Reactivation capacity by latency-reversing agents *ex vivo* correlates with the size of the HIV-1 reservoir

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Objective: HIV-1 reservoirs are the major hurdle to virus clearance in combination antiretroviral therapy (cART)-treated patients. An approach to eradicating HIV-1 involves reversing latency in cART-treated patients to make latent cells visible to the host immune system. Stimulation of patient cell cultures with latency-reversing agents (LRAs) *ex vivo* results in heterogeneous responses among HIV-infected patients. Determinants of this heterogeneity are unknown and consequently important to determine.

Design and methods: Here, we grouped and retrospectively analyzed the data from our two recent HIV-1 reactivation studies to investigate the role of the HIV-1 reservoir size in the reactivation capacity by LRAs in *ex vivo* cultures of $CD8^+$-depleted peripheral blood mononuclear cells (PBMCs) isolated from 54 cART-treated patients and of resting $CD4^+$ T cells isolated from 30 cART-treated patients.

Results: Our results established a statistically relevant positive correlation between the HIV-1 reservoir size measured by total cell-associated HIV-1 DNA and the frequency of positive HIV-1 recovery measurements in response to various LRAs in *ex vivo* cultures of cells isolated from cART-treated HIV^+ aviremic patients. HIV-1 reservoir size also correlated with the extracellular HIV-1 RNA median level measured in supernatants of cell cultures following LRA treatments. However, we identified HIV^+ patients whose positive measurements frequency and median level of extracellular HIV-1 RNA deviated from linearity relative to their corresponding HIV reservoir size.

Conclusion: We demonstrated that the reservoir size is one predictive marker of LRA effectiveness but this parameter alone is not sufficient. The identification of other predictive markers is necessary to predict the success of HIV anti-latency approaches.

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Keywords: anti-HIV strategy, HIV reactivation, HIV reservoir size, latency-reversing agent, predictive markers of latency-reversing agent effectiveness

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Introduction

HIV-1 reservoirs impede virus clearance in patients under combination antiretroviral therapy (cART) [1]. Activation of HIV-1 expression from latent reservoirs is a part of proposed strategies that may potentially lead to a decrease in the size of the reservoirs [2]. Cells harboring induced proviruses could then be lysed by viral cytopathic effects or host cytolytic effector mechanisms, whereas new rounds of infection are blocked by cART [3]. Latency-reversing agents (LRAs) have been identified through different mechanism-based approaches and include histone deacetylase inhibitors (HDACis), DNA methyltransferase inhibitors, protein kinase C (PKC) agonists and bromodomain and extra-terminal (BET) inhibitors (BETi) [4]. We and others have demonstrated the HIV-1 reactivation potential of combined treatments with different classes of LRAs ([5–8], reviewed in [4]). These studies highlight a high diversity of responses among ex-vivo patient cell cultures in terms of reactivation capacity following treatment with LRAs. The understanding of the determinants involved in these patient-specific reactivation variations would be a key progress made toward the identification of means to reverse latency. Notably, little is known about the HIV-1 reservoir size role in the reactivation of patient cell cultures by LRAs. In this report, we analyzed patient data from two of our most recent reactivation studies to reach a number of patients sufficient for an accurate and statistically relevant analysis of the results [8,9]. In the first study, we have shown that combined treatments of PKC agonists with compounds releasing active positive transcription factor b (P-TEFb) synergistically reactivate HIV-1 in ex-vivo cultures of primary cells isolated from blood of cART-treated HIV$^+$ aviremic patients [8]. In our second recently published study performed with the same postintegration latency models, we have demonstrated that combined treatments of the nucleoside analogue DNA methylation inhibitor 5-aza-2′-deoxycytidine (5-AzadC (Sigma, Belgium), marketed as Dacogen) with clinically tolerable HDACis synergistically reactivates HIV-1 from latency [9]. Importantly, this synergy between 5-AzadC and HDACis was stronger when a sequential treatment was applied compared with the corresponding simultaneous combined treatment, highlighting for the first time the importance of treatment time schedule for LRAs combinations [9].

Here, we grouped the data from these two studies to investigate the role of the HIV-1 reservoir size in the reactivation capacity by LRAs in ex-vivo cultures of CD8$^+$-depleted PBMCs isolated from 54 cART-treated patients and of resting CD4$^+$ T cells isolated from 30 cART-treated patients.

Methods

Study participants

We grouped the data from our two most recent reactivation studies to allow for an accurate and statistically relevant analysis of the results [8,9]. Indeed, the addition of our data from reactivation assays performed in these two studies allowed us to investigate the role of the HIV-1 reservoir size in the reactivation capacity by LRAs in three ex-vivo cellular models for HIV-1 postintegration latency:

(1) Ex-vivo patient cell cultures of 6 million CD8$^+$-depleted PBMCs (at 2 million cells/ml) from 43 cART-treated HIV$^+$ patients, in the absence of cART [24 patients (P1–P24) from [8] and 19 patients (P25–P43) from [9]].
(2) Ex-vivo patient cell cultures of 0.5 million resting CD4$^+$ T cells (at 0.8 million cells/ml) isolated from 30 cART-treated HIV$^+$ patients [14 patients (P44–P57) from [8] and 16 patients (P58–P73) from [9]].
(3) Ex-vivo patient cell cultures of 12 million CD8$^+$-depleted PBMCs (at 2 million cells/ml) isolated from 11 cART-treated HIV$^+$ patients, in the presence of cART antiretrovirals [efavirenz (4624, the AIDS Research and Reference Reagent Program, National Institutes of Health (NIH) Bethesda, Maryland, USA; 100 nmol/l); zidovudine (3485, the AIDS Research and Reference Reagent Program, NIH; 180 nmol/l); raltegravir (11680, the AIDS Research and Reference Reagent Program, NIH; 200 nmol/l)] (P74–P84) [8].

