Associations between recent thymic emigrants and CD4$^+$ T-cell recovery after short-term antiretroviral therapy initiation

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Objective: Around 20–30% of HIV-infected individuals (HIV+) on successful antiretroviral therapy (ART) fail to normalize their CD4$^+$ T-cell counts. Various factors could contribute to the lack of immune reconstitution, one of them being thymic insufficiency. We aimed to explore associations between recent thymic emigrants (RTEs) and CD4$^+$ T-cell recovery.

Design: ART-naive HIV+ individuals who started ART with advanced AIDS were selected. Good versus poor immune reconstitution was defined by CD4$^+$ gains above or below 100 CD4$^+$ T cells/μl. The follow-up period was 6 months.

Methods: Peripheral blood mononuclear cells were isolated and flow cytometry was used to characterize RTEs as the fraction of naive CD4$^+$ T cells expressing CD31$^+$, the platelet endothelial cell adhesion molecule. Markers of cellular activation, senescence, exhaustion and cycling were also assessed.

Results: After 6 months on ART, HIV+ individuals with good immune reconstitution had higher absolute numbers of RTEs, compared with those with poor immune reconstitution, and these strongly correlated with CD4$^+$ gains in those individuals with good immune reconstitution but not with poor immune reconstitution. We also found that CD8$^+$ T-cell immune activation decreased as early as 2 months post-ART initiation in individuals with good immune reconstitution, but only at month 6 post-ART in individuals with poor immune reconstitution. Levels of immune activation were inversely correlated with the absolute numbers of RTEs in both groups, but more strongly so in individuals with poor immune reconstitution.

Conclusion: We show that RTEs are linked to CD4$^+$ T-cell recovery and that the degree of immune reconstitution is not directly linked to persistent immune activation.

Introduction

A fair number of antiretroviral (ART)-experienced HIV-infected individuals (HIV+) do not recover their CD4$^+$ T-cell counts despite complete suppression of HIV viremia [1,2]. Failure to normalize CD4$^+$ T-cell counts to levels above 500 cells/μl is associated with poor clinical outcome [3−5] and has been attributed to impaired peripheral and lymphoid tissue homeostasis [6], accelerated T-cell death, chronic immune activation [7−9] and thymic insufficiency [10−12].

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Recent thymic emigrants (RTEs) are thought to represent newly produced peripheral naive CD4$^+$ T cells and have been extensively used as a surrogate marker to quantify thymic export [13,14]. RTEs can be measured by flow cytometry in peripheral blood, allowing for their phenotypic and functional characteristics to be examined [15−17]; and are defined as the fraction of naive CD4$^+$ T cells that express CD31$^+$, the platelet endothelial cell adhesion molecule (PECAM-1) [18]. Numerous studies have shown that RTEs contribute towards CD4$^+$ T-cell recovery and their frequency and absolute numbers in peripheral blood predict immune restoration in ART-experienced individuals [12,19−28]. Indeed, HIV+ individuals who achieve good immune reconstitution (immunological responders, defined by absolute CD4$^+$ T-cell counts above 350 cells/μl after 2 years of undetectable plasma viremia) have frequencies of RTEs matching to those of HIV seronegative individuals (HIV−), as opposed to those who do not achieve immune reconstitution (immunological nonresponders, CD4$^+$ T cells below 350) [23,29,30].

Much work has been done in order to assess thymic reactivation and its association with immune reconstitution using markers of thymic output, such as RTEs and T-cell excision circles (TREC) [14]. However, and to our knowledge, detailed phenotypic characterization of RTEs has not been fully investigated in untreated and short-term-treated HIV infection [31].

Therefore, we thought to examine changes in the expression of markers of activation (HLADR, CD38), senescence (CD57), exhaustion (PD-1) and cycling (Ki67) on RTEs as HIV+ individuals transition from untreated to treated HIV infection. We also explored associations between the expression of these markers and CD4$^+$ T-cell recovery. Our study included two longitudinal cohorts, an HIV+ cohort, primarily HIV+ individuals with advanced AIDS and an HIV− cohort, with three-time point measurements, and a follow-up of 6 months. Both cohorts included individuals who were naive to therapy (baseline) and started therapy, ART for HIV+ and preexposure prophylaxis (PrEP) for HIV−. Our aim was to understand associations between RTEs and early CD4$^+$ T-cell recovery. We sought to identify if there were differences in frequencies, absolute numbers and/or phenotypic characteristics of RTEs from HIV+ individuals based on their degree of immune reconstitution, here assessed by CD4$^+$ gains.

Methods

Ethics statement
Our study was approved by the ethics committee of the National Institute of Respiratory Diseases, Mexico City, Mexico, and was conducted according to the principles expressed in the Declaration of Helsinki. All individuals were adults (over 18 years old). All participants provided written consent.

Study cohorts
Our study design is summarized in Supplementary Figure 1, http://links.lww.com/QAD/B593. A total of 55 individuals were included in this study.

