

## Partial recovery of disturbed V-J pairing profiles of T-cell receptor in people living with HIV receiving long-term antiretroviral therapy

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Chronic human immunodeficiency virus (HIV) infection not only causes a gradual loss of CD4<sup>+</sup> T cells but also leads to a disturbance of the T cell receptor (TCR) repertoire. In people living with HIV (PLWH), monitoring TCR repertoire is challenged by the inconsistency of complementarity determining region 3 (CDR3) and limited cell numbers in clinical samples. Thus, a quantitative method is necessary for monitoring the TCR repertoire in PLWH. We characterized the TCR V-J pairing profile of naïve and memory CD4<sup>+</sup> T cells in healthy donors, HIV-infected antiretroviral therapy (ART)-naïve patients and long-term (over 5 years) ART-experienced patients by performing TCR sequencing. We developed a V-J index with 18 parameters which were subdivided into five categories (expression coverage, cumulative percentage of the top tenth percentile, diversity, intra-individual similarity and inter-individual similarity). In ART-naïve patients, 14 of the 18 parameters were significantly altered. Long-term ART recovered ten parameters. The four unrecovered parameters were related to inter-individual similarity. Therefore, these findings indicate that long-term ART could only partially recover TCR V-J pairs and introduce newly impacted V-J pairs. Moreover, these results provide new insights into the V-J pairing of the TCR and into the disturbance of TCR repertoire in HIV infection.

**acquired immune deficiency syndrome, human immunodeficiency virus, antiretroviral therapy, T cell receptor, V-J pairing**

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## INTRODUCTION

T cells mediate immune responses to a wide variety of pa-

thogenic antigens via a large repertoire of unique T cell receptors (TCRs) (Alcover et al., 2018; Turner et al., 2006; Turner et al., 2009). Human immunodeficiency virus (HIV) may destroy CD4<sup>+</sup> T cells via virological and immunological mechanisms (Doitsh et al., 2014). Progressive loss of CD4<sup>+</sup> T cells may further drive the TCR repertoire of CD4<sup>+</sup> T cells

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away from a healthy status. The disturbed TCR repertoire of CD4<sup>+</sup> T cells leads to immunosurveillance defects, resulting in opportunistic pathogen infections and tumours, namely the acquired immune deficiency syndrome (AIDS)-defining illnesses (Baum et al., 2012; Heather et al., 2015). Long-term antiretroviral therapy (ART) successfully restores CD4<sup>+</sup> T cell counts in people living with HIV (PLWH). However, even after ART, the risks of certain infections and cancers, especially the non-AIDS-defining cancers, are still higher in PLWH than in the healthy population (Anglemyer et al., 2014; Deeken et al., 2012; Dominguez-Molina et al., 2016; Mocroft et al., 2010), suggesting the existence of potential defects in immunosurveillance in PLWH.

In humans, most mature T lymphocytes are T cells expressing the  $\alpha\beta$  heterodimer of TCR (Alcover et al., 2018; Kreslavsky et al., 2010). The generation of TCR diversity occurs in the precursor T cells in the thymus by the random and imprecise somatic rearrangement of segregated germline variable (V) and joining (J) gene segments for the TCR  $\alpha$  chain and segregated germline V, diversity (D) and J gene segments for the TCR  $\beta$  chain (Call et al., 2006; Mallis et al., 2015; McDonald et al., 2015). Since the hypervariable complementarity determining region 3 (CDR3) of TCRs determines the antigenic specificity of one given T cell (Call et al., 2004; Dash et al., 2017; Glanville et al., 2017), the sequences coding CDR3 have been extensively studied in the fields focusing on TCR specificity, such as anti-tumour immunity, vaccine development, and autoimmune diseases (Billam et al., 2011; Farge et al., 2017; Jiang et al., 2018; McNeel, 2016; Miyasaka et al., 2019; Ochsenreither et al., 2012; Zhao et al., 2016).

However, facing the dramatic loss of CD4<sup>+</sup> T cells in PLWH, the diversity rather than the specificity is more able to indicate the severe and widespread changes in the TCR repertoire (Heather et al., 2015). The clinical usage of CDR3 has been hampered in PLWH because of the considerable genetic variation and low consistency of CDR3 among different individuals, as well as the gap between the tremendous

amount of CDR3 diversity and the limited number of T cells in clinical samples (Memon et al., 2012; Wettstein et al., 2014).

Since the sequences encoding CDR3 locate at the joint of rearranged V and J segments, V-J pairing is the cornerstone of TCR diversity (Currier and Robinson, 2001; Eason et al., 2004; Venturi et al., 2008). Recently, a number of studies with immune repertoire sequencing technology revealed conservative preferential expression patterns of V and J segments in healthy individuals (Li et al., 2015; Liu et al., 2014; Migalska et al., 2018). In the present study, we aimed to quantitatively characterize the defect in TCR V-J pairing profile caused by chronic HIV infection, and the recovery of T cell receptors after long-term ART.

## RESULTS

### Sequencing characteristics

The clinical characteristics of 17 healthy donors, 17 chronically ART-naïve HIV-infected patients and 15 chronically HIV-infected ART-experienced patients are listed in Table 1. The sequenced samples of TCR  $\alpha$ - and  $\beta$ -chains from sorted T cell subpopulations are summarized in Table S1 in Supporting Information. The median number of V-J paired reads was  $0.95 \times 10^5$  per sample (range,  $0.27 \times 10^5$ – $8.43 \times 10^5$ ).

