International Journal of Infectious Diseases 86 (2019) 178–187

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

Two types of poor immunological responder showing distinct responses to long-term HAART

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ARTICLE INFO

Article history:
Received 10 June 2019
Received in revised form 29 July 2019
Accepted 31 July 2019
Corresponding Editor: Eskild Petersen, Aarhus, Denmark

Keywords:
Poor immunological responders
Subdivision
Thymic output
Long-term HAART

ABSTRACT

Objectives: Most previous studies on poor immunological responders (PIRs) have been performed on one cohort at one time-point following highly active antiretroviral therapy (HAART). The aim of this study was to investigate whether there are different subtypes of PIR and whether a certain population might achieve better immune reconstitution following longer HAART.
Methods: This study was designed as an ambispective cohort study, including a 4–5-year retrospective study and a 2-year prospective follow-up investigation. Thymic output, activated T cell and regulatory T cell (Treg) subset frequencies, expression levels of interferon-stimulated genes, and plasma concentrations of neopterin were determined at 4–5 years and 6–7 years following HAART initiation.
Results: PIRs were subdivided into two populations after 4–5 years of HAART, according to the kinetics of T cell recovery. Type II PIRs exhibited a significantly lower percentage of naïve $CD4^+$ T cells and $CD31^+$ naïve $CD4^+$ T cells compared with type I PIRs. After an additional 2 years of HAART treatment, type I PIRs showed a better outcome than type II PIRs. Furthermore, it was found that 2 years of additional HAART could persistently improve thymic output.
Conclusions: The two PIR subgroups are different in terms of immune characteristics and the response to prolonged HAART.

Introduction

In recent decades, highly active antiretroviral therapy (HAART) has dramatically reduced the mortality of HIV-infected patients by diminishing viral replication and increasing $CD4^+$ cell counts (Cohen et al., 2011). However, a proportion of patients (from 15% to 30%) fail to reach optimal $CD4^+$ T cell recovery, despite the full suppression of viral replication after several years of HAART (Kelley et al., 2009; Lok et al., 2010; Marchetti et al., 2006; Moore and Keruly, 2007). These poor immunological responders (PIRs) have an increased risk of acquired immunodeficiency syndrome (AIDS)-related and non-related illnesses and death (Achhra et al., 2010; Engsig et al., 2014; Lichtenstein et al., 2010; Monforte et al., 2008). In developed countries, poor CD4 cell recovery has gradually attracted less attention with early HAART initiation. In developing countries, where more than 94% of HIV-infected patients live, a late diagnosis of HIV infection and delayed treatment after diagnosis remain major barriers to starting early HIV/AIDS treatment at a high CD4 baseline (Lahuerta et al., 2013; The, 2017; UNAIDS, 2017). In 2015, the median CD4 cell count at HAART initiation was approximately 250/μl in the Asia-Pacific, including China, but close to 500 cells/μl in North America (World Health Organization, 2016). Thus, PIRs remain a major problem in developing countries.

T cell recovery is a long-term, complicated and dynamic process. The accumulated data have shown that poor CD4 cell recovery is correlated with a number of factors that either cause an increase in $CD4^+$ T cell depletion or impair T cell production

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https://doi.org/10.1016/j.ijid.2019.07.037

(Gaardbo et al., 2012; Li et al., 2011; Marchetti et al., 2010). Among these factors, reduced thymic output has been regarded as a major cause of poor CD4 cell recovery after long-term HAART. A 3-year follow-up study suggested that it is difficult to achieve the recovery of defective thymic output after HAART in the majority of patients (Li et al., 2011). Moreover, a series of studies indicated that a CD4 cell count plateau may occur after 4–5 years of successful HAART treatment (Garcia et al., 2004; Kaufmann et al., 2003; Mocroft et al., 2006; Moore and Keruly, 2007; Tarwater et al., 2001). Thus, PIRs in a number of previous studies have been defined at around 4 years following HAART (Erikstrup et al., 2010; Kelley et al., 2009; Lok et al., 2010; Marchetti et al., 2010; Moore and Keruly, 2007). However, some cohort studies have shown that certain PIR patients can still achieve immune reconstitution after prolonged HAART treatment (5.5–9.7 years) (Kelley et al., 2009, Lok et al., 2010). Thus, it would appear necessary to identify those patients who may still experience successful immune reconstitution by evaluating the clinical and immunological characteristics of PIRs at 4 years following HAART.

Accordingly, we designed an ambispective cohort study, which included a retrospective study based on a review of hospital records at 4–5 years after HAART initiation and a 2-year prospective follow-up investigation. PIRs at 4–5 years after HAART initiation were subdivided into two populations according to the kinetics of T cell recovery: PIRs who displayed a persistent defect in $CD4^+$ T cell growth and PIRs who had a T cell count of more than 200 cells/μl after 2 years of therapy, but who failed to show further improvement at the 4-year time-point. These two PIR subtypes showed different immune characteristics, and more importantly, distinct clinical outcomes after an additional 2 years of HAART.

Materials and methods

Study participants

This ambispective cohort study was approved by the Ethics Committee at Beijing Ditan Hospital, Capital Medical University in Beijing, China. All human blood samples were collected with informed consent. The present study examined the immunological indices in 43 healthy control subjects and 182 patients who had been on first-line HAART for 4–5 years and had a good viral response and compliance. Those patients who maintained a good viral response were defined by a plasma viral load that decreased to an undetectable level (<50 copies/ml) that was then maintained at <50 copies/ml at all time points thereafter. The clinical characteristics of all of the study patients are described in Table 1. In the longitudinal study, all 49 PIRs received HAART and were followed up for 2 years.

Plasma HIV-1 viral load and $CD4^+$ T cell count

The plasma HIV-1 RNA copy number was measured using a Standard Amplicor HIV Monitor assay, version 1.5 (Roche Diagnostics, Indianapolis, IN, USA), with a lower detection limit of 40 copies/ml. The $CD4^+$ T cell count was determined by standard flow cytometry technique with a TruCOUNT tube in routinely equipped laboratories (BD Biosciences, San Jose, CA, USA).

