Combined ART started during acute HIV infection protects central memory CD4+ T cells and can induce remission

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Background: Therapeutic control of HIV replication reduces the size of the viral reservoir, particularly among central memory CD4+ T cells, and this effect might be accentuated by early treatment.

Methods: We examined the effect of ART initiated at the time of the primary HIV infection (early ART), lasting 2 and 6 years in 11 and 10 patients, respectively, on the HIV reservoir in peripheral resting CD4+ T cells, sorted into naive (TN), central memory (TCM), transitional memory (TTM) and effector memory (TEM) cells, by comparison with 11 post-treatment controllers (PTCs).

Results: Between baseline and 2 years, CD4+ T cell subset numbers increased markedly (P = 0.004) and HIV DNA levels decreased in all subsets (P = 0.009). TTM cells represented the majority of reservoir cells at both timepoints, T cell activation status normalized and viral diversity remained stable over time. The HIV reservoir was smaller after 6 years of early ART than after 2 years (P = 0.019), and did not differ between PTCs and patients treated for 6 years. One patient, who had low reservoir levels in all T cell subsets after 2 years of treatment similar to the levels in PTCs, spontaneously controlled viral replication during 18 months off treatment.

Conclusions: Early prolonged ART thus limits the size of the HIