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Limited restoration of T cell subset distribution and immune function in older people living with HIV-1 receiving HAART

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Abstract

Background Older people living with HIV-1 (PLWH) experience a dual burden from the combined effects of aging and HIV-1 infection, resulting in significant immune dysfunction. Despite receiving HAART, immune reconstitution is not fully optimized. The objective of this study was to investigate the impact of aging and HAART on T cell subsets and function in PLWH across different age groups, thereby providing novel insights into the prognosis of older PLWH.

Method This study was conducted at Yunnan AIDS Care Center, China, to explore the immunological responses of old PLWH to HAART and compared with the middle-age and the younger. Blood samples were collected from 146 PLWH to analyze T cell subsets and their functions, with a particular emphasis on markers related to T cell differentiation, activation, exhaustion, inflammation, and cellular function, using multicolor flow cytometry analysis.

Results Older age may have a greater effect on long-term $CD4^+$T cell recovery. Compared with young and middle-aged PLWH, older PLWH presented distinct alterations in their immune profile, including a decline in the Naïve $CD4^+$T and $CD8^+$T cell subsets, an expansion of effector memory cells, and other potential immune risk phenotypes, such as activation, exhaustion, and up-regulation of aging markers. In addition, we observed a significant association between the CD4 + EM3 subset and the CD8 + EM2 subset with HIV-1 progression, independent of age, suggesting their potential as reliable markers for assessing immune reconstitution in all PLWH.

Conclusion Our study extends previous findings showing that older participants exhibit a wide range of late differentiation, senescence, or exhaustion phenotypes in cells, including all the $CD4^+$T and $CD8^+$T subsets, consistent with an immunosenescent phenotype. This may accelerate poor immune recovery in older PLWH. Identifying new strategies to improve the immune risk phenotypes of older PLWH may help improve their immune reconstitution outcomes. The CD4 + EM3 subset and the CD8 + EM2 subset should be studied as additional markers of late presentation.

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Keywords HIV-1, Aging, T cell subsets, Immunosenescence, Immunophenotyping, Flow cytometry

Introduction

The global population of older people living with HIV-1 (PLWH) is steadily growing, whether they are aging with HIV-1 or contracting the infection at the age of 50 or older. According to the Joint United Nations Programme on HIV/AIDS (UNAIDS), the worldwide number of people aged 50 years and above with HIV-1 is estimated to reach 8.1 million [1]. A recent study by Fu et al. on the burden and trends of AIDS and other sexually transmitted infections among people aged 60–89 years globally, regionally, and nationally between 1990 and 2019 found that in 2019, among older PLWH globally, there were an estimated 77,327 (95% uncertainty interval 59443 to 97648) new cases of HIV (age-standardised incidence rate 7.6 [5.9 to 9.6] per 100000 population) [2]. In China, the older has experienced the greatest annual increase in HIV infections [3, 4]. Data from the Chinese Center for Disease Control and Prevention (CDC) revealed that between 2007 and 2018, the rate of new diagnoses in individuals aged 60 and over increased 10.31 times for males (from 2.17 to 22.37) and 10.81 times for females (from 0.57–6.16) [5]. Chen et al. found that individuals aged ≥ 50 years account for 48.1% of new cases. Specifically, the incidence rate among males increased from 0.56 per 100,000 in 2004 to 20.78 per 100,000 in 2018, while among females, it rose from 0.28 per 100,000 to 7.84 per 100,000. By 2030, the projected incidence rates are expected to reach 96.25 per 100,000 in men and 44.90 per 100,000 in women, with corresponding mortality rates projected at 48.27 and 13.67 per 100,000, respectively [6].

Highly active antiretroviral therapy (HAART) has significantly prolonged the life expectancy of PLWH. However, older PLWH initiating HAART have been shown to exhibit a poorer immunological response compared to their younger counterparts [7]. Specifically, old PLWH presented slower recovery rates of $CD4^+$T cells and lower CD4/CD8 ratios, as corroborated by our retrospective study [8]. Furthermore, older PLWH has been reported to experience elevated mortality rates and a higher incidence of non-AIDS-associated comorbidities, including neurocognitive disorders, cardiovascular disease, renal disease, bone implications, and cancer [7, 9–11]. Despite the advantages of effective HAART and achieving undetectable viral loads, addressing these comorbidities remains a substantial challenge [12]. In-depth studies of aging model systems have clearly demonstrated that the functional and phenotypic characteristics of T cells undergo significant dynamic changes with age. These alterations are believed to contribute to a wide range of age-related clinical manifestations [13]. Notable irregularities include dysregulated T cell subsets, persistent immune activation, chronic inflammation, exhaustion, and immune senescence [14]. Importantly, these compromised states can persist in older PLWH even with relatively high $CD4^+$T cell counts [15]. One of the primary challenges in immune reconstitution is the limited understanding of the immune characteristics and mechanisms involved in the reconstitution of T cells in older PLWH. We proposed that immune dysregulation, as a driving factor in the progression of HIV/AIDS and the onset of aging, may provide a more accurate reflection of the prognosis of older PLWH and even predict immune reconstitution after HAART. While the literature has compared differences in immune responses between young and old PLWH, highlighting the impact of age-related factors on the immune system, there remains a notable absence of comprehensive and in-depth studies that meticulously classify T-cell subsets and examine their functional changes across adult, middle-aged, and old PLWH [16]. Further research is necessary to determine whether these variances can provide valuable insights into distinguishing and elucidating the disparate responses to HAART in younger and older individuals.

Our objective was to provide a refined analysis of parameters commonly associated with immune dysregulation in PLWH, considering their HAART status (i.e., untreated or treated with HAART for more than 1 year) and age (i.e., young, middle-aged, and older). Multicolor flow cytometry was employed to classify $CD4^+$T and $CD8^+$T cells into 9 subsets [17] and comprehensively characterize the immunophenotyping of activation, inflammation, exhaustion, senescence, and T cell function within each subset. Additionally, we examined the differential expression of 11 plasma cytokines in parallel. In conjunction with comprehensive clinical data, we conducted an analysis to identify the characteristics that may render T cells in older individuals particularly vulnerable to HIV-1 infection and depletion.

Materials and methods

Study participants

The study subjects consisted of two cohorts. A retrospective study was first conducted to identify the clinical indicators that impact the progression of HIV/AIDS after HAART. The methodology of this study has been detailed in previous research [8]. Specifically, the study included PLWH who had received HAART for over three years between January 2010 and December 2019 in Yunnan Province, China. The inclusion criteria were as follows: (1) be at least 18 years old, (2) have received HAART for a period exceeding three years, and (3) have complete records for no less than three follow-up visits.

Participants with incomplete demographic, baseline, or follow-up data were excluded. Follow-up information for each participant was retrieved from the National Comprehensive AIDS Control Information System (NCACIS), ensuring that any personally identifiable information is excluded through the use of the participant's unique corresponding HAART number. Routine physical examination data were collected in Microsoft Excel spreadsheets and categorized into four groups: age characteristics (age at diagnosis, age at HAART initiation, and age at examination), baseline immune outcomes ($CD4^+$T cell count, $CD8^+$T cell count, and CD4/CD8 ratio), hematological parameters (including white blood cell count (WBC) hemoglobin (HGB), platelet (PLT), total bilirubin (TBIL), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (Cr); blood glucose (Glu), triglyceride (TG), and total cholesterol (TC)), and treatment details (including the treatment interval from HIV-1 diagnosis to treatment initiation, duration on HAART, initial HAART regimen, and most recent HAART regimen). The first physical examination conducted after a participant is diagnosed as HIV-positive and prior to the initiation of HAART is defined as the baseline. Full data extraction was completed by 1 reviewer and verified by a second reviewer.

The second cohort consists of a cross-sectional study that enrolled 146 patients. The objective of this study is to comprehensively characterize T-cell homeostasis and function in PLWH of varying ages, both with and without HAART, and to identify factors influencing immune reconstitution in old PLWH. Participants were consecutively recruited from the AIDS Care Centre of Yunnan Infectious Diseases Hospital between March 2021 and December 2021, based on specified criteria. For the HAART group, inclusion criteria were: (1) confirmed HIV infection through both primary screening and confirmatory tests; and (2) receiving HAART for at least six months, with a viral load maintained below the lower detection limit. For the untreated group, inclusion criteria were: (1) confirmed HIV infection through both primary screening and confirmatory tests; and (2) not currently receiving any form of antiretroviral therapy. The main exclusion criterion for all groups included: (1) pregnancy, planned pregnancy, breastfeeding, alcoholism, mental illness, and speech or intellectual disabilities; (2) individuals participating in clinical trials of other medications or undergoing immunomodulatory therapy; (3) Participants with severe underlying conditions affecting the heart, liver, brain, kidneys, endocrine system, or metabolism; (4) those experiencing serious opportunistic infections leading to vital organ failure; (5) Participants with poor adherence to antiviral therapy and those unable to attend regular follow-up visits; and (6) individuals lacking test records and available data. The 146 PLWH were divided into three groups: adult (18–35 years), middle-aged (35–50 years), and older (50 years or older).

Sample collection

Peripheral blood samples were collected in ethylenediaminetetraacetic acid (EDTA)-treated tubes during routine blood collection. The blood was centrifuged at 1500 rpm for 10 min (Beckman Coulter, CA, USA). The plasma was divided into aliquots and stored in separate tubes to avoid multiple freeze-thaw cycles. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque (TBD science, China) through differential density gradient centrifugation. The PBMCs were resuspended in a serum-free cryopreservation medium (NCM Biotech, Suzhou, China). Both the plasma and PBMCs were immediately stored at -80 °C until analysis.

Flow Cytometry

Absolute lymphocyte counts were initially measured. 50 μL fresh blood was coincubated with fluorescein-conjugated antibodies for 30 min on ice. RBC Lysis/Fixation Solution (BioLegend San Diego, USA) and Precision Count Beads (BD Biosciences, New Zealand, USA) were added to lyse and fix red blood cells. A FACS Verse flow cytometer (BD Biosciences, New Zealand, USA) was used to determine the absolute number of lymphocytes.