Characteristics of the patients for which we have previously isolated and stimulated, in the absence of cART, CD8$^+$-depleted PBMCs or resting CD4$^+$ T cells are presented in Table 1. Characteristics of the patients for which we have previously isolated CD8$^+$-depleted PBMCs and performed reactivation assays, in the presence of cART, are presented in Table 2. Isolation and stimulation of CD8$^+$-depleted PBMCs and of resting CD4$^+$ T cells as well as quantification of viral RNA have been performed in the same lab for the whole patient cohort, as previously described [10]. Briefly, 1 day after isolation, cells were mock-treated or treated with anti-CD3 + anti-CD28 antibodies as a positive control or by various LRAs for 6 days. HIV-1 RNA levels in culture supernatants were next determined using the reverse transcription quantitative polymerase chain reaction (RT-qPCR)-based Generic HIV Charge Virale kit (Biocentric, Bandol, France). Cell cultures exhibiting reactivation level of supernatant HIV-1 RNA higher than 15, 150 or 200 copies/ml (depending on the tested supernatant volumes) have been considered as positive measurements (Tables 1 and 2). We defined for each patient a frequency of positive measurements as the ratio between the number of

Table 1. Characteristics of HIV-1-infected individuals from whom $CD8^+$-depleted PBMCs were isolated, named P1–P43 and from whom resting $CD4^+$ T cells were isolated, named P44–P73.