The first cohort was composed of 41 HIV+ individuals with advanced AIDS who were selected based on five criteria: being ART-naive at study entry, starting their first ART regimen, having a good virological response to ART at month 6 defined by plasma HIV-1 RNA less than 400 copies/ml, having CD4$^+$ T-cell count and plasma viral load (pVL) measurements available at all three time points of interest: pre-ART (T0, Baseline), 2-months (T2) and 6-months (T6) after ART initiation, and having stored peripheral blood mononuclear cells (PBMCs) available at all three time points (T0, T2 and T6).

The second cohort, our control cohort, was composed of HIV-uninfected individuals, who were eligible to start oral preexposure prophylaxis (PrEP, tenofovir disoproxil fumarate/emtricitabine, 300/200 mg) because they were at high-risk of HIV infection. To evaluate the risk of HIV acquisition, a risk behavior assessment questionnaire based on sexual practices was administered [32]. HIV testing was performed at study entry and at month 3 (T3) and month 6 (T6) after PrEP initiation. For this study, HIV-1-uninfected individuals were selected based on three criteria: having CD4$^+$ T-cell count measurements available at baseline (pre-PrEP, T0), having an HIV negative test at month 3 (T3) and month 6 (T6) after PrEP initiation, having stored PBMCs available at all three time points (T0, T3 and T6). A total of 14 individuals were included. Note that for this cohort, CD4$^+$ T-cell counts were not available at T3 and T6.

HIV-1 RNA plasma viral load and lymphocyte populations
HIV-1 RNA plasma viral load was determined by automated real-time PCR using m2000 system (Abbot, Abbott Park, Illinois, USA). Lymphocyte populations were obtained by flow cytometry using Trucount in a FACSCanto II (BD Biosciences, San Jose, California, USA).

Peripheral blood mononuclear cell isolation
Ethylene diamine tetraacetic acid (EDTA)-blood was quantified by venipuncture at all three visits for HIV+ and HIV-uninfected individuals. PBMCs were isolated as previously described [33].

Flow cytometry
PBMC from the same individual (T0, T2/3, T6) were thawed, stained and analyzed in the same experiment. Briefly, PBMC were thawed and washed in PBS, and then stained with a single cocktail of antibodies, which are

listed in Supplementary Table 1, http://links.lww.com/QAD/B593, for 15 min at room temperature (RT). PBMC were then washed twice with PBS and permeabilized with the eBiosciences FOXP3/fixation permeabilization kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) following manufacturer's instructions. Next, PBMC were stained intracellularly for Ki-67 for 60 mins at RT, washed twice, fixed in 300 μl of 1% paraformaldehyde (Sigma–Aldrich, St. Louis, Missouri, USA), stored at 4 °C and acquired within 24 h of fixing. Live/Dead Aqua dye was used to exclude dead cells. A dump gate was used to exclude unwanted populations (see Supplementary Table 1, http://links.lww.com/QAD/B593). A minimum of 1.5 million events were acquired on a BD LSRFortessa (BD Biosciences, San Jose, California, USA) and analyzed using FlowJo V10 software (Tree Star, Ashland, Oregon, USA). FMO (fluorescence minus one) was used as gating control. Quality control was performed for each experiment using BD cytometer Setup & Tracking Beads (BD biosciences, San Jose, California, USA). A compensation matrix was calculated and applied individually to each experiment using BD Comp Beads (BD Biosciences). The gating strategy is shown in Supplementary Figure 2, http://links.lww.com/QAD/B593. Research staff performing the flow cytometry experiments were blinded to all information pertaining to group allocation and clinical data.

Statistical analysis of flow data

Mann–Whitney, Kruskal–Wallis and Friedman nonparametric tests were used as appropriate. All *P* values were corrected for multiple comparisons using Dunn's multiple comparisons test. Corrected *P* values below 0.05 were considered significant. Correlations were performed using a nonparametric Spearman test. Graphs and statistics were performed in GraphPad Prism version 7

(San Diego, California, USA) and STATA/SE 14 (STATA Corp. LP, College Station, Texas, USA).

Results

Study cohort

Table 1 shows the clinical and demographic characteristics of all individuals included in this study. This cohort is characterized by HIV+ individuals with advanced AIDS and very low pre-ART CD4$^+$ T-cell counts. During the first 6 months on ART, HIV+ individuals gained a median of 172 [77–260] CD4$^+$ cells/μl (T6-T0, ΔCD4). For this study, we defined a good versus poor immune reconstitution (GIR vs. PIR) based on a ΔCD4$^+$ value of 100 CD4$^+$ T cells/μl and this arbitrary value was chosen according to our cohort characteristics and after reviewing the literature. Accordingly, we divided our HIV+ cohort into individuals with GIR ($n = 28$, ΔCD4 ≥100 cells/μl) and PIR ($n = 13$, ΔCD4 <100 cells/μl). As expected, their median absolute CD4$^+$ T-cell count at T6 was different ($P = 0.0042$) but not their baseline median absolute CD4$^+$ T-cell count (GIR: 57.50 [26.25–263.5] vs. PIR: 143 [55–252.5] cells/μl, $P ≥ 0.05$). There were no differences between GIR and PIR with regards to age, BMI, nadir CD4$^+$, duration on ART, ART regimen and co-infections (Table 1 and Supplementary Table 2, http://links.lww.com/QAD/B593).