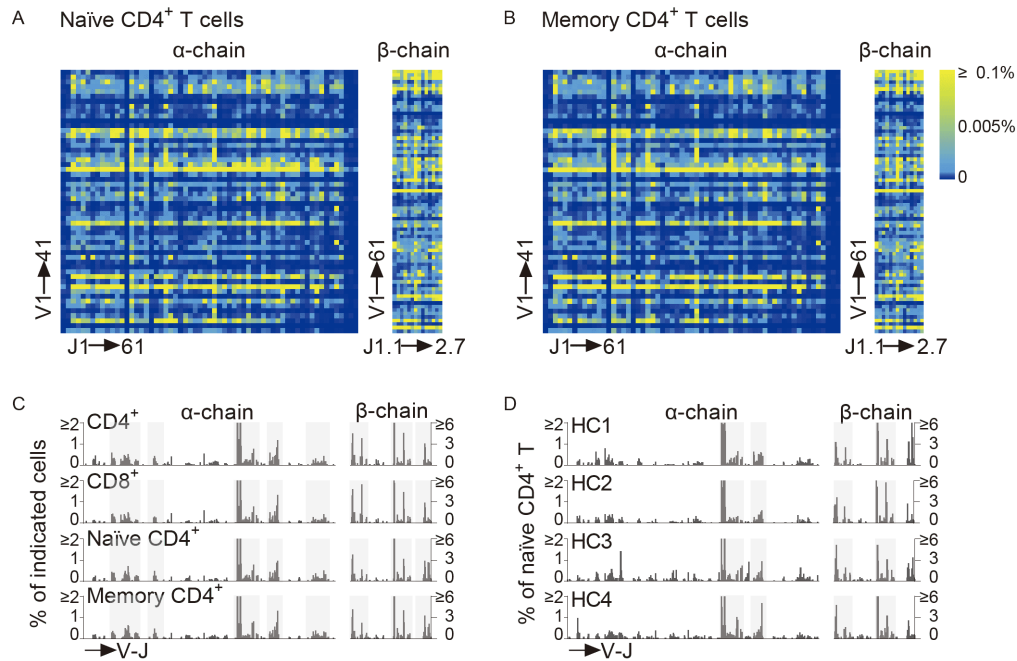
### Healthy donors exhibited conserved preference and high similarity of V-J pairing

The numbers of theoretical pairing types were 3,294 in the  $\alpha$ -chain and 1,050 in the  $\beta$ -chain. In healthy donors, and the V-J pairing reads covered more than 85% in the  $\alpha$ -chain and the  $\beta$ -chain of naïve and memory CD4<sup>+</sup> T cells (Table 2). Heatmap analysis revealed a remarkable preference of V-J pairing in TCR  $\alpha$ - and  $\beta$ -chains in both naïve and memory CD4<sup>+</sup> T cells (Figure 1A and B). The cumulative percentage

**Table 1** Characteristics of healthy controls, ART-naïve and ART-experienced HIV-infected patients (Median, range)<sup>a)</sup>

	Healthy donors (N=17)	ART-naïve (N=17)	ART-experienced (N=15)
Age, years	31 (23–40)	29 (20–43)	33 (25–41)
Gender (Male/Female)	17 (15/2)	17 (15/2)	15 (12/3)
Baseline CD4 <sup>+</sup> T cell counts (cells $\mu\text{L}^{-1}$ )	–	318 (243–443)	307 (212–416)
CD4 <sup>+</sup> T cell counts (cells $\mu\text{L}^{-1}$ )	849 (556–1224)	318 (243–443)	719 (635–1112)
Baseline CD8 <sup>+</sup> T cell counts (cells $\mu\text{L}^{-1}$ )	–	1103 (645–1963)	1220 (523–1618)
CD8 <sup>+</sup> T cell counts (cells $\mu\text{L}^{-1}$ )	772 (542–1263)	1103 (645–1963)	911 (436–1950)
Baseline VL ( $\log_{10}$ copies $\text{mL}^{-1}$ )	–	4.8 (4.2–5.5)	4.8 (4.0–5.6)
pVL ( $\log_{10}$ copies $\text{mL}^{-1}$ )	–	4.8 (4.2–5.5)	TND
ART duration (weeks)	–	0	384 (342–420)

a) pVL: plasma viral load; ART: active antiretroviral therapy; TND: the target could not be detected.



**Figure 1** The preferences of TCR V-J pairs in CD4<sup>+</sup> T cells of healthy donors. A and B, Heat maps of TCR  $\alpha$ - $\beta$ -chain V-J pairing usages in naïve (A) and memory (B) CD4<sup>+</sup> T cells. The percentages of V-J pairs are the median of healthy donors (HD). Blue and yellow represent low or high percentages of observations in the healthy donors, respectively. Colour density indicates the relative frequency level, from 0 to over 0.1%. C, Similar distribution of V-J pairing profiles among different CD4<sup>+</sup> T cell subsets. The grey bar indicates the median percentages of frequency in  $\alpha$ - and  $\beta$ -chains from different T cell subsets. D, Distributions of the V-J pairing frequency of naïve CD4<sup>+</sup> T cell subsets in four representative individuals. The bars indicate the frequencies of presented V-J pairs in naïve CD4<sup>+</sup> T cells. The V-J pairs with similar percentage distributions among different T cell subsets (C) or different individuals (D) are highlighted in grey.

**Table 2** TCR parameters of VJ-index in Healthy donors, HIV-infected ART-naïve and experienced patients (median, range)<sup>a)</sup>