	Patients	Year of birth	Year of HIV diagnostics	HIV subtype	$CD4^+$ nadir (cells/μl)	Year of first treatment	Year of undetectable status	Year of blood sampling	$CD4^+$ T-cell count (cells/μl)	Last treatment	Year of the beginning of the ongoing treatment	HIV DNA log_{10} copies/10^6 cells	HIV DNA copies/10^6 cells	Number of tested conditions	Number of reactivated patient cell cultures	Frequency of HIV-1 recovery (%)	Median level of HIV RNA copies/condition
$CD8^+$ -depleted PBMCs	P1	1966	1994	X	384	1997	1998	2013	905	ABC/3TC/ZDV	2004	2.833	680	16	9	56.3	300
	P2	1957	2004	X	45	2004	2005	2013	351	EFV/TDF/FTC	2010	3.085	1217	16	1	6.3	5
	P3	1972	1994	X	138	1994	2002	2013	747	TDF/FTC/FPV/r	2010	1.845	70	16	11	68.8	584
	P4	1961	2007	X	364	2011	2011	2013	659	ABC/3TC/ATV/r	2013	2.544	350	15	12	80.0	394
	P5	1971	2008	X	603	2008	2011	2013	758	EFV/TDF/FTC	2008	3.251	1782	16	12	75.0	3235
	P6	1971	2012	X	477	2012	2012	2013	607	TDF/FTC/ATV/r	2012	2.825	669	16	15	93.8	1792
	P7	1982	2008	X	620	2009	2009	2013	889	TDF/FTC/RPV	2009	2.826	670	14	9	64.3	378
	P8	1965	1995	05 DF	11	1995	2006	2013	990	DRV/r/RAL	2011	2.893	782	16	10	62.5	421
	P9	1939	2001	X	267	2001	2006	2013	1198	EFV/ABC/3TC	2006	2.928	847	16	7	43.8	51
	P10	1964	1998	X	1	2005	2006	2013	506	TDF/FTC/FPV/r	2009	2.538	345	16	6	37.5	0
	P11	1977	2008	02 AG	143	2009	2009	2013	494	EFV/TDF/FTC	2010	3.157	1435	16	12	75.0	984
	P12	1965	1996	X	11	1996	2010	2013	657	TDF/FTC/NVP	2012	2.976	947	16	7	43.8	0
	P13	1953	1995	X	199	1995	2004	2013	847	ABC/3TC/SQV/r	2007	2.403	253	16	5	31.3	0
	P14	1961	1996	X	92	1996	2005	2013	770	EFV/TDF/FTC	2010	3.261	1823	16	9	56.3	189
	P15	1960	1995	X	314	1997	2003	2013	633	ABC/3TC/FPV/r	2012	2.591	390	16	6	37.5	1
	P16	1953	2006	X	340	2006	2007	2013	641	ABC/3TC/NVP	2008	2.531	340	16	6	37.5	0
	P17	1973	1997	X	395	1997	2010	2013	585	EFV/TDF/FTC	2010	2.420	263	16	6	37.5	0
	P18	1955	2003	X	77	2012	2012	2013	313	TDF/FTC/ATV/r	2012	2.522	333	16	13	81.3	1033
	P19	1959	2009	X	331	2009	2010	2013	815	EFV/TDF/FTC	2010	2.272	187	16	5	31.3	0
	P20	1965	2009	X	316	2010	2010	2013	895	TDF/FTC/ATV/r	2011	3.092	1236	16	12	75.0	849
	P21	1968	1995	X	185	1995	2000	2013	890	TDF/FTC/NVP	2009	2.778	600	17	8	47.1	11
	P22	1952	2005	X	133	2005	2005	2013	319	EFV/TDF/FTC	2011	2.867	737	17	10	58.8	869
	P23	1964	1996	X	435	1996	2001	2013	531	ABC/3TC/ZDV	2002	2.580	380	16	10	62.5	1062
	P24	1963	1990	X	140	1990	2003	2014	752	TDF/FTC/ATV/r	2010	2.307	203	17	8	47.1	0
	P25	1950	1999	X	290	2000	2000	2014	948	ABC/3TC/ZDV	2002	1.041	11	17	1	5.9	0
	P26	1961	1997	B	224	1999	2009	2014	590	TDF/FTC/ETR/RAL	2011	2.826	670	13	4	30.8	0
	P27	1960	1997	X	185	1997	2003	2014	428	EFV/TDF/FTC	2010	2.667	464	16	5	31.3	0
	P28	1963	2006	X	256	2006	2006	2014	633	TDF/FTC/ETR/RAL	2009	2.769	587	17	8	47.1	80
	P29	1971	1997	D	255	1997	2009	2014	786	3TC/ZDV/RAL/MVC	2009	2.860	724	17	9	52.9	314
	P30	1969	2011	F2	364	2012	2012	2014	723	ABC/3TC/ATV/r	2012	3.407	2552	17	9	52.9	216
	P31	1969	1998	X	138	1999	2012	2014	723	ABC/3TC/ATV/r	2012	2.739	548	17	3	17.6	0
	P32	1973	2005	X	71	2005	2005	2014	513	TDF/FTC/FPV/r	2009	3.037	1089	13	4	30.8	0
	P33	1963	1996	X	360	1996	1998	2014	777	ABC/3TC/ATV/r	2014	2.097	125	13	4	30.8	0
	P34	1951	1990	X	144	1991	1997	2014	629	TDF/FTC/NVP	2014	2.872	744	13	3	23.1	0
	P35	1976	2005	X	422	2012	2012	2014	603	TDF/FTC/ATV/r	2014	0.699	5	15	1	6.7	0
	P36	1947	1998	B	151	1998	2006	2014	881	ATV/r	2009	2.943	876	17	7	41.2	22
	P37	1959	2000	B	193	2000	2000	2014	547	EFV/TDF/FTC	2011	2.936	863	17	10	58.8	200
	P38	1972	2000	X	146	2000	2002	2014	625	TDF/FTC/NVP	2009	1.756	57	15	2	13.3	0
	P39	1967	2006	X	289	2006	2006	2014	1175	FTC/RPV/TDF	2011	2.410	257	17	4	23.5	0
	P40	1966	2007	X	371	2010	2011	2014	739	TDF/FTC/ATV/r	2011	3.195	1567	17	4	23.5	0
	P41	1968	2008	X	192	2008	2008	2014	354	EFV/TDF/FTC	2010	2.959	909	13	8	61.5	229
	P42	1972	1996	X	373	1997	2009	2014	1015	EFV/TDF/FTC	2010	3.652	4486	17	15	88.2	513
	P43	1980	2006	X	230	2006	2006	2014	623	ABC/3TC/NVP	2012	3.035	1083	9	9	100	504
Resting $CD4^+$ T cells	P44	1949	1995	X	399	1997	2007	2014	869	EFV/TDF/FTC	2011	3.079	1200	9	3	33.3	0
	P45	1966	1998	X	558	1998	1999	2014	670	3TC/ZDV/NVP	1999	3.072	1179	9	3	33.3	0
	P46	1973	2004	X	746	2004	2010	2014	848	TDF/FTC/LPV/r	2008	2.049	112	9	7	77.8	676
	P47	1966	2006	X	215	2006	2007	2014	418	EFV/TDF/FTC	2009	3.645	4414	9	5	55.6	503
	P48	1960	1989	C	53	1999	1999	2014	401	TDF/FTC/NVP	2009	3.250	1777	9	3	33.3	0
	P49	1954	1984	X	61	1996	2011	2014	856	3TC/ZDV/NVP	2002	3.718	5220	8	5	62.5	570
	P50	1973	2004	X	294	2004	2004	2014	736	ABC/3TC/NVP	2007	2.960	911	9	2	22.2	0
	P51	1956	1985	B	92	1991	2005	2014	818	DRV/r/FTC/MVC	2010	3.584	3841	8	5	62.5	454
	P52	1995	1998	X	251	1998	2006	2014	663	EFV/TDF/FTC	2010	2.976	946	9	6	66.7	392
	P53	1969	2001	X	192	2001	2004	2014	928	ABC/3TC/ZDV	2002	2.720	525	9	2	22.2	0
	P54	1948	2009	X	354	2009	2009	2014	817	ABC/3TC/NVP	2013	3.183	1523	9	3	33.3	0
	P55	1952	2000	01 AE	424	2002	2007	2014	1091	EFV/TDF/FTC	2010	3.966	9248	9	6	66.7	453
	P56	1969	2011	F2	364	2012	2013	2013	723	ABC/3TC/ATV/r	2012	3.686	4849	9	6	66.7	412
	P57	1967	1992	X	362	1994	2000	2014	845	TDF/FTC/NVP	2009	3.071	1177	9	6	66.7	218
	P58	1975	2004	B	292	2006	2006	2014	561	EFV/TDF/FTC	2006	2.880	758	9	3	33.3	0
	P59	1948	2009	X	354	2009	2009	2014	817	ABC/3TC/NVP	2013	3.183	1523	7	2	28.6	0
	P60	1955	2000	X	157	2000	2000	2014	579	TDF/FTC/ATV/r	2009	3.340	2186	5	4	80.0	472
	P61	1978	1996	X	0	1996	2010	2014	493	TDF/FTC/LPV/r	2009	3.671	4691	7	3	42.9	172
	P62	1967	1992	X	362	1994	2000	2014	845	TDF/FTC/NVP	2009	3.071	1177	7	1	14.3	81
	P63	1975	2004	B	292	2006	2006	2014	561	EFV/TDF/FTC	2010	2.880	758	7	0	0.0	0
	P64	1959	2009	B	629	2009	2009	2014	994	EFV/TDF/FTC	2010	2.500	316	7	3	42.9	0
	P65	1970	1996	B	331	1998	2010	2014	1079	ABC/3TC/ATV/r	2010	3.499	3157	7	4	57.1	200
	P66	1976	2004	X	288	2007	2007	2014	819	DRV/r	2014	2.634	431	6	2	33.3	0
	P67	1945	1988	B	9	1997	2002	2014	491	DRV/r/RAL	2011	2.928	847	7	1	14.3	0
	P68	1965	2001	X	182	2002	2002	2014	789	EFV/TDF/FTC	2012	3.539	3460	7	3	42.9	118
	P69	1969	2011	F2	364	2012	2012	2014	723	ABC/3TC/ATV/r	2012	3.686	4849	7	6	85.7	788
	P70	1945	2000	X	141	2000	2000	2014	469	EFV/TDF/FTC	2010	3.627	4232	7	7	100.0	2390
	P71	1970	2005	B	291	2005	2012	2014	493	TDF/FTC/ATV/r	2011	3.509	3226	7	7	100.0	782

Table 1 (*continued*)

Patients	Year of birth	Year of HIV diagnostics	HIV subtype	$CD4^+$ nadir (cells/μl)	Year of first treatment	Year of undetectable status	Year of blood sampling	$CD4^+$ T-cell count (cells/μl)	Last treatment	Year of the beginning of the ongoing treatment	HIV DNA log_{10} copies/10^6 cells	HIV DNA copies/10^6 cells	Number of tested conditions	Number of reactivated patient cell cultures	Frequency of HIV-1 recovery (%)	Median level of HIV RNA copies/ condition
P72	1963	2005	X	237	2005	2008	2014	414	EFV/TDF/FTC	2009	3.714	5177	7	6	85.7	627
P73	1970	2001	Not-B	264	2002	2007	2014	697	ABC/3TC/RAL	2012	3.492	3101	7	3	42.9	0

Characteristics (age, $CD4^+$ T-cell count, $CD4^+$ nadir, antiviral regimens, duration of therapy, duration with undetectable plasma HIV-1 RNA level and HIV-1 subtypes) of patients from the CHU St-Pierre Hospital (Brussels, Belgium) are presented. 'X' indicates not reported. The total HIV-DNA level, the number of reactivated patient cell cultures (genomic viral RNA in culture supernatants higher than 150 or 200 copies/ml depending on tested supernatant volumes), the number of tested conditions, the frequency of positive measurements expressed in percentage and the median level of HIV RNA in the supernatants of cell cultures are also indicated.

positive measurements observed following the diverse LRA treatments and the total number of tested conditions including the mock condition and the positive control (anti-CD3 + anti-CD28 antibodies). We also calculated a median level of extracellular HIV-1 RNA levels measured in supernatants following diverse LRAs, positive control and mock treatments for each patient (Tables 1 and 2).