The phenotype and function of the T cells were assessed. Panels A and B provide information on activation and proliferation markers, including CD38, Human Leukocyte Antigen DR (HLA-DR), and Ki67; exhaustion markers, such as Programmed cell death protein 1 (PD-1) and T cell immunoreceptor with Ig and ITIM Domains (TIGIT); cytotoxicity indicators, including myxovirus resistance 1 (MX1), Granzyme B, Perforin, and T-box expressed in T cells (T-bet); as well as immunosenescence markers, CD57 and CD31. For cell surface staining, PBMCs were thawed and incubated with antibodies at 37 °C for 30 min. For intracellular marker staining, the cells were fixed and permeabilized using the Cytofix/CytoPerm Kit (BD Biosciences, New Zealand, USA) for 20 min at room temperature and then washed twice with PermWash buffer (BD Biosciences, New Zealand, USA). The cells were then mixed with appropriate intracellular antibodies overnight at 4 °C. If the initial staining was performed with a biotin-conjugated antibody, a fluorescein-conjugated antibody with streptavidin was used for the second staining step. The cells were washed with PermWash buffer before and after each step to ensure proper staining. Finally, the cells were resuspended in a standing buffer and analyzed by an LSRFortessa flow cytometer (BD Biosciences, New Zealand, USA).

The function of T cells was determined in Panel C by analyzing their ability to secrete cytokines such as

Interleukin-2 (IL-2), Interferon-gamma (IFN-γ), Tumor Necrosis Factor-alpha (TNF-α), and Interleukin-17 (IL-17) in response to stimulation with phorbol myristate acetate (PMA) and ionomycin. The cells were resuspended in prewarmed RPMI-1640 medium (Gibco, CA, USA) containing 10% fetal bovine serum (FBS, Gibco, CA, USA), 100 U/mL penicillin (Sigma Aldrich, USA) and 100 μg/mL streptomycin (Solarbio, Beijing, China). Then, the cells were cocultured with Cell Stimulation Cocktail (Invitrogen, CA, USA, which contains PMA, ionomycin, brefeldin A, and monensin) for 6 h at 37 °C in a 5% CO_2 humidified incubator. Surface marker and intracellular staining were performed as described above. The data were collected via an LSRFortessa flow cytometer (BD Biosciences, New Zealand, USA) and analyzed with FlowJo v10.5.3 software (BD Biosciences, New Zealand, USA). The details for the antibodies are provided in Table S1. All flow cytometry experiments were conducted with rigorous quality control measures to ensure the high quality and reliability of the experimental data. These measures include Zombie UV (BioLegend, San Diego, USA) stains the dead cells, stringent compensation adjustments, the application of FlowAI for data cleaning, the inclusion of isotype controls, and the use of flowStats to correct for batch effects.

Human inflammation mediators assay

Plasma levels of inflammatory mediators were evaluated using the LEGENDplex Human Inflammation Panel 2 multi-analyte flow assay kit (Biolegend, San Diego, USA), following the manufacturer's instructions. The sensitivity, or limit of detection (LOD), for each analyte in serum was determined as follows: soluble suppression of tumorigenicity 2 (sST2) at 26.62 pg/mL, soluble receptor for advanced glycation end products (sRAGE) at 4.40 pg/mL, soluble CD40 ligand (sCD40L) at 5.51 pg/mL, soluble fms-like tyrosine kinase-1 (sFlt-1) at 34.51 pg/mL, tumor necrosis factor-alpha (TNF-α) at 1.18 pg/mL, interleukin-6 (IL-6) at 0.78 pg/mL, interleukin-18 (IL-18) at 1.48 pg/mL, interleukin-10 (IL-10) at 0.59 pg/mL, and chemokine ligand 2 (CCL2) at 1.30 pg/mL. All sample measurements exceeded these LODs, ensuring the accuracy and reliability of the results. Samples were analyzed using an FACSVerse flow cytometer (BD Biosciences, New Zealand, USA), and the data were processed with LEGENDplex data analysis software (Biolegend, San Diego, USA). The results are reported as picograms per milliliter (pg/mL) of plasma.

HIV-1 viral load testing

Viral RNA was purified using the High Pure Viral RNA Kit (Roche, Basel, Switzerland) following the manufacturer's instructions. Viral RNA quantification was immediately performed with RNA-direct Real-Time PCR Master Mix (TOYOBO, Osaka, Japan) on a ViiA7 Real-Time PCR System (Applied Biosystems, Darmstadt, Germany). HIV-1 RNA was quantified using gag primers. The detection system was previously reported [18]. The primer sequences were as follows: 5'-CAT GTT TTC AGC ATT ATC AGA AGG A-3'and 5'-TGC TTG ATG TCC CCC CAC T-3'. The TaqMan probe sequence was 5'-FAM-CCA CCC CAC AAG ATT TAA ACA CCATGC TAA-TAMRA-3'.

Assay for serum HBV, HCV, CMV and EBV markers

All markers were measured using the enzyme-linked immunosorbent assay (ELISA). Diagnostic kits for hepatitis B virus (HBV) surface antigen and hepatitis C virus (HCV) antibody diagnostic kits were purchased from WanTai BioPharm, China. ELISA kits for anti-CMV (cytomegalovirus) IgG and anti-EBV (Epstein-Barr virus) IgG were obtained from Bioroyee, China. The experimental procedures were conducted in accordance with the manufacturer's instructions. HCV infection was defined by positivity for HCV antibody. A positive result for HBV surface antigen (HBsAg) indicates HBV infection. A CMV IgM- or EBV-positive sample was defined as a sample with an OD value greater than or equal to the cutoff value.

Statistical analyses

Data were collected using Microsoft Excel (Microsoft Excel 2010). GraphPad Prism version 9.4.1 (GraphPad Software, San Diego, USA) and R software were used for data analysis and visualization. The variance decomposition method developed by Garcia-Palacios et al. [19] was used to determine the contributions of clinical factors to the $CD4^+$T cell count and CD4/CD8 ratio. Akaike Information Criterion (AIC) was used to assess support for different models using the package "MuMIn" [20]. The time-dependent receiver operating characteristic curve (ROC) and Area under ROC (AUC) were generated with the R package "timeROC". For multi-group comparison, *P* values were derived from the Kruskal-Wallis test (for continuous variables) or the Chi-squared test or Fisher's exact test (for categorical variables). Tukey's HSD multiple comparisons test was conducted following a two-way ANOVA using the R package "rstatix" to determine differences between groups at various time points. The correlation matrix was visualized by using the R packages "corrplot" and "psych". For all comparisons, $P<0.05$ was considered statistically significant.

Results

Predictive advantage of age and baseline CD4+T cell count in immune status

A multiple regression model was employed to assess the explanatory power of four clinical categories on both the

$CD4^+$T cell count and the CD4/CD8 ratio. The most suitable model was identified based on a ΔAICc ≤ 2 [21]. The findings indicated that clinical factors exerted a more substantial influence on the $CD4^+$T cell count ($R^2 = 0.53$) compared to the CD4/CD8 ratio ($R^2 = 0.18$). Age and baseline measurements accounted for 61.9% and 24.8% of the variance in the $CD4^+$T cell count, respectively, which was greater than the contributions of hematological parameters (10.6%) and treatment details (2.7%). Among these factors, age at examination and baseline $CD4^+$T cell count were identified as the most significant contributors to the variance in the $CD4^+$T cell count ($p < 0.01$) (Fig. 1a). Our findings were consistent with previous studies, highlighting the critical role of baseline $CD4^+$T cell counts and age in immune recovery [22–24]. The ability of baseline $CD4^+$T cell count and age at examination to predict the risk of low $CD4^+$T cell count and low CD4/CD8 was compared separately by the ROC analysis. A larger area under the curve (AUC) indicates better discriminant power [25]. Figure 1b shows that the baseline $CD4^+$T cell count had a significantly better ability to distinguish low $CD4^+$T cell count and low CD4/CD8 than did age at examination, with an AUC above 0.7 after 4 years of HAART, whereas the AUC for age at examination never exceeded 0.6 after 7 years of HAART. The results indicated that the baseline $CD4^+$T cell count was a more reliable predictor of immune status than the age at examination. A closer inspection of the results revealed that the ROC curves of the baseline $CD4^+$T cell count in predicting the risk of low $CD4^+$T cell count and a low CD4/CD8 ratio were significantly influenced by age. Specifically, only PLWH over the age of 60 years exhibited an AUC greater than 0.9, indicating a strong predictive value. However, as the age of the PLWH decreased, the AUC decreased, dropping to an AUC lower than 0.6 for individuals under the age of 40 years (Fig. 1c). Similarly, the ROC curves for HGB, PLT, and Cr in predicting low $CD4^+$T cell and low CD4/CD8 risk were significantly influenced by age at examination to various degrees (Figure S1). These observations further indicated that the considerable variability in the $CD4^+$T cell count caused by age at examination is primarily mediated by synergistic effects with other clinical indicators.

Delayed and limited immune reconstitution of CD4+T cells in older PLWH

This study investigates the impact of age on immune reconstitution by evaluating the dynamics of CD4+T cell counts and CD4/CD8 recovery in adults aged < 35 years ($n = 392$) and older PLWH aged > 50 years ($n = 396$) over a 7-year follow-up period. The participant's characteristics are detailed in Table S2. Our findings indicate that old PLWH had a significantly lower baseline $CD4^+$T cell count (246.2 cells/μL) compared to adult PLWH (402.8 cells/μL, $p < 0.0001$). While HAART effectively restored $CD4^+$T cell counts across the different age groups, more pronounced changes were observed in the adult cohort compared to the old PLWH. Notably, $CD4^+$T cell counts were consistently higher in the adult than in the old throughout the 7 years of follow-up ($p < 0.0001$). To account for differences in baseline $CD4^+$T cell counts, we calculated relative CD4+T cell counts, representing the increase in $CD4^+$T cells following HAART relative to baseline. Results showed that relative $CD4^+$T cell counts in the adult group consistently outperformed those in the old group. Further analyses revealed that the differences between the two groups reached statistically significant levels at the 1st, 2nd, 4th, and 5th year follow-up time points. Additionally, over the 5-year HAART period, the old PLWH exhibited a slower growth rate (211.8 cells/μL/year) compared to the adult group (266.3 cells/μL/year, $p < 0.0001$). It took 5 years for the overall $CD4^+$T cell count in old PLWH to exceed 500 cells/μL, whereas adult PLWH reached this milestone after only 1 year of HAART. Similarly, the baseline CD4/CD8 ratio was significantly lower in the old (0.29) than in the adult (0.41, $p = 0.00869$); With the use of HAART, there was a gradual increase in the CD4/CD8 ratio, which remained consistently higher in the younger group than in the older group, achieving statistical significance at 2, 3, and 4 years after HAART. However, the relative CD4/CD8 were not significantly different, and neither group achieved a mean CD4/CD8 ratio exceeding 0.8 after HAART (Fig. 1d**).** These results revealed that older PLWH tend to have lower $CD4^+$T count at baseline and poorer recovery of $CD4^+$T cell count following HAART than younger counterparts, and the differences in $CD4^+$T cell recovery between age groups increased over time, indicating that the negative impact of older age on $CD4^+$T cell recovery becomes more pronounced with extended follow-up.