VJ-index	Cell type & $\alpha/\beta$ chain	Healthy donor	ART-naïve	ART-experienced	Recover or not
Coverage (%)	Naïve $\alpha$	88.1 (72.0–98.8)	77.8 (47.6–97.4)*	87.8 (69.5–98.1)	√
	Memory $\alpha$	89.9 (66.5–89.9)	87.8 (51.7–98.0)	86.7 (67.8–97.0)	-
	Naïve $\beta$	98.3 (81.9–99.5)	93.0 (83.9–98.8)*	96.0 (90.4–99.4)	√
	Memory $\beta$	97.9 (84.0–99.4)	95.0 (80.9–100.0)	96.0 (91.3–99.7)	-
Cumulative frequency (% top 10 percentile)	Naïve $\alpha$	84.3 (66.1–97.3)	96.4 (92.7–97.7)***	91.6 (76.4–98.4)	√
	Memory $\alpha$	85.3 (64.2–98.3)	96.2 (86.8–97.7)***	87.0 (76.4–98.6)	√
	Naïve $\beta$	82.7 (76.0–97.7)	91.8 (77.1–95.4)*	91.4 (72.5–97.1)	√
	Memory $\beta$	87.6 (70.4–97.3)	91.1 (80.0–96.5)	90.6 (71.6–95.0)	-
Diversity	Naïve $\alpha$	5.72 (3.41–6.68)	4.42 (3.03–5.12)***	5.47 (1.78–6.21)	√
	Memory $\alpha$	5.71 (3.15–6.77)	4.85 (3.14–5.77)***	4.53 (1.55–6.17)	√
	Naïve $\beta$	4.81 (2.64–5.25)	4.17 (2.93–5.10)*	4.54 (2.90–5.84)	√
	Memory $\beta$	4.64 (2.76–5.43)	4.09 (3.54–4.98)	4.19 (3.15–5.35)	-
Intra-individual similarity	Naïve	0.76 (0.25–0.89)	0.55 (0.44–0.75)***	0.64 (0.42–0.84)	√
	Memory	0.83 (0.26–0.93)	0.64 (0.08–0.88)**	0.83 (0.48–0.90)	√
Inter-individual similarity	Naïve $\alpha$	0.47 (0.09–0.82)	0.33 (0.13–0.66)***	0.28 (0.10–0.80)***	×
	Memory $\alpha$	0.40 (0.07–0.87)	0.35 (0.11–0.64)**	0.27 (0.11–0.81)**	×
	Naïve $\beta$	0.59 (0.07–0.89)	0.49 (0.01–0.79)***	0.30 (0.07–0.85)***	×
	Memory $\beta$	0.48 (0.06–0.89)	0.34 (0.02–0.81)***	0.28 (0.07–0.87)***	×

a) \*\*\*:  $P < 0.001$ , \*\*:  $P < 0.01$ , \*:  $P \leq 0.05$ ;  $P$  values were calculated by Mann-Whitney U tests vs. Healthy donor. √: recovered in ART-experienced patients; ×: unrecovered in ART-experienced patients; -: parameters were not affected by HIV infection ( $P > 0.05$ , ART-naïve vs. Healthy donor).

of the top tenth percentile accounted for approximately 85% of the total V-J pairing reads (Table 2). The top 10 most

frequently used V-J pairs in the samples from healthy donors are listed in Table S2 in Supporting Information. Similar

preferences of V-J pairing were also observed in the naïve and memory fractions of CD8<sup>+</sup> T cells (Figure S1 in Supporting Information).

Of note, the V-J pairing profiles of naïve and memory fractions of CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells displayed high similarity of V-J pairing profiles in each healthy donor individual (named intra-individual similarity, Figures 1C and S1C in Supporting Information). The similarity of V-J pairing profiles was also observed among different healthy individuals (named inter-individual similarity, Figures 1D and S1D in Supporting Information), especially in high frequency V-J pairs. Among the top 10 most frequently used V-J pairs of TCR  $\alpha$ - and  $\beta$ -chains, the naïve and memory fractions of CD4<sup>+</sup> T cells shared 8 and 9 V-J pairs, respectively (Table S2 in Supporting Information).

Based on the conserved preferences and high similarity of the V-J pairing profiles, we proposed 18 parameters as a V-J pairing index to evaluate the TCR repertoire in HIV-infected patients (Table 2). These parameters cover the following five categories: (i) the coverage of V-J pairing, (ii) cumulative V-J pairing percentage of the top tenth percentile, (iii) the diversity of V-J pairing, (iv) intra-individual similarity of V-J pairing (the similarity between naïve and memory CD4<sup>+</sup> T cells within each individual), and (v) inter-individual similarity of V-J pairing (the similarity among individuals).

### Alteration of the V-J pairing index in ART-naïve PLWH

Next, we analysed the alteration of the V-J pairing index in ART-naïve patients. The median coverage of TCR V-J pairing from naïve CD4<sup>+</sup> T cells was 77.8% (range, 47.6%–97.4%) in the  $\alpha$ -chain and 93.0% (range, 83.9%–98.8%) in the  $\beta$ -chain, which were significantly lower than those in healthy donors (median, 88.1% in the  $\alpha$ -chain and 98.3% in the  $\beta$ -chain,  $P < 0.05$ ) (Table 2). Meanwhile, ART-naïve patients displayed an increased preference for V-J pairing (Figure 2A) in both the  $\alpha$ -chain of naïve and memory CD4<sup>+</sup> T cells and the  $\beta$ -chain of naïve CD4<sup>+</sup> T cells. Consistently, the cumulative percentages of the top tenth percentile V-J pairs of ART-naïve patients were significantly higher in TCR the  $\alpha$ -chain (96.4%) and  $\beta$ -chain (91.6%) of naïve CD4<sup>+</sup> T cells and the  $\alpha$ -chain of memory CD4<sup>+</sup> T cells (91.8%) (Table 2).

We further calculated the Shannon entropy index, which is commonly used for the measurement of diversity, and found that HIV-infected patients exhibited a significantly decreased value for V-J pairing in TCR  $\alpha$ - and  $\beta$ -chains in naïve CD4<sup>+</sup> T cells, as well as in the TCR  $\alpha$ -chain of memory CD4<sup>+</sup> T cells ( $P < 0.05$ , Figure 2B).

In agreement with the dramatic decrease in the percentage of naïve CD4<sup>+</sup> T cell counts (Table 1), we observed a decrease in intra-individual similarity in ART-naïve patients (Figure 2D). By evaluating Pearson correlation coefficients, we also found a significant reduction in inter-individual si-

ilarity of the TCR  $\alpha$ - and  $\beta$ -chains in both naïve and memory CD4<sup>+</sup> T cells in ART-naïve patients (Figure 2C).