Total HIV-1 DNA was quantified by the Generic HIV DNA cell kit based on a TaqMan PCR (Biocentric, Bandol, France) [11].

Statistical analyses

Spearman's coefficient (r_s) and corresponding P values were calculated two tailed and with a confidence interval of 95%. Analyses were performed using Prism version 6.0 (GraphPad software, San Diego, California, USA). Statistical significance interpreted as P value 0.05 or less.

Results

Reactivation capacity by latency-reversing agents in ex-vivo cultures of $CD8^+$-depleted peripheral blood mononuclear cells positively correlates with total HIV-1 DNA reservoir size

We first studied the role of the HIV-1 reservoir size in the reactivation capacity by LRAs in ex-vivo cultures of $CD8^+$-depleted PBMCs isolated from 43 cART-treated patients, in the absence of cART in the cell cultures.

As shown in Fig. 1a, we observed a statistically relevant positive correlation between the reservoir size measured by total HIV-1 DNA and the frequency of positive HIV-1 recovery measurements in response to various LRAs, assessed with the Spearman correlation coefficient ($r_s = 0.3730$; $P = 0.0138$). We next examined the correlation between the reservoir size and the median level of extracellular HIV-1 RNA measured in the supernatant of tested conditions for each patient. We also observed a statistically relevant positive correlation ($r_s = 0.4125$; $P = 0.0060$) (Fig. 1b).

Table 2. Characteristics of HIV-1-infected individuals from which $CD8^+$-depleted PBMCs were isolated and stimulated in the presence of combination antiretroviral therapy, named P74–P84.

Patients	Year of birth	Year of HIV diagnostics	HIV subtype	$CD4^+$ nadir (cells/μl)	Year of first treatment	Year of undetectable status	Year of blood sampling	$CD4^+$ T-cell count (cells/μl)	Last treatment	Year of the beginning of the ongoing treatment	HIV DNA log_{10} copies/10^6 PBMCs	HIV DNA copies/10^6 PBMCs	Number of tested conditions	Number of reactivated patient cell cultures	Frequency of HIV-1 recovery (%)	Median level of HIV RNA copies/ condition
P74	1967	2002	X	482	2002	2009	2015	534	FTC/TDF/NVP	2009	3.243	1750	5	5	100.0	1053
P75	1952	1998	X	316	1998	1999	2015	832	FTC/TDF/NVP	2009	2.580	380	5	4	80.0	75
P76	1944	1991	X	15	1991	2002	2015	859	ATV/r/ABC/3TC	2008	3.004	1010	5	5	100.0	63
P77	1969	1996	X	200	1996	1997	2015	554	ABC/3TC/EFV	2006	2.646	443	5	5	100.0	56
P78	1966	1994	X	384	1997	2011	2015	778	EFV/ATV/r	2011	2.958	907	7	7	100.0	769
P79	1960	1993	B	187	1996	2011	2015	830	LPV/r/NVP	2010	2.926	843	7	7	100.0	257
P80	1939	1994	X	272	1994	2002	2015	880	3TC/ZDV/EFV	2002	3.430	2693	5	5	100.0	762
P81	1975	2011	X	10	2011	2012	2015	453	DRV/3TC/ZDV	2012	2.859	723	7	5	71.4	24
P82	1967	1990	B	587	1990	2001	2015	2112	ABC/3TC/ZDV	2002	2.919	830	7	4	57.1	15
P83	1990	2013	X	515	2013	2014	2015	1400	DTG/ABC/3TC	2013	3.074	1187	7	7	100.0	656
P84	1979	2008	B	135	2009	2009	2015	463	EFV/c/TDF/FTC	2014	2.831	677	7	7	100.0	227

Characteristics (age, $CD4^+$ T-cell count, $CD4^+$ nadir, antiviral regimens, duration of therapy, duration with undetectable plasma HIV-1 RNA level, and HIV-1 subtypes) of patients from the CHU St-Pierre Hospital (Brussels, Belgium) are presented. 'X' indicates not reported. The total HIV-DNA level, the number of reactivated patient cell cultures (genomic viral RNA in culture supernatants higher than 15 copies/ml), the number of tested conditions, the frequency of positive measurements expressed in percentage and the median level of HIV RNA in the supernatants of cell cultures are also indicated.