The characteristics of study population

A total of 146 PLWH were recruited and the participant's characteristics are detailed in Table 1. Participants were categorized into distinct groups based on their age and HAART status. The study included PLWH aged between 18 and 83 years. Among those receiving HAART, the ages for the adult, middle-aged, and old groups were 28, 41, and 58 years, respectively. In contrast, for the untreated PLWH, the mean ages for the adult, middle-aged, and old groups were 23, 44, and 54 years, respectively. The participants were predominantly male (67.8%, $N = 99$), with no significant inter-group differences in sex distribution observed ($p = 0.339$). Marital status exhibited a significant difference among the groups, with comparable values ($p < 0.001$); 48.6% ($N = 71$) of the participants were unmarried or single. Among adult PLWH receiving HAART, most were unmarried or single, comprising

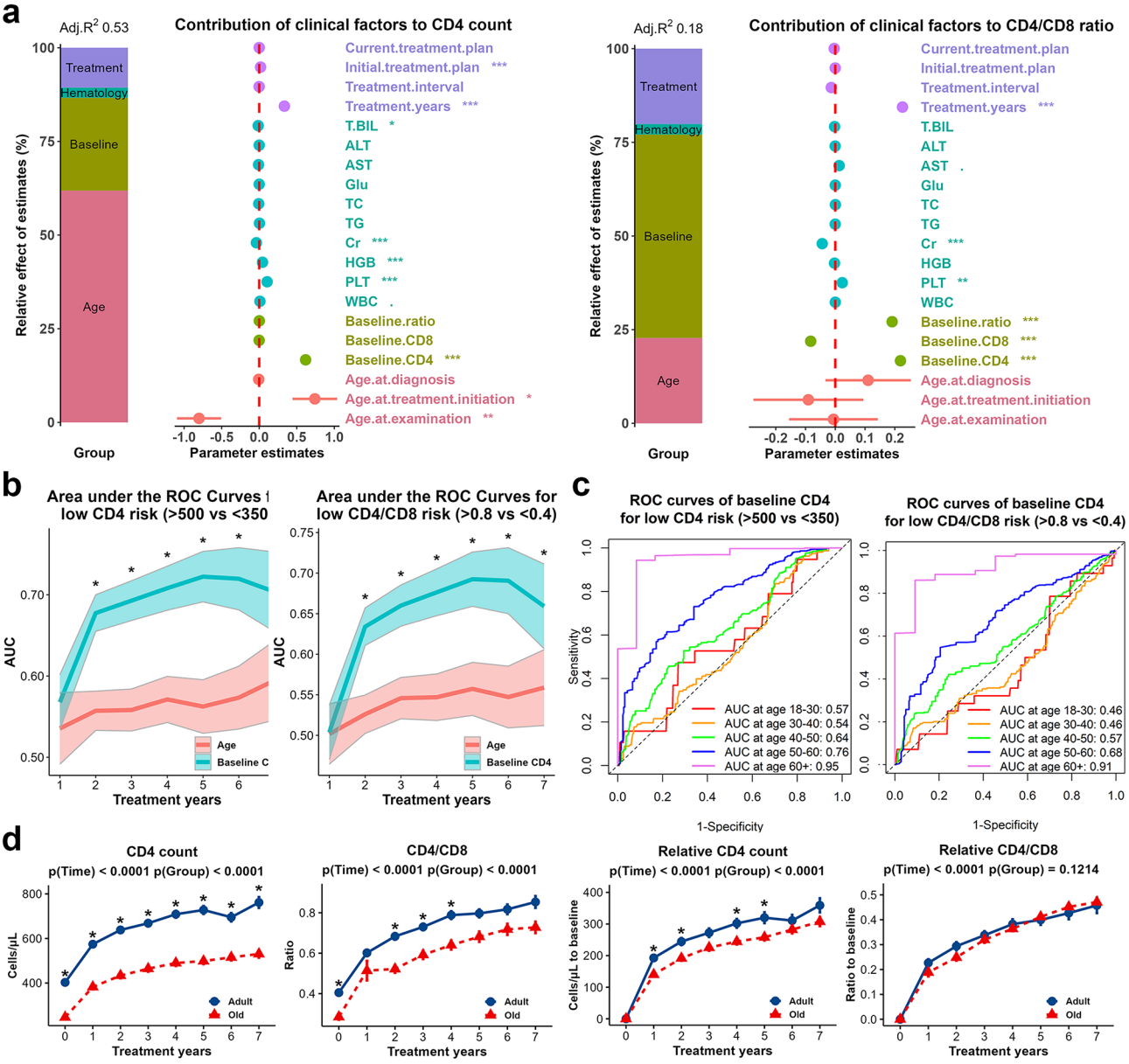

Fig. 1 Effect of age on immune reconstitution in PLWH. (**a**) Relative effect of clinical factors on CD4$^+$T cell count and CD4/CD8 ratio. Variables are grouped into 4 components: red = age characteristics (age at diagnosis, age at treatment initiation, and age at examination), green = baseline immune outcomes (CD4$^+$T, CD8$^+$T cell counts, and CD4/CD8 ratio), blue = hematological parameters (WBC, HGB, PLT, T.BIL, ALT, AST, Cr, Glu, TG, TC), purple = treatment details (treatment interval, treatment years, initial and current treatment plan). The averaged parameter estimates (standardized regression coefficients) for the model predictors are presented alongside their corresponding 95% confidence intervals and the relative importance of each predictor, expressed as a percentage of the explained variance. The graph illustrates the best model selected based on the AICc criterion. The relative effects of the predictors and their interactions can be calculated by taking the ratio of the parameter estimate of each predictor to the sum of all parameter estimates, with the result expressed as a percentage. (**b**) Time-dependent AUCs for baseline CD4$^+$T cell count and age to predict the risk of CD4$^+$T cell count <350 cells/μL and CD4/CD8 < 0.4. Shaded region indicates the 95% confidence interval. (**c**) ROC curve analysis for predicting the risk of low CD4$^+$T cell or low CD4/CD8 based on baseline CD4$^+$T cell counts across different age groups (18–30, 30–40, 40–50, 50–60 and > 60 years old). (**d**) Dynamics of CD4$^+$T cell counts, CD4/CD8 ratios, relative CD4$^+$T cell counts, and relative CD4/CD8 ratios over the 7-year treatment period in the adult group (age < 35 years, $n = 392$) and the older group (age > 55 years, $n = 396$). Relative CD4$^+$T cell count or relative CD4/CD8, represents the increase in the number of CD4$^+$T cells or CD4/CD8 relative to baseline after HAART. Data are shown as means ± SEs. The p values are calculated by two-way ANOVA. * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$. WBC: white blood cells, HGB: hemoglobin, PLT: platelets, T.BIL: total bilirubin, ALT: alanine aminotransferase, AST: aspartate aminotransferase, Cr: creatinine, Glu: glucose, TG: triglycerides, TC: total cholesterol, AICc: akaike information criterion corrected, AUC: area under the curve, ROC: receiver operating characteristic curve, HAART: highly active antiretroviral therapy

