 1). The major subsets of CD8+ T cells were naïve cells (CCR7+ SELLP+ CD27+), cm cells (CD28+ IL7R+GPR183+), em cells (CCR7- CD27- CXCR3+), emra cells (CCR7- CD27- GPR183- FGFBP2+ KLRG1+), and MAIT cells.
SLC4A10 + TRAV1-2 + gdT cells (TRGV9 +). Two naïve CD8+ T-cell clusters were identified: naïve_LEF1 (LEF1+) and naïve_ISG (IFI16+IFI44L+). Two CD8+ cm clusters were identified: cm_GPR183 (GPR183hi IL7R+ SIPRI+1+) and cm_CCL4 (CCL4+ PDCD1+ TOX+). Two CD8+ emra clusters were identified: emra_EOMES (EOMES+) and emra_KIR (KIR2DLs+ KIR3DLs+). The proportions of CD8+ T-cell clusters in 3 individuals were presented in Supplementary Figure 6C.

Interestingly, the emra_KIR and cm_CCL4 subsets identified in scRNA-seq were characterized with robust expression of CCL5 and KIRs in T_EMRA and CCL4 in T_CM, resembling T_VM and CCL4+ T_CM cells, respectively. The emra_KIR cells were also featured by high expression of cytotoxicity related genes, including GZMB, GNLY and TBX21 (Figure 4B), which is in accordance with the phenotype at protein levels in T_VM cells as previously reported (28). GO analysis indicated that emra_KIR cluster was highly activated and characterized with upregulation of lymphocyte mediated immunity and T cell activation pathways, whereas cm_CCL4 cluster was potentially suppressive and characterized with downregulation of T cell activation pathways (Supplementary Figure 6D). To be specific, compared with cm_CCL4, emra_KIR up-regulated multiple effector molecules, such as GZMB, PRF1, GZMH and CCL5, supporting its superior antiviral function (Figure 4C and Supplementary Table 2). We further performed SCENIC analysis to identify potential TFs in emra_KIR and cm_CCL4 subsets. The results indicated that the responsible TFs in emra_KIR were RUNX3 and T-bet (encoded by TBX21) and those in cm_CCL4 were cAMP responsive element modulator (CREM) and JUNB (Figures 4D, E and Supplementary Figure 6E). RUNX3 and T-bet have been reported in regulating the function of CD8+ T cells and their expression are recognized as cardinal features of T_VM (28, 40, 41). In contrast, JUNB and CREM were proposed to exert suppressive functions in cm_CCL4, as indicated in CD4+ regulatory T cells (42, 43). Gene regulatory networks between TFs and DEGs in these two clusters were reconstructed (Figure 4E; Supplementary Figure 6E and Supplementary Table 3). RUNX3 and T-bet regulated the expression of several important effector molecules, such as PRF1, CX3CR1 and GZMA (Figure 4E); whereas CREM and JUNB regulate gene expression maintaining T cell exhaustion, such as PDCD1, BTG1 (44) and ZFP36L2 (45) (Supplementary Figure 6E). These results supported the pro-activation roles of RUNX3 and T-bet in emra_KIR and suppressive function of CREM and JUNB in cm_CCL4.

CCL5-Producing T_VM Cells are Negatively Correlated With HIV-1 Reservoir Size

The frequency of T_VM correlated negatively with HIV-1 DNA and HIV-1 CA usRNA (Figure 5). The T_VM count (calculated according to its ratio with CD4+ T cells and the CD4 T cell count) and percentage in CD8+ T cells correlated negatively with HIV-1 DNA ($r = -0.4902$, $p < 0.0001$ and $r = -0.3653$, $p = 0.0041$, respectively; Figures 5A, B). The correlation between the T_VM count and HIV-1 CA usRNA was significantly negative ($r = -0.4530$, $p = 0.0003$), and the T_VM percentage in CD8+ T cells tended to negatively correlate
with HIV-1 CA usRNA (r = -0.2270, p = 0.0812) (Figures 5A, B). In addition, total CCL5 producing T VM percentage correlated negatively with HIV-1 DNA (r = -0.3522, p = 0.0222) and HIV-1 CA usRNA (r = -0.7316, p < 0.0001) (Figure 5C). Associations between CCL4 and CCL5 producing polyfunctional T VM cells with HIV-1 reservoir were analyzed. CCL5 producing T VM subset (including CCL4-CCL5+CCL3- and CCL4-CCL5+CCL3+) percentages negatively correlated with HIV-1 reservoir sizes, whereas CCL4 producing T VM subset (including CCL4+CCL5-CCL3- and CCL4+CCL5+CCL3-) percentages positively correlated with HIV-1 reservoir sizes (Supplementary Figure 7). These data support the anti-HIV-1 roles of CCL5-secreting T VM cells.