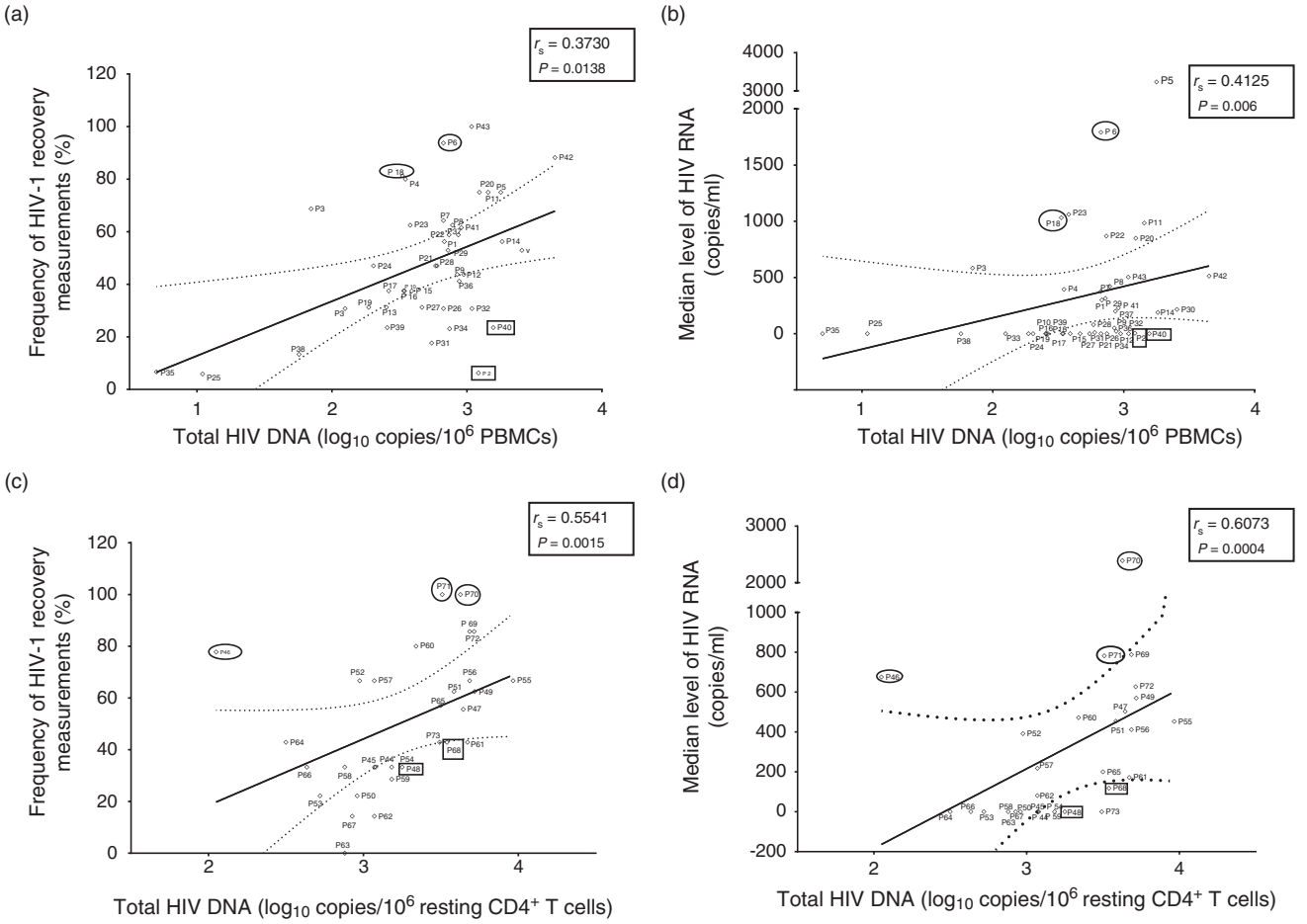

Fig. 1. Representation of the frequency of positive measurements observed in ex-vivo cultures and the median level of HIV RNA (copies/ml) in the supernatants of cell cultures following LRAs, positive control and mock treatments related to total HIV DNA. (a) Representation of the frequency of positive measurements observed in ex-vivo cultures of $CD8^+$-depleted PBMCs following latency-reversing agents, positive control and mock treatments related to total HIV DNA (43 patients from Table 1, P1–P43). We observed a statistically relevant correlation as indicated. We also identified patients with high capacities of cell reactivation despite low level of total HIV-DNA (e.g. P6 and P18, indicated by circles), whereas others presented low cell reactivation capacities relative to their high total HIV-DNA level (e.g. P2 and P40, indicated by rectangles). (b) Representation of the median level of HIV RNA (copies/ml) in the supernatants of cell cultures of $CD8^+$-depleted PBMCs following latency-reversing agents, positive control and mock treatments related to total HIV DNA. The analysis was performed on cultures described in (a) (P1–P43). We observed a statistically relevant correlation as indicated. Patients emphasized in (a) are highlighted in the same way. (c) Representation of the frequency of positive measurements observed in ex-vivo cultures of resting $CD4^+$ T cells following latency-reversing agents, positive control and mock treatments related to total HIV DNA (30 patients from Table 1, P44–P73). We observed a statistically relevant correlation. We also identified patients with high capacities of cell reactivation despite low level of total HIV-DNA (e.g. P46, P70 and P71, indicated by circles), whereas others presented low cell reactivation capacities relative to their high total HIV-DNA level (e.g. P68 and P48 indicated by rectangles). (d) Representation of the median level of HIV RNA (copies/ml) in the supernatants of cell cultures of resting $CD4^+$ T cells following latency-reversing agents, positive control and mock treatments related to total HIV DNA. The analysis was performed on cultures described in (c) (P44–P73). We observed a statistically relevant correlation as indicated. Patients emphasized in (c) are highlighted in the same way.

Reactivation capacity by latency-reversing agents in ex-vivo cultures of resting $CD4^+$ T cells positively correlates with total HIV-1 DNA reservoir size

We confirmed the positive correlations that we observed in $CD8^+$-depleted PBMCs between the HIV-1 reservoir size and either the frequency or the level of reactivation in ex-vivo cultures of resting $CD4^+$ T cells isolated from 30 cART-treated HIV^+ aviremic patients (Fig. 1c and d, respectively). In this case, the correlations were even statistically stronger ($r_s = 0.5541$; $P = 0.0015$ and $r_s = 0.6073$; $P = 0.0004$, respectively) than the correlations we observed in $CD8^+$-depleted PBMCs ($r_s = 0.3730$; $P = 0.0138$ and $r_s = 0.4125$; $P = 0.0060$, respectively).

Altogether, our data strongly established positive correlations between the HIV-1 reservoir size and the ex-vivo capacity of HIV-infected patient cell cultures to be reactivated in response to different classes of LRAs.

However, the correlations were far from perfect correlations. Indeed, we identified HIV$^+$ patients whose frequency of positive measurements and median level of extracellular HIV-1 RNA deviated from linearity relative to their corresponding HIV reservoir size. For example, patients P6 and P18 exhibited a high frequency of positive measurements and a high median level of HIV-1 RNA relative to their corresponding HIV-1 DNA level (circles in Fig. 1a and b, respectively). In contrast, patients P2 and P40 exhibited opposite reactivation capacity relative to their corresponding HIV-1 DNA level (rectangles in Fig. 1a and b). We observed similar data in the resting CD4$^+$ T-cell population for patients P46, P70 and P71 (Fig. 1c and d, circles) and for patients P48 and P68 (Fig. 1c and d, rectangles).