Table 1 Demographic and clinical baseline characteristics of study participants

		HAART			Pre-HAART			
Parameter	**Overall (*N* = 146)**	**Adult (*N* = 38)**	**Middle (*N* = 15)**	**Old (*N* = 29)**	**Adult (*N* = 27)**	**Middle (*N* = 18)**	**Old (*N* = 19)**	***P***
Age (years)	39.5 (28.0, 52.0)	28.0 (26.0, 30.0)	41.0 (39.0, 44.0)	58.0 (53.0, 65.0)	23.0 (21.0, 28.5)	43.5 (41.0, 48.0)	54.0 (51.0, 57.0)	< 0.0001
Gender (%)								0.339
Male	99 (67.8)	25 (65.8)	9 (60.0)	18 (62.1)	23 (85.2)	13 (72.2)	11 (57.9)	
Female	47 (32.2)	13 (34.2)	6 (40.0)	11 (37.9)	4 (14.8)	5 (27.8)	8 (42.1)	
Marriage (%)								< 0.001
Divorced or separated	21 (14.4)	0 (0.0)	2 (13.3)	5 (17.2)	1 (3.7)	5 (27.8)	8 (42.1)	
Married or live togerther	48 (32.9)	3 (7.9)	5 (33.3)	24 (82.8)	1 (3.7)	7 (38.9)	8 (42.1)	
Never married or single	71 (48.6)	35 (92.1)	8 (53.3)	0 (0.0)	24 (88.9)	3 (16.7)	1 (5.3)	
Unclear	6 (4.1)	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.7)	3 (16.7)	2 (10.5)	
Infection route(%)								< 0.001
Homosexual	35 (24.0)	16 (42.1)	3 (20.0)	0 (0.0)	14 (51.9)	0 (0.0)	2 (10.5)	
Heterosexual	85 (58.2)	21 (55.3)	6 (40.0)	22 (75.9)	11 (40.7)	11 (61.1)	14 (73.7)	
Intravenous drug use.	9 (6.2)	0 (0.0)	2 (13.3)	4 (13.8)	0 (0.0)	3 (16.7)	0 (0.0)	
Others/Unclear*	17 (11.6)	1 (2.6)	4 (26.7)	3 (10.3)	2 (7.4)	4 (22.2)	3 (15.8)	
Initial HAART regimen (%)								0.654
NRTIs + NNRTIs	136 (93.2)	35 (92.1)	13 (86.7)	29 (100.0)	25 (92.6)	16 (88.9)	18 (94.7)	
PIs	4 (2.7)	2 (5.3)	1 (6.7)	0 (0.0)	1 (3.7)	0 (0.0)	0 (0.0)	
INSTIs	6 (4.1)	1 (2.6)	1 (6.7)	0 (0.0)	1 (3.7)	2 (11.1)	1 (5.3)	
Current HAART regimen								0.052
NRTIs + NNRTIs	110 (75.3)	28 (73.7)	8 (53.3)	20 (69.0)	22 (81.5)	14 (77.8)	18 (94.7)	
PIs	21 (14.4)	9 (23.7)	4 (26.7)	3 (10.3)	3 (11.1)	1 (5.6)	1 (5.3)	
INSTIs	15 (10.3)	1 (2.6)	3 (20.0)	6 (20.7)	2 (7.4)	3 (16.7)	0 (0.0)	
Anti-HBsAg								
Negative	142 (97.3)	37 (97.4)	14 (93.3)	27 (93.1)	27 (100.0)	18 (100.0)	19 (100.0)	0.4728
Positive	4 (2.7)	1 (2.6)	1 (6.7)	2 (6.9)	0 (0.0)	0 (0.0)	0 (0.0)	
Anti-HCV								
Negative	137 (93.8)	38 (100.0)	13 (86.7)	25 (86.2)	27 (100.0)	16 (88.9)	18 (94.7)	0.0974
Positive	9 (6.2)	0 (0.0)	2 (13.3)	4 (13.8)	0 (0.0)	2 (11.1)	1 (5.3)	
Anti -CMV#								
Negative	123 (87.2)	33 (86.8)	10 (66.7)	25 (86.2)	22 (91.7)	17 (94.4)	16 (94.1)	0.1725
Positive	18 (12.8)	5 (13.2)	5 (33.3)	4 (13.8)	2 (8.3)	1 (5.6)	1 (5.9)	
Anti -EBV#								
Negative	119 (84.4)	33 (86.8)	10 (66.7)	25 (86.2)	22 (91.7)	14 (77.8)	15 (88.2)	0.3495
Positive	22 (15.6)	5 (13.2)	5 (33.3)	4 (13.8)	2 (8.3)	4 (22.2)	2 (11.8)	
BMI	21.0 (19.4, 23.4)	20.0 (18.9, 21.2)	21.8 (18.9, 23.6)	21.2 (19.9, 23.2)	20.6 (18.8, 23.3)	23.4 (21.6, 24.3)	21.8 (19.8, 23.4)	0.0535
WBC (×109/L)	5.56 (4.35, 6.84)	5.6 (4.4, 7.3)	6.2 (5.5, 7.8)	5.1 (4.1, 6.3)	5.8 (4.9, 7.0)	5.8 (4.2, 6.4)	4.7 (3.8, 5.6)	0.1318
PLT (×109/L)	246.0 (199.0, 283.0)	255.5 (225.0, 277.8)	254.0 (222.0, 287.0)	195.0 (143.0, 249.0)	264.0 (213.0, 322.0)	246.0 (201.0, 292.0)	258.0 (191.0, 283.0)	0.0023
Hb (g/L)	155.0 (142.0, 173.0)	170.5 (146.5, 180.0)	151.0 (142.5, 157.5)	153.0 (137.0, 163.0)	160.0 (144.5, 172.4)	152.0 (108.0, 161.0)	146.0 (130.0, 157.0)	0.004
Cre (μmol/L)	69.0 (59.0, 78.0)	68.0 (58.2, 76.5)	67.0 (58.5, 74.5)	72.0 (61.0, 78.0)	71.0 (62.5, 77.8)	68.0 (65.0, 73.0)	62.0 (54.0, 83.0)	0.7362
TG (mmol/L)	1.6 (1.2, 2.2)	1.5 (1.2, 2.1)	2.0 (1.2, 3.1)	1.6 (1.1, 2.4)	1.4 (1.2, 1.9)	1.7 (1.2, 2.5)	1.9 (1.4, 2.0)	0.7203
CHO (mmol/L)	4.7 (4.2, 5.5)	4.8 (4.4, 5.4)	4.9 (4.3, 5.6)	5.1 (4.2, 6.1)	4.3 (3.9, 4.9)	5.0 (4.4, 5.4)	4.5 (4.2, 5.4)	0.1923
Glu (mmol/L)	5.4 (5.0, 5.8)	5.2 (4.9, 5.4)	5.3 (5.1, 5.7)	5.6 (5.2, 6.3)	5.3 (5.0, 5.8)	5.7 (5.4, 5.7)	6.0 (5.2, 6.6)	0.0025
ALT (U/L)	22.0 (15.5, 32.5)	20.0 (14.2, 33.8)	25.0 (19.0, 27.0)	23.0 (19.0, 30.0)	19.0 (14.0, 25.0)	25.0 (20.0, 39.0)	28.0 (13.0, 31.0)	0.4659
AST (U/L)	22.0 (18.0, 27.0)	21.0 (18.0, 25.0)	22.0 (20.0, 25.0)	24.0 (19.0, 32.0)	18.0 (16.0, 21.5)	26.0 (20.0, 31.0)	25.0 (18.0, 32.0)	0.0191

Table 1 (continued)

		HAART			Pre-HAART			
Parameter	**Overall (*N*=146)**	**Adult (*N*=38)**	**Middle (*N*=15)**	**Old (*N*=29)**	**Adult (*N*=27)**	**Middle (*N*=18)**	**Old (*N*=19)**	***P***
TBIL (μmol/L)	9.0 (6.7, 12.1)	8.9 (6.7, 12.5)	9.0 (7.2, 14.7)	10.4 (8.6, 13.5)	10.1 (7.2, 12.2)	7.2 (6.1, 9.9)	6.7 (5.6, 8.6)	0.0255
b.CD4$^+$T (cells /μL)$^{\&}$	285 (175, 419)	325 (251, 455)	202 (85, 282)	200 (97, 314)	380 (262, 524)	227 (166, 312)	260 (129, 448)	< 0.001
b.CD8$^+$T (cells /μL)$^{\&}$	1041 (745, 1514)	860 (713, 1502)	820 (490, 1537)	833 (594, 1175)	1448 (1163, 1730)	1092 (843, 1359)	1082 (803, 1593)	0.014
b.CD4/CD8$^{\&}$	0.22 (0.14, 0.43)	0.37 (0.21, 0.49)	0.20 (0.09, 0.23)	0.16 (0.13, 0.36)	0.28 (0.18, 0.45)	0.17 (0.14, 0.23)	0.22 (0.14, 0.38)	0.023

Figures are n (%) or median (IQR). *Others/Unclear included blood transfusion, mother to child transmission, and unknown. #Due to unavailability of plasma samples, 5 PLWH had to be excluded for the CMV and EBV screen. &b.Representation of the baseline. HAART: Highly active antiretroviral treatment, NRTIs: nucleoside reverse transcriptase inhibitors, NNRTI: non-nucleoside reverse transcriptase inhibitor, PI: protease inhibitor, INSTI: integrase strand transfer inhibitor, WBC: white blood cell count, PLT: platelet, Hb: hemoglobin, Cre: creatinine, CHO: cholesterol, TG: triglycerides, Glu: blood glucose, ALT: alanine transaminase, AST: aspartate transaminase, TBIL: total bilirubin, Anti-BsAg: hepatitis B surface antigen, Anti-HCV: antibodies against the hepatitis C virus, Anti-CMV: antibodies against cytomegalovirus; Anti-EBV: antibodies against cytomegalovirus

92.1% (*N*=35). In contrast, old PLWH were predominantly married or cohabiting, accounting for 82.8% (*N*=24). Sexual transmission was identified as the primary route of HIV infection, with heterosexual transmission responsible for 58.2% (*N*=85) and homosexual transmission for 24.0% (*N*=35) of all participants. In terms of treatment, NRTIs combined with NNRTIs were the predominant therapies, representing 93.2% (*N*=136) for the initial regimen and 75.3% (*N*=110) for the current regimen, respectively. Previous evidence has shown that co-infection status induces broad and strong T-cell responses in HIV-infected individuals, particularly in those who are positive for CMV and EBV [26]. In light of this, we conducted a comprehensive screening of the co-infection status among the enrolled patients for pathogens including HCV, HBV, CMV, and EBV. The results indicated a low overall percentage of co-infections among the 146 participants. Specifically, 2.7% (*N*=4) were positive for HBV, 6.2% (*N*=9) for HCV, 12.8% (*N*=18) for CMV, and 15.6% (*N*=22) for EBV. Comparisons between groups revealed that HBV- and HCV-positive participants were most prevalent in the HAART-treated old group, with 6.9% (*N*=2) and 13.8% (*N*=4), respectively. In contrast, the HAART-treated middle-aged group exhibited the highest positivity rates for both CMV and EBV, with 33.3% (*N*=5) for each. Although there were differences in positivity rates among the groups, these differences were not statistically significant. All participants underwent a comprehensive health check-up before the commencement of the study, which included routine laboratory tests for blood counts and blood biochemistry. The results indicated no significant differences in WBC, Cre, TG, CHO, and ALT when comparing adult, middle-aged, and old participants on HAART with their untreated counterparts in a two-by-two analysis. Notably, PLT in the HAART-treated old groups were 195.0 (143.0, 249.0)×10^9/L, which was significantly lower than those in the treated adult group at 255.5 (225.0, 277.8)×10^9/L and the middle-aged group at 254.0 (222.0, 287.0)×10^9/L. Glu in HAART-treated old groups were recorded at 5.6 (5.2, 6.3) mmol/L, significantly higher than the 5.2 (4.9, 5.4) mmol/L observed in the HAART-adult group. Additionally, Hb, TBIL, and AST were significantly different between the groups. According to the Standard reference intervals for blood cell analysis of the People's Republic of China's health industry. (WS/T 405–2012, WS/T 404–2012), although numerical differences were observed among the three groups, all values remained within the normal range.