Individuals with poor immune reconstitution have fewer naive CD4$^+$ T cells than individuals with good immune reconstitution pre-antiretroviral therapy but not after 6 months on antiretroviral therapy

First, we analyzed the distribution of CD4$^+$ T-cell maturation subsets based on the expression of the

Table 1. Demographic and clinical characteristics of our cohort at baseline and at follow-up (6 months postantiretroviral therapy).

	Baseline			Follow-up		
	GIR	PIR	*P* value	GIR	PIR	*P* value
Number	28	13		28	13	
Age (years)[a]	34.5 (20–64)	37 (26–55)	NS			
BMI (kg/m^2)	21.65 [19.88–23.78]	22.50 [20.05–25.20]	NS			
Sexual preference[b]						
MSM	23 (82)	11 (85)	NS			
MSWM	5 (18)	2 (15)				
CD4$^+$ T (cells/μl)	57.50 [26.25–263.5]	143 [55–252.5]	NS	336 [232.5–627]	154 [96–310]	0.0042
CD4$^+$ (%)	9 [5–16]	10 [4–16]	NS	18.50 [10–27.75]	10 [5.5–21]	NS
CD8$^+$ T (cells/μl)	499 [315–1076]	878 [611.5–1715]	0.055	1070 [619.3–1577]	928 [546.5–1429]	NS
CD8$^+$ (%)	58 [45–67]	68 [57–75]	0.028	53 [39.50–62]	59 [52–74.50]	0.038
CD4$^+$/CD8$^+$ ratio	0.17 [0.09–0.27]	0.13 [0.065–0.24]	NS	0.325 [0.18–0.602]	0.18 [0.08–0.385]	0.06
Nadir CD4$^+$ (cells/μl)	57.50 [26.25–263.5]	143 [55–252.5]	NS			
Log pVL (copies/ml)	5.29 [4.78–5.68]	4.68 [4.26–5.50]	NS	1.5 (1.5–2.4)	1.5 (1.5–2.0)	NS
CD4 gains (cells/μl)				213.5 [162.3–365.3]	50 [11.50–77]	<0.0001
Time on ART (days)				184 [180.5–194.8]	178 [171–187]	NS

Values are given as median [interquartile range, IQR] unless stated otherwise. ART, antiretroviral therapy; CD, cluster of differentiation; GIR, good immune reconstitution; HIV+, HIV-infected; log, logarithmic scale; MSWM, men who have sex with women or men; PIR, poor immune reconstitution; pVL, plasma viral load; NS, not significant ($P ≥ 0.05$).
[a]Median (minimum–maximum).
[b]Number (percentage).

canonical markers CCR7 and CD45RO (expressed as frequencies). We found that HIV+ individuals have a skewed phenotype with increased frequencies of effector memory and terminally differentiated (TEMRA) and decreased frequencies of naive and central memory $CD4^+$ T cells (Supplementary Figure 3, http://links.lww.com/QAD/B593). We found that the percentage of central memory was equally depleted in GIR and PIR, conversely, the percentage of effector memory was equally increased in GIR and PIR compared with HIV− during the first 6 months on ART (Fig. 1a–c). However, the proportion of naive $CD4^+$ T cells were significantly lower in PIR when comparing with GIR ($P = 0.0026$) and HIV− ($P = 0.0029$) at baseline (T0) (Fig. 1a). When looking at the distribution of $CD4^+$ T-cell maturation subsets expressed as absolute counts at baseline, we found that all four $CD4^+$ T-cell subsets were decreased in HIV+ compared to HIV− (Supplementary Figure 4A, http://links.lww.com/QAD/B593). This pattern remained similar when dichotomizing the HIV+ cohort in individuals with GIR and PIR (Supplementary Figure 4A, http://links.lww.com/QAD/B593). Interestingly, at T6 (6 months post-ART), both GIR and PIR had significantly lower frequencies of naive $CD4^+$ T cells compared with HIV− ($P = 0.0192$ and $P = 0.0028$, respectively) but similar levels between them (Fig. 1c). However, we found that PIR had significantly lower absolute number of naive $CD4^+$ T cells compared with GIR at T2 and T6 ($P = 0.0116$ and $P = 0.0026$, respectively, Supplementary Figure 5, http://links.lww.com/QAD/B593). We were unable to compare absolute numbers between HIV− and HIV+ GIR and PIR after ART initiation as absolute $CD4^+$ T-cell counts were not available for the HIV− cohort at follow-up. There were no changes in the frequency of $CD4^+$ T-cell differentiation subsets (naïve, central memory, effector memory, TEMRA) across HIV− and HIV+ (GIR and PIR) individuals during the entire follow-up period (Supplementary Figure 6, http://links.lww.com/QAD/B593).

Individuals with poor immune reconstitution have lower absolute number of recent thymic emigrants pre-antiretroviral therapy and after 6 months on antiretroviral therapy

As the main focus of our work was to understand the contribution of the thymus to immune recovery following ART initiation, we measured the fraction of naive $CD4^+$ T cells (CCR7+ CD45RO−) that expressed $CD31^+$, defined here as RTEs in terms of frequencies and absolute numbers. We found no differences in the frequency of RTEs when comparing HIV− with HIV+ or when comparing GIR and PIR (Supplementary Figure 7, http://links.lww.com/QAD/B593). However, we found that at study entry, HIV+ individuals, irrespective of having a GIR or PIR, had significantly fewer absolute numbers of RTEs compared to HIV− ($P < 0.0001$, Fig. 2a). After ART initiation, individuals with GIR had significantly more RTEs than

PIR at T2 and T6 ($P = 0.026$ and $P = 0.033$, respectively, Fig. 2b). When we looked at changes in RTEs (absolute numbers and frequencies) throughout the 6-month study period, we found that GIR showed an increase in RTEs between T0 and T2 ($P = 0.0025$), whereas PIR increased their RTEs between T2 and T6 ($P = 0.0320$) (Fig. 2c and d). Importantly, we found that the absolute number of RTEs correlated with $CD4^+$ gains ($\Delta CD4^+$) in GIR group (rho $= 0.54$, $P = 0.0029$) but not in the PIR group at the 6-month time point (Table 2). This association remained significant when analyzing $CD4^+$ recovery continuously without subdividing the HIV+ cohort in GIR and PIR (rho $= 0.486$, $P = 0.0013$, Supplementary Table 3, http://links.lww.com/QAD/B593). However, when adjusting for pre-ART $CD4^+$ T-cell counts, differences observed in the absolute number of RTEs in GIR and PIR at 2 and 6 months, respectively, were lost (unadjusted $P = 0.009$; 95% CI 89.700−13.392 and adjusted $P = 0.763$; 95% CI 14.035−10.316). This is most probably because of the fact that individuals with lower pre-ART $CD4^+$ cell counts have a more robust $CD4^+$ recovery.

$CD4^+$ and $CD8^+$ T-cell activation inversely correlate with the absolute number of recent thymic emigrants