Median level of extracellular HIV-1 RNA after treatment by latency-reversing agents in ex-vivo cultures of CD8$^+$-depleted peripheral blood mononuclear cells in the presence of combination antiretroviral therapy positively correlates with total HIV-1 DNA reservoir size

The fact that some patients escape from the correlations could be explained by the fact that in addition to the size of the HIV-1 reservoir, additional elements could be involved in the reactivation capacity of patient cell cultures, such as variations in the strength of HIV-1 transcriptional repression but also different amplifications of newly produced viruses in cell cultures due to variable capacities of cells to get reinfected by HIV-1 (an effect which is possibly LRA-induced) or to different viral fitness. Therefore, we took advantage of our previous published reactivation experiments in CD8$^+$-depleted PBMCs isolated from 11 aviremic patients (Table 2), performed in the presence of cART to prevent de novo infection by newly produced virions [8], to explore the correlation between the median level of extracellular HIV-1 RNA following LRA treatments and the reservoir size. The correlation between the reservoir size and the frequency of positive HIV-1 recovery measurements was not analyzed as the sensitivity of the HIV-1 RNA quantification in these assays in the presence of cART allowed us to detect very low levels of viral production in almost all the cell cultures.

Interestingly, we observed again a statistically relevant positive correlation ($r_s = 0.6455$; $P = 0.0368$) (Fig. 2). Moreover, this correlation was even stronger than the one observed in the absence of cART. Nevertheless, in these assays in the presence of cART, we also identified patients with high or low capacities of cell reactivation relative to their corresponding level of total HIV-1 DNA (P78 indicated by a circle and P76 indicated by a rectangle, respectively, in Fig. 2).

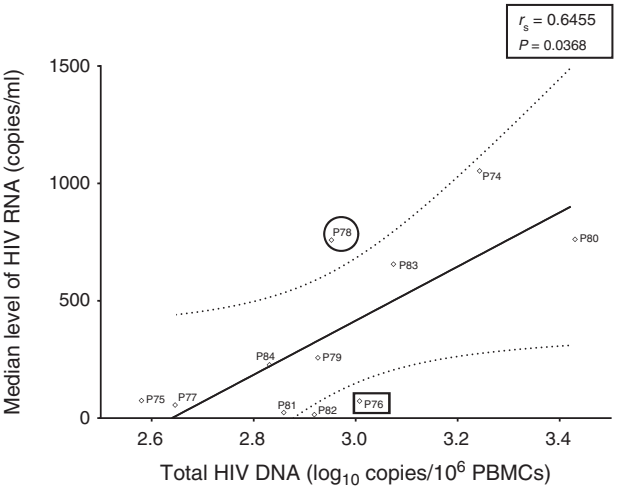

Fig. 2. Representation of the median level of HIV RNA (copies/ml) in the supernatants of CD8$^+$-depleted PBMCs following latency-reversing agents, positive control and mock treatments in the presence of combination antiretroviral therapy related to total HIV DNA (11 patients from Table 2, P74–P84). We observed a statistically relevant correlation as indicated. We identified patients with high or low capacity of cell reactivation relative to their level of total HIV-DNA (P78 indicated by a circle and P76 indicated by a rectangle, respectively).

Consequently, we demonstrated a positive correlation between the HIV-1 reservoir size and the ex-vivo capacity of HIV-infected patient cell cultures to be reactivated in response to different classes of LRAs in the three ex-vivo postintegration latency models evaluated in the present study. However, in these three models, we identified patients who escape from the correlation. The comparison of the results observed in the presence and in the absence of cART supported that these patient-specific variations were likely due to differences in the strength of HIV-1 transcriptional repression and not to different amplifications of newly produced viruses.

Ex-vivo reactivation assays performed in CD8$^+$-depleted peripheral blood mononuclear cells allow the isolation of a greater number of latently infected cells compared with the ex-vivo assay performed in resting CD4$^+$ T cells

The small numbers of latently infected cells found *in vivo* hinders reactivation studies and forces researchers to withdraw great volumes of fresh whole blood from aviremic patients to perform ex-vivo assays. In the two studies [8,9] retrospectively analyzed in this report, we isolated either CD8$^+$-depleted PBMCs or resting CD4$^+$ T cells from 120 ml of blood of cART-treated HIV$^+$ patients. Our reactivation experiments were designed in such a way that we first performed the reactivation assays

in $CD8^+$-depleted PBMCs and selected the most potent and promising LRAs and combinations of LRAs for the ex-vivo reactivation assays performed in resting $CD4^+$ T-cell cultures.

Here, we calculated the median frequency of HIV-1 recovery (median number of the frequency of HIV-1 recovery calculated for each patient and expressed in percentage) in both ex-vivo assays, and we surprisingly found that the percentage of median frequency of HIV-1 recovery was similar in $CD8^+$-depleted PBMCs (47%) and in resting $CD4^+$ T cells (43%) despite the fact that, in the latter cell type, we tested the most potent LRAs and combinations of LRAs (Table 3) and we would therefore expect to reach higher median frequency of HIV-1 recovery. This observation of similar median values obtained in both cell types is due, at least partially, to the higher number of infected cells seeded in the $CD8^+$-depleted PBMCs ex-vivo assays (Table 3). Indeed, the median number of seeded HIV-1 copies per tested condition, obtained by the multiplication of total HIV-1 DNA copies/10^6 cells ($CD8^+$-depleted PBMCs or resting $CD4^+$ T cells) and of the number of plated cells per tested condition, was 4.9 times higher in cultures of $CD8^+$-depleted PBMCs than in cultures of resting $CD4^+$ T cells (Table 3).

In addition, the seeding of $CD8^+$-depleted PBMCs in medium provided a more physiological culture environment. Indeed, the production of cytokines or growth factors by cell types not present in cultures of resting $CD4^+$ T cells could be another possible explanation for the higher frequency of HIV recovery in the $CD8^+$-depleted PBMCs ex-vivo cultures compared with the resting $CD4^+$ T-cell ex-vivo cultures.