Absolute counts of peripheral lymphocytes and chronic inflammatory state in participants

To achieve a comprehensive understanding of the differences in T-cell immunophenotype and immune function among PLWH of varying ages, we conducted a specific experimental procedure, as illustrated in Fig. 2a. PBMCs were analyzed using a 16-parameter flow cytometer to identify the phenotype and function of subsets within CD4$^+$T and CD8$^+$T cells. Plasma samples were collected to assess the levels of various inflammatory cytokines. We proceeded to evaluate the peripheral lymphocytes of the enrolled participants. As shown in Fig. 2b, there were no statistically significant differences in the percentage and absolute numbers of CD4$^+$T cells, CD8$^+$T cells, CD4/CD8 ratio, or viral load among the untreated groups. HAART successfully restored the absolute numbers of CD4$^+$T cells and the percentage of CD8$^+$T cells, resulting in a higher CD4/CD8 ratio and a dramatic reduction in the plasma viral load for PLWH who had received HAART ($p<0.001$). The CD4$^+$T cell counts in the treated adults were notably higher than the older, whereas no significant differences were observed in CD8$^+$T cell count, percentage, the CD4/CD8 ratio, or viral load. The levels of inflammatory factors were subsequently assessed. Our

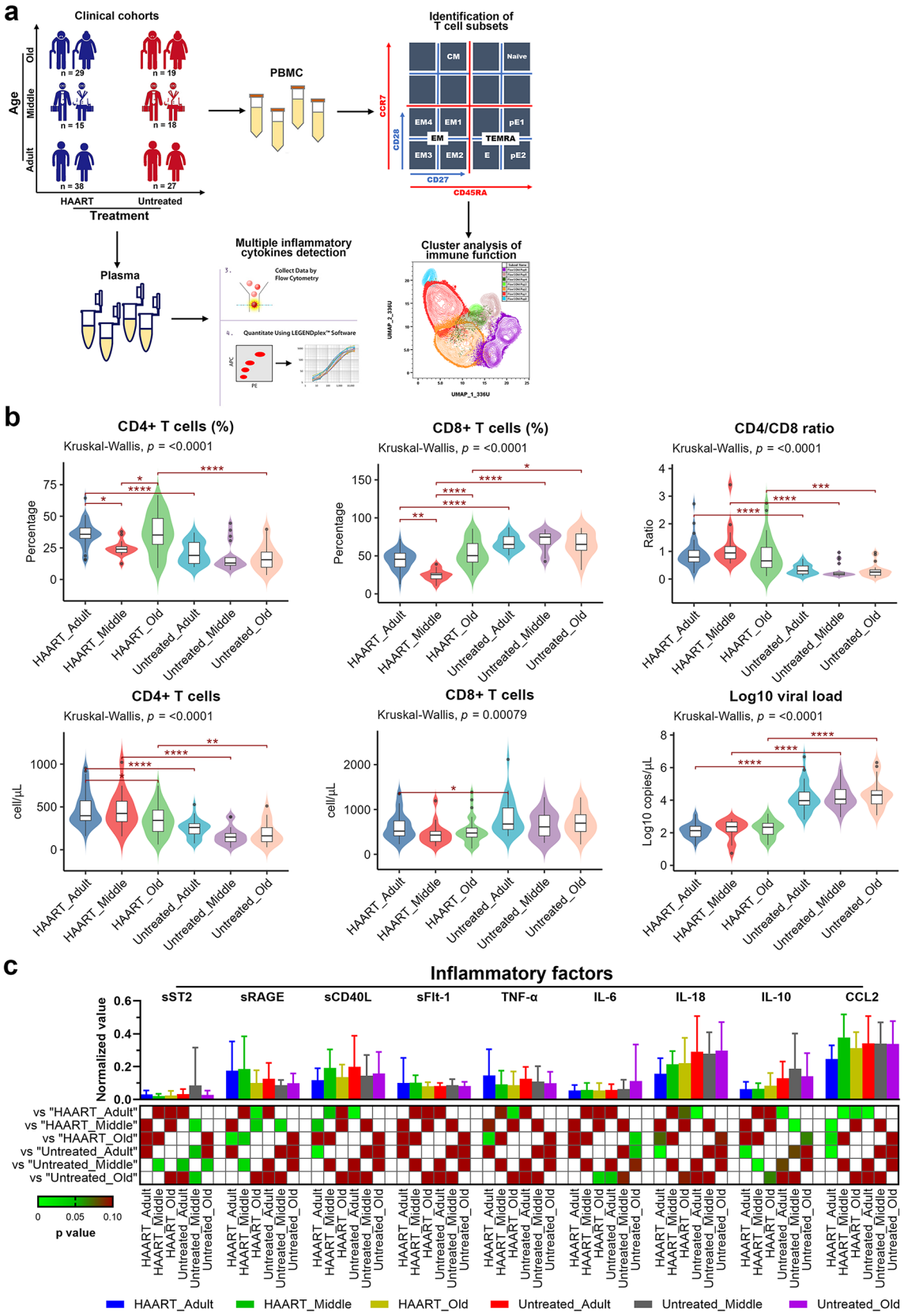

Fig. 2 (See legend on next page.)

(See figure on previous page.)

Fig. 2 Study design and characteristics of study participants. (**a**) Schematic showing the overall study design. The study involved 146 PLWH who were grouped based on their age and whether they received HAART. Flow cytometry and multiple inflammatory cytokines were applied to their PBMCs and plasma, respectively. (**b**) The percentages and absolute numbers of $CD4^+$T and $CD8^+$T cells, the CD4/CD8 ratio, and viral load in PLWH stratified by age and HAART status. Flow cytometry was employed to assess the T cell count in PBMCs, while viral load was measured in plasma by qPCR. The data distribution is visually presented through violin plots. Statistical analyses were conducted using the nonparametric Kruskal-Wallis test, with significance levels denoted as: * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. (**c**) The quantitative results of multiple inflammatory mediators in plasma samples determined by cytometric bead array. The data are expressed as means ± SDs and analyses were performed using Two-way ANOVA test. The heatmap utilizes color intensity to convey the *p*-values for pairwise comparisons. A green color indicates statistical significance at $P<0.05$, while red signifies no significant difference (refer to the color bar). PBMCs: peripheral blood mononuclear Cells, sST2: soluble suppression of tumorigenicity 2, sRAGE: soluble receptor for advanced glycation end products, sCD40L: soluble CD40 ligand, sFlt-1: soluble fms-like tyrosine kinase-1, TNF-α: tumor necrosis factor-alpha, IL-6: interleukin-6, IL-18: interleukin-18, IL-10: interleukin-10, CCL2: chemokine ligand 2

observations indicated that HAART significantly diminishes IL-10 levels in the plasma of PLWH when compared to those who remain untreated. Previous reports indicate that successful HAART reduces plasma IL-10 levels, which corresponds with a decrease in viral load among PLWH. A lack of decline in plasma IL-10 levels after HAART administration has been associated with virologic treatment failure [27, 28]. Furthermore, HAART-induced reduction in the number of Th2 cells may be directly responsible for the significant decrease in IL-10 levels [29–31]. Additionally, we discovered that following HAART, CXCL2 levels were notably lower in adults compared to their middle-aged and older counterparts (Fig. 2c). The overall similarity in immunological characteristics indicated that all PLWH appeared to derive benefits from HAART without any discernible impact of age on immune recovery. This consistency prompted further analysis of T cell subsets and rendered our subsequent analyses more comparable.

Significant restoration of T cell subsets by HAART with distinct patterns in older PLWH

T cell subsets have been characterized in detail as previously reported by Sven Koch. et al. [17]. A representative flow cytometry gating strategy for immune cell subsets was illustrated in Figure S2. As shown in Fig. 3a and c, the $CD4^+$T cells of untreated PLWH were predominantly composed of effector memory (EM) cells, which accounted for approximately 49.1-50.9% of the overall $CD4^+$T cell pool. $CCR7^+CD4^+$T cells constituted roughly 37.0%-47.9% of the $CD4^+$T cell composition. Compared with untreated middle-aged and older PLWH, untreated adults exhibited the highest proportion of Naïve $CD4^+$T cells (29.4% vs. 15.5% vs. 18.3%, $p<0.01$), and the lowest proportion of EM1 $CD4^+$T cells (14.0% vs. 19.8% vs. 18.3%, $p<0.01$). Conversely, untreated older PLWH demonstrated a significantly higher proportion of the terminally differentiated effector memory (TEMRA) subset than untreated middle-aged and young PLWH (3.0% vs. 3.0% vs. 7.3%, $p<0.01$), with particularly notable differences observed between the CD4+E and CD4+PE2 subsets ($P<0.01$). HAART effectively reversed the decline in the CCR7+CD4 subset, and there was a significant increase in Naïve $CD4^+$T cells after HAART in both adults (from 29.4 to 47.6%, $p<0.0001$) and middle-aged individuals (from 15.5 to 39.1%, $p<0.001$). However, no significant change was observed among the older(from 18.3 to 25.4%, $p=0.086$). There was also a decrease in the EM subset in treated PLWH, especially for the EM3 subset ($p<0.001$), which exhibited significantly lower levels than untreated PLWH. Notably, the proportions of central memory (CM) subsets were significantly higher in the treated older PLWH than in the treated younger and middle-aged groups (23.5% vs. 18.3% vs. 30.2%, $p<0.001$).

In contrast to $CD4^+$T cell subsets, $CCR7^+$T cells comprised the smallest subset (4.8-12.0%) of $CD8^+$T cells in untreated PLWH, while EM exhibited the highest frequency (64.0-69.0%), closely followed by TEMRA (21.8-31.0%). Similar to the $CD4^+$T cells, the untreated older PLWH showed significantly lower percentages of Naïve $CD8^+$T cells (11.4% vs. 6.5% vs. 3.7%, $p<0.001$) and higher percentages of TEMRA $CD8^+$T cells (24.0% vs. 21.8% vs. 31.0%, $p<0.001$) than the untreated adult and middle-aged PLWH. HAART-induced changes in the $CD8^+$T cell compartment appear to be more pronounced. Compared with untreated PLWH, all treated PLWH experienced a notable increase in the proportion of Naïve, EM1, and PE1 subsets, whereas the proportion of EM2 subsets significantly decreased ($p<0.001$). Nevertheless, Naïve $CD8^+$T cells were consistently the lowest in older PLWH (27.9% vs. 21.7% vs. 13.6%, $p<0.001$) after HAART (Fig. 3b and d).