Flow cytometry cell phenotype analysis

The peripheral blood mononuclear cells (PBMCs) were separated from freshly collected samples and were stained for the analysis of thymic output, immune activation, and regulatory T cell (Treg) subsets by flow cytometry (Zhou et al., 2013). The antibodies (CD4-FITC, CD4-PE, CD8-PerCp-Cy5.5, CD8-FITC, CD45RA-APC, CD45RA-FITC, CD31 PE, CD25 PE-Cy5, HLA-DR Percp, and CD38 APC) were purchased from BD Biosciences. Data acquisition was performed on a FACS-Calibur flow cytometer (BD Biosciences) and data were analyzed using FlowJo Software (Tree Star, Ashland, OR, USA).

Real-time PCR for the detection of interferon-stimulated genes (ISGs)

Total RNA was extracted from PBMCs and real-time PCR was performed as described previously (Fernandez et al., 2011). All primers used for detection are listed in Supplementary material Table S1.

Measurement of neopterin

The plasma levels of neopterin were quantified by enzyme-linked immunosorbent assay (IBL International, Hamburg, Germany).

Table 1
Demographic and clinical characteristics of the HIV-infected patients.

Characteristics	IRs	PIRs		*p*-Value		
		Type I	Type II	IRs vs. type I	IRs vs. type II	Type I vs. type II
All, *n* (%)	133 (73.08)	25 (13.74)	24 (13.18)	–	–	–
Sex, male/female	103/30	18/7	23/1	0.555	0.071	0.062
Age (years), mean ± SD	37 ± 9	38 ± 11	40 ± 11	0.915	0.276	0.471
Transmission route, *n*				0.036	0.810	0.490
MSM	72	12	13	–	–	–
Heterosexual	30	2	4	–	–	–
Blood	9	6	2	–	–	–
Other/unknown	22	5	5	–	–	–
AZT in ART regimen, *n* (%)	111 (83.46)	21 (84)	19 (79.17)	>0.999	0.608	0.946
HBV/HCV co-infection, *n* (%)	13 (9.77)	1 (4)	1 (4.17)	0.583	0.618	1.000
Baseline CD4 count (cells/mm^3), median (IQR)	149 (56–211)	159 (96–195)	45 (20–87)	0.905	<0.001	<0.001
Baseline CD8 count (cells/mm^3), median (IQR)	746 (518–970)	704 (440–872)	497 (378–779)	<0.001	0.015	<0.001
Baseline WBC ($\times 10^9$/l), median (IQR)	4.51 (3.60–5.68)	4.35 (3.14–5.30)	4.55 (3.26–5.08)	0.332	0.228	0.749
Baseline LY ($\times 10^9$/l), median (IQR)	1.30 (1.00–1.79)	1.47 (1.10–1.78)	1.06 (0.67–1.49)	0.484	0.024	0.032
Baseline PLT ($\times 10^9$/l), median (IQR)	177 (141–211)	168 (146–206)	180 (143–231)	0.766	0.061	0.536
Baseline Hb (g/l), median (IQR)	144 (125–154)	140 (121–156)	126 (111–143)	0.473	0.002	0.065
Baseline AST (U/l), median (IQR)	22.2 (17.9–29.9)	21.9 (18.8–31.2)	24.0 (18.9–39.1)	0.790	0.286	0.531
Baseline ALT (U/l), median (IQR)	22.4 (15.8–34.8)	17.7 (12.5–25.4)	23.2 (15.3–31.9)	0.060	0.876	0.159
Pretreatment OI	30	3	10	0.356	0.048	0.043

IRs, immunological responders; PIRs, poor immunological responders; SD, standard deviation; MSM, men have sex with men; AZT, zidovudine; ART, antiretroviral therapy; HBV, hepatitis B virus; HCV, hepatitis B virus; IQR, interquartile range; WBC, white blood cell count; LY, lymphocyte count; PLT, blood platelet count; Hb, hemoglobin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; OI, opportunistic infection.

Statistical analysis

Data are expressed as the mean (standard deviation (SD)), median (interquartile range (IQR)), or percentage (frequency). In the case of two normally distributed data types, the comparison of variables was performed using the unpaired or paired Student *t*-test, as appropriate. When the data were not normally distributed, the comparison of variables was performed by Mann–Whitney *U*-test or Wilcoxon matched-pairs signed rank test. To compare two more independent samples, one-way analysis of variance (ANOVA) (for normally distributed data) or the Kruskal–Wallis test (for non-normally distributed data) was applied. The comparisons of patient characteristics were analyzed using Fisher's exact test (categorical variables) or the Kruskal–Wallis test (continuous variables). Spearman's correlation coefficients were used to evaluate correlations between activated T cell frequencies and plasma neopterin concentrations. For all analyses, a *p*-value of <0.05 was considered statistically significant.

Results

The two subgroups of patients with poor CD4 cell recovery according to the kinetics of CD4$^+$ T cell recovery

The flow diagram of participant enrollment throughout the study is shown in Figure 1. Among the 3033 HIV-infected individuals treated with HAART at Beijing Ditan Hospital from January 2008 to January 2014, 274 who experienced first-line therapy for 4–5 years achieved and maintained a good viral response. Eighteen patients were excluded due to incomplete longitudinal clinical data and 74 patients refused to participate in the study. The other 182 were enrolled, including 133 (73.08%) immunological responders (IRs; CD4 cell count ≥350 cells/μl after 4–5 years of HAART) and 49 (26.92%) PIRs (CD4 cell count <350 cells/μl after 4–5 years of HAART) (Table 1).

Next, according to their CD4 cell count at 2 years of treatment, these 49 PIRs were divided into two subtypes: 25 PIRs (13.74%) displayed a CD4 cell count that was no less than 200 cells/μl after experiencing 2 years of HAART (type I PIRs), while the other 24 patients (13.19%) had a CD4 cell count lower than 200 cells/μl at the same time-point (type II PIRs) (Figure 2). There was no statistically significant difference among the IRs and two types of PIRs with regard to age, sex, transmission route, zidovudine (AZT) usage, or co-infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) (Table 1). However, compared with the IRs and type I PIRs, the type II PIRs exhibited significantly lower baseline levels of several hematopoietic parameters, including the CD4 cell count, CD8 cell count, total lymphocytes, and hemoglobin. Consistently, a significantly higher rate of type II PIRs was found in the low-baseline CD4 group (baseline CD4 < 100 cells/μl) compared with the high-baseline CD4 group (baseline CD4 ≥ 200 cells/μl): 19/76 (25%) vs. 1/44 (2.27%), $p = 0.0165$; Supplementary material Table S2).