The lack of immune reconstitution has been linked to chronic immune activation. Next, we measured the level of immune activation by using $CD38^+$ and HLADR on total $CD4^+$ and $CD8^+$ T cells. As expected, the frequency of activated total $CD4^+$ and $CD8^+$ T cells was higher in HIV+ individuals (both GIR and PIR) compared with HIV− (Supplementary Figure 8 A–F, http://links.lww.com/QAD/B593). After ART initiation, the frequency of activated total $CD4^+$ and $CD8^+$ T cells decreased in HIV+ individuals (both GIR and PIR), but not to levels comparable with HIV− (Supplementary Figure 8 A–F, http://links.lww.com/QAD/B593). Interestingly, when looking at T-cell activation over the entire follow-up period (6 months) within each group, we found that $CD8^+$ T-cell immune activation decreases at T2 ($P = 0.0012$) and T6 ($P < 0.0001$) compared with baseline for GIR. For PIR, the decrease in $CD8^+$ T-cell activation was only seen after 6 months on ART ($P < 0.0001$) (Supplementary Figure 9 A–F, http://links.lww.com/QAD/B593). We found that $CD4^+$ and $CD8^+$ T-cell immune activation were inversely correlated with the absolute number of RTEs for both GIR and PIR, and when analyzing $CD4^+$ recovery continuously (Table 2 and Supplementary Table 3, http://links.lww.com/QAD/B593). When examining the relationship between RTEs and $CD4^+$ recovery adjusting for $CD8^+$ T-cell activation, we found that this relationship was not significantly attenuated (unadjusted P value: $P < 0001$; 95% CI: 0.250–0.315, adjusted P value: $P < 0.0001$; 95% CI 0.254–0.327). Also, we found no correlation between $CD8^+$ T-cell activation and immune recovery ($\Delta CD4$) in the GIR or PIR group or when analyzed continuously.
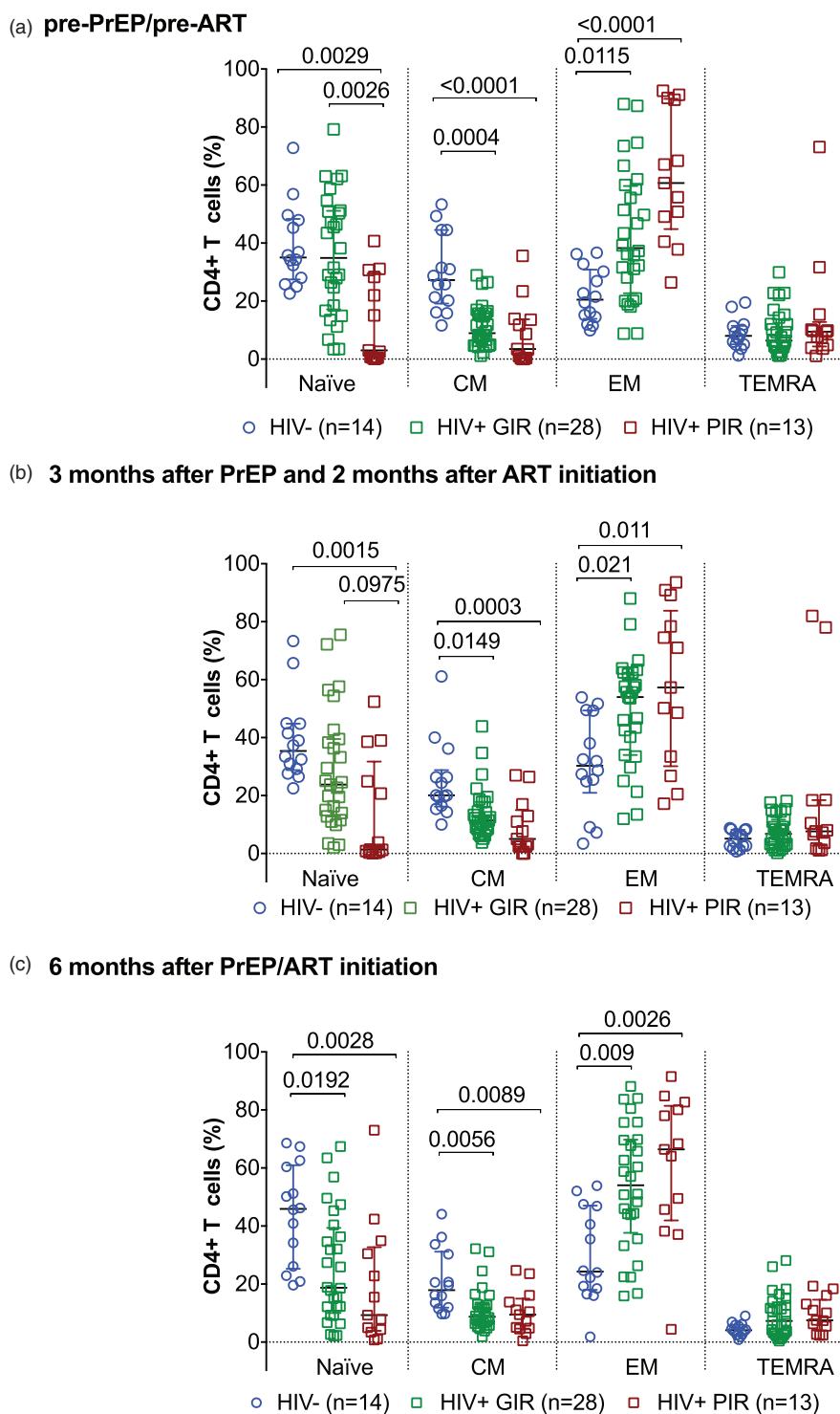
Fig. 1. PIR have fewer naive CD4$^+$ T cells than GIR pre-ART but not after 6 months on ART. Frequencies (%) of peripheral blood naïve (CD45RO- CCR7+), central memory (CM, CD45RO+ CCR7+), effector memory (EM, CD45RO+ CCR7-) and effector memory RA+ (TEMRA, CD45RO- CCR7-) T cells were identified by flow cytometry in GIR (green squares, $n = 28$), PIR (red squares, $n = 13$) and HIV- (blue, rounds $n = 14$) individuals. Frequency (%) of the CD4$^+$ T-cell subpopulations were measured (a) before ART/PrEP, (b) 2/3 months and (c) 6 months after ART/PrEP initiation. Scatter plots were used to