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Long-Term Spontaneous Control of HIV-1 Is Related to Low Frequency of Infected Cells and Inefficient Viral Reactivation

Nicolas Noel,[a,b,c,d] Ruth Peña,[e] Annie David,[f] Veronique Avettand-Fenoel,[g,h] Itziar Erkizia,[e] Esther Jimenez,[e] Camille Lecuroux,[a,c] Christine Rouzioux,[g,h] Faroudy Boufassa,[i] Gianfranco Pancino,[j] Alain Venet,[a] Carine Van Lint,[k] Javier Martinez-Picado,[e,l,m] Olivier Lambotte,[a,b,c,d] Asier Sáez-Cirión,[f] Julia G. Prado,[e] for the ANRS CO21 Cohort

Université Paris Sud, UMR-1184, Le Kremlin Bicêtre, France[a]; CEA, DSV/iMETI, Division of Immuno-Virology, IDMIT, Fontenay-aux-Rose, France[b]; INSERM, U1184, Center for Immunology of Viral Infections and Autoimmune Diseases, Le Kremlin Bicêtre, France[c]; APHP, Service de Médecine Interne-Immunologie Clinique, Hôpitaux Universitaires Paris Sud, Le Kremlin Bicêtre, France[d]; AIDS Research Institute-IrsiCaixa, Institut d'Investigació en Ciències de la Salut Germans Trias i Pujol, Universitat Autònoma de Barcelona, Badalona, Spain[e]; Institut Pasteur, Unité HIV Inflammation et Persistance, Paris, France[f]; APHP, Hôpital Necker, Service de Virologie, Paris, France[g]; Université Paris Descartes, Sorbonne Paris Cité, Faculté de Médecine, EA7327, Paris, France[h]; INSERM, U1018, Faculté de Médecine Paris Sud, Le Kremlin-Bicêtre, France[i]; Institut Pasteur, Unité de Régulation des Infections Rétrovirales, Paris, France[j]; Service de Virologie Moléculaire, IBMM, Université Libre de Bruxelles (ULB), Gosselies, Belgium[k]; Universitat de Vic, Barcelona, Spain[l]; Institució Catalana de Recerca i EstudisAvançats (ICREA), Barcelona, Spain[m]

ABSTRACT

HIV establishes reservoirs of infected cells that persist despite effective antiretroviral therapy (ART). In most patients, the virus begins to replicate soon after treatment interruption. However, a low frequency of infected cells at the time of treatment interruption has been associated with delayed viral rebound. Likewise, individuals who control the infection spontaneously, so-called HIV-1 controllers (HICs), carry particularly low levels of infected cells. It is unclear, however, whether and how this small number of infected cells contributes to durable viral control. Here we compared 38 HICs with 12 patients on effective combined antiretroviral therapy (cART) and found that the low frequency of infected cells in the former subjects was associated both with less efficient viral reactivation in resting CD4$^+$ T cells and with less efficient virion production *ex vivo*. We also found that a potent HIV-specific CD8$^+$ T cell response was present only in those HICs whose CD4$^+$ T cells produced virus *ex vivo*. Long-term spontaneous control of HIV infection in HICs thus appears to be sustained on the basis of the inefficient reactivation of viruses from a limited number of infected cells and the capacity of HICs to activate a potent HIV-specific CD8$^+$ T cell response to counteract efficient viral reactivation events.

IMPORTANCE

There is a strong scientific interest in developing strategies to eradicate the HIV-1 reservoir. Very rare HIV-1-infected patients are able to spontaneously control viremia for long periods of time (HIV-1 controllers [HICs]) and are put forward as a model of HIV-1 remission. Here, we show that the low viral reservoirs found in HICs are a critical part of the mechanisms underlying viral control and result in a lower probability of HIV-1 reactivation events, resulting in limited HIV-1 release and spread. We found that those HICs in whom viral reactivation and spread from CD4$^+$ T cells *in vitro* were the most difficult were those with diminished CD8$^+$ T cell responses. These results suggest that, in some settings, low HIV-1 reservoirs decisively contribute to at least the temporary control of infection without antiretroviral therapy. We believe that this work provides information of relevance in the context of the search for HIV-1 remission.

So-called human immunodeficiency virus type 1 (HIV-1) controllers (HICs) provide a valuable model of natural, durable control of HIV-1 infection (1). A better understanding of the mechanisms underlying this viral control could help with the development of therapeutic interventions capable of achieving HIV-1 remission in other patients. Numerous reports point to a prominent role of CD8$^+$ T cells in the control of infection observed in HICs. Indeed, many HICs have high frequencies of CD8$^+$ T cells that exert multiple effector functions in response to HIV-1 antigens (2–4). In particular, CD8$^+$ T cells from many HICs efficiently eliminate infected CD4$^+$ T cells *ex vivo* (4). Certain HLA class I alleles, such as B*57 and B*27, are overrepresented in HICs (4–7), but efficient anti-HIV CD8$^+$ T cell responses are not restricted to individuals carrying these alleles (8). In addition, potent HIV-specific CD8$^+$ T cell responses are not found in all HICs, at least during the chronic phase of infection (8, 9). We have found that HIV-specific CD8$^+$ T cell responses in some HICs enrolled in the ANRS CO21 cohort wane over time, yet the plasma viral load remains undetectable (unpublished observations). Similar observations have been made in macaques spontaneously controlling simian immunodeficiency virus (SIV) SIVmac251 infection (10). In HICs, highly responsive CD8$^+$ T cells tend to have an effector phenotype (4, 8, 11), whereas weakly responsive CD8$^+$ T cells tend to have a resting

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Address correspondence to Asier Sáez-Cirión, asier.saez-cirion@pasteur.fr, or Julia G. Prado, jgarciaprado@irsicaixa.es.

A.S.-C. and J.G.P. contributed equally to this article.

memory phenotype (8, 9). Weakly responsive CD8$^+$ T cells from HICs can regain their effector functions upon antigen stimulation (12), but their role in HIV-1 control *in vivo* is unclear. These results suggest that several factors probably contribute to long-term spontaneous HIV-1 control, acting in synergy or relieving each other during the period of control.

We and others have previously shown that despite the presence of replication-competent viruses (13–15), HICs are characterized by low levels of CD4$^+$ T cell-associated HIV DNA (16, 17). Although this may be the consequence of viral control, different results indicate that the low frequencies of HIV-1-infected CD4$^+$ T cells might also contribute to the maintenance of such control. The stochastic nature of HIV-1 reactivation from latency suggests that very low HIV-1 reservoirs might result in at least the temporary control of infection without therapy (18). Along this line, the control of HIV-1 viremia or a delayed viral rebound after the discontinuation of antiretroviral therapy (ART) has consistently been associated with low levels of cell-associated HIV DNA at the time of treatment interruption (19–22), even when a specific anti-HIV immune response was not present (23).

In the present study, we analyzed what the low frequency of HIV-1-infected CD4$^+$ T cells found in HICs may represent in terms of virus reactivation and its contribution to the control of infection. We found that the low number of HIV-1-infected cells in HICs was associated with the less frequent and inefficient reactivation of HIV-1 infection *in vitro* and impaired viral spread. We also found that HICs whose CD4$^+$ T cells did not produce HIV-1 proteins *in vitro* had a diminished HIV-specific CD8$^+$ T cell response, suggesting that inefficient viral reactivation may suffice to maintain, at least temporarily, control of infection in the absence of antiretroviral treatment.