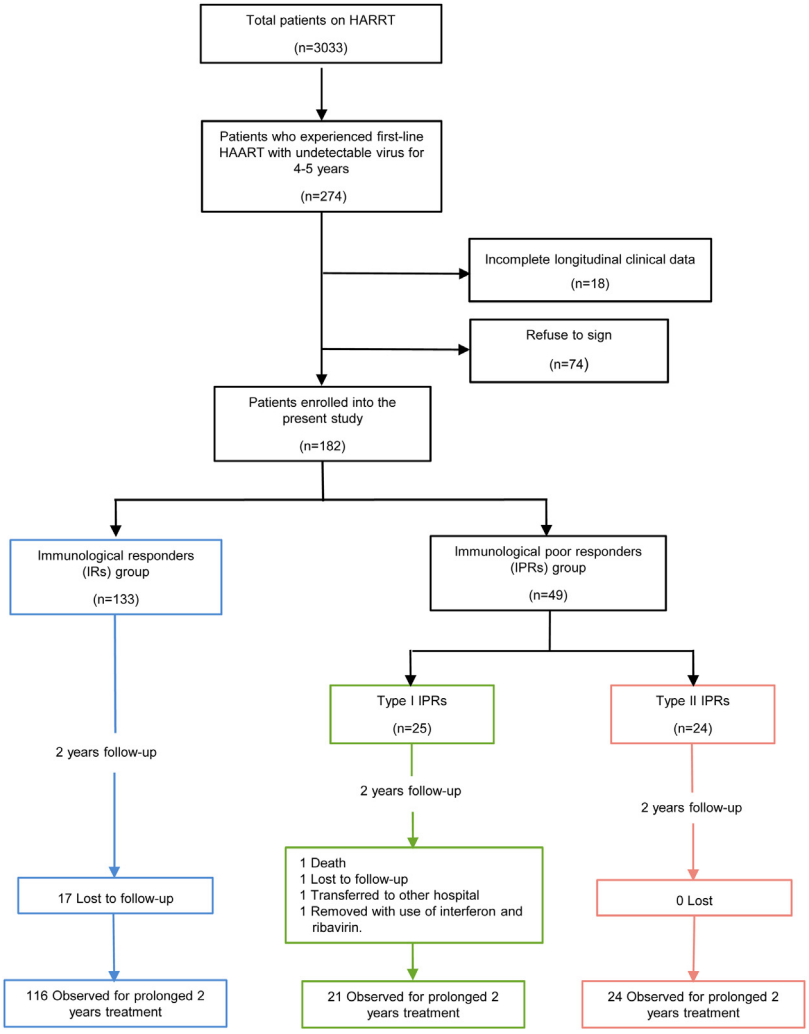

Figure 1. Flow diagram of patient enrollment in the study. Diagram of enrolled patients, number of follow-ups, and numbers included in the analysis.

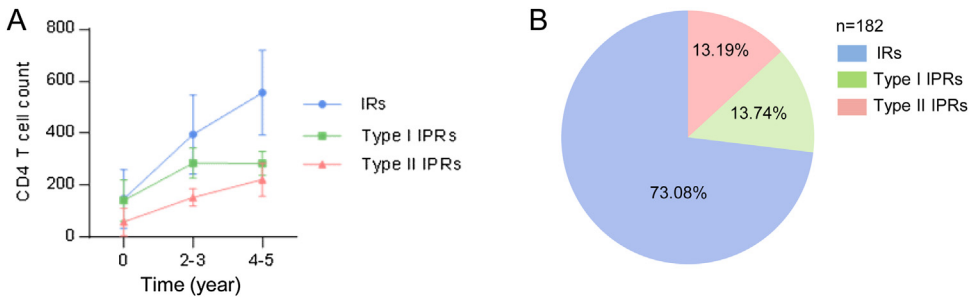

Figure 2. Definition and distribution of type I and type II poor immunological responders (PIRs). (A) The 182 HIV-infected patients on HAART were subgrouped on the basis of the CD4 cell count at 2–3 years and 4–5 years after the initiation of HAART. (B) The frequencies of immunological responders (IRs), type I PIRs, and type II PIRs are shown for the 182 patients.

Type II PIRs displayed impaired thymic output

Considering the persistently lower CD4 cell count in the type II PIRs, it was hypothesized that these patients would show deteriorating defects in thymic output. To test this hypothesis, the percentage of $CD4^+$ naïve T cells (T_N) was examined in these patients at 4–5 years following HAART initiation (Figure 3A). The type I PIRs showed comparable $CD4^+$ T_N frequency to the IRs and healthy controls, while the type II PIRs exhibited a significantly lower percentage of $CD4^+$ T_N (Figure 3B). Along with a defect in naïve T cell recovery, the ratio of naïve to memory $CD4^+$ T cells was also decreased in the type II PIRs (Figure 3C). To further evaluate the newly exported T cells from the thymus, the expression levels of CD31, a marker for new emigrants from the thymus, on naïve $CD4^+$ T cells were analyzed. A lower percentage of $CD31^+$ T_N was observed in type II PIRs (Figure 3D), while the $CD31^+$ memory T cells (T_M) were comparable in the IRs and PIRs (Figure 3E). Collectively, these results suggest a more dramatic defect in the recovery of T cell output from the thymus in type II patients.

Interferon gamma-related T cell activation in type I and type II PIRs

In addition to thymic output, the overactivation of T cells is also considered a contributor to poor CD4 cell recovery by causing T cell loss. Thus, the expression levels of CD38 and HLA-DR on T cells were assessed to evaluate the T cell activation in the patients at 4–5 years following HAART initiation. Both the IRs and the two types of PIRs exhibited higher percentages of circulating $CD38^{hi}HLA\text{-}DR^{hi}$ fractions in both $CD4^+$ and $CD8^+$ T cells when compared with the healthy controls (Figure 4A, B). Notably, the PIRs (type I and type II) displayed comparably high levels of $CD38^{hi}HLA\text{-}DR^{hi}$ cells with IRs, suggesting that residual and general T cell activation still occurred despite the viral suppression.