Cytokine dysregulation and immune dysfunction of T cells in older PLWH

Cytokine dysregulation and inflammation have been recognized as the primary drivers of $CD4^+$T cell exhaustion and immune dysfunction during the progression of HIV-1 disease [32]. Subsequently, T cells were stimulated with PMA and ionomycin, enabling the investigation of age-related variations in cytokine profiles. Our findings demonstrated comparable expression levels of IL-2, Ki67, and MX1 across untreated age groups. Notably, untreated older PLWH exhibited significantly elevated levels of CD57, CXCR3, HLA-DR, IFN-γ, T-bet, and TIGIT compared to untreated younger PLWH ($p<0.05$). The

administration of HAART resulted in a significant reduction in the levels of HLA-DR, Ki67, and MX1 across all PLWH. In both adult and middle-aged PLWH, HAART resulted in a reduction of CD57 and PD-1 expression. However, when comparing older PLWH who received treatment with those who did not, no significant differences were observed ($p>0.05$). Similar to the untreated status, markers of cell activation and exhaustion, such as CXCR3, HLADR, and TIGIT, continue to exhibit higher expression in the treated older PLWH compared to treated adult and middle-aged controllers (Fig. 4a).

Correlation analysis revealed positive associations between the expression of CXCR3, HLADR, and TIGIT with age and viral load. Conversely, a negative correlation was observed with $CD4^+$T cell count, CD4/CD8 ratio, and duration of treatment(Fig. 4b). Furthermore, HLA-DR, CXCR3, and TIGIT were strongly positively correlated with each other and positively correlated with PD-1 and Ki67. Mantel's tests revealed that the expression of HLA-DR, CXCR3, and TIGIT was closely associated with EM subsets. Specifically, CXCR3 was significantly associated with EM1 subsets (Mantel's $r=0.22$, $p<0.01$), while TIGIT was significantly associated with EM2 subsets (Mantel's $r=0.33$, $p<0.01$). The markers that were positively correlated with HLA-DR, including PD-1, T-bet, and GranzymeB, were closely associated with the EM3 subset (Fig. 4c). These results indicate that old PLWH exhibit elevated levels of cytokines and immune markers, such as CXCR3, HLA-DR, and TIGIT when stimulated in vitro, compared to their adult counterparts. While these molecules are implicated in the immune response, they are also associated with immune activation, inflammation, and T-cell exhaustion. The increased levels observed in old PLWH suggest a chronic state of immune activation and inflammation, which may be detrimental to immune function and could contribute to accelerated immune senescence and impaired $CD4^+$T cell reconstitution, and HAART does not alleviate the persistent heightened activation of $CD4^+$T cells in old PLWH. Moreover, the $CD4^+$T EM subset is characterized by elevated levels of exhaustion markers (TIGIT, PD-1), an activation marker (HLA-DR), and inflammation markers (CXCR3, TNF-α). The accumulation of the EM $CD4^+$T subset in older PLWH may impede $CD4^+$T cell reconstitution.

The same strategy was employed for $CD8^+$T cells. As shown in Fig. 4d, the expression levels of Granzyme B, HLA-DR, MX1, PD-1, and T-bet did not show significant differences among all untreated PLWH. However, CD57, perforin, and TNF-α were expressed at significantly higher levels in untreated older PLWH compared to untreated young and middle-aged PLWH. Following HAART, the expression levels of CD38, CD57, Granzyme B, HLA-DR, MX1, PD-1, T-bet, TIGIT, and TNF-α were significantly reduced across all PLWH. Particularly in the young and middle-aged PLWHs, there was also a more pronounced decrease in perforin expression. Comparisons of PLWH across different age groups after treatment revealed no significant differences in the expression of CD38, HLA-DR, perforin, or T-bet. However, the expression levels of CD57, Granzyme B, and TIGIT were significantly higher in older PLWH compared to their younger and middle-aged counterparts. Additionally, the MX1 and TNF-a expression levels were significantly higher than the young PLWH (Fig. 4d). Consistently, immune activation markers (CD38 and HLA-DR), cytotoxic phenotypes (Granzyme B, T-bet, and MX1), exhaustion markers (TIGIT and PD1) and the proliferation marker (Ki67) were positively associated with the viral load and significantly negatively correlated with $CD4^+$T cell count, CD4/CD8 ratio, and duration of treatment (Fig. 4e). These markers were positively correlated with each other and were the strongest correlates of both the Naïve and EM2 subsets (Mantel's $r=0.19–0.58$, $p<0.01$), moderately correlated with the E and PE1 subsets (Mantel's $r=0.04–0.30$, $p<0.05$) (Fig. 4f).

Discussion

In this study, a comprehensive analysis was conducted to investigate the impact of HIV-1 infection and HAART on the distribution of T cell subsets and immune function in PLWH across various age groups. By conducting a detailed examination of the results, we gained a deeper understanding of potential variations in immune reconstitution and treatment outcomes among older PLWH.

Extensive research has explored the effects of HAART on immune recovery in older PLWH, with a specific focus on whether comparable virological and immunological outcomes can be achieved by older individuals compared with their younger counterparts [9, 33–38]. Consistent with previous studies by Zhang et al. [7] and Ocheretyaner et al. [36], our retrospective cohort study suggested that older PLWH may experience diminished immune recovery, as evidenced by a notable decrease in $CD4^+$T cell count recovery, restoration of the CD4/CD8 ratio, and relative $CD4^+$T cell count and CD4/CD8 ratio after HAART compared to younger PLWH. Chen et al. [38] developed a semimechanistic population model to elucidate the longitudinal recovery trajectories of Naïve and memory $CD4^+$T cells in PLWH. They revealed the correlation between older age and impaired restoration of Naïve T cells, as well as the detrimental impact of aging on long-term reconstitution of $CD4^+$T cell count. Additionally, numerous studies have consistently demonstrated the significance of baseline $CD4^+$T cell count and CD4/CD8 ratios in assessing HIV/AIDS progression, evaluating HAART efficacy, and predicting immune reconstitution [39–42]. However, our findings

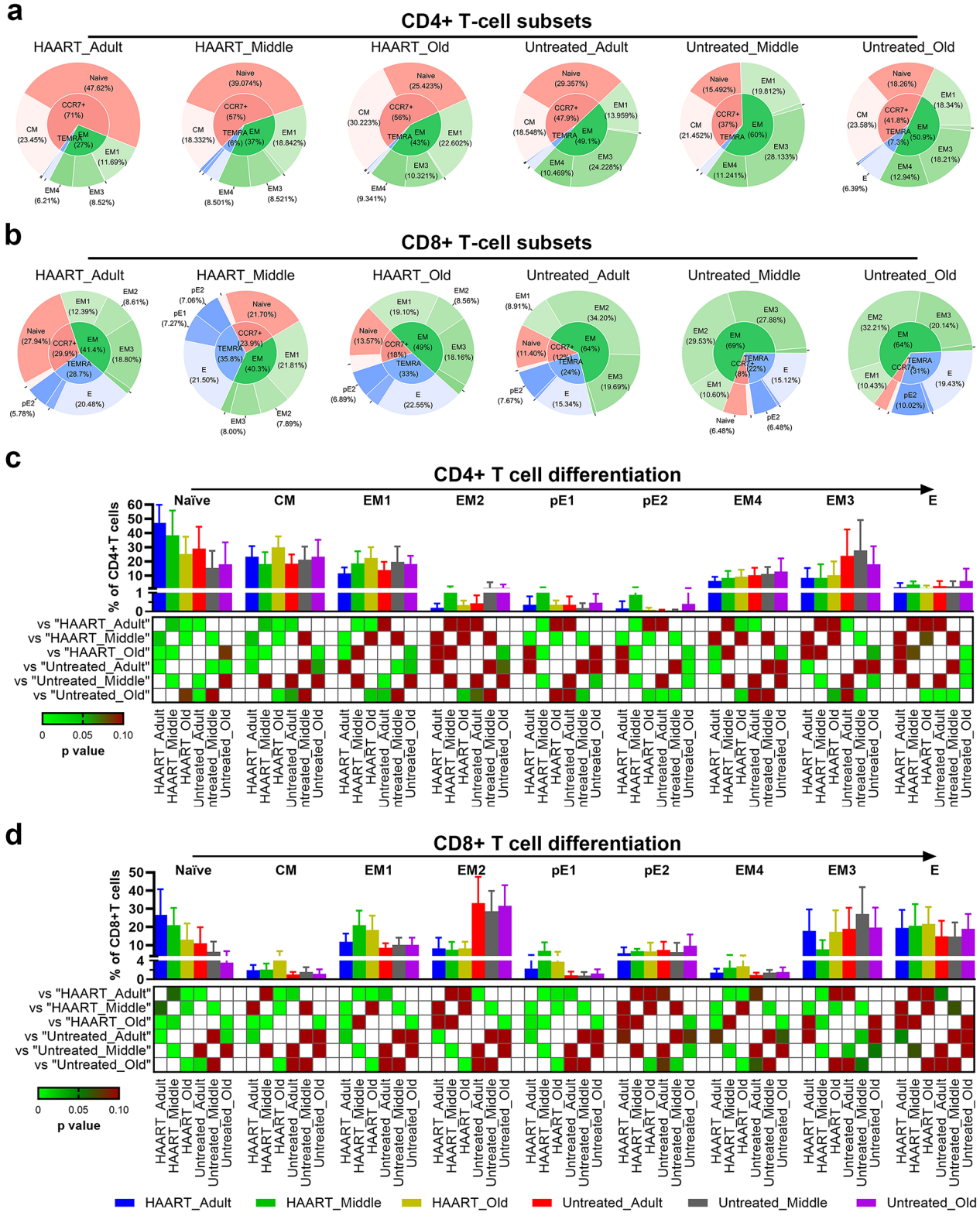

Fig. 3 Distribution of CD4$^+$T and CD8$^+$T cell subsets in PLWH of different ages. (**a**-**b**) Distribution of the CD4$^+$T and CD8$^+$T cell subsets at different ages of PLWH. Percentage data in the graphs were accurately determined by flow cytometry and reflect the average proportion of each cell subset in the total CD4$^+$T cells or CD8$^+$T cells poor. (**c**-**d**) Differences in the frequencies of CD4$^+$T and CD8$^+$T cell subsets were compared between the HAART and untreated PLWH across the three age groups. The groups are represented in different colors. The data are presented as means ± SDs, and analyses were conducted using the Kruskal-Wallis test. The heatmap illustrates the *p*-values for pairwise comparisons. A green color indicates statistical significance at $P < 0.05$, while red denotes no significant difference (see color bar). CM: central memory cells, EM: effector memory cells, CCR7: C-C Chemokine Receptor 7