Finally, the median number of tested conditions was higher in the $CD8^+$-depleted PBMCs ex-vivo assays as the number of isolated cells was much higher (Table 3). This constitutes another advantage for the use of $CD8^+$-depleted PBMCs instead of resting $CD4^+$ T cells as ex-vivo HIV-1 latency model.

In conclusion, in addition to providing a more physiological culture environment, the ex-vivo assays performed with $CD8^+$-depleted PBMCs allowed us to test a higher number of conditions containing a larger amount of proviruses. Therefore, this model should probably be favored in ex-vivo experiments when many conditions have to be tested.

Discussion

In this report, we grouped and retrospectively analyzed the data from our two very recent HIV-1 reactivation studies [8,9] to assess the role of the HIV-1 reservoir size in the reactivation capacity by LRAs in ex-vivo cultures

Table 3. Comparison of ex-vivo assays in $CD8^+$-depleted PBMCs and in resting $CD4^+$ T cells.

	Year of birth	Year of HIV diagnostics	Median $CD4^+$ nadir	Year of first treatment	Year of undetectable status	Year of blood sampling	Median $CD4^+$ T-cell count (cells/μl)	Year of the beginning of the ongoing treatment	HIV DNA $\log_{10}$ copies/10^6 cells	Median HIV DNA copies/10^6 cells (PBMCs or resting $CD4^+$ T cells, respectively)	Median number of HIV DNA copies/patient cell culture	Median number of tested conditions	Median number of reactivated patient cell cultures	Median frequency of HIV-1 recovery (%)
Medians calculated from cultures of $CD8^+$-depleted PBMCs	1965	2000	230	2000	2006	2013	659	2010	2.826	670	4020	16	8	47
Medians calculated from cultures of resting $CD4^+$ T cells	1967	2001	292	2002	2007	2014	730	2010	3.217	1650	825	8	3	43

Medians were calculated for all patient characteristics from Table 1. Median number of HIV DNA copies/patient cell culture [obtained by the multiplication of total HIV-1 DNA copies/10^6 cells (PBMCs or resting $CD4^+$ T cells, respectively) and of the number of plated cells per tested condition], median numbers of tested conditions, reactivated patient cell cultures and frequency of HIV-1 recovery are also indicated.

of $CD8^+$-depleted PBMCs and of resting $CD4^+$ T cells isolated from cART-treated patients.

We measured the HIV-1 reservoir size using a qPCR-based method for proviral DNA rather than culture-based assays. Indeed, each of these assays has its specific advantages and drawbacks [12]. Culture-based assays and PCR-based assays, respectively, underestimate and overestimate the size of HIV-1 reservoir [12,13]. Even if these assays have their limitations, Kiselinova *et al.* [14] have recently reported important correlations between the viral outgrowth assay and total HIV-1 DNA measures, demonstrating that the total pool of HIV-1 DNA predicts the size of the replication-competent virus in cART-suppressed patients. In addition, multiple recent treatment interruption trials have revealed a correlation between total HIV-1 DNA levels and time to viral rebound following cART interruption [15,16]. Altogether, these studies indicate that PCR-based assays provide an accurate and reproducible estimate of the HIV-1 reservoir size.

We strongly established a statistically significant positive correlation between the size of the HIV-1 reservoir and the frequency of positive HIV-1 recovery measurements in response to various LRAs in ex-vivo cultures of $CD8^+$-depleted PBMCs. We next demonstrated a statistically relevant positive correlation between reservoir size and the median level of extracellular HIV RNA measured in the supernatants of tested conditions for each patient and confirmed these two correlations in ex-vivo cultures of resting $CD4^+$ T cells. To the best of our knowledge, this report constitutes the first demonstration of a strong and statistically relevant positive correlation between the reservoir size and the reactivation capacity by LRAs *ex vivo*, relying on a very large amount of patients ($n = 84$).

However, the correlations were far from perfect correlations. Indeed, we identified HIV^+ patients whose frequency of positive measurements and median level of extracellular HIV-1 RNA deviated from linearity relative to their corresponding HIV reservoir size. We obtained similar results in the presence and in the absence of cART, supporting the notion that the heterogeneity observed between patients was not due to different viral amplifications in cell cultures.

The possibility that the outliers could be due to alterations in primer/probe binding sites is highly unlikely. Indeed, the HIV DNA Biocentric kit we used is based on a TaqMan PCR with primers and internal probe targeting a conserved consensus region in the long terminal repeat of the HIV-1 major group. These primers and probe have proved their ability to quantify the HIV-1 genome of different clades in large cohorts of HIV^+ patients from various resource-limited countries in which several HIV-1 subtypes or circulating recombinant forms are present [17].

Consequently, the heterogeneity that we observed between patients in terms of reactivation capacity of their ex-vivo cell cultures indicates that patients with similar reservoir sizes assessed by total HIV-1 DNA may have differences in the strength of HIV-1 transcriptional repression resulting from the establishment of different molecular mechanisms of viral persistence. For instance, these mechanisms might vary depending on the T-cell subsets, which have been found to be impacted by patient history [18–20]. In this regard, we can imagine that not only the patient characteristics (e.g. genetic background, time to treatment initiation, duration and type of therapy) but also viral specificities and virus–host interaction features may have a significant role in the establishment and the maintenance of the latent reservoirs and their capacity to produce viruses, leading, at the molecular level, to the establishment of a multitude of mechanisms that regulate latency and probably vary from one patient to the other and even from one cell to the other in a single patient.

The patient-to-patient diversity that we observed and which constitutes a major finding of this report raises the possibility that a molecular-based or clinical-based individualized LRA treatment could be more efficient than a nonpersonalized treatment, even if this later treatment is potent ex-vivo. It emphasizes the need to evaluate the efficacy of an LRA first *ex vivo* in cell cultures from a given patient before the administration of this LRA to this given patient *in vivo* in the context of a clinical trial. The efficacy of an LRA in a given patient cannot be generalized to a cohort of patients. In the vast majority of clinical trials aimed at reactivating HIV-1 from latency, a preselection based on HIV-1 DNA reservoir size and on the ex-vivo reactivation assays has not been performed. It might partially explain the failure of these clinical trials. The possibility that the molecular mechanisms of latency vary even from one cell to the other in a single patient also explains that the extent of latency reversal currently achieved in clinical trials using an LRA targeting a single mechanism of viral persistence is insufficient to mobilize significant proportions of the latent reservoir.