**T VM Cells Restricted HIV-1 Reactivation via CCL5 Secretion**

Since both the frequency and the ability of CCL5 secretion in T VM cells negatively correlated with HIV reservoir size, we further plotted every individual according to the T VM percentage in CD8+ T cells and CCL5 production in T VM. As shown in Figure 6A, the individuals could be divided into 3 groups: Group 1 with low T VM percentage and high CCL5 production; Group 2 with low T VM percentage but high CCL5 production; Group 3 with high T VM percentage and high CCL5 production. Compared with those in Group 1, the HIV-1 DNA size in Group 2 tended to be smaller (p = 0.0847), HIV-1 CA usRNA size in Group 2 was smaller (p < 0.0001) and these reservoir sizes were both significantly smaller in Group 3 (p = 0.0004, and p < 0.0001, respectively) (Figure 6A). No significant difference was found between Group 2 and Group 3 for HIV DNA, while Group 3 tended to have smaller CA usRNA size (p = 0.0968). This implies the crucial role of CCL5-secreting T VM cells in restraining HIV reservoir.

To further confirm the viral inhibitory function of T VM cells, we assayed viral inhibition ex vivo as described (32–34). The levels of HIV replication were evaluated by intracellular P24 staining using a single antibody (KC57 clone), which was easier to detect and positively correlated with a stricter HIV FLOW analysis (Supplementary Figure 9) (46). P24+ CD4+ T cells were significantly increased following PMA/Iono activation, but this condition was significantly suppressed by co-culture with CD8+ T cells (p < 0.05) (Figures 6B, C). Further results of viral inhibition assays showed that the T VM subset suppressed HIV-1 replication more efficiently than the non-T VM subset (p < 0.05; Figure 6D). The suppressive effects were partially reversed by αCCL5 (p < 0.05), but not by αCCL3 (Figure 6E).

**DISCUSSION**

This study explored the relationship between the HIV-1 reservoir and CD8+ T-cell subsets and functionality in PLWH undergoing long-term ART. Our study identified that CCL5-secreting virtual memory subset inversely correlated with HIV-1 reservoir size and might be utilized to shrink HIV-1 reservoir.

Given that the determinant roles of antigen-specific CD8+ T cells in controlling primary HIV-1 infection have been established, accumulating evidence suggested that the capability of HIV-1-specific CD8+ T cells is limited in eliminating latent cells carrying provirus, and shaping the viral epitope landscape in individuals undergoing ART (21–23). Alternatively, CD8+ T cells can exert anti-HIV-1 effects via a bystander mechanism (18, 20, 47, 48). The incapability of antigen-specific CD8+ T cells might be due to the escape mutation of HIV-1 epitopes (49), whereas viral killing mediated by bystander CD8+ T cells did not need MHC matching (18, 19). Our recent findings supported this notion. We found that T VM cells TCR-independently and KIR-dependently restrain the HIV-1 DNA reservoir (28). KIRs are thought to function by releasing T VM from functional restriction when target cells down-regulate MHC-I expression, like “misset” activation in NK cells. The inherent resistance of the reservoir to TCR-dependent antigen-specific recognition might be due to the downregulation of MHC-I by Nef expression following activation (50). However, such action of Nef would enable T VM with killing capability. The present study further clarified that T VM cells that were enriched in the classic T EMRA subset inversely correlated with the size of the HIV-1 reservoir. We showed that T VM cells restrained the HIV-1 reservoir more effectively than non-T VM cells, which might be partially due to their abundant CCL5 secretion. Together, our findings suggest that T VM cells could restrain latently infected cells in PLWH undergoing ART.