represent the data. Horizontal lines indicate median values. Each symbol represents one individual. Groups were compared using Kruskal–Wallis test correcting for multiple comparisons using Dunn's multiple comparisons test. Only significant corrected *P* values are shown in the graphs. ART, antiretroviral therapy; GIR, good immune reconstitution; HIV+, HIV-infected; HIV−, HIV-uninfected; NS, not significant; PIR, poor immune reconstitution; PrEP, preexposure prophylaxis.
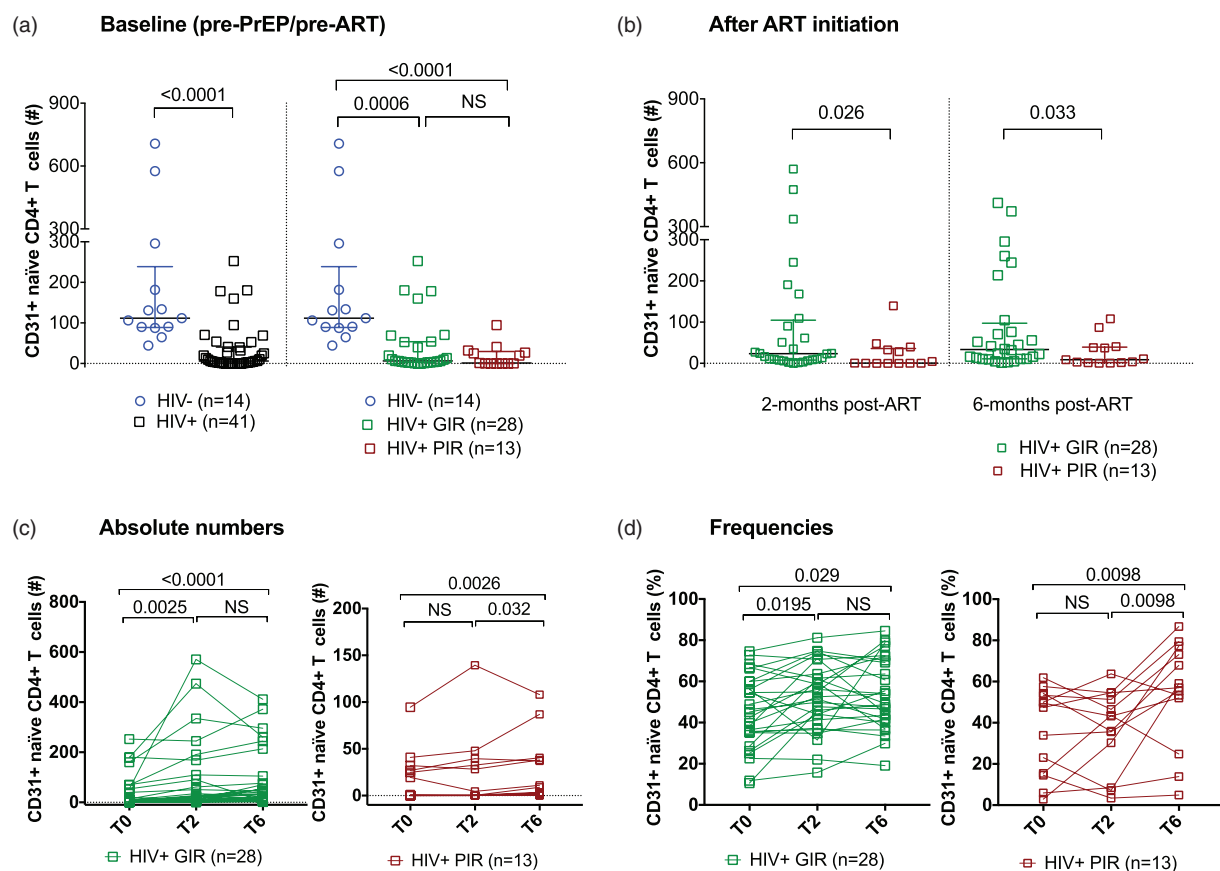
Fig. 2. Individuals with PIR have lower absolute number of recent thymic emigrants pre-ART and after 6 months on ART. The absolute number of recent thymic emigrants (RTEs) was calculated as described elsewhere [34]. (a) Comparison of the absolute number (#) of RTEs in HIV− (blue rounds, $n = 14$), HIV+ (black squares, $n = 41$) individuals and in HIV− (blue rounds, $n = 14$), GIR (green squares, $n = 28$), PIR (red squares, $n = 13$) before antiretroviral treatment (baseline). Groups were compared using Mann–Whitney test (HIV− vs. HIV+) and Kruskal–Wallis test correcting for multiple comparisons using the Dunn's multiple comparisons test (HIV−, GIR and PIR). (b) Comparison of GIR (green squares, $n = 28$) and PIR (red squares, $n = 13$) at 2 and 6 months post-ART initiation. Groups were compared using Mann–Whitney test. Absolute number of RTEs could not be calculated for HIV− individuals at 3 and 6 months post-PrEP initiation as absolute CD4+ T-cell counts were not available to us. Scatter plots were used to represent the data. Horizontal lines indicate median values. Each symbol represents one individual. (c) Dynamics in the absolute numbers of RTEs (#) for GIR (green squares, $n = 28$) and PIR (red squares, $n = 13$) during the follow-up period is shown. (d) Dynamics in the frequencies of RTEs (#) for GIR (green squares, $n = 28$) and PIR (red squares, $n = 13$) during the follow-up period is shown. Each symbol and line represents one individual. Matched samples were compared using Friedman test correcting for multiple comparisons using Dunn's multiple comparisons test. Only significant corrected P values are shown in the graphs. ART, antiretroviral therapy; GIR, good immune reconstitution; HIV+, HIV-infected; HIV−, HIV-uninfected; NS, not significant; PIR, poor immune reconstitution; PrEP, preexposure prophylaxis.