In conclusion, the reservoir size is one predictive marker of LRA effectiveness but this parameter alone is not sufficient. The identification of other predictive markers is necessary to predict the success of HIV antilatency approaches. This could allow the selection of the HIV^+ cART-treated patients who could respond to 'shock' strategies.

In this report, we were not able to identify patient characteristics implicated in the patient-specific reactivation variations. Therefore, well designed prospective studies aimed at understanding the potential determinants involved in this interpatient variability are absolutely needed and could lead to identification of key elements in

the race for HIV durable remission or hopefully for HIV cure.

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Conflicts of interest

There are no conflicts of interest.

References

1. Colin L, Van Lint C. **Molecular control of HIV-1 postintegration latency: implications for the development of new therapeutic strategies.** *Retrovirology* 2009; **6**:111.
2. Richman DD, Margolis DM, Delaney M, Greene WC, Hazuda D, Pomerantz RJ. **The challenge of finding a cure for HIV infection.** *Science* 2009; **323**:1304–1307.
3. Shan L, Deng K, Shroff NS, Durand CM, Rabi SA, Yang HC, *et al*. **Stimulation of HIV-1-specific cytolytic T lymphocytes facilitates elimination of latent viral reservoir after virus reactivation.** *Immunity* 2012; **36**:491–501.
4. Van Lint C, Bouchat S, Marcello A. **HIV-1 transcription and latency: an update.** *Retrovirology* 2013; **10**:67.
5. Jiang G, Mendes EA, Kaiser P, Wong DP, Tang Y, Cai I, *et al*. **Synergistic reactivation of latent HIV expression by ingenol-3-angelate, PEP005, targeted NF-kB signaling in combination with JQ1 induced p-TEFb activation.** *PLoS Pathog* 2015; **11**:e1005066.
6. Reuse S, Calao M, Kabeya K, Guiguen A, Gatot JS, Quivy V, *et al*. **Synergistic activation of HIV-1 expression by deacetylase inhibitors and prostratin: implications for treatment of latent infection.** *PLoS One* 2009; **4**:e6093.
7. Laird GM, Bullen CK, Rosenbloom DI, Martin AR, Hill AL, Durand CM, *et al*. **Ex vivo analysis identifies effective HIV-1 latency-reversing drug combinations.** *J Clin Invest* 2015; **125**:1901–1912.
8. Darcis G, Kula A, Bouchat S, Fujinaga K, Corazza F, Ait-Ammar A, *et al*. **An in-depth comparison of latency-reversing agent combinations in various in vitro and ex vivo HIV-1 latency models identified bryostatin-1+JQ1 and ingenol-B+JQ1 to potently reactivate viral gene expression.** *PLoS Pathog* 2015; **11**:e1005063.
9. Bouchat S, Delacourt N, Kula A, Darcis G, Van Driessche B, Corazza F, *et al*. **Sequential treatment with 5-aza-2′-deoxycytidine and deacetylase inhibitors reactivates HIV-1.** *EMBO Mol Med* 2015; **8**:117–138.
10. Bouchat S, Gatot JS, Kabeya K, Cardona C, Colin L, Herbein G, *et al*. **Histone methyltransferase inhibitors induce HIV-1 recovery in resting CD4+ T cells from HIV-1+ HAART-treated patients.** *AIDS* 2012; **26**:1473–1482.
11. Avettand-Fenoel V, Chaix ML, Blanche S, Burgard M, Floch C, Toure K, *et al*. **LTR real-time PCR for HIV-1 DNA quantitation in blood cells for early diagnosis in infants born to seropositive mothers treated in HAART area (ANRS CO 01).** *J Med Virol* 2009; **81**:217–223.
12. Bruner KM, Hosmane NN, Siliciano RF. **Towards an HIV-1 cure: measuring the latent reservoir.** *Trends Microbiol* 2015; **23**:192–203.
13. Ho YC, Shan L, Hosmane NN, Wang J, Laskey SB, Rosenbloom DI, *et al*. **Replication-competent noninduced proviruses in the latent reservoir increase barrier to HIV-1 cure.** *Cell* 2013; **155**:540–551.
14. Kiselinova M, De Spiegelaere W, Buzon MJ, Malatinkova E, Lichterfeld M, Vandekerckhove L. **Integrated and total HIV-1 DNA predict ex vivo viral outgrowth.** *PLoS Pathog* 2016; **12**:e1005472.
15. Azzoni L, Foulkes AS, Papasavvas E, Mexas AM, Lynn KM, Mounzer K, *et al*. **Pegylated Interferon alfa-2a monotherapy results in suppression of HIV type 1 replication and decreased cell-associated HIV DNA integration.** *J Infect Dis* 2013; **207**:213–222.
16. Williams JP, Hurst J, Stohr W, Robinson N, Brown H, Fisher M, *et al*. **HIV-1 DNA predicts disease progression and posttreatment virological control.** *Elife* 2014; **3**:e03821.
17. Rouet F, Chaix ML, Nerrienet E, Ngo-Giang-Huong N, Plantier JC, Burgard M, *et al*. **Impact of HIV-1 genetic diversity on plasma HIV-1 RNA Quantification: usefulness of the Agence Nationale de Recherches sur le SIDA second-generation long terminal repeat-based real-time reverse transcriptase polymerase chain reaction test.** *J Acquir Immune Defic Syndr* 2007; **45**:380–388.
18. Cheret A, Bacchus-Souffan C, Avettand-Fenoel V, Melard A, Nembot G, Blanc C, *et al*. **Combined ART started during acute HIV infection protects central memory CD4+ T cells and can induce remission.** *J Antimicrob Chemother* 2015; **70**:2108–2120.
19. Chomont N, El-Far M, Ancuta P, Trautmann L, Procopio FA, Yassine-Diab B, *et al*. **HIV reservoir size and persistence are driven by T cell survival and homeostatic proliferation.** *Nat Med* 2009; **15**:893–900.
20. Descours B, Avettand-Fenoel V, Blanc C, Samri A, Melard A, Supervie V, *et al*. **Immune responses driven by protective human leukocyte antigen alleles from long-term nonprogressors are associated with low HIV reservoir in central memory CD4 T cells.** *Clin Infect Dis* 2012; **54**:1495–1503.