MATERIALS AND METHODS

Patients and samples. We studied 38 HICs from the ANRS CO21 CODEX cohort and 12 patients receiving combined antiretroviral therapy (cART patients) from the Kremlin-Bicêtre University Hospital (France) and the Germans Trias i Pujol Hospital (Badalona, Spain). The HICs were patients who had been infected with HIV-1 for at least the previous 5 years and whose last five consecutive viral loads were below 400 HIV RNA copies/ml of plasma. Their median age at the time of the study was 49 years (interquartile range [IQR], 36 to 74 years), their median CD4$^+$ T cell count was 786 cells/mm^3 (IQR, 515 to 1,203 cells/mm^3), and their median RNA load was <50 HIV RNA copies/ml (IQR, <50 to 213 HIV RNA copies/ml). The cART patients were individuals in whom ongoing antiretroviral treatment had suppressed their viral load (<50 copies HIV RNA/ml) for at least the past 2 years. Their median age at the time of the study was 56 years (IQR, 52 to 57 years), and their median CD4$^+$ T cell count was 677 cells/mm^3 (IQR, 433 to 1,165 cells/mm^3). A detailed description of the patients is provided in Table 1.

Blood samples from HIV-seronegative donors used for viral amplifications experiments were obtained through the collaborative programs between the Institute Pasteur or the AIDS Research Institute-IrsiCaixa and the respective national blood banks.

Ethics statement. All the subjects provided their written informed consent to participate in the study. The CO21 CODEX cohort and this substudy were funded and sponsored by ANRS and approved by the Ile de France VII Ethics Committee. The study was conducted according to the principles expressed in the Declaration of Helsinki.

HIV-1 reactivation from resting CD4$^+$ T cells. Peripheral blood mononuclear cells (PBMCs) were collected by Ficoll gradient centrifugation of 50 ml of fresh EDTA-treated blood. A first negative isolation step for total CD4$^+$ T cells was performed with anti-CD8, -CD14, -CD16 (a and b), -CD19, -CD36, -CD56, -CD123, and -CD235a antibodies together with magnetic Dynabeads (Life Technologies, Foster City, CA, USA). A second negative isolation step was performed after CD25$^+$ CD69$^+$ HLADR$^+$ cell depletion with unlabeled anti-CD25 (clone MA251), anti-CD69 (clone FN50), and anti-HLADR (clone L243) (all from BD Bioscience), followed by Dynabeads separation (Life Technologies, Foster City,

TABLE 1 Clinical and epidemiological characteristics of the study groups[a]

Subject identifier	Sex	Age (yr)	No. of CD4 cells/mm^3	VL (no. of RNA copies/ml)	No. of HIV DNA copies/10^6 PBMCs	Time with VL of <400 RNA copies/ml (yr)
5002	M	54	1,314	<50	11	13
18004	M	47	690	<50	12	14
19001	F	52	702	<50	<10	13
23001	F	53	803	<50	11	13
23002	F	52	649	<50	<10	14
30004	F	41	1,202	<50	10	11
34003	F	45	857	<50	16	12
34006	M	55	554	<40	NA	19
34009	M	40	676	<50	<10	10
34014	F	44	931	79	22	10
34015	M	59	NA	<50	<10	2
34017	F	32	995	<50	<10	7.5
38002	F	42	1,060	<50	<10	11
41002	M	64	756	<50	<10	12
41003	M	55	776	<50	45	11
47003	F	26	620	<50	11	6
51001	F	44	520	<50	<10	12
54001	F	34	892	36	45	12.5
57002	M	48	1,150	42	<10	13
58001	F	56	465	<50	<10	11
58003	F	36	649	<50	17	11
60005	M	48	931	<50	<10	13.5
72002	F	45	1,154	<50	34	15
72003	M	42	495	<50	<10	9
78001	M	55	934	<50	<10	12.5
81003	F	38	882	<50	<10	11
81004	M	50	942	<50	25	11
85002	M	49	1,003	26	30	12
86001	M	72	575	72	<10	11
86003	F	34	1,144	<50	NA	8
86005	F	39	775	<50	11	12
86006	M	52	NA	<50	3	8
86007	F	36	667	<50	NA	13
88001	F	64	752	<50	NA	8
114002	M	65	979	<50	11	10
134001	M	43	1,164	<50	187	13
146001	F	39	464	81	<10	13
171001	M	50	437	<50	<10	9
ART1	M	65	281	<50	NA	21
ART2	M	58	774	<50	NA	16
ART3	M	33	580	<50	NA	6
ART4	F	53	1,151	<50	NA	14
ART5	M	60	437	<50	317	6.5
ART6	M	57	564	<50	183	9.5
ART7	M	56	1,058	<50	<13	6.5
ART8	M	57	420	<50	1,010	9.5
ART9	M	56	385	<50	120	12
ART10	M	50	1,243	<50	233	4.5
ART11	M	55	1,419	<50	103	7.5
ART12	M	47	1,205	<50	167	5.5

[a] NA, data not available; M, male; F, female; VL, viral load.

CA, USA). The purity of the quiescent $CD4^+$ $CD25^-$ $CD69^-$ $HLADR^-$ cells thus isolated was >97%.

The cells were resuspended in RPMI medium with 10% fetal calf serum (R10 medium) at 10^6 cells/ml, and 500,000 cells/well were cultured under the following conditions: prostratin (2.5 μM; Enzo Life Sciences), chaetocin (90 nM; Sigma-Aldrich), suberoylanilide hydroxamic acid (SAHA; 2.5 μM; Alexis Biochemicals), 5-aza-2′deoxicytidine (1 μM; Sigma-Aldrich), 10-[(3-hydroxy-4-methoxybenzylidene)]-9(10H)-anthracenone (HMBA; 5 mM; Sigma-Aldrich), and interleukin-7 (IL-7; 10 ng/ml; R&D Systems). As positive controls, we stimulated resting $CD4^+$ T cells under two conditions: with anti-CD2/anti-CD28 antibodies (monoclonal antibody [MAb] 39C1.5 [rat IgG2a, anti-CD2] plus MAb 6F10.3 [mouse IgG1, anti-CD2] and MAb 28.2 [mouse IgG1, anti-CD28]) (24) and with phytohemagglutinin (PHA; 0.25 mg/ml) plus IL-2 (25 ng/ml). PHA-preactivated allogeneic CD8-depleted T cells from healthy volunteers were used for specific activation. On days 1, 3, and 6, 200 μl of supernatant was collected and replenished with medium alone on day 1 and with medium together with reactivating agents on days 3 and 6. At days 1, 3, 6, and 14, supernatants were collected and stored at −80°C. Viral RNA was extracted from the culture supernatant, and HIV RNA quantification was done in five PCR runs using the ANRS assay (HIV-1 Generic Charge Virale; Biocentric, Bandol, France). The PCR targets a conserved consensus region in the long terminal repeat (LTR) of the HIV-1 major group and has proved suitable for quantification of HIV-1 isolates of different clades (25). The sequences of the forward primer (primer NEC005) and the reverse primer (primer NEC131) were 5′-GCCTCA ATAAAGCTTGCC-3′ and 5′-GGCGCCACTGCTAGAGATTTT-3′, respectively. The internal HIV-1 TaqMan probe (MLC1) MGB LTR (5′-AAGTRGTGTGTGCCC-3′) carried the 5′ reporter 6-carboxyfluorescein and the 3′ quencher 6-carboxytetramethylrhodamine (Applied Biosystems, Foster City, CA). Real-time PCR was performed on a CFX96 device (Bio-Rad), and experimental values were derived from the kit standards as described by the manufacturer.