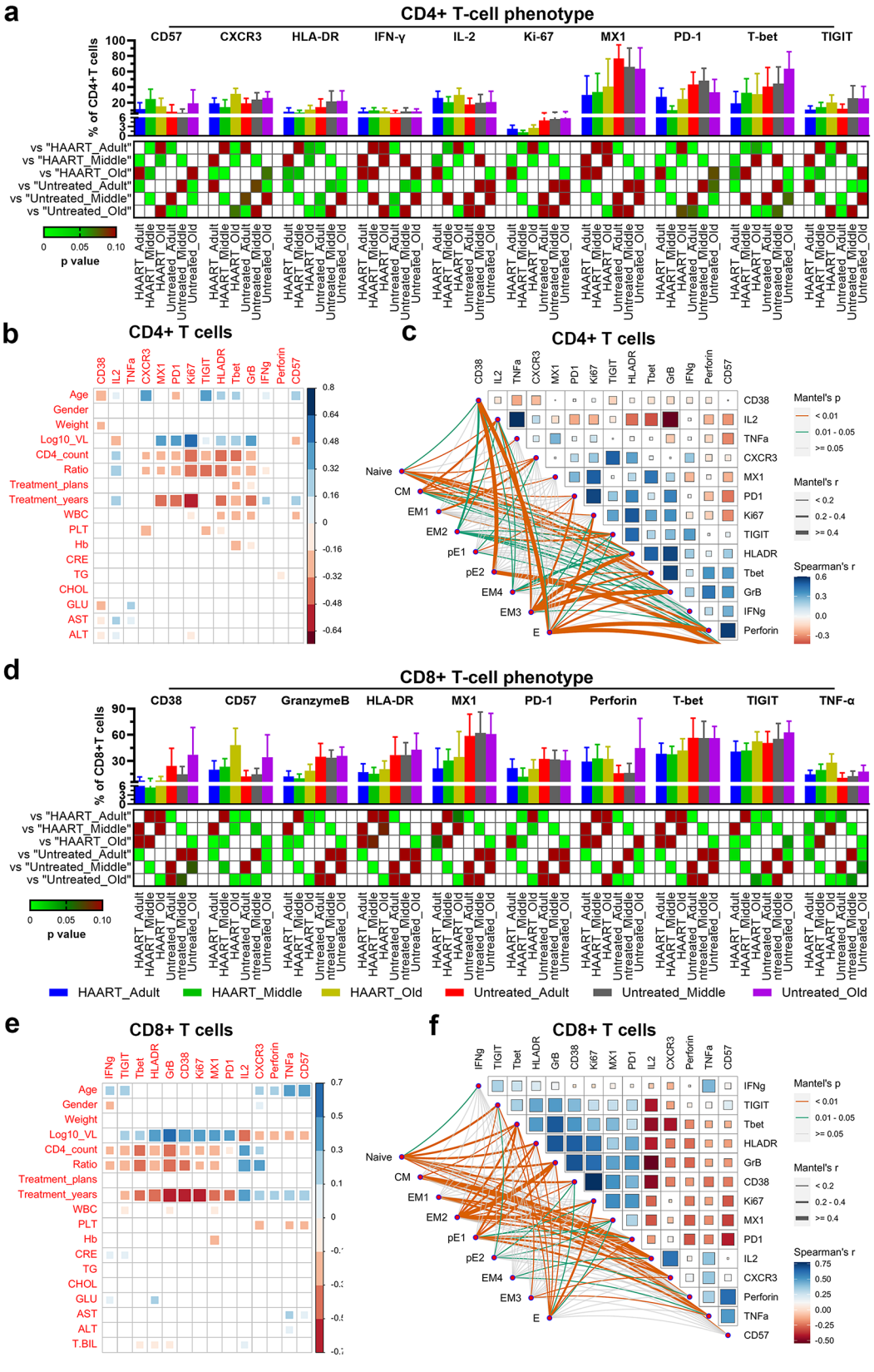

Fig. 4 (See legend on next page.)

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Fig. 4 Characterisation of T cell subsets and their correlation with clinical characteristics. (**a**, **d**) Expression of activation, senescence and exhaustion molecules by CD4 (**a**) and CD8$^+$T (**d**) lymphocytes from the PLWHs by flow cytometry. Statistical analysis was performed using Kruskal-Wallis. The heatmap illustrates the *p*-values for pairwise comparisons. A green color indicates statistical significance at $P < 0.05$, while red denotes no significant difference (see color bar). (**b**, **e**) The correlation matrix shows the associations between the clinical characteristics and immune markers within the CD4$^+$T subsets (**b**) and CD8$^+$T subsets (**e**). The colors signify negative correlations (red) and positive correlations (blue). The size of the squares and the saturation of the color denote the absolute values of the Spearman coefficients. (**c**, **f**) Multidimensional correlation analysis between T cell subsets and parameters of immune function within CD4$^+$T subsets (c) and CD8$^+$Tsubsets (**f**). The rectangular boxes represent Spearman's correlations between the different immune markers, with red indicating negative correlations and blue indicating positive correlations, with darker colors and larger color blocks indicating stronger correlations. The relationship between each subset of T cells and each immune variable was determined via Mantel's test, with lines of different thicknesses related to Mantel's r statistic, while different ranges of *P* values are indicated by lines of different colors. VL: viral load, WBC: white blood cells, HGB: hemoglobin, PLT: platelets, T.BIL: total bilirubin, ALT: alanine aminotransferase, AST: aspartate aminotransferase, GLU: glucose, TG: triglycerides, CHOL: total cholesterol, CM: central memory cells, EM: effector memory cells, TNF-α: tumor necrosis factor-alpha, IL-2: interleukin-2, CXCR3: C-X-C motif chemokine receptor 3, MX1: murine myxovirus resistance 1, PD-1: programmed cell death protein 1, TIGIT: T cell Immunoreceptor with Ig and ITIM domains, HLA-DR: human leukocyte antigen DR, T-bet: T-box expressed in T cells, GrB: Granzyme B, IFNg: IFN-γ

indicated that the accuracy of these indicators in predicting immune reconstitution among PLWH is significantly influenced by age. Specifically, they exhibit excellent predictive ability primarily among older PLWH but fail to accurately predict immune reconstitution in individuals aged 18–30 years. These findings emphasize the intricate and persistent impact of age on immune recovery in PLWH, highlighting the importance of considering age-related factors when assessing immune reconstitution.

The aging process results in notable changes in the immune system, referred as immunosenescence [43]. These changes are important because they increase the likelihood and severity of infectious diseases, diminish the efficacy of vaccinations, and potentially contribute to autoimmune disorders and cancer development [44]. One of the most prominent features of immunosenescence, which is associated with a decline in immunological responsiveness among individuals, is the alteration in the composition of the T cell compartment [44]. Therefore, our focus lies in investigating the major age-related changes encompassing modifications in both T cell subsets and functionality within a cohort consisting of 146 PLWH who exhibited similar immune characteristics. The results revealed that older aging led to a significant decrease in the proportion of Naïve CD4$^+$ T and CD8$^+$T cells, accompanied by an increase in memory T cell subsets such as CM and TEMRA cells within both CD4$^+$T and CD8$^+$T cell subsets, when compared with young and middle-aged individuals. The observed phenotypic changes were consistent with our previous findings in old Chinese rhesus monkeys (ChRMs) with acute SIV infection. Importantly, these immunosenescence signatures are strongly correlated with indicators of HIV/AIDS progression. Old ChRM shows a rapid surge in the plasma viral load accompanied by a swift decline in CD4$^+$T cells, leading to severe CD4/CD8 inversion, which further exacerbates the progression of early death in this population [45]. Specifically, a prominent observation is that older PLWH have increased numbers of CD4$^+$T and CD8$^+$T cells that highly express CD57, a marker associated with replicative senescence and prolonged chronic infections [46, 47]. These findings are also consistent with other studies on the T cell compartment observed during normal aging, which are believed to be caused by repeated immune activation over time [45, 48].

The initiation of HAART is crucial for the restoration of T cell subsets. Research has demonstrated that HAART results in a significant increase in the frequency of Naïve CD4$^+$T and CD8$^+$T cells over time, ultimately reaching levels comparable to those observed in healthy individuals [48, 49]. However, this improvement in the Naïve T cell subset appears to be offset by the deleterious effects of aging. Our study revealed that older PLWH, both those receiving HAART and those not receiving HAART, presented lower frequencies of Naïve CD4$^+$T and CD8$^+$T cells than young and middle-aged PLWH did, despite having comparable total T cell counts. Furthermore, no significant effect on the Naïve CD4$^+$T cell subset was observed in treated PLWH compared with untreated older PLWH. This undoubtedly reflects an insufficient generation of Naïve T cells in PLWH due to thymic involution associated with aging [50], which has been demonstrated to impede the recovery of CD4$^+$T cells by decelerating the proliferation of Naïve T cells [38]. This finding could also be attributed to the fact that older PLWH may demonstrate increased susceptibility to sustained and severe immune activation, resulting in rapid and elevated turnover of lymphocytes and a transition toward an immunosenescence phenotype [51]. Our study observed increased numbers of HLADR$^+$CD4, CD38$^+$CD8, and HLADR$^+$CD8 cells in the older PLWH, providing additional evidence supporting this hypothesis [52]. We investigated the correlation between the expression of activation markers on T cells and various clinical parameters. Specifically, we found positive associations with age and the viral load, but negative associations with the CD4$^+$T cell count and CD4/CD8 ratio. These results indicate a potential impairment of T cell function and clinical deterioration due to immunosenescence and sustained immune activation [53]. The enhancement of immunosenescence and the regulation of aberrant

immune responses in older PLWH have significant implications for enhancing their quality of life.