Constant low-dose interferon (IFN) in plasma has been demonstrated to correlate with immune activation during HIV infection (Fernandez et al., 2011; Jacquelin et al., 2009). Since the plasma levels of IFN-α and IFN-γ are usually lower than the detection limit in most HIV patients with viral repression, we investigated the host response to IFN-α exposure by measuring

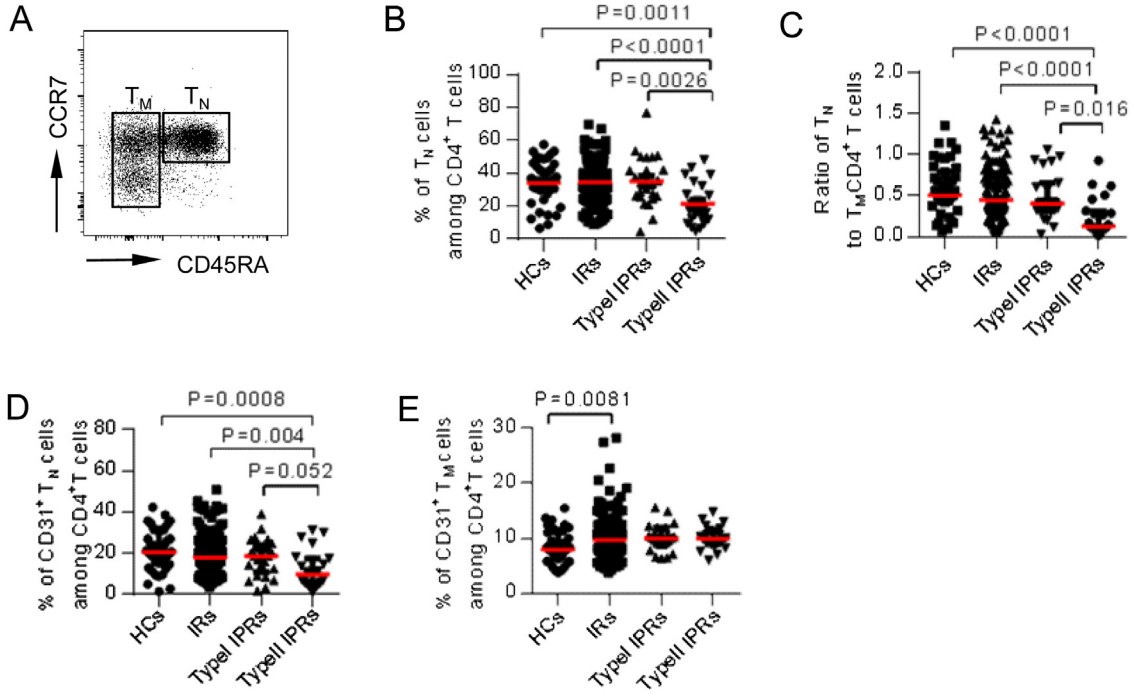

Figure 3. Analysis of T cell subsets in healthy controls and the subgroups of HIV-infected patients on HAART. (A) Representative flow data of T cell subsets gated on CD4 in healthy donors are shown. (B–E) The frequency of naïve $CD4^+$ T cells (B), the ratio of naïve to memory $CD4^+$ T cells (C), and the percentages of $CD31^+$ naïve $CD4^+$ T cells (D) and $CD31^+$ memory $CD4^+$ T cells (E) in healthy controls ($n = 43$), immunological responder (IRs) ($n = 133$), type I poor immunological responders (PIRs) ($n = 25$), and type II PIRs ($n = 24$).