HIV DNA quantification. Total cell-associated HIV DNA in PBMCs from cART patients and in leukocytes or purified resting $CD4^+$ T cells from HICs was quantified using an ultrasensitive ANRS real-time PCR assay (generic HIV DNA cell kit) as previously described (26). The primers and the probe were the same as those used in the real-time PCR for HIV-1 RNA quantitation. All runs were performed with a 50-ml volume containing DNA extract (1 mg of total DNA), primers and probe (a 200 nM concentration of each), 1× PCR buffer (Platinum 1 quantitative PCR SuperMix-UDG; Invitrogen, Cergy-Pontoise, France), and carboxy-X-rhodamine reference dye (500 nM; Invitrogen). Thermocycling conditions were 2 min at 50°C and 10 min at 95°C, followed by 50 cycles at 95°C for 15 s and 60°C for 1 min. Each entire DNA extract was tested in two to four PCR runs. The results are reported either as the actual HIV DNA copy number per million cells or as 50% of the detection limit for samples in which HIV DNA was undetectable. The detection limit varied according to cell availability (27).

UCA. An ultrasensitive coculture assay (UCA) was applied to purified $CD4^+$ T cells isolated by negative selection from total cryopreserved PBMCs from HICs and cART patients (Miltenyi, Spain). Purified $CD4^+$ T cells were stimulated with a pool of allogeneic irradiated PBMCs at a ratio of 1:5 (20,000 $CD4^+$ cells and 100,000 allogeneic PBMCs per well) in 96-well plates in the presence of PHA (1 μg/ml) and IL-2 (100 U/ml) (Novartis, Spain) for 72 h. Simultaneously, PBMCs from three HIV-seronegative donors were isolated and $CD8^+$ T cells were depleted with RosetteSep human $CD8^+$ depletion cocktail (Stemcell Technologies, France). Pooled CD8-depleted PBMCs were then stimulated under three different conditions (referred to here as 3×3 cells) as previously described (28, 29). After 72 h, the PHA was washed off and the 3×3 cells were cultured in R10 medium containing IL-2 (100 U/ml). On day 7, the culture was scaled up to 48-well plates with the addition of 200 μl of fresh medium. During the following 2 weeks, to maximize viral outgrowth, the cocultures were fed once a week with fresh medium and once a week with 3×3 cells. After 21 days in culture, the supernatants were tested for p24 by enzyme-linked immunosorbent assay (ELISA; PerkinElmer, Spain). p24-negative wells were given a value of zero. Supernatants and cells from positive wells were stored at −80°C.

HIV-1 spread, viral titration, and p24 input assay. PBMCs from three HIV-1-seronegative donors were depleted of $CD8^+$ T cells and stimulated as described above. After 72 h of activation, pellets of 1×10^6 cells were spinoculated for 2 h at 2,000 × *g* and 22°C with UCA supernatants containing ≥10 pg/ml p24 or with HIV_{NL43} as a positive control in the presence of dextran (25 μg/ml). After spinoculation, the cells were resuspended and cultured in R10 medium with IL-2 (100 U/ml; Novartis, Spain). Virus spread in the supernatants was monitored on days 0, 1, 7, and 10 postchallenge. Supernatants containing at least 1×10^6 pg/ml p24 were stored at −80°C for further analysis.

The numbers of 50% tissue culture infective doses ($TCID_{50}$s) per milliliter of three primary viral isolates (two from cART patients and one from an HIC) and the HIV_{NL43} laboratory reference strain were measured in TZM-bl cells. In addition, 3×3 cells were spinoculated with 10-fold serial dilutions of virus (10^5 to 1 pg/ml p24) in the presence of dextran (25 μg/ml). After spinoculation, the cells were extensively washed and cultured in R10 medium with IL-2 (100 U/ml). Virus production in the supernatants was monitored by a p24 ELISA on days 3, 7, and 10 postchallenge.

Sequencing of HIV-1 *nef*. Total DNA was extracted from UCA-positive wells with over 20 pg/ml of p24 for samples from HICs and patients receiving cART by using a QIAamp DNA minikit (Qiagen, Spain), and 721 bp of the *nef* gene was amplified with specific primers as previously described (56). Taking into account that we estimated that less than one cell per well carried proviral DNA in the UCA with $CD4^+$ T cells from HICs and that we performed the PCRs in replicates ($n = 8$), we approximated that each viral sequence underwent a single genome amplification event. In addition, total viral RNA was extracted from virus expanded in the UCA by reverse transcription-PCR with the pairs of primers described above. The sequences were obtained by Sanger reactions using the Macrogen Service (Netherlands) and analyzed with the Sequencher program (version V; Gene Codes). Evolutionary analyses were conducted in MEGA (version 5) software. The number of APOBEC signatures for each patient was calculated with the Highlighter tool from the Los Alamos National Laboratory database, and the sequences were compared to HIV-1 consensus clade B sequences.

Analysis of the HIV-specific $CD8^+$ T cell response. The capacity of $CD8^+$ T cells to suppress infection of autologous $CD4^+$ T cells *ex vivo* was measured as previously described (30). The capacity of $CD8^+$ T cells to suppress HIV-1 replication was calculated as the log of the decrease in the level of p24 production in the presence of $CD8^+$ T cells relative to the level of p24 production in the presence of $CD4^+$ T cells cultured alone.

The frequency of HIV-specific (gamma interferon [IFN-γ]-secreting) $CD8^+$ T cells was estimated with an enzyme-linked immunosorbent spot assay after a brief stimulation with pools of optimal HIV peptides.

Statistical analysis. *P* values were calculated by the Mann-Whitney test. Only significant values ($P < 0.05$) are shown in the figures. Spearman's correlation coefficient (*r*) and the associated two-tailed *P* value were also calculated.

RESULTS

A low frequency of viral reactivation correlates with a low number of latently infected resting $CD4^+$ cells. We first compared the capacity of resting $CD4^+$ T cells from HICs and the capacity of resting $CD4^+$ T cells from patients receiving antiretroviral treatment to reactivate HIV-1 replication. Because of the complex regulation of HIV-1 latency, the reactivation of all fully competent integrated provirus requires repeated stimulation of the cells (31) or the use of different stimuli (14, 32, 33). Therefore, we evaluated HIV-1 reactivation in isolated resting ($HLADR^-$ $CD69^-$ $CD25^-$)