Additionally, the accumulation of scientific evidence in the past decade has shown that advanced age and persistent viral infection both significantly lead to T cell exhaustion, which has been confirmed to be one of the main mechanisms causing rapid AIDS progression, early death and failure of immune reconstitution in HIV-1 infection [54]. Phenotypically, exhausted T cells upregulate the expression of inhibitory receptors such as PD-1, TIGIT, cytotoxic T lymphocyte antigen 4 (CTLA4) and T cell immunoglobulin domain and mucin domain-3 (TIM-3) [55]. As expected, we observed that the level of TIGIT was upregulated on $CD8^+$T and $CD4^+$T cells in older PLWH. The upregulation of TIGIT during chronic HIV-1 infection can enhance viral replication and immune activation while also leading to reduced cytotoxic function of $CD8^+$T cells, hastening the progression to AIDS [56]. Experimental studies have shown that blocking the TIGIT pathway can reverse this effect, restoring the ability of HIV-1-specific $CD8^+$T cells to combat the virus and suggesting its potential as an immunological therapy for HIV-1 infection [57].

An intriguing finding from our study is that compared with treated young and middle-aged PLWH, older PLWH receiving HAART exhibited a higher proportion of CM subsets among both CD4 and CD8 cells. CM T cells are characterized by CD45RA-CD28+CD27+CCR7+. This study found that CD4+CM cells exhibit high expression of MX1, TNF-α, IL-2, and PD-1, whereas CD8+CM cells highly express IFN-γ, TNF-α, IL-2, and CXCR3. The CM subset was associated with temporal factors such as age and treatment duration. CM $CD8^+$T cells were negatively correlated with the viral load but positively correlated with the patient's current $CD4^+$T cell count and CD4/CD8 ratio. Current research is inconsistent regarding the role of CM subsets in HIV/AIDS progression. Several studies have shown that a relatively high frequency of PD-1 expressing $CD4^+$T CM cells is linked to incomplete $CD4^+$T cell reconstitution and increased HIV-1 DNA content during HAART [58]. Additionally, research has shown that CM T cells, especially lymph node CM $CD4^+$T cells, act as the primary cellular reservoirs for latent viruses in PLWH undergoing HAART due to their extended survival. The homeostatic proliferation of these latently infected cells may contribute to the regeneration and maintenance of the viral reservoir [59–61]. On the other hand, some studies have suggested that the balance of CM cells is crucial in maintaining the asymptomatic stage of HIV-1 infection, with the disruption of CM cell homeostasis being associated with progression to AIDS [62, 63]. Therefore, further research on the phenotypes and functions of CM subsets that occur in older PLWH is needed.

Surprisingly, we observed the $CD4^+$T EM3 subset and the $CD8^+$T EM2 subset, where they were found at appreciable frequencies in $CD4^+$T and $CD8^+$T cells. The $CD4^+$T EM3 subset is characterized by high levels of T-bet, Granzyme B and PD-1 expression. The $CD8^+$T EM2 subset exhibited increased expression of the activation markers HLA-DR and CD38, as well as the exhaustion markers PD-1 and TIGIT. The $CD4^+$T EM3 subset and the $CD8^+$T EM2 subset all have the same features and have the same characteristics. Both subsets decreased in frequency after HAART initiation. They both strongly negatively correlated with $CD4^+$T cell counts, CD4/CD8 ratios, and treatment duration but positively correlated with the viral load. Importantly, cellular frequencies were not found to be influenced by age. These findings suggest that they could serve as surrogate markers for clinical monitoring to evaluate the efficacy of HAART.

In contrast to previous works, we presented a more comprehensive classification of T cell subsets. While conventional investigations typically categorize T cells into four subsets (Naïve, CM, EM, and TEMRA) [46, 64], our study expands this classification to encompass 9 distinct subsets based on the differentiation order [17]. This detailed categorization and analysis of T cell subsets could offer valuable mechanistic insights into HIV-1 pathogenesis and immune recovery that may not be captured through the examination of bulk T cells alone. A key point to note is the exclusion of co-infection status from the potential effects on T cell phenotype and function in our study. Chronic inflammation is strongly associated with morbidity and mortality in both the elderly and PLWH, with chronic viral infections (e.g., HCV and CMV) playing a significant role in persistent inflammation. Naeger et al. found that, even after prolonged HAART, PLWH exhibited high levels of CMV-specific effector T cells, a characteristic typically observed in the older [65]. Kovacs et al. demonstrated that PLWH co-infected with HCV experience a significantly heightened state of immune activation, which accelerates the AIDS process [66]. Such chronic infections can induce lifelong antigenic stimulation, resulting in terminal T-cell differentiation, apoptosis tolerance, and limited proliferative potential. This, in turn, diminishes the immune system's ability to recognize neoantigens and increases the risk of age-related diseases [67]. Given the prevalence of co-infection among PLWH and its substantial impact on immune function and clinical outcomes, we systematically screened samples for co-infections with HCV, HBV, EBV, and CMV. Our findings indicate that these co-infections were uniformly distributed across groups and were present at low overall levels, with no significant differences observed between the groups. This finding is critical as it enables us to focus more clearly on the influence

of age factors on T cell phenotype and function, eliminating concerns regarding potential confounding effects of co-infection. By rigorously controlling for co-infection as a variable, we have been able to more accurately elucidate age-associated changes in T cells, thereby providing strong support for a deeper understanding of the relationship between age and immune system function in HIV-infected individuals. Consequently, our findings, after excluding the confounding effect of co-infection, offer a clearer and more reliable perspective for investigating the impact of age on T-cell immune status.

However, our study has certain limitations. First, this cross-sectional study was unable to track long-term changes in participant T cell subsets and function over time, and this study design did not allow for establishing causality. Future longitudinal studies with more frequent sampling are crucial for gaining a better understanding of these mechanisms. Additionally, the lack of direct matched comparisons with HIV-1 negative individuals based on participant characteristics hindered the accurate assessment of treatment effects and potentially impacted the interpretation of the results. A future study is planned to include comparisons with HIV-1 negative individuals or healthy controls to thoroughly evaluate immunophenotypic speciation and validate the experimental results. Moreover, although detailed medical history and clinical characteristics were recorded during the study, other clinical factors such as active opportunistic infection or malignancy, compliance and HAART regimen were not adequately considered in relation to immune function. These omissions may limit the generalizability of our findings.

Conclusion

Overall, our study has a larger sample size and the most detailed pattern of T cell subset changes, depicting the impact of aging in HIV-1 infection and HAART on T cell subsets. Our findings validate and expand upon previous observations that older PLWH exhibits a highly dysregulated immunity with an expansion of central memory and TEMRA cells, as well as a wide range of late differentiated, senescent, or exhausted phenotypes across all $CD4^+$T and $CD8^+$T cell subsets. In this context, the immune function of older PLWH can become significantly compromised over time, thereby increasing their susceptibility to various diseases and potentially exerting a substantial impact on their quality of life. Personalized health management strategies are crucial in addressing the challenges of HIV-1 aging.

Abbreviations

AIC	Akaike information criterion
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
AUC	Area under roc
CCL2	Chemokine ligand 2
CCR7	C-C chemokine receptor 7
CD38	Cluster of differentiation 38
CDC	Center for disease control and prevention
ChRMs	Chinese rhesus monkeys
CM	Central memory
Cr	Creatinine
CTLA4	Cytotoxic t lymphocyte antigen 4
CXCR3	C-X-C motif chemokine receptor 3
EDTA	Ethylenediaminetetraacetic acid
EM	Effector memory
FBS	Fetal bovine serum
Glu	Glucose
HAART	Highly active antiretroviral therapy
HGB	Hemoglobin
HLA-DR	Human leukocyte antigen DR
IFN-γ	Interferon-gamma
IL-10	Interleukin-10
IL-17	Interleukin-17
IL-18	Interleukin-18
IL-2	Interleukin-2
IL-6	Interleukin-6
MX1	Myxovirus resistance 1
PBMCs	Peripheral blood mononuclear cells
PD-1	Programmed cell death protein 1
PLT	Platelet
PLWH	People living with HIV-1
PMA	Phorbol myristate acetate
ROC	Receiver operating characteristic curve
sCD40L	Soluble CD40 ligand
sFlt-1	Soluble fms-like tyrosine kinase-1
sRAGE	Soluble receptor for advanced glycation end products
sST2	Soluble suppression of tumorigenicity 2
T-bet	T-box expressed in t cells
TBIL	Total bilirubin
TC	Total cholesterol
TEMRA	Terminally differentiated effector memory
TG	Triglyceride
TIGIT	T Cell Immunoreceptor with Ig and ITIM domains
TIM-3	T Cell Immunoglobulin domain and mucin domain-3
TNF-α	Tumor necrosis factor-alpha
UNAIDS	Joint united nations programme on HIV/AIDS
WBC	White blood cell count

Supplementary Information

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Supplementary Material 1

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Author contributions

The authors' contributions in this study are as follows: Professors Zhi-Qiang Shen and Yong-Tang Zheng proposed the initial conception and design of the study, providing invaluable guidance throughout the design and implementation process. Professors Xing-Qi Dong and Mi Zhang ensured the acquisition of clinical data and blood samples. Author Hong-Yi Zheng contributed to the data analysis, the graphical presentation of the results, and ensuring the appropriateness of the statistical methods used. Author Na Li, Wei Li and Xiao-Yan He contributed to the experimental procedures and drafted the initial version of the paper. Authors Xia Li and Ren-Rong Tian upervised data analysis and manuscript revising. All the authors read and approved the paper.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
The study was approved by the Ethics Committee of Yunnan Infectious Diseases Hospital (approval number: 202115 and 2022004). For the retrospective study, informed consent from participants was waived because of the use of anonymous data. For blood sample collection, we provided detailed information to patients and obtained written informed consent.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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