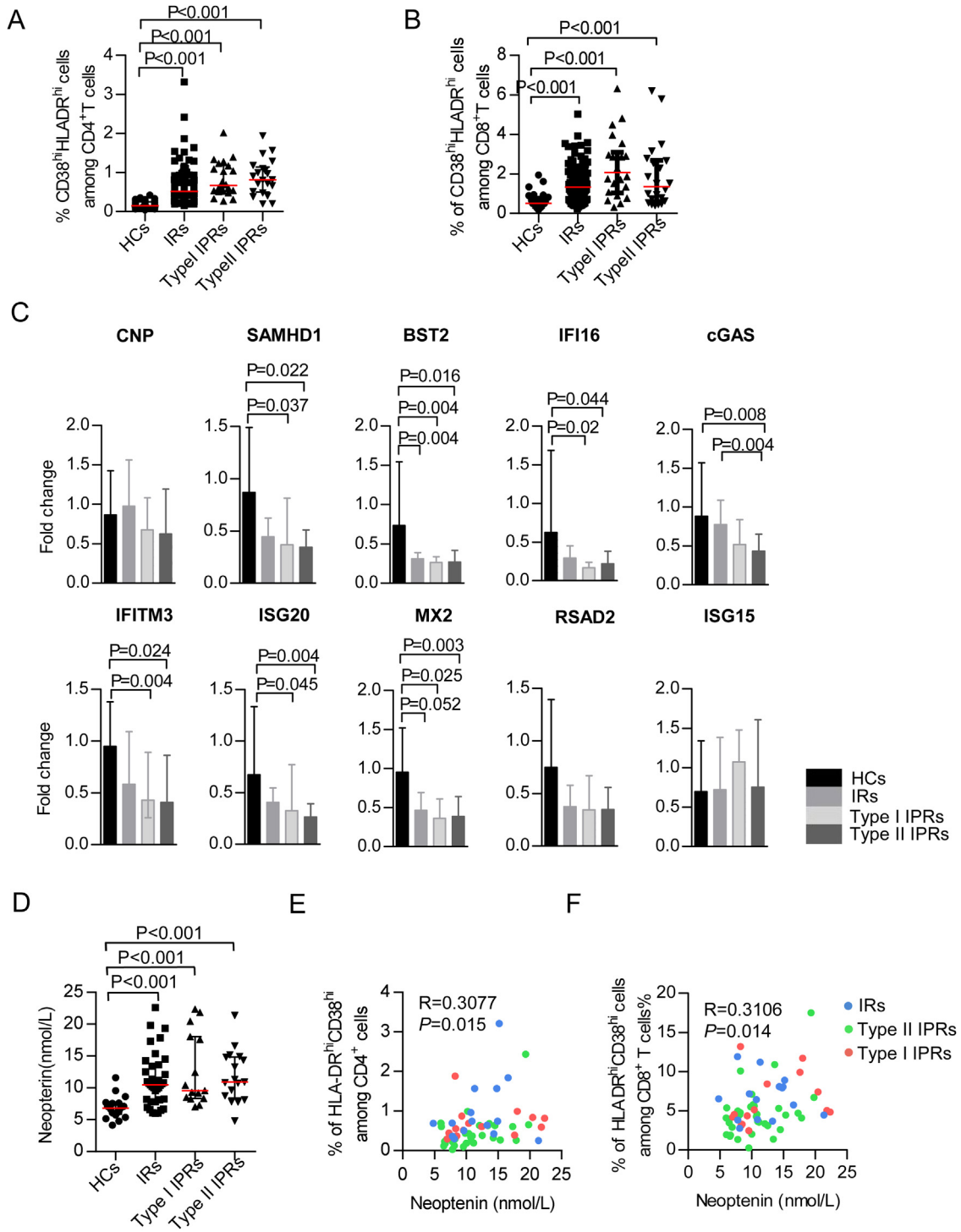

Figure 4. Analysis of T cell activation in healthy controls and subgroups of HIV-infected patients on HAART. (A, B) Frequencies of activated $CD38^{hi}HLA\text{-}DR^{hi}$ $CD4^{+}$ (A) and $CD8^{+}$ T cells (B) in healthy controls ($n = 43$), immunological responder (IRs) ($n = 133$), type I poor immunological responders (PIRs) ($n = 25$), and type II PIRs ($n = 24$). (C) The expression of interferon-stimulated gene (ISG) mRNA in healthy controls ($n = 19$), IRs ($n = 82$), type I PIRs ($n = 23$), and type II PIRs ($n = 24$). (D) The plasma concentrations of neopterin in healthy controls ($n = 21$), IRs ($n = 37$), type I PIRs ($n = 15$), and type II PIRs ($n = 17$). (E, F) Correlation analysis of the plasma concentrations of neopterin with the percentages of $CD38^{hi}HLA\text{-}DR^{hi}$ $CD4^{+}$ (E) and $CD8^{+}$ T cells (F) in IRs and PIRs.

the expression levels of ISGs in PBMCs (Figure 4C). However, no increase in the tested ISGs was observed in IRs and PIRs compared with the healthy controls. Next, the plasma concentration of neopterin, a surrogate marker reflecting the IFN-γ activity, was assessed; significantly higher plasma neopterin levels were found in PIRs (type I and type II) and IRs than in the healthy controls (Figure 4D). Moreover, the percentages of activated $CD4^{+}$ and $CD8^{+}$ T cells were both tightly correlated with the plasma concentration of neopterin (Figure 4E, F). Thus, IFN-γ might generally contribute to residual immune activation in HIV-infected patients.

Disturbed homeostasis of circulating Treg subpopulations in type II PIRs

Since HIV/AIDS patients show disturbed homeostasis among their Treg subpopulations, we tested whether increased T cell activation in PIRs is correlated with a decreased frequency of circulating Treg cells. The two types of PIRs exhibited comparable frequencies of classically defined Treg cells ($CD4^+Foxp3^+$) compared with IRs at 4–5 years following HAART initiation (Figure 5A). Next, the $CD4^+$ Treg cells were divided into three subsets based on the expression patterns of CD45RA and CD25, as described previously: naïve Treg (nTreg, $CD25^{low}CD45RA^+$), activated Treg (aTreg, $CD25^{high}CD45RA^-$), and cytokine-secreting non-suppressive T cells (fraction III, $CD25^{low}CD45RA^-$) (Figure 5B). Unlike the type I PIRs, the type II PIRs exhibited significantly decreased proportions of nTreg cells ($p = 0.001$, Figure 5C), but increased proportions of aTreg cells and inflammatory fraction III cells (Figure 5D, E), when compared with the IRs. Taken together, these data show that the balance between immunosuppressive subsets and inflammatory subsets in Tregs is disturbed in type II PIRs.

Type I PIRs showed a better outcome than type II PIRs after an additional 2 years of HAART treatment

Since type I PIRs exhibited higher levels of thymic output than the type II PIRs at 4–5 years after HAART initiation, the question arose as to whether type I PIRs would have a better clinical outcome than type II PIRs after prolonged HAART treatment. These cohorts were followed for an additional 2 years and a continuous increase in CD4 cell count was found in each group, including the type II PIRs (Supplementary material Figure S1A). Type I PIRs exhibited a significantly higher CD4 cell gain after the additional 2 years of HAART when compared to the type II PIRs ($p = 0.013$, Supplementary material Figure S1B). At 6–7 years after initiating HAART, four patients among the 25 type I PIRs were excluded for the reasons indicated. Sixteen subjects (76.19%) showed CD4 cell counts higher than 350 cells/μl and only five patients (23.81%) still maintained a low-level CD4 count of less than 350 cells/μl. By contrast, only six patients (25.00%) among the 24 type II PIRs showed a CD4 count recovery of more than 350 cells/μl and 18 patients (75.00%) still had failed immune reconstitution (Figure 6A).