As summarized in Table 2, 14 of 18 V-J pairing parameters were significantly altered in ART-naïve HIV-infected patients.

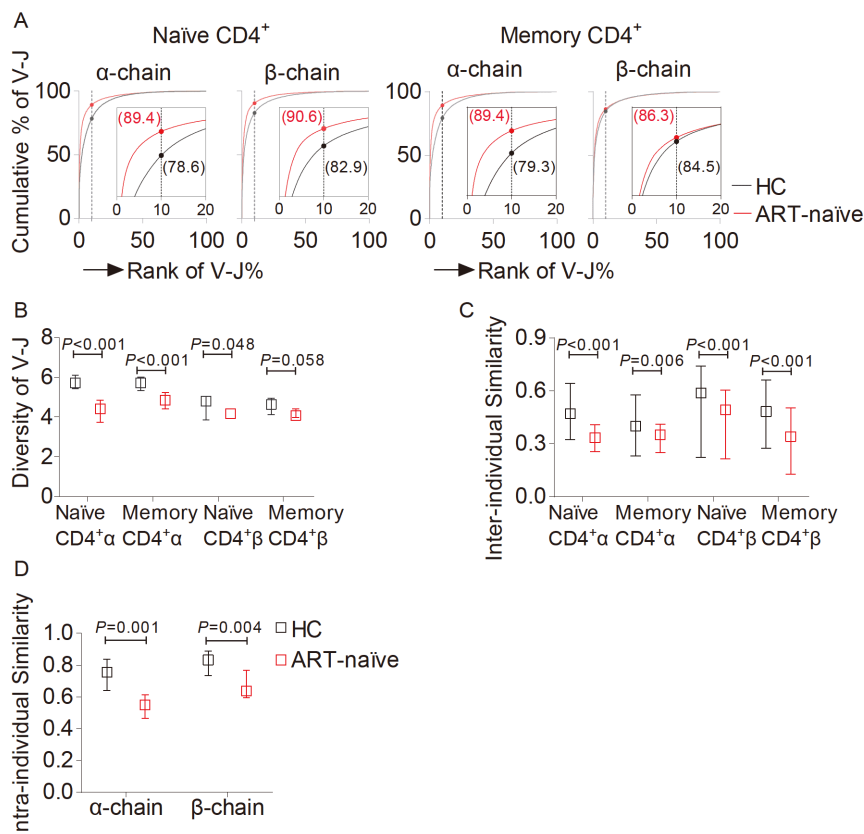
### Partial recovery of the V-J pairing index after long-term ART

Next, we investigate whether long-term ART could successfully recover the disturbed V-J pairing index. Along with the recovery of CD4<sup>+</sup> T cell counts, the coverage of TCR V-J pairs from naïve CD4<sup>+</sup> T cells returned to 87.8% (range, 69.5%–98.1%) and 96.0% (range, 90.4%–99.4%,  $P > 0.05$ , Table 2). The preferences of TCR  $\alpha$ - and  $\beta$ -chains decreased towards healthy status (Figure 3A). In addition, the Shannon entropy diversity index of TCR  $\alpha$ - and  $\beta$ -chain V-J pairs in CD4<sup>+</sup> T cell subsets rose to a level comparable to healthy donors (Figure 3B). The similarity coefficients of intra-individual similarities of V-J pairing profiles returned towards the values of healthy donors (Figure 3C). Taken together, the results of the Mann-Whitney test showed that among 14 parameters that were altered in ART-naïve patients, 10 parameters had recovered after long-term ART (Table 2). In addition, among 1,401 significantly impacted V-J pairs ( $P < 0.01$ ) in ART-naïve patients, 1,113 (79.4%) recovered after over 5 years of therapy (Figure 4).

Strikingly, we observed that 4 parameters related to the inter-individual similarity coefficients of both naïve and memory CD4<sup>+</sup> T cells in ART-experienced patients remained lower than those in healthy donors (Figure 3D, Table 2). All these parameters were related to the inter-individual similarity coefficients of both naïve and memory CD4<sup>+</sup> T cells, showing certain defects in the inter-individual similarity of V-J pairing profiles, in the patients, whose CD4<sup>+</sup> T cell count recovered after long-term ART.

### Unrecovered and newly impacted V-J pairs after ART or HIV infection

Next, we analysed the V-J pairs that differently expressed in ART-experienced patients and ART-naïve patients (cut-off:  $P < 0.01$ , Figure 4). Among 705 (sum of all the V-J pairs in panel II and III of Figure 4B and C) disturbed V-J pairs in ART-experienced patients, 288 (sum of all the V-J pairs in panel II of Figure 4B and C, 40.9%) were those unrecovered from the significantly impacted V-J pairs in ART-naïve patients. The remaining 417 (sum of all the V-J pairs in panel III of Figure 4B and C, 59.1%) V-J pairs were comparable between healthy donors and ART-naïve patients, and became significantly different from healthy donors after ART ( $P < 0.01$ , lower right quarter of the scatter-plots in Figure 4), suggesting that the disturbance of these V-J pairs might be



**Figure 2** Preference, diversity, and intra- and inter-individual similarities of TCR V-J pairing in CD4<sup>+</sup> T cells from HIV-infected ART-naïve patients. A, Preference of TCR V-J pairing in naïve and memory CD4<sup>+</sup> T cells from chronic HIV infections. The curves show the accumulated frequency (median) of TCR V-J pairs presented along the rank percentage of V-J pairs in healthy donors (HD, black) and HIV-infected ART-naïve patients (red). Dashed lines and the number in brackets correspond to the cumulative percentages of the top tenth percentile of highest frequencies of V-J pairing. B, Diversities of V-J pairing in CD4<sup>+</sup> T cells from patients with chronic HIV infection without ART. C, Inter-individual similarity of TCR V-J pairing in CD4<sup>+</sup> T cells from HIV-infected ART-naïve patients. D, Intra-individual similarity of TCR V-J pairing in CD4<sup>+</sup> T cells from HIV-infected ART-naïve patients. Red boxes indicate the diversity or similarities of V-J pairs from HIV-infected ART-naïve patients, and black boxes indicate these parameters in the healthy donors. *P* values were calculated by Mann-Whitney U tests.

introduced by ART.