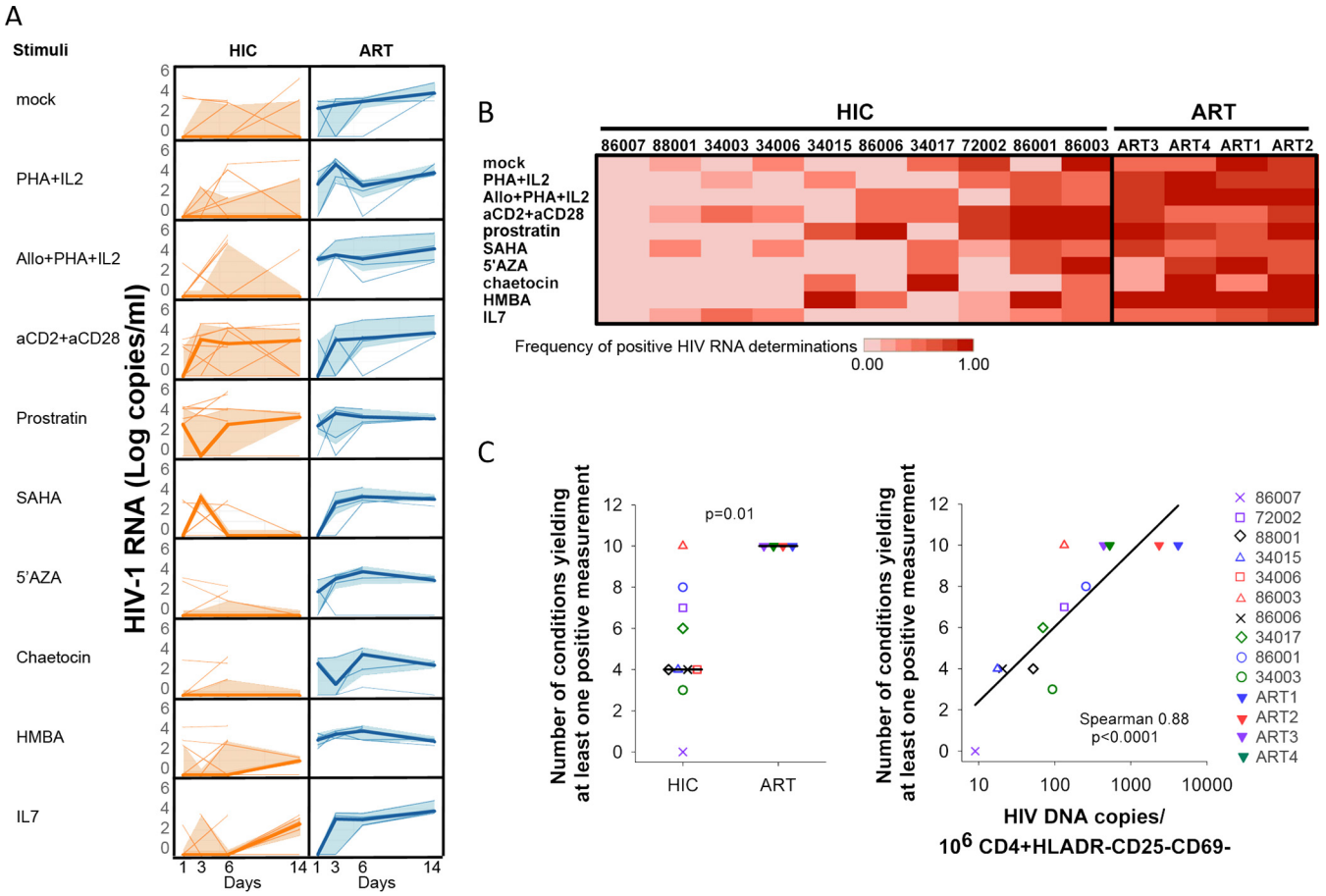


FIG 1 Viral reactivation in resting CD4$^+$ T cells from HICs and cART patients. (A) HIV RNA levels produced by CD4$^+$ T cells from 10 HIV controllers and 4 cART-treated patients over time under the different culture conditions tested. Thin lines, data for individual subjects; thick lines and colored areas, the median and the IQR per group, respectively. (B) The heat map represents the frequency of positive HIV RNA determinations assessed in culture supernatants of CD4$^+$ T cells from HICs and cART patients at different times of culture (days 1, 3, 6, and 14) in the presence of various stimuli. (C) Differences in the number of culture conditions resulting in at least one positive HIV RNA determination at any given time (days 1, 3, 6, and 14) with cells from HICs and cART patients (left) and correlation with the number of HIV DNA copies per million resting CD4$^+$ T cells (right). Each symbol represents one patient (open symbols, HICs; filled symbols, cART patients). The results for HIC 34006 are not included in the correlation due to a technical problem during the quantification of the HIV DNA. Allo, allogeneic cells; 5′AZA, 5-azacytidine.

CD4$^+$ T cells from 10 HICs and 4 cART patients cultured under the following conditions: no stimulus (mock) or stimulus with PHA plus IL-2 with or without allogeneic CD8-depleted PBMCs, anti-CD2 plus anti-CD28, IL-7 (34), prostratin (24, 35), HMBA (36), 5-azacytidine (37), chaetocin (38), and SAHA (24, 39). All the stimuli were periodically replenished in the cultures. The efficiency of viral reactivation, measured in terms of positive HIV-1 RNA levels in the culture supernatants, varied among individuals and conditions. The total proportions of positive reactivations at days 1, 3, 6, and 14 were 0.48, 0.73, 0.9, and 0.93, respectively, for cART patients and 0.22, 0.23, 0.34, and 0.35, respectively, for HICs. Among the positive reactivations, higher median HIV-1 RNA levels per patient were measured at day 14 for cART patients (median number of HIV-1 RNA copies per milliliter at days 1, 3, 6, and 14, 1,126.5 [IQR, 412.5 to 1,530.8], 1,875 [IQR, 857 to 5,323.8], 2,120 [IQR, 618.8 to 13,285], and 5,023.5 [IQR, 1,601.7 to 5,0678.5], respectively; $P = 0.012$) and at day 6 for HICs (median number of HIV-1 RNA copies per milliliter at days 1, 3, 6, and 14, 1,044 [IQR, 311 to 2,536], 2,963[IQR, 2,002.8 to 17,151.8], 5,514.5 [IQR, 697.3 to 41,643.8], and 2,225 [IQR, 1,093.5 to 8,061.5], respectively; $P = 0.3$), although the differences over time were not statistically significantly different for HICs. These results are in agreement with reports showing that repeated stimulation is necessary to induce maximal reactivation (31). While HIV-1 RNA production in cultures of CD4$^+$ T cells from cART patients was usually sustained and HIV-1 RNA accumulated over time, in the case of HICs, HIV-1 RNA was often detected at a single time point or the level of HIV-1 RNA production did not increase over time (Fig. 1A).

Viral reactivation was observed in cells from all the HICs (except HIC 86007) under at least one condition tested (Fig. 1B). The anti-CD2 plus anti-CD28 stimulus often resulted in viral reactivation events in HICs (median proportion of positive reactivations per HIC, 0.5 [IQR, 0.19 to 0.81; $n = 10$]; at least one reactivation event occurred in 8 of 10 HICs), but this difference was not statistically significantly higher than that under the other conditions tested. Viral reactivation occurred in resting CD4$^+$ T cells from cART patients at least at one time point under all conditions tested (Fig. 1B). Overall, the number of conditions activating virus was much higher in cART patients than HICs (Fig. 1C, left) and