We further compared the clinical and immunological parameters at 4–5 years after initiating HAART between the recovered and non-recovered patients in the PIR cohorts. No statistically significant difference was found between the recovered and non-recovered type I PIR patients with regards to the clinical parameters (Table 2). However, recovered type II PIR patients were significantly younger ($p = 0.007$; Table 2) and exhibited significantly higher CD4 counts at 4–5 years after the initiation of HAART ($p = 0.0001$; Table 2) when compared to the non-recovered type II PIR patients. Other hematopoietic parameters including the CD8 count and blood platelet count in these recovered patients showed a similar trend, which was close to statistical significance (CD8 count: $p = 0.052$; platelet count: $p = 0.066$; Table 2). Moreover, regardless of whether they were type I or type II PIRs, the recovered patients exhibited a higher percentage of naïve CD4 T cells and $CD31^+$ naïve CD4 T cells than the non-recovered patients

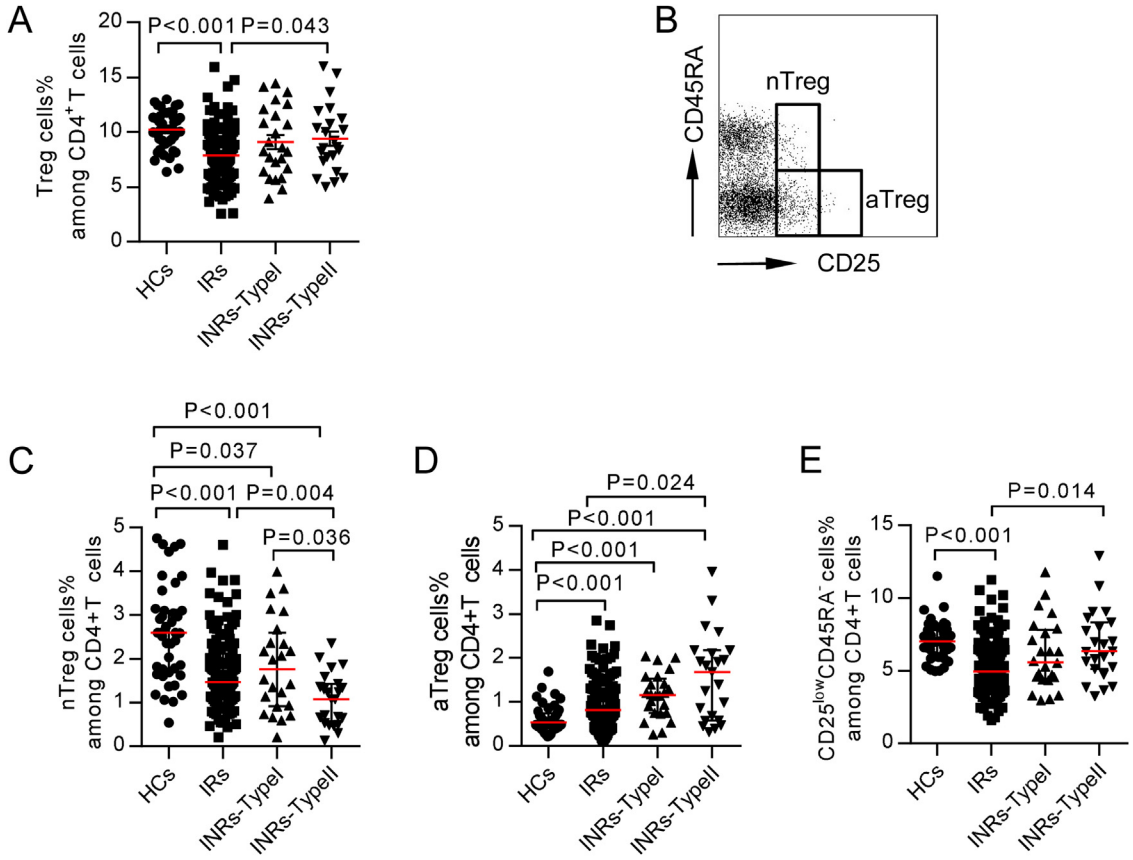

Figure 5. Analysis of Treg cells in healthy controls and subgroups of HIV-infected patients on HAART. (A) The frequency of the total Treg cells in the healthy controls ($n = 43$), immunological responder (IRs) ($n = 126$), type I poor immunological responders (PIRs) ($n = 25$), and type II PIRs ($n = 24$). (B) Representative flow data of Treg subsets gated on CD4 in healthy donors are shown. (C–E) The frequencies of the total nTreg (C), aTreg (D), and cytokine-secreting non-suppressive T cells (E) in the healthy controls ($n = 43$), IRs ($n = 126$), type I PIRs ($n = 25$), and type II PIRs ($n = 24$).