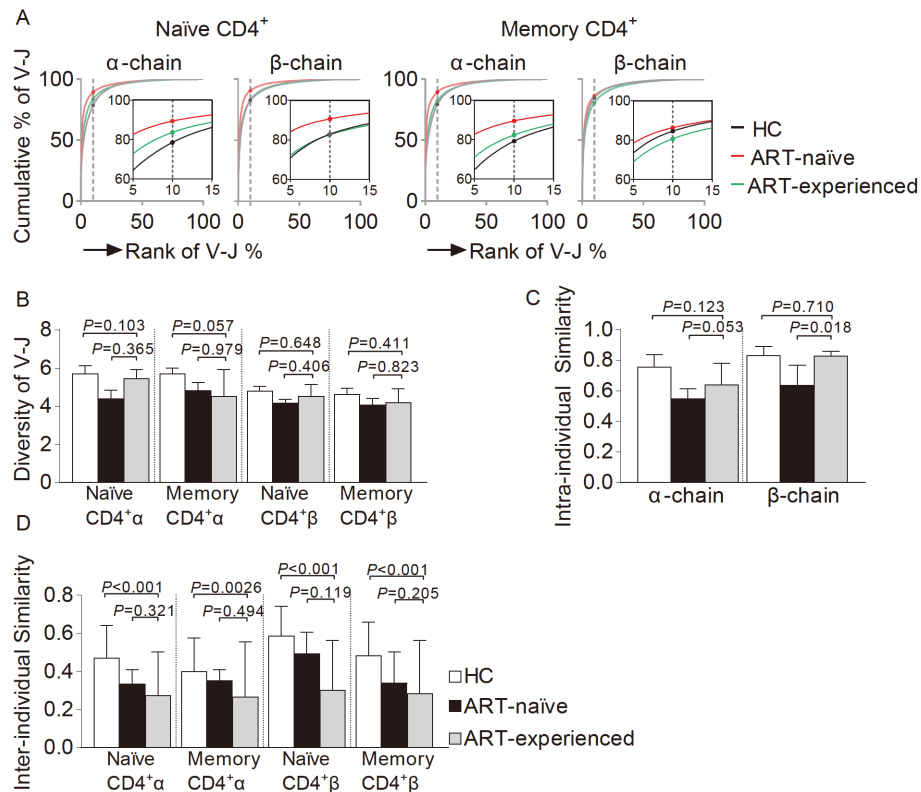
Among the 288 uncovered V-J pairs, 240 (83.3%) resided in the TCR α-chain of naïve CD4<sup>+</sup> T cells, 40 (13.9%) in the TCR α-chain of memory CD4<sup>+</sup> T cells, and only 5 (1.74%) and 3 (1.04%) V-J pairs were found in the TCR β-chain of naïve and memory CD4<sup>+</sup> T cells, respectively (Figure 4B and C). Most unrecovered V-J pairs (282 grey rings of total 288 rings in Figure 4A and B, 97.9%) had significantly lower frequencies (*P*<0.01) than those in healthy donors. In addition, 56.3% (135/240) of unrecovered V-J pairs in naïve CD4<sup>+</sup> T cells were concentrated into TRAV38-2/DV8 (43), TRAV8-2 (31), TRAV8-4 (26), TRAV38-1 (19) and TRAV12-1 (16), while 45.0% (18/40) of unrecovered V-J pairs in memory CD4<sup>+</sup> T cells were concentrated into TRAV8-2 (Figures 4B and C and 5A). All these concentrated V segments belong to highly frequently used V segments in healthy donors (the rank of frequencies: TRAV8-2, 2; TRAV8-4, 4; and TRAV38-2/DV8, 11; Table S3 in Supporting Information). These V segments clustered in the co-occurrence networks, which suggested the relevance of their

expressions (Figure 5B). By contrast, 417 V-J pairs in the panel III of Figure 4B and C were newly impacted after ART, and they were randomly dispersed in V or J segment usage (naïve CD4<sup>+</sup> T cell: 72 V-J pairs increased, 48 decreased; memory CD4<sup>+</sup> T cells: 23 V-J pairs increased, 102 decreased; Figure 5C and D).

## DISCUSSION

In the present study, based on the conservative profile of V-J pairing of TCRs, we established a quantitative system and successfully evaluated the TCR repertoire in PLWH before and after ART. Among eighteen parameters of V-J pairing, the expression coverage, the cumulative percentage of the top tenth percentile and the diversity of V-J pairing quantitatively describe the diversity of TCR repertoire of an individual, while the intra-individual similarity and inter-individual similarity of V-J pairing represent the difference in the types of TCR V-J pairing between PLWH and the