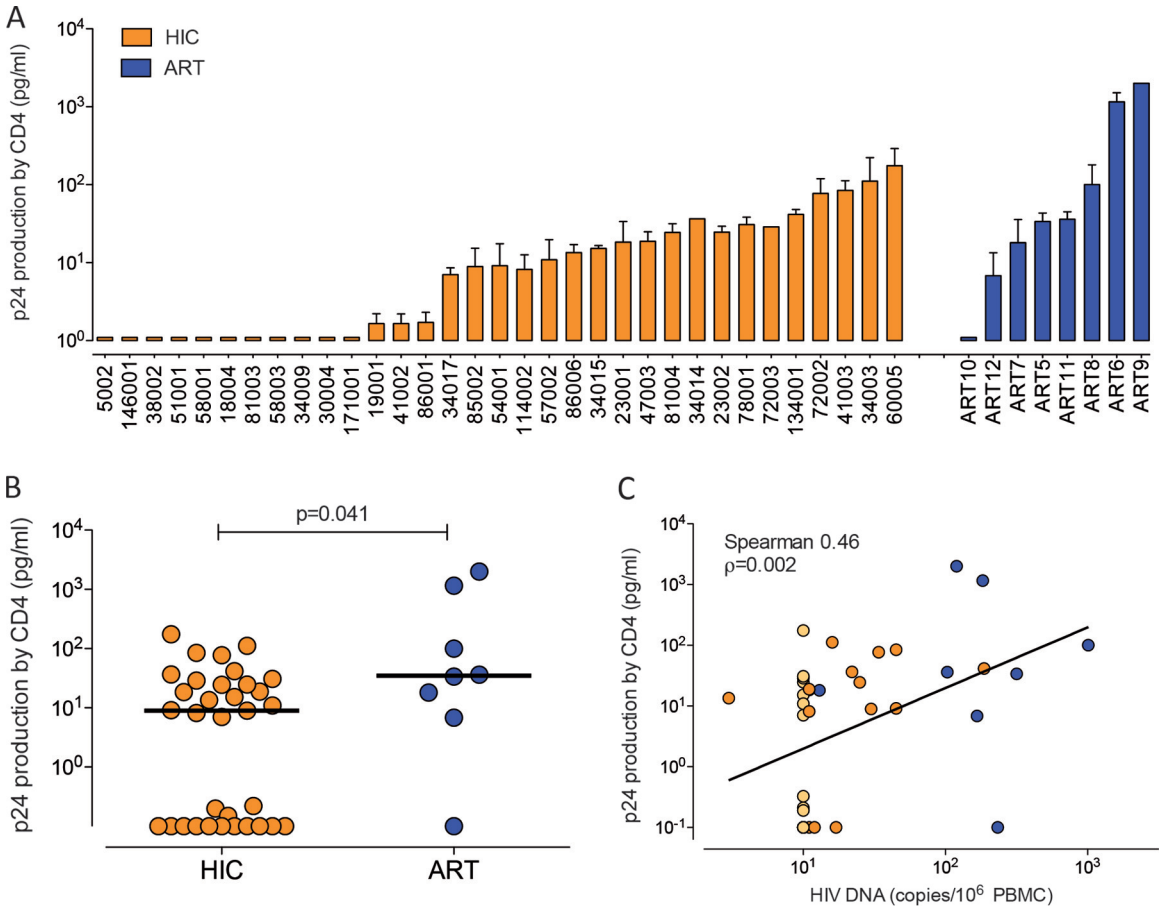


FIG 2 UCA. The UCA was performed with $CD4^+$ T cells isolated by negative selection from cryopreserved PBMCs from 33 HICs and 8 cART patients. (A) Each bar corresponds to one subject and represents the median level of HIV-1 p24 in positive wells. (B) Comparison of levels of p24 production in the UCA with cells from HICs ($n = 33$; orange) and cART patients ($n = 8$; blue). Each circle represents one subject, and the lines indicate mean values. The *P* value was calculated using the Mann-Whitney test. (C) Correlation between the PBMC HIV DNA load and the level of p24 production by $CD4^+$ T cells. Light-colored symbols, cells with HIV DNA levels below the limit of detection, corresponding to HICs; line, data fit to a linear regression. The frequency of p24 positivity was calculated as follows: (number of p24-positive wells/total number of wells tested) × 100. Virus production in the UCA was calculated as the median level of p24 production in positive wells.

was directly related to HIV DNA levels in resting $CD4^+$ T cells for all subjects (Spearman $r = 0.88$, $P < 0.0001$) (Fig. 1C, right) and in the HIC subgroup (Spearman $r = 0.783$, $P = 0.009$).

HIV-1 outgrowth is reduced in HICs and limits viral spread. Besides the difference in the frequency of infected cells, additional factors might contribute to fewer reactivation events in HICs: (i) the differential capacity to produce viral particles, (ii) the resistance of HIC cells to HIV-1 infection (17, 40), or (iii) the lower replicative capacity of the newly produced virions. Although because of sample limitations we could not perform a standard limiting dilution assay, we used an ultrasensitive coculture assay (UCA) to monitor *ex vivo* HIV-1 production by $CD4^+$ T cells from 33 HICs and 8 cART patients. We estimated that less than one cell per well in the UCA of $CD4^+$ T cells from HICs carried HIV-1 DNA, based on the number of cells per well used (median, 0.8 $CD4^+$ T cells carrying HIV-1 DNA per culture [IQR, 0.8 to 1.2 $CD4^+$ T cells carrying HIV-1 DNA per culture], considering that all the HIV-1 DNA measured in the PBMCs was in $CD4^+$ T cells, which made up ~25% of the PBMCs, and that only one copy was present per infected cell). We detected the p24 antigen in the culture supernatants of $CD4^+$ T cells from 22 HICs (66.6%) and 7 cART patients (87.6%) (Fig. 2A). Although fewer HIC $CD4^+$ T cells than cART patient $CD4^+$ T cells were analyzed in total (860,000 [IQR, 490,000 to 1,700,000) versus 1,880,000 [IQR, 1,700 000 to 2,240,000]), because of the limited availability of cells from HICs, no difference in the frequency of p24-positive cultures was found (medians, 4.8% [IQR, 0 to 9.1%] and 4.1% [IQR, 2.2 to 9.3%] for HIC and cART patients, respectively; $P = 0.73$). In contrast, cells from HICs produced significantly less p24 antigen than cells from cART patients (medians, 9.03 pg/ml p24 [IQR, 9.29 to 35.8 pg/ml p24] for HICs and 34.97 pg/ml p24 [IQR, 208.7 to 1,047 pg/ml p24] for cART patients; $P = 0.041$) (Fig. 2B). A trend toward a lower level of p24 production by cells from HICs was also observed when only p24-positive cultures were considered (medians, 18.6 pg/ml p24 [IQR, 8.7 to 37.7 pg/ml p24] and 36.2 pg/ml p24 [IQR, 18.1 to 1,158 pg/ml p24] for HICs [$n = 22$] and cART patients [$n = 7$], respectively; $P = 0.1$). A direct correlation between the PBMC HIV DNA load and the amount of p24 produced in culture was found (Spearman $r = 0.46$, $P = 0.002$) (Fig. 2C). These data show that HIV-1 can be reactivated from HIV-1 controllers' cells in the presence of heterologous cells but that virion production is often inefficient.

To further evaluate if differences in virus reactivation and production in HICs and cART patients affect viral spread, we infected preactivated primary allogeneic CD8 cell-depleted PBMCs with supernatants from the UCA containing ≥10 pg/ml of p24. Viral spread in cells that were in culture for a week was monitored by a p24 ELISA. Our data showed a lower frequency of spread of infections in samples from HICs than samples from cART patients (3% for HICs versus 21.4% for cART patients; $P = 0.045$) (Fig. 3A and B). To evaluate if viral spread was associated with a threshold of virus production from $CD4^+$ T cells, we performed a p24 limiting dilution infectivity assay and tested the growth of virus from one HIC (HIC 60005) and two cART patients (patients ART5 and ART8) in cells in comparison with that of HIV_{NL43}. We found a direct correlation between viral input and viral outgrowth (Spearman $r = 0.77$, $P < 0.0001$) (Fig. 3C), where sustained viral outgrowth was observed only at p24 values of $>10^3$ pg/ml, confirming the existence of a p24 threshold for successful viral spread. Additionally, by using a nonlinear fit of the data, we estimated that 380.18 pg/ml of p24 is the half-maximal effective concentration (EC_{50}) required to achieve viral spread in 50% of cultures (Fig. 3D). This amount is well above the median level of production observed for $CD4^+$ T cells in HICs (9.03 pg/ml) and further supports the suggestion that low viral spread from HIC $CD4^+$ T cell cultures is limited by low levels of particle release after reactivation.

To evaluate whether differences in viral spread in HICs and cART patients could be due to the presence of defective virus, we extracted DNA and RNA from UCA wells containing cells from HIC 60005 and cART patients ART5 and ART8, whose viruses were able to infect allogeneic cells. We also analyzed cells from four additional subjects, three HICs (HICs 34009, 134001, and 57002) and one cART patient (patient ART8), whose virus did not spread. To increase the chances of amplification, we focused on the gene *nef*. As shown in Fig. 3E, we observed monophyletic groups of viruses with a lower mean population diversity in HICs than cART patients (0.022 for HICs versus 0.061 for cART patients). These results corroborate previous experimental data that identified in HICs monoclonal populations with low levels of diversity (41). Moreover, no defects in the *nef* reading frame were found in any of the study groups. An exception was HIC 34009, where two large deletions in *nef* (19 and 16 amino acids) were observed (Fig. 3E, right). No differences in APOBEC signatures were found between viruses from HICs and cART patients (not shown), dismissing the potential role of hypermutation in the differential spread potential of the viruses from both groups. Taken together, our data show that the production of fit viruses by $CD4^+$ T cells is stochastic and highly dependent on the total frequency of infected cells. Viral reactivation in cells from HICs was difficult and resulted in only the transient release of viral particles that was not sufficient to ensure sustained viral spread.

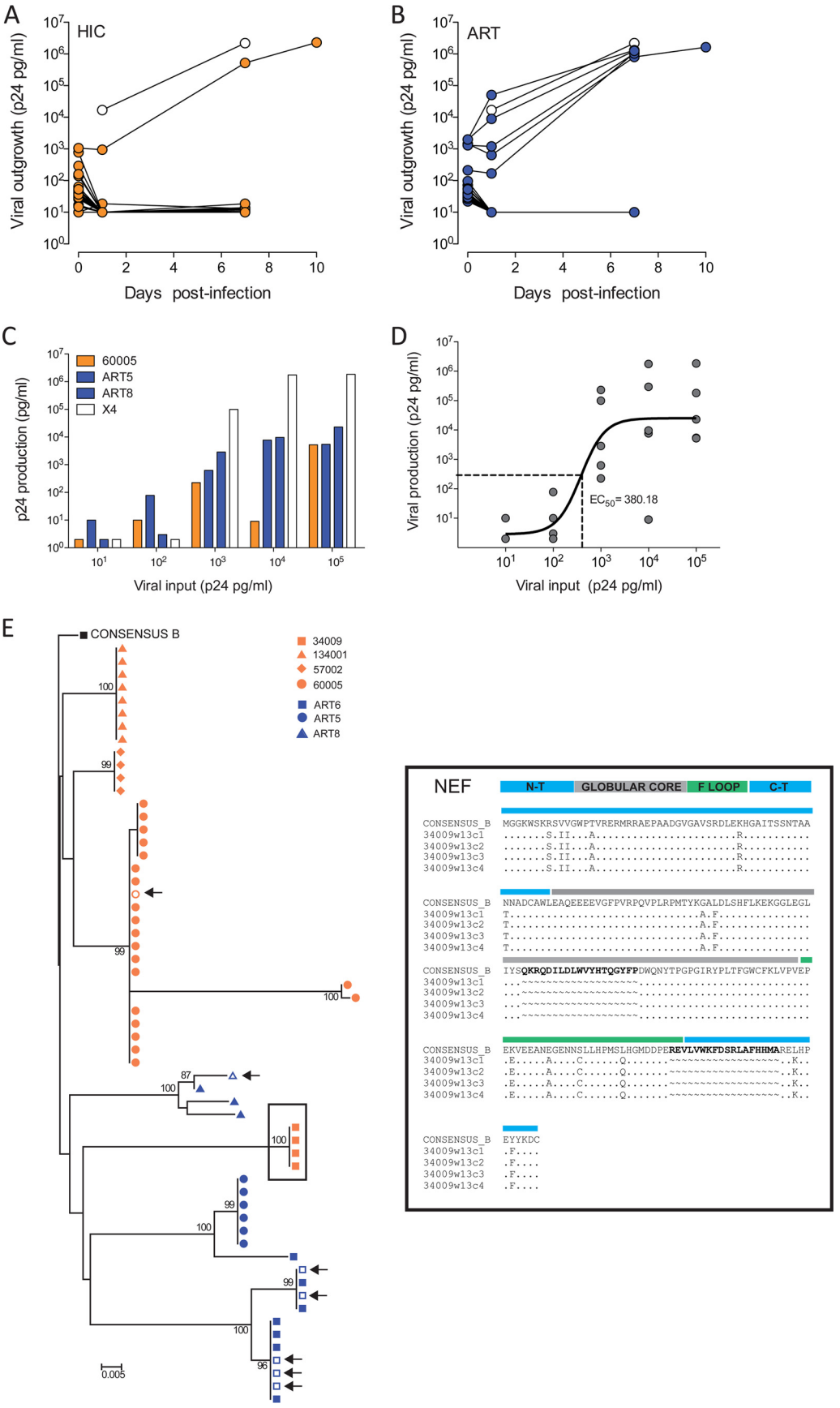
The amplitude of $CD8^+$ T cell responses is reduced in HIC virus nonproducers. We then examined whether the different amplitudes of HIV-specific $CD8^+$ T cell responses that we and others have observed in HICs (8, 9, 42) were related to the capacity of these patients' $CD4^+$ T cells to produce viral particles *ex vivo*. Viral producers were defined as those HICs with p24 values in the supernatant over the limit of detection of the UCA. We found that the HIV-specific $CD8^+$ T cell responses of HICs whose $CD4^+$ T cells did not produce viral particles in the UCA did not differ from those of cART patients (9, 43). In particular, $CD8^+$ T cells from the latter HICs did not efficiently suppress HIV-1 infection of autologous $CD4^+$ T cells (Fig. 4A), a property considered characteristic of $CD8^+$ T cells from HICs (4, 30, 44). Similarly, the frequency of circulating HIV-specific (IFN-γ-producing) $CD8^+$ T cells in the same HICs was low (Fig. 4B). In contrast, HICs whose $CD4^+$ T cells efficiently produced viral particles *ex vivo* had potent $CD8^+$ T cell responses (Fig. 4A and B). Positive correlations were found between the capacity of $CD8^+$ T cells from HICs to suppress HIV-1 infection and the level of p24 produced by their $CD4^+$ T cells *in vitro* (Fig. 4B) and between the HIV-suppressive capacity or the frequency of HIV-specific $CD8^+$ T cells and the frequency of p24-positive cultures (Spearman $r = 0.48$ [$P = 0.006$] and Spearman $r = 0.46$ [$P = 0.007$], respectively; not shown).

DISCUSSION

The results reported in this study provide functional evidence of the stochastic nature of HIV-1 reactivation and how it may contribute to the maintenance of virological control in the absence of antiretroviral treatment. In particular, we show that the lower that the frequency of infected resting $CD4^+$ T cells in HICs is, the lower that the probability of viral reactivation, viral production, and viral spread *in vitro* compared to that in cART patients is.