Table 2. Correlations between the absolute number of recent thymic emigrants, CD4+ gains and immune activation after 6 months on antiretroviral therapy.

	GIR (rho and P)		PIR (rho and P)
Absolute numbers of RTEs and CD4+ gains at T6			
# RTEs	Rho = 0.542; P = 0.029	ΔCD4	Rho = −0.22; P = 0.461
Absolute number of RTEs and immune activation			
# RTEs	Rho = −0.486; P = 0.0087	%CD38+ HLADR+ CD8+	Rho = −0.802; P = 0.0016
# RTEs	Rho = −0.400; P = 0.0349	%CD38+ HLADR+ CD4+	Rho = −0.824; P = 0.0009

Correlations were performed using nonparametric Spearman test. #, absolute numbers; %, frequency; CD, cluster of differentiation; ΔCD4+, CD4+ gains between T6 and T0; GIR, good immune reconstitution; PIR, poor immune reconstitution; RTEs, recent thymic emigrants.

Recent thymic emigrants from HIV+ individuals with good immune reconstitution and poor immune reconstitution are cycling and expressing more programmed death-1 than HIV−

Next, we explored the phenotypic characteristics of RTEs, and we focused on the expression of four markers: Ki67 (cycling), CD57 (senescence), PD-1 (exhaustion) and CD38$^+$ and HLADR (cellular activation). There were no significant differences in the level of expression for CD57$^+$ and HLADR/CD38$^+$ between GIR or PIR at T0, T2 nor T6 (Supplementary Figure 10, http://links.lww.com/QAD/B593). As shown in Fig. 3, at baseline (T0), the percentage of RTEs expressing Ki67 (cycling) is significantly higher in HIV+ compared with HIV− ($P = 0.003$) and in both GIR and PIR compared with HIV− ($P = 0.0143$, $P = 0.0012$. respectively) (Fig. 3a). After ART initiation, cycling remains high in GIR vs. HIV− at T2 ($P = 0.0296$) and between PIR and HIV− at T6 ($P = 0.0167$) (Fig. 3b and c). There is a significant increase in PD-1 expression at T0 and T6 in HIV+ compared with HIV− (T0 $P = 0.0336$; T6 $P < 0.0001$; Fig. 3d and f) and for GIR and PIR compared with HIV− at T6 ($P = 0.0008$, $P = 0.0002$, respectively, Fig. 3f). The proportion of RTEs expressing Ki67, PD-1, CD57 and HLADR/CD38 did not change over the period of follow-up (Supplementary Figure 11, http://links.lww.com/QAD/B593).

Discussion

Antiretroviral therapy has one main goal, suppress HIV plasma viremia to undetectable levels; the second therapeutic goal is to reverse the damage incurred to the immune system at the hands of HIV. The lack of immune reconstitution is common among HIV+ treated individuals [3,35,36]. As previously reported [3,36], and as observed in our cohort, we found that one in three HIV+ individuals failed to achieve good immune reconstitution after 6 months on ART. Understanding the mechanisms associated with CD4$^+$ T-cell recovery or the lack thereof, is important so that therapeutic options might be thought of. In this study, we aimed to evaluate early immune responses after ART initiation focusing on thymic output. Our results are in line with other studies [37,38] showing that HIV+ individuals have a skewed phenotype towards higher circulating levels of effector memory and TEMRA and lower circulating levels of naive and central memory CD4$^+$ T cells, and that this skewed phenotype is not immediately normalized after ART initiation. Interestingly, HIV+ individuals with PIR had fewer naive CD4$^+$ T cells at baseline and 2 months after ART, compared with HIV+ individuals with GIR when expressed as frequencies. Absolute numbers of naive CD4$^+$ T cells were significantly lower in PIR compared with GIR at 2 and 6 months after ART.

As elegantly shown by others [19], the naive CD4$^+$ peripheral T-cell pool is maintained by homeostatic proliferation of mature naive CD4$^+$ T cells and the production of new naive CD4$^+$ T cells by the thymus. Thymic export can be inferred by assessing naive CD4$^+$ T cells expressing CD31$^+$, known as RTEs. RTEs are phenotypically and functionally distinct from mature naive CD4$^+$ T cells [14]. We found that HIV+ individuals had fewer absolute numbers of RTEs compared with the HIV− group, as previously reported [19−22,23,24]. As others have also shown [23,29,30], HIV+ with GIR have increased levels of circulating RTEs compared with those with PIR after ART initiation. Furthermore, our data shows that the increase in circulating RTEs can be detected as early as 2 months after ART initiation in the GIR group, whereas there seems to be a delay in thymic output in HIV+ individuals with PIR. Whether RTEs are a true reflection of thymic reactivation or a redistribution of RTEs from lymphoid tissues is unknown to us. RTEs have been shown to preferentially home to lymphoid tissues [39] with a greater bias for their retention in the tissues [40]. We found that the absolute number of RTEs was directly associated with the gain of CD4$^+$ T cells at 6 months post-ART [41,42], further evidencing that RTEs are key participants in maintaining CD4$^+$ T-cell pool homeostasis during treated HIV infection.