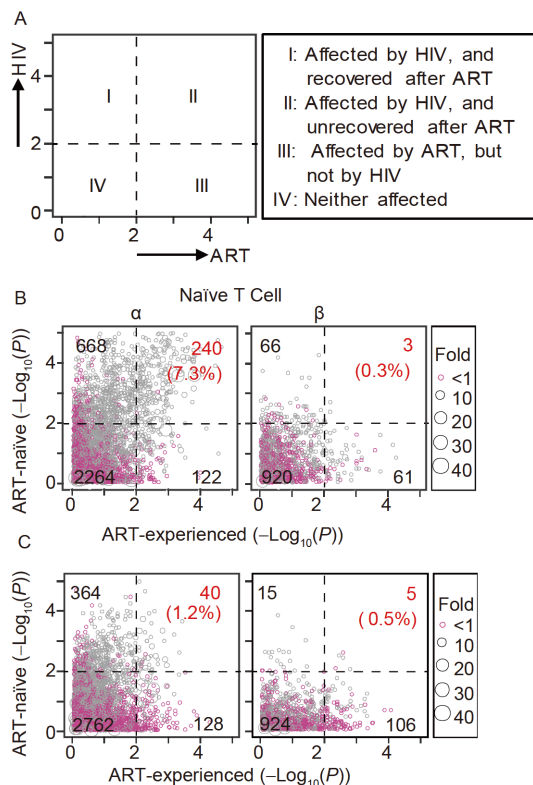
**Figure 3** Changes in preference, diversity, and intra- and inter-individual similarity of CD4<sup>+</sup> T cells from HIV-infected ART-experienced patients. **A**, Preference of TCR V-J pairing in naïve and memory CD4<sup>+</sup> T cells from HIV-infected ART-experienced patients. Black, red, and green curves show the accumulation of the percentage (median) of presented TCR V-J pairs along the rank percentage of V-J pairs in healthy donors (HD), HIV-infected ART-naïve patients and HIV-infected ART-experienced patients, respectively. **B**, Diversities of V-J pairing in CD4<sup>+</sup> T cells from HIV-infected ART-experienced patients. **C**, Intra-individual similarity of TCR V-J pairing in CD4<sup>+</sup> T cells from HIV-infected ART-experienced patients. Grey bars indicate the diversity or similarities of V-J pairs from HIV-infected ART-experienced patients. Black and white bars indicate the similarities in HIV-infected ART-experienced patients and healthy donors, respectively. *P* values were calculated by Mann-Whitney U tests.

healthy donors. By using V-J pairing index, we explored the depressed coverage, heightened preference of total V-J pairs, increased cumulative percentages of the top tenth percentile, as well as decreased inter- and intra-individual similarities in ART-naïve patients. More importantly, although many studies have concluded that the current ART regimen may extend the life expectancy of PLWH close to that of healthy people (Wandeler et al., 2016), our data points out the limitations of the current ART regimen, i.e., partial restoration of TCR V-J pairs in CD4<sup>+</sup> T cells.

To our knowledge, the only study on TCR repertoires reported by Heather and colleagues showed that short-term ART (3 months) could not restore TCR diversity and the usage patterns of V and J segments (Heather et al., 2015). However, it has been acknowledged that the increase in CD4<sup>+</sup> T cell counts in blood at the early stage after ART was mainly accounted for by the emigration of CD4<sup>+</sup> T cells from lymphoid organs (Bosch et al., 2006). Since it usually takes four to five years for CD4<sup>+</sup> T cells to achieve a stable plateau after initiating ART (Pantazis et al., 2019), we recruited a cohort with long-term ART (more than 5 years) whose

median CD4<sup>+</sup> T cell counts successfully increased from a baseline of 307  $\mu\text{L}^{-1}$  to a plateau of 719  $\mu\text{L}^{-1}$ . As expected, most (10/14) of the dysregulated V-J pairing indexes that were observed in ART-naïve patients shifted towards and recovered to healthy status, including the preference, coverage, diversity, and intra-individual similarity of V-J pairs. In general, ART could partially recover preferential TCR V-J pairing in AIDS patients.

Surprisingly, long-term ART failed to recover the inter-individual similarity, and the median values in ART-experienced patients even much lower than ART-naïve patients. Two factors might contribute to this phenomenon. First, approximately 20% of disturbed V-J pairs in ART-naïve patients did not reach the normal level after long-term ART. Most of these unrecovered V-J pairs resided in the naïve fraction, concentrated into several highly correlated V segments of the TCR  $\alpha$ -chain (TRAV38-2/DV8, TRAV8-2 and TRAV8-4) with a decreased frequency. This finding suggests that some CD4<sup>+</sup> T cell subpopulations with certain V segments and V-J gene usage still have potential defects at the early stage of T cell development, which might be related



**Figure 4** The V-J pairs impacted by chronic HIV infection and long-term ART. A, The schematic diagram of the V-J pairing distribution. All V-J pairs are divided into four panels with the dash representing a cut-off of  $P$  value=0.01 ( $-\log_{10}(P)=2$ ). The panel I represents the V-J pairs only impacted by HIV infections, the panel II represents the V-J pairing only impacted by ART, the panel III represents the V-J pairing impacted by both HIV infections and ART, and the left panel IV represents the V-J pairing changed neither by HIV infections nor ART. B and C, The distribution of V-J pairs of naïve (B) and memory (C)  $CD4^+$  T cells in patients by  $P$  values during HIV infection and ART. The x-axis shows the  $-\log_{10}(P)$  between healthy donors (HD) vs. ART-experienced patients. The  $P$  value was calculated by the Mann-Whitney test. The y-axis shows the  $-\log_{10}(P)$  between HD vs. ART-naïve patients. Red and grey rings indicate the decrease and increase in V-J frequencies in ART-experienced patients and HD, respectively. The sizes of the rings represent the fold changes in V-J pair percentage by HD/ART-experienced patients.