The association between CD8+ T-cell effector molecules and HIV-1 reservoirs has not been determined in detail. Prodger et al. (7) found that TNFα+ and IL-2+ CD8+ T cells are respectively positive and negative determinants of reservoir size measured by quantitative viral outgrowth assay (QVOA). In this study, we
observed a significantly inverse correlation between IL-2+ CD8+ T cell percentage and HIV-1 reservoir sizes. Our polyfunctional analysis of CD8+ T cells revealed a positive correction between the frequency of TNFα+IFNγ-IL-2- CD8+ T cells and the size of the HIV-1 reservoir. Moreover, we did not observe a meaningful connection between IFNγ secretion by CD8+ T cells and HIV-1 reservoir size, which is consistent with the fact that the functionality of IFNγ secretion by CD8+ T cells does not correlate with HIV-1 controls (51, 52).

For beta chemokines, we consistently found that CCL5 was negative determinants of reservoir size in both CD8+ T cells and TVM cells. In sharp contrast to CCL3 and CCL5, CCL4+ CD8+ T cells correlated positively with HIV-1 CA usRNA, indicating heterogeneity among CD8+ T cells that secrete β-chemokines. Accordingly, distinctive CCL4 and CCL5-producing CD8+ T cell subsets with different regulators were identified in primary human T cells by genome-wide CRISPR screening (53). Indeed, our flow cytometry detection and scRNA-seq analysis identified a unique CCL4+CCL5- subpopulation within the classic TCM subset, the frequency of which correlated positively with HIV-1 reservoir size. Similarly, CD8+ T cells that produce CCL4 are expanded in individuals with a poor immune reconstitution who undergo ART (16). A recent study on melanoma found CCL4-expressing CD8+ T cells were robust expressors of Lag-3, which supports the notion that this subset might be exhausted (54). Using scRNA-seq data, we performed SCENIC analysis and identified CREM and JUNB as potential TFs in cm_CCL4. Further studies are needed to precisely characterize the phenotypes and functions of CCL4+ CD8+ T cells, especially in chronic viral infection. Moreover, in the ex vivo assay, we observed that CCL5 blockade diminished the virus-inhibitory effect of TVM cells. Since ART was not included in the assay, CCL5 might be multi-functional in HIV inhibition, either by preventing of transmission or by directly inhibiting of
viral replication. The latter could be possibly explained by CCL5 induced activation of signal transduction cascades through its receptors, such as CCR5, CCR1 and CCR3 (55).

Our study has several limitations. We measured the HIV-1 reservoir as HIV-1 DNA and CA usRNA, but not as being replication-competent by QVOA or as intact provirus by full-length sequencing or intact proviral DNA assays. Despite these indicators were highly correlated (56), the characteristics of latent reservoirs cannot be fully determined by HIV-1 DNA or CA usRNA. The HIV-1 reservoir was not measured at multiple timepoints. Thus, the dynamics and decay of the HIV-1 reservoir could not be determined. We also did not exclude HIV-1-specific CD8+ T cells from the analyzed CD8+ T cells. Nevertheless, our findings indicated that CCL5-secreting TVM cells could limit the size of the HIV-1 reservoir, which might be helpful to design CD8+ T cell-based therapeutic strategies for the cure of the disease.

**DATA AVAILABILITY STATEMENT**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material

**ETHICS STATEMENT**

The studies involving human participants were reviewed and approved by the Fifth Medical Center of the Chinese PLA General Hospital and the Fourth People’s Hospital of Nanning. The patients/participants provided their written informed consent to participate in this study.

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**AUTHOR CONTRIBUTIONS**

F-SW, and CZhang conceived the study, supervised the work performed, wrote the manuscript, and constructed the figures with WH, Y-JL, CZh, and Y-YW. The participants were enrolled by H-HH, JZ and Y-QZ. The PBMC samples were collected and isolated by G-CH, S-RM, Y-QQ, and F-YW. Clinical data were collected by WH, Y-YW, and J-HJ. Flow cytometry experiments were performed by WH, Y-YW, JI, M-JZ, Y-LF, X-YL, and X-HY, with technical support of C-BZ, and J-HY. Experiment to quantify viral RNA was performed by TY and X-WW. scRNA-seq data was analyzed by CZhen and PZ. J-WS, XF, Y-MJ, R-NX, J-YZ, C-BZ, and LH edited the manuscript and provided comments and feedback. F-SW, and MS managed the study team and oversaw data analysis. 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: https://www.frontiersin.org/articles/10.3389/fimmu.2022.897569/full#supplementary-material
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