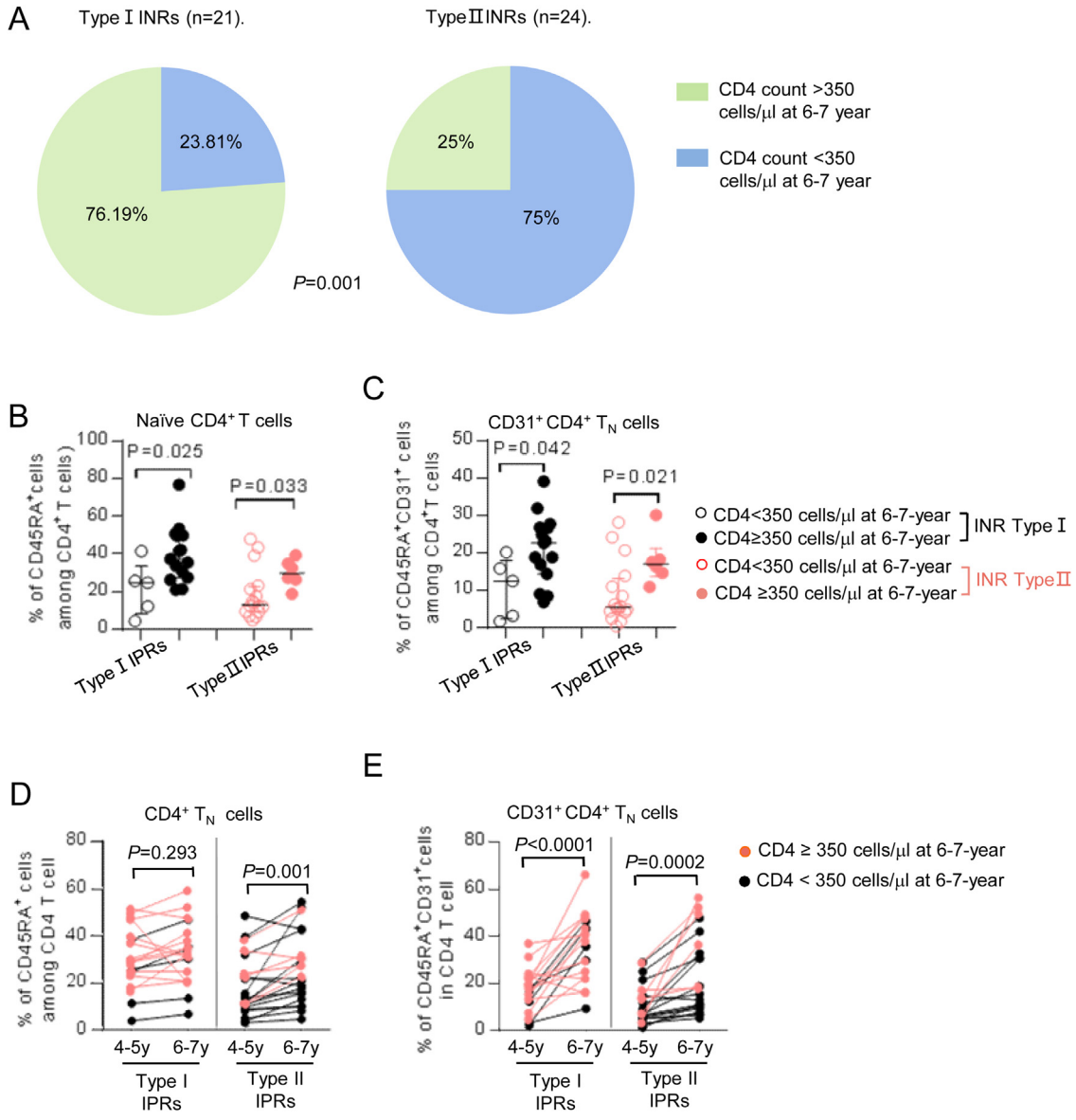

Figure 6. Thymic output in subgroups of poor immunological responders (PIRs) with a distinct clinical outcome after 2 years of additional HAART. (A) The percentages of recovered and non-recovered patients in the type I ($n = 21$) and type II PIRs ($n = 21$). (B, C) The frequencies of naïve $CD4^+$ T cells (B) and $CD31^+$ naïve $CD4^+$ T cells (C) at 4–5 years in recovered (CD4 count <350 cells/ml at 6–7 years) and non-recovered (CD4 count ≥350 cells/ml at 6–7 years) patients among the type I PIRs and type II PIRs. (D, E) Dynamic analysis of naïve $CD4^+$ T cells (D) and $CD31^+$ naïve $CD4^+$ T cells (E) from 4–5 years to 6–7 years in recovered and non-recovered patients among type I PIRs and type II PIRs.

Table 2
Demographic and clinical characteristics of the two subgroups of poor immunological responders.

Characteristics (at 4–5 years after HAART)	Type I (defined at 4–5 years)			Type II (defined at 4–5 years)		
	CD4 at 6–7 years (cells/mm^3)		*p*-Value	CD4 at 6–7 years (cells/mm^3)		*p*-Value
	<350	≥350		<350	≥350	
All	5	16	–	18	6	–
Sex, male/female	3/2	12/4	0.935	17/1	6/0	>0.999
Age (years), mean ± SD	40 ± 13	37 ± 12	0.719	43 ± 10	31 ± 5	0.007
Transmission route, *n*			0.149			>0.999
MSM	2	8	–	9	4	–
Heterosexual	0	1	–	3	1	–
Blood	3	2	–	2	0	–
Other/unknown	0	5	–	4	1	–
AZT in ART regimen, *n* (%)	3 (60)	15 (93.75)	0.250	5/13	0/5	0.472
HBV/HCV co-infection, *n* (%)	0 (0)	1 (6.25)	1.000	18/0	5/1	0.250

HAART, highly active antiretroviral therapy; SD, standard deviation; MSM, men have sex with men; AZT, zidovudine; ART, antiretroviral therapy; HBV, hepatitis B virus; HCV, hepatitis B virus.

(Figure 6B, C). However, all of the recovered patients showed a comparable percentage of active $CD38^{hi}HLA\text{-}DR^{hi}$ T cells and neopterin concentration to non-recovered ones (Supplementary material Figure S2A–C). Notably, it was found that two additional years of HAART could hardly reduce the residual T cell activation, but it did persistently improve the thymic output (Figure 6D, E; Supplementary material S2D, E). Surprisingly, the percentages of $CD31^+$ naïve $CD4^+$ T cells in IRs and type I PIRs at 6–7 years after HAART initiation were even higher than those of healthy controls (data not shown), suggesting that long-term HAART can enhance thymic output.

Discussion

Previous studies have attempted to anticipate the T cell recovery after HAART based on baseline parameters. Consistent with previous findings, the present study found a significantly higher incidence of PIRs in the low baseline CD4 group. However, 67.1% of the patients with a baseline CD4 count of <100 cells/μl achieved successful immune reconstitution, while 13.63% of the patients with a baseline CD4 count of >200 cells/μl failed to reach optimal CD4 cell recovery after 4–5 years of HAART. Thus, it is challenging to make an early prediction of T cell recovery based on the CD4 nadir. In the present study, based on the dynamics of T cell recovery, the PIRs were defined by their response after 4–5 years of HAART and divided into two populations; the immune parameters were reevaluated in these two groups. Compared to the type I PIRs, the type II PIRs displayed comparable levels of residual immune activation but more dramatic defects in thymic output. Accordingly, the type II PIRs showed better immune reconstitution after the additional 2 years of treatment.