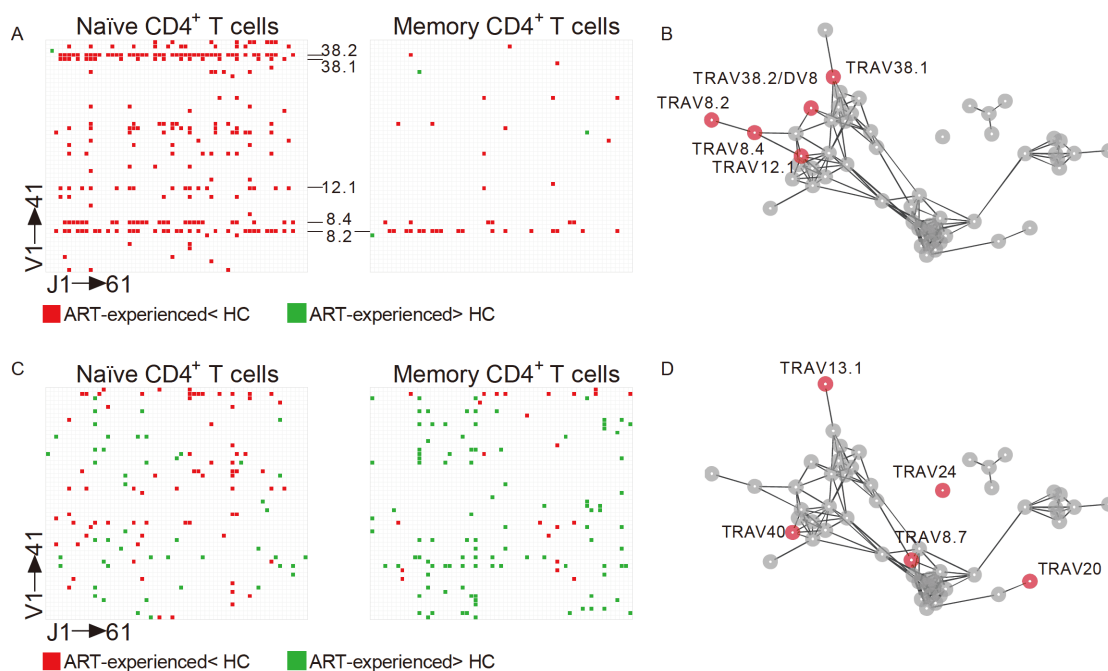
with some kind of pathological processes. Whether this observation is caused by residual HIV virus or local immune activation needs further investigation. Second, ART causes a more widely disturbance of V-J pairing homeostasis in both naïve and memory  $CD4^+$  T cells, which may drives the inter-individual similarity even far away from the normal status. It has been reported that zidovudine, a nucleoside analogue, can cause myelosuppression and can inhibit lymphocyte development (Rachlis and Fanning, 1993). Whether other drugs might affect TCR V-J pairing requires further studies with clinical cohort or animal experiments. Minimizing these kinds of interferences to V-J pairing profiles should be taken into consideration for developing novel ART strategy.

Specificity and diversity are two important characteristics of the TCR repertoire in the adaptive immune system (Al-

cover et al., 2018; Turner et al., 2006; Turner et al., 2009). Specificity describes the subtle details of T cell clones, whereas diversity provides a more general view of the entire T cell population. In most studies on the TCR repertoire, high-throughput sequencing of the CDR3 coding region has successfully described the specificity of TCR for recognizing specific peptides of pathogens, tumour cells and auto-antigens (Billam et al., 2011; Farge et al., 2017; Jiang et al., 2018; McNeel, 2016; Miyasaka et al., 2019; Ochsenreither et al., 2012; Zhao et al., 2016). Unlike the diseases related to mono- or oligo-clonal changes of T cells, the disturbance of  $CD4^+$  T cells caused by HIV infection is severe and broad (Baum et al., 2012). Thus, it is necessary to describe the changes in TCR repertoire diversity for the clinical evaluation of immune status. However, the clinical usage of the CDR3 sequence has been hindered by two factors. First, the tremendous amount of variation in the CDR3 sequences causes high inconsistency, even among healthy individuals, and leads to uncertainty in the theoretical value of CDR3 quantity (Memon et al., 2012). Second, the clinical samples represent only a very small fraction of  $CD4^+$  T cells in the theoretically estimated TCR repertoire based on the CDR3 sequence ( $10^6$  cells  $mL^{-1}$  vs.  $10^{12}$  to  $10^{19}$ ) (Alcover et al., 2018; Arstila et al., 1999; Dupic et al., 2019; Goronzy and Weyand, 2017). The V-J pairing index exhibits three advantages. First, the coverage of V-J pairing can be calculated precisely with the finite number of 3,294 V-J pairs in the  $\alpha$ -chain and 1,050 V-J pairs in the  $\beta$ -chain (Bowerman et al., 2014; Liu et al., 2014). Second, the intra-individual similarity between naïve and memory fractions may help illustrate the difference in TCR diversity of different T cell subsets within a single person. Third, the high inter-individual similarity allows us to successfully describe the differences in the TCR repertoire of a certain T cell subset in a given population.

Unlike previous study in which total  $CD4^+$  cells were used for TCR analysis, we investigated V-J pairing index of naïve and memory fraction of  $CD4^+$  T cells. Notably, the naïve fraction of  $CD4^+$  T cells displayed a greater disturbance of TCR V-J pairs than the memory fraction. This finding is not surprising because HIV preferentially invades and depletes naïve  $CD4^+$  T cells in blood (Rainwater-Lovett et al., 2014). The uneven distribution of naïve and memory  $CD4^+$  T cells also explains the decreased intra-individual similarity of V-J pairing in ART-naïve patients. Furthermore, this finding also suggested that the defects of TCR V-J pairing are not mainly mediated via HIV antigen-specific mechanisms because the naïve  $CD4^+$  T cells are not antigen-experienced.

We sequenced 314 samples of different T cell subsets from 49 participants, and generated about 270 G sequencing data. The clinical sample size in this study is still limited. However, from the present exploratory research, these results may promote us to carry out a study on large clinical sample size.



**Figure 5** Characteristics of impacted TCR V-J pairs in CD4<sup>+</sup> T cells after long-term ART in HIV-infected patients. A and C, Heat maps of unrecovered (A) and newly impacted (C) V-J pairs in the CD4<sup>+</sup> T cell TCR  $\alpha$ -chain of HIV-infected ART-experienced patients. Each square in the heat maps represents a unique V-J pair. Red and green squares show healthy donors (HD)>ART-experienced and HD<ART-experienced percentages of V-J pairs with a cut-off *P* value<0.01, respectively. The red and green squares in (A) and (C) represent the V-J pairs in the upper right panel of  $\alpha$ -chains from naïve and memory CD4<sup>+</sup> T cells in Figure 4. B and D, Correlations among the highly concentrated Va segments of unrecovered (B) and newly impacted (D) TCR  $\alpha$ -chain V-J pairs in HIV-infected ART-experienced patients. Each V segment is shown as a bubble, and the line between two adjacent V segment bubbles indicates a high correlation (>0.5), which was calculated by Pearson correlations. The types of unrecovered and newly impacted V-J pairs in HIV-infected ART-experienced patients were counted, and the top 5 paired Va segments in their own section are shown in red.