Our results suggest that natural control of HIV-1 infection can be maintained at least at two different levels. Strong but incomplete viral control would result in low-level virion release from infected cells, sustaining the strong $CD8^+$ T cell responses observed in many HICs. This response, in turn, readily controls the infection, maintaining a steady state between infected cells and the immune system (Fig. 5A), which may result in residual systemic activation and mild progression in the absence of therapy (45–47). The virtuous circle between the virus and the host response would be interrupted when the frequency of latently infected $CD4^+$ T cells falls to a very low level, hindering the stochastic reactivation of fit viruses (18) and leading to fewer viral amplification events. This would result in the contraction of $CD8^+$ T cell responses, at least transiently, due to the absence of the antigenic boost (Fig. 5B), as has indeed been reported in some HICs after ART initiation (48). This implies that a low frequency of latently infected cells may be sufficient to transiently prevent viral outgrowth, even in the absence of a major contribution of the immune system, as recently reported in two patients in Boston, MA, and a baby in Mississippi (23, 49). This phenomenon may be particularly relevant and efficient in the long term in HICs, whose target cells are also strongly resistant to HIV-1 infection, particularly at low infectious doses (17, 40), providing an additional barrier to viral amplification. In addition, weakly responsive memory $CD8^+$ T cells in HICs are ready to react and expand promptly in response to stochastic viral amplification events (12, 50) (Fig. 5C), thereby ensuring natural control of HIV-1 infection in the very long term.

The levels of cell-associated HIV-1 DNA have been shown to predict progression to disease and the capacity to control infection after treatment interruption (19, 22, 51–53). Although measurement of total HIV-1 DNA levels may take into account many copies of replication-deficient viruses, it is likely that under conditions of very low levels of viral replication, as in HICs, differences in the levels of HIV-1 DNA probably maintain proportionality between replication-competent and noncompe-


A
HIC
Viral outgrowth (p24 pg/ml)
Days post-infection
B
ART
Viral outgrowth (p24 pg/ml)
Days post-infection
C
60005
ART5
ART8
X4
p24 production (pg/ml)
Viral input (p24 pg/ml)
D
Viral production (p24 pg/ml)
EC50= 380.18
Viral input (p24 pg/ml)
E
CONSENSUS B
34009
134001
57002
60005
ART6
ART5
ART8
0.005
NEF
N-T
GLOBULAR CORE
F LOOP
C-T
CONSENSUS_B MGGKWSKRSVVGWPTVRERMRRAEPAADGVGAVSRDLEKHGAITSSNTAA
34009w13c1 .....S.II...A.....R.....
34009w13c2 .....S.II...A.....R.....
34009w13c3 .....S.II...A.....R.....
34009w13c4 .....S.II...A.....R.....
CONSENSUS_B NNADCAWLEAQEEEEVGFPVRPQVPLRPMTYKGALDLSHFLKEKGGLEGL
34009w13c1 T.....A.F.....
34009w13c2 T.....A.F.....
34009w13c3 T.....A.F.....
34009w13c4 T.....A.F.....
CONSENSUS_B IYSQKRQDILDLWVYHTQGYFPDWQNYTPGPGIRYPLTFGWCFKLVPVEP
34009w13c1 ...~~~~~~~~~~~~~~~~~~~.....
34009w13c2 ...~~~~~~~~~~~~~~~~~~~.....
34009w13c3 ...~~~~~~~~~~~~~~~~~~~.....
34009w13c4 ...~~~~~~~~~~~~~~~~~~~.....
CONSENSUS_B EKVEEANEGENNSLLHPMSLHGMDDPEREVLVWKFDSRLAFHHMARELHP
34009w13c1 .E.....A....C.....Q.....~~~~~~~~~~~~~~~~~~..K..
34009w13c2 .E.....A....C.....Q.....~~~~~~~~~~~~~~~~~~..K..
34009w13c3 .E.....A....C.....Q.....~~~~~~~~~~~~~~~~~~..K..
34009w13c4 .E.....A....C.....Q.....~~~~~~~~~~~~~~~~~~..K..
CONSENSUS_B EYYKDC
34009w13c1 .F....
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34009w13c3 .F....
34009w13c4 .F....

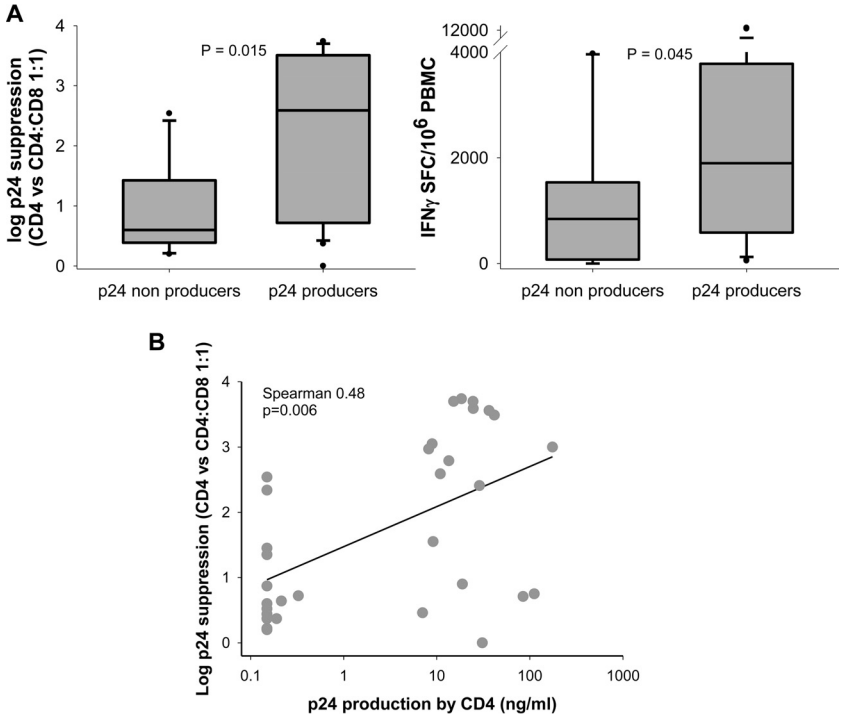

FIG 4 HIC HIV-specific $CD8^+$ T cell responses according to the amount of p24 produced by their $CD4^+$ T cells *in vitro*. The responses of HIC $CD8^+$ T cells tested in the UCA were analyzed *ex vivo*. HICs were classified as p24 producers or nonproducers depending on whether or not p24 was produced by their $CD4^+$ T cells in the UCA. (A) (Left) $CD8^+$ T cell capacity to suppress infection of autologous $CD4^+$ T cells, as measured by the decrease in the level of p24 production in the presence of unstimulated autologous $CD8^+$ T cells ($n = 32$; one value is missing, owing to unsuccessful $CD4^+$ T cell infection). (Right) Frequency of IFN-γ-producing cells after PBMC stimulation with optimal HIV-1 peptides. The box plots represent the medians; 10%, 25%, 75%, and 90% ranges; and outliers. SFC, spot-forming cells. (B) Correlation between $CD8^+$ T cell capacity to suppress HIV-1 infection and p24 production by $CD4^+$ T cells. Each symbol represents one HIC. The line indicates data fit to a linear regression.

tent proviruses, as recently observed in treated patients (54). This assumption is supported, despite limitations in the total numbers of $CD4^+$ T cells tested, by the direct correlation between the levels of cell-associated HIV-1 DNA and HIV-1 reactivation, measured by determination of the levels of HIV-1 RNA or p24 production in culture supernatants, that was found when $CD4^+$ T cells were split into parallel cultures and were subjected to different repeated stimuli (with polyclonal or allogeneic stimuli or the provision of latency-reversing agents).

We did not find significant differences in the hypermutation rates or defective sequences between the viruses from HICs and those from cART patients. However, the difference in the number of infected cells between HICs and cART patients resulted in different levels of virion production after activation, which ultimately would result in a lower probability of the spread of infections for HICs. The reduced cellular susceptibility to HIV-1 infection in HICs may contribute to the lower levels of infected cells found in these patients (17). Moreover, the block of HIV-1 replication in HIC $CD4^+$ T cells has been shown to affect preintegration steps of the replication cycle (17, 40, 55) but also mRNA transcription from the integrated viruses (40), which could explain the reduced levels of viral particle production observed in HICs. Further mechanistic studies on HIV-1 transcriptional steps or particle production in HICs will contribute to a clarification of these findings.