Immune activation is one of the main mechanisms driving CD4$^+$ T-cell depletion, and although this decreases significantly after ART initiation, it rarely normalizes to levels comparable with those seen among HIV− [43−46]. Our data mirrors the previously established concepts, with both CD4$^+$ and CD8$^+$ T-cell activation remaining high after ART initiation. We also showed that both CD4$^+$ and CD8$^+$ T-cell activation was inversely correlated with the absolute number of RTEs. However, CD8$^+$ T-cell activation did not correlate with immune recovery (ΔCD4), suggesting that factors others than immune activation might be influencing thymic output (#RTEs) and immune recovery (ΔCD4). Immune activation has also been shown to drive phenotypic alterations in both CD4$^+$ and CD8$^+$ T cells, with increased expression of senescence and exhaustion markers [47−49]. We found that the frequency of PD-1-expressing RTEs was significantly elevated in HIV+, with no differences between HIV+ with GIR and PIR. Indeed, RTEs are exposed to sustained levels of immune activation in the periphery and during passage through lymph nodes, and in order to down-regulate this stimulatory environment, they express PD-1 [47,50−52].

Postthymic proliferation of RTEs is necessary for maintaining CD4$^+$ naive T-cell homeostasis, especially during lymphopenic conditions, such as HIV infection [53,54]. We found that RTEs from HIV+ individuals cycled more (increased expression of Ki67) compared with HIV− group. With our current results, we cannot

Fig. 3. **Recent thymic emigrants from individuals with good immune reconstitution and poor immune reconstitution are cycling and expressing more programmed death-1 than HIV−.** Frequencies (%) of peripheral blood RTEs (defined as CD3⁺ CD4⁺ CD45RO- CCR7+ CD31⁺) that express Ki67 (a–c) or PD-1 (d–f) were identified by flow cytometry in HIV- (blue rounds, $n = 14$), HIV+ (black squares, $n = 41$), GIR (green squares, $n = 28$) and PIR (red squares, $n = 13$) individuals, before ART (T0, a and d) and 2/3 months (b and e) and 6 months (c and f) after ART/PrEP initiation. Graphs scatter plots were used to represent the data. Horizontal lines indicate median values. Each symbol represents one individual. Groups were compared using Mann–Whitney test (HIV− vs. HIV+) and Kruskal–Wallis test correcting for multiple comparisons using the Dunn's multiple comparisons test (HIV−, GIR and PIR). Only significant corrected *P* values are shown in the graphs. ART, antiretroviral therapy; GIR, good immune reconstitution; HIV+, HIV-infected; HIV−, HIV-uninfected; NS, not significant; PIR, poor immune reconstitution; PrEP, preexposure prophylaxis.

comment as to whether the observed increase in RTE cycling might be caused by compensatory response to an accelerated loss of the pool of naive CD4$^+$ T cells [55–57], driven by continuous stimulation by cytokines or low-avidity T-cell receptor (TCR) antigens [58] or by direct cytopathic effect of HIV [21,55]. As immune reconstitution relies on the appropriate activation, clonal expansion and homeostatic proliferation of the naive CD4$^+$ T-cell compartment, quantitative and qualitative alterations within this compartment, in particular RTEs, are likely to impact the ability of HIV+ individuals to successfully reconstitute their immune system.

We fully acknowledge that our study has several limitations, including a short follow-up period. Also, we didn't assess thymic size by computed tomography scan nor did we quantify TCR excision circles (TREC) in sorted CD31$^+$ naive CD4$^+$ T cells to accurately define RTEs. To the best of our knowledge, surface expression of CD31$^+$ on naive CD4$^+$ T cells define a peripheral T-cell subset that recently completed thymic development and egress [59]. Our definition of poor versus good immune reconstitution was driven by our cohort characteristics and after reviewing the literature, and was based on CD4$^+$ T cells gains rather than an absolute CD4$^+$ T-cell count above a specific threshold. Currently, there is no consensus on what defines suboptimal immunological responses nor the specific period of time that has to pass on ART. When adjusting for pre-ART CD4$^+$ cell counts, differences observed between GIR and PIR arm with regards to the absolute number of RTEs were lost, showing that early immune reconstitution is confounded by many factors. Conversely, our study is strengthened by the inclusion of longitudinal follow-up of both HIV+ and HIV− individuals and the inclusion of men only, eliminating the influence of sex on differences in both immune responses to ART and absolute CD4$^+$ T-cell counts [60].

In conclusion, no differences in the expression of Ki67, CD57$^+$, PD-1, CD38$^+$ and HLADR were observed between HIV+ individuals with good and poor immune reconstitution. We also found that CD8$^+$ T-cell immune activation decreased as early as 2 months post-ART initiation in individuals with GIR, and only at month 6 post-ART in individuals with PIR. We show that RTEs are linked to CD4$^+$ T-cell recovery and that the degree of immune reconstitution is not directly linked to persistent immune activation.

Conflicts of interest

There are no conflicts of interest.

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