Among the multiple factors correlated with poor CD4 cell recovery, defective thymic output has been recognized as one of the most important factors (Ferrando-Martinez et al., 2017; Li et al., 2011; Zakhour et al., 2016). Two strategies have been considered to translate this observation into clinical practice. First, starting HAART at a high CD4 baseline is necessary to secure a good clinical outcome (Le et al., 2013; Zhang et al., 2011). In 2003, only patients with a CD4 count <200 cells/μl were covered by the national free antiretroviral therapy program in China (Zhang et al., 2007, 2011). Since June 2016, free antiretroviral therapy has been made available to all HIV/AIDS individuals in China regardless of their CD4 count. In the present study it was found that the patients with a baseline CD4 count <100 cells/μl had a higher risk of becoming a type II PIR, indicating that earlier HAART treatment to aggressively control viral infection and maintain higher CD4 cell count levels is crucial to improve the clinical outcome. Second, prolonged HAART might be an alternative approach to benefit this patient population. However, Li et al. have reported that the application of HAART alone is unlikely to reverse the existing thymus damage in the majority of patients (Li et al., 2011). Of note, their observation period was within 3 years after the initiation of therapy. In the present study, we observed an increase in the frequencies of newly generated $CD31^+$ $CD4^+$ naïve T cells in most PIRs after prolonged treatment. This finding indicates that the thymic output could be further improved after long-term HAART. Thus, a longer follow-up (e.g., 4–7 years) may be required after HAART to assess the status of CD4 cell recovery. In addition, the study results highlight the importance of long-term HAART because it has persistent effects on thymic improvement.

This study demonstrated that, compared with the IRs and the type I PIRs, the type II PIRs not only displayed a decrease in $CD31^+$ $CD4^+$ naïve T cells, but they also showed a broad decrease in a number of hematopoietic parameters: the baseline CD4 cell count, CD8 cell count, lymphocyte count, and hemoglobin. It has been reported that a defective thymic output could occur at the intrathymic lymphopoiesis stage or at the extrathymic lymphopoiesis stage (Kong et al., 2016; Sauce et al., 2011). A series of studies showed that HIV could infect long-lived hematopoietic stem cells (HSCs) and other multipotent hematopoietic progenitor cells, suggesting that there were defects in early hematopoiesis (Carter et al., 2011; Carter et al., 2010; Nixon et al., 2013; Pace and O'Doherty, 2013). HIV proteins result in the aberrant activation of inflammatory pathways, which could impact the bone marrow niche and subsequently exert indirect effects on HSCs (Kuller et al., 2008). Hematopoietic abnormalities, including the development of all the hematopoietic lineages, are common manifestations during HIV infection, and the degree of hematopoietic pathology correlates with the stage of disease progression (Moses et al., 1998). Thus, the multiple defects in the hematopoietic parameters of type II PIRs might indicate an impaired early hematopoiesis, which would explain why type II PIRs had less of an ability than type I PIRs (approximately 25% versus 75% in our cohort) to achieve satisfactory T cell recovery after the additional 2 years of therapy.

Importantly, the study findings provide a strong rationale for targeting HIV-induced defects during early hematopoiesis rather than thymopoiesis for optimal HIV treatment. A series of clinical trials have been performed to promote T cell homeostasis and thymopoiesis through the administration of cytokines (e.g., interleukins IL-7 and IL-2) and growth hormone (Group et al., 2009; Levy et al., 2009; Napolitano et al., 2002). However, these cytokines primarily act at the intrathymic stage of lymphopoiesis or T cells in the secondary lymphoid organ, which might explain the limited effects on type II PIRs. In addition, no clinical benefits were observed in the PIRs, according to the latest clinical guidelines for HIV-1-infected adults and adolescents (Department of Health and Human Services).

Many factors may cause chronic immune activation during HAART therapy. These factors include persistent antigen stimulation by residual viruses, excess levels of various pathogens, the destruction of mucosal surfaces, and a loss of immunoregulatory mucosal cells (Paiardini and Muller-Trutwin, 2013). Based on these observations, a series of preclinical studies and clinical trials have been conducted to improve T cell recovery by using immunosuppressive agents, such as chloroquine, hydroxychloroquine, cyclosporine A, mycophenolic acid, rapamycin, and cytokine neutralization (tumor necrosis factor alpha (TNF-α), IL-6) (Coull et al., 2001; Krown et al., 2012; Lederman et al., 2006; Markowitz et al., 2010; Paton et al., 2012). Unfortunately, the clinical benefits of these interventions are limited. Here, we observed that after long-term HAART treatment, IRs and PIRs showed comparable excessive chronic immune activation, as manifested by the similar expression of IFN-γ. Notably, two additional years of HAART did not improve the chronic immune activation. Therefore, the full recovery of thymic output is necessary for successful treatment by immune suppression. Implementing immunosuppressive therapy randomly regardless of immune status may be detrimental by aggravating immune instability in patients.

A limitation of this study is that baseline thymic output data were not available due to the ambispective study design. Therefore whether the thymic output at the initiation of HAART predicts the clinical outcome remains unclear. Our ongoing prospective study, in which baseline samples are obtained for the evaluation of thymic output function, will help to address this important question in the near future.

In summary, this study demonstrated a distinct role of thymic output in PIRs and further separated them into type I and type II PIRs. Prolonged HAART was much more effective in type I PIRs compared with type II PIRs. In this study, the classification of PIRs was based on the kinetics of T cell recovery, which allows us to predict the therapeutic effects of HAART and subsequently provide aggressive treatment to patients with type II PIRs. The immune

characteristics of the type II PIRs discovered in this study will provide pivotal information for developing novel, effective HIV treatments that can overcome the limitations of HAART.

Ethical approval

The study protocol was approved by the Ethics Committee at Beijing Ditan Hospital, Capital Medical University in Beijing, China. All human blood samples were collected with informed consent. The study was conducted in accordance with the tenets of the Declaration of Helsinki.

Conflict of interest

None of the authors have any conflicts of interest to declare.

Author contributions

H. Zeng and H. Zhao designed the study; H. Zeng and Y.K. drafted the article; H. Zeng, F.Z., and H. Zheng revised the manuscript; Y.K., Y.T., and Y.H. analyzed and interpreted the data; Y. T., Y.H., X.C., C.S., J.H., and G.D. performed the experiments; J.X., D. Y., and H. Zhao collected the patients.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (81672000), the Thirteen-Fifth Key Project (2018ZX10715-005), and Capital Clinical Characteristic Application (Z171100001017052).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijid.2019.07.037.

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