It is also of interest to investigate whether the defects in TCR V-J pairs correlate with high risks of non-AIDS-defining cancers or certain infections in PLWH after ART. We are also interested in the studies to verify the relevance between the preference of V-J pairing and the incidence of tumors or infections. Future studies on novel long-term ART regimens are needed to improve the efficacy in recovering the TCR repertoire. In addition, we expect that the V-J pairing index may provide new quantitative parameters for clinical monitoring of the TCR repertoire in patients with other conditions causing violent disturbances of the TCR repertoire, such as sepsis, bone marrow transplantation and chemotherapy.

## MATERIALS AND METHODS

### Study population

The present study was approved by the Committee of Ethics at Beijing Ditan Hospital, Capital Medical University, China. All human blood samples were collected with informed consent. We recruited 17 age- and gender-paired healthy donors, 17 chronically HIV-infected ART-naïve patients, and 15 long-term (over 5 years) HIV-infected ART-experienced patients (Table 1). All HIV-infected patients (ART-naïve and

-experienced patients) had baseline counts of CD4<sup>+</sup> T cells between 200 and 450  $\mu\text{L}^{-1}$ , with a median baseline plasma viral load (pVL) of 4.8  $\log_{10}$  copies  $\text{mL}^{-1}$  (range, 4.0–5.6  $\log_{10}$  copies  $\text{mL}^{-1}$ ). The ART-experienced patients had a baseline of CD4<sup>+</sup> T cell counts and pVL comparable to ART-naïve patients, the level of pVL had been lower than 40 copies  $\text{mL}^{-1}$  for at least five years, and the CD4<sup>+</sup> T cell counts were more than 600  $\mu\text{L}^{-1}$  at the time point of sample collection (Table 1).

### Sorting of the T cell subpopulation

Fresh peripheral blood mononuclear cells (PBMCs) of all the participants were isolated with Lymphoprep<sup>TM</sup> (Stemcell, Canada). Then, the total CD4<sup>+</sup>, total CD8<sup>+</sup>, naïve and memory CD4<sup>+</sup> T cells were sorted with were further sorted with magnetic beads from the MagniSort<sup>TM</sup> kits (Invitrogen, USA), including the Human CD4 naïve T Cell Enrichment Kit, the Human CD4 Memory T Cell Enrichment Kit, the Human CD4 T Cell Enrichment Kit and the Human CD8 T Cell Enrichment Kit (Ugolini et al., 2018). The purity of each sorted T cell subsets from healthy donors and ART-naïve and -experienced patients was detected by flow cytometry, and the median purities was 95.9% (range, 92.0%–99.6%) (Figure S2 in Supporting Information). Approximately



$1 \times 10^5$  sorted cells from each sample were used for total RNA extraction with the ReliaPrep™ miRNA Miniprep System (Promega, USA).

### RACE PCR, library preparation and high-throughput sequencing

Total RNA from each sample was subjected to 5' rapid-amplification of cDNA ends (RACE) to obtain the unbiased amplified sequences of variable regions of both the TCR  $\alpha$ - and  $\beta$ -chains as described previously (Bowerman et al., 2014; Ozawa et al., 2008). This strategy included three continuous polymerase chain reactions (PCRs), i.e., one reverse transcription PCR (RT-PCR) and two nested PCRs. With the Superscript™ II (Invitrogen) system, the cDNA was primed by a TCR C gene segment specific primer (GSP1) and a template switching oligo-dG. The first steps of nested PCR were priming by adaptor primer 1 (AP1) and annealing of another primer (GSP2) to the 3' end of GSP1, and these steps were performed with Platinum™ Pfx polymerase (Invitrogen). The second nested PCR enhanced the specificity by using another adaptor primer (AP2) and annealing the other primer (GSP3) to a site located upstream of GSP2 in the C segment.

The PCR products were run on a 2% agarose gel, and the objective fragments in the 350–500 bp range were excised and purified using the Gel Extraction Kit (Promega). The sequencing libraries were constructed with Illumina adaptor ligation according to the manufacturer's protocol. Then, the samples were sequenced using a HiSeq 2500 platform (Illumina, USA). The sequences of each individual were generated as 250 bp paired-end reads.

### Statistical analysis of sequencing data

The final TCR repertoire sequencing reads were used to identify all V and J gene segments of TCR  $\alpha$ - and  $\beta$ -chains by the bioinformatics workflow of TCRklass (Li et al., 2015; Yang et al., 2015). The output data on expression percentages of each kind of V and J segment and V-J pairs, as well as their subsequent supported data, were processed by statistical analysis.

Shannon entropy indexes of chronically HIV-infected ART-experienced patients, ART-naïve patients and healthy donors were calculated to measure V-J pairing diversity. The similarity between naïve and memory CD4<sup>+</sup> T cells within an individual was named “intra-individual similarity”, and the similarity of naïve or memory CD4<sup>+</sup> T cells among different individuals within the same population was named “inter-individual similarity”. These two similarities of V-J pairing preference were evaluated by Pearson correlation coefficients. The Mann-Whitney test was performed to test the differences in intra-individual similarity and inter-individual

similarity among the three groups. All statistical analyses were performed using PRISM version 7 (GraphPad) and R scripts.

### Data availability

FASTQ data about the present study have been deposited in BIG (Beijing Institute of Genomics) Data Center, Chinese Academy of Sciences, accessible at <https://bigd.big.ac.cn/gsa>.

**Compliance and ethics** *The author(s) declare that they have no conflict of interest.*

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