Our study has some limitations. First, our analysis focused on

FIG 3 HIV-1 spread from UCA wells and viral p24 limiting dilution input assay. (A and B) Viral spread in UCA wells containing >10 pg/ml of p24 from activated CD8-depleted PBMCs was monitored on days 0, 1, 7, and 10 postinfection by p24 ELISA of the supernatants of cultures of cells from HICs (A) and cART patients (B). (C) The outgrowth of HIV-1 from subjects 60005, ART5, and ART8 and the NL43 (X4) virus strain was measured in cells receiving 10-fold dilutions of input p24 ranging from 10^5 to 1 pg/ml. The viral outgrowth represented in the graphs was calculated as follows: (amount of p24 at day 7 postinfection) – (amount of p24 at day 3 postinfection), where the amount of p24 is given in picograms per milliliter. (D) Nonlinear regression of p24 input and production. Line, fit of the data; dashed line; half-maximal effective concentration (EC_{50}). (E) (Left) Phylogenetic tree determined by the neighbor-joining method of *nef* sequences from HICs (HICs 34009, 134001, 57002, and 60005) and cART patients (cART patients ART5, ART6, and ART8). Sequences were obtained from proviral DNA (filled symbols) from UCA wells and viral RNA in successfully spreading infections, indicated by arrows (empty symbols). Sequences were aligned to the consensus clade B sequence using the BioEdit package, and the neighbor-joining tree was built with MEGA (version 5) software over 1,000 replicates. Mean evolutionary diversity analyses for the entire population were conducted using the maximum composite likelihood model. The analysis involved 33 nucleotide sequences for HICs and 22 nucleotide sequences for cART patients. Codon positions included were the 1st, 2nd, and 3rd positions and the noncoding sequence. All positions containing gaps and missing data were eliminated. There were a total of 610 positions in the final data set. The tree is rooted to the consensus clade B sequence. Only bootstrap values over 70 are represented. (Right) Nef amino acid sequence alignment of 34,009 clonal sequences shown at the top. Bold, deletions compared to the consensus sequence; colored regions, functional domains of the Nef protein (blue, N-terminal and C-terminal regions; gray, globular core; green, flexible [F] loop).

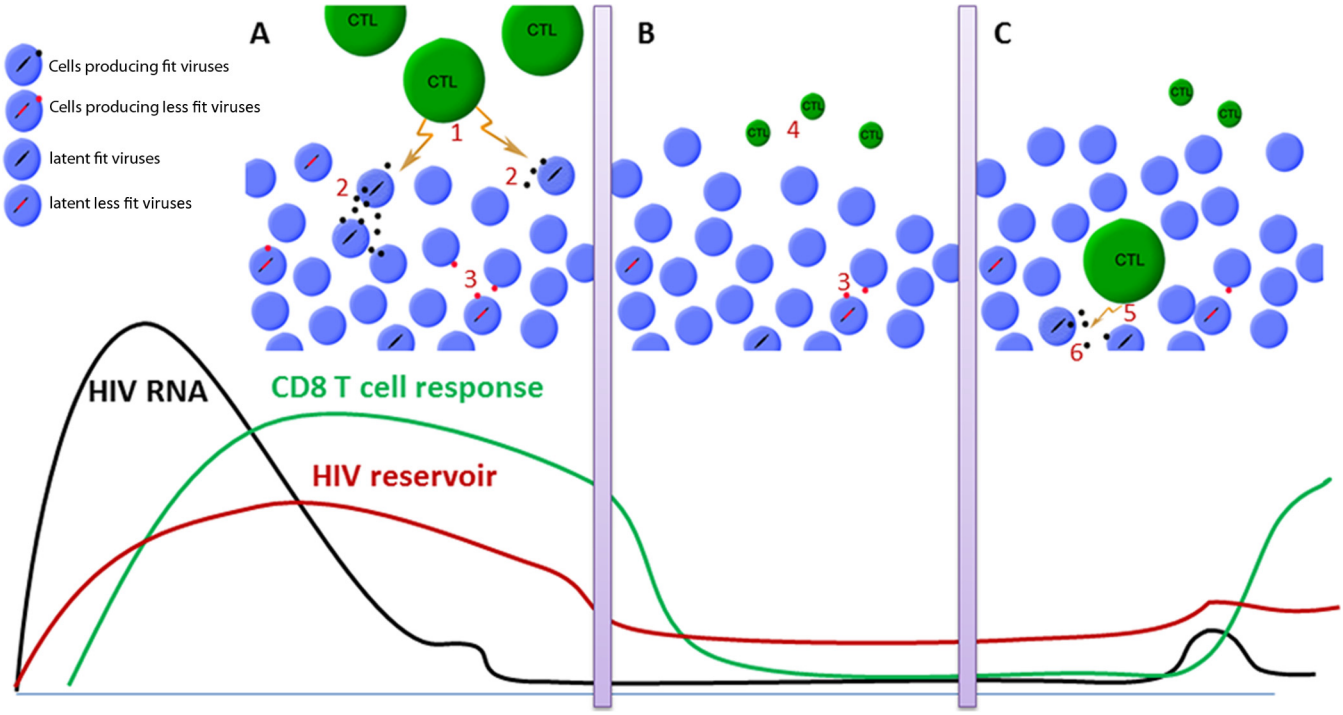

FIG 5 (A) Most HICs are able to generate potent HIV-specific effector CD8$^+$ T cell responses (marker 1) that efficiently eliminate HIV-infected cells, allowing the control of viral replication and avoiding the replenishment of the viral reservoirs (marker 2). A virtuous circle between low-level viral replication and the CD8$^+$ T cell response that ensures long-term control of infection is established. (B) We hypothesize that in some HICs most cells carrying fit viruses are intensely eliminated by a combination of the cytopathogenic effects of the viruses and the HICs' efficient CD8$^+$ T cell responses. Considering the stochastic character of viral activation, the reactivation of fit viruses in these HICs would be rare, and more often, activation events would involve less fit viruses that result in a small viral burst insufficient to transmit infection (marker 3). In HICs, this phenomenon is reinforced by the intrinsic resistance of their cells to infection with low doses of viruses. In such a context, the control of infection would be related to rare and inefficient viral reactivation events and the CD8$^+$ T cell response, which would no longer be required to maintain control, shrink, and deactivate (marker 4). (C) These cells, however, retain the capacity to expand and again exert effector functions (marker 5) if a viral amplification event occurs (marker 6). CTL, cytotoxic T lymphocyte.

CD4$^+$ T cells not expressing activation markers *ex vivo*, which excluded other cell types that may contribute to the reservoir size. Second, our findings are limited to peripheral blood. The level/frequency of reactivatable competent viruses in the tissues of some HICs with low levels of viremia (i.e., HIC 146001) might be different from that in peripheral blood and might explain the absence of virus induction *in vitro*. Our analyses of viral sequences were limited to a small number of sequences and capture the diversity associated only with the *nef* region after *ex vivo* culture. Therefore, additional defects in other regions of the viral sequence were missed and potential artifacts due to *in vitro* viral expansion may have been introduced. However, other, deeper studies have not detected, in general, major defects in the viruses from HICs (15).

Overall, our results suggest that limiting the frequency of infected cells could suffice to achieve temporary control of infection in the absence of antiretroviral treatment. However, additional intrinsic and/or immune mechanisms are needed to reinforce this effect and ensure HIV-1 control in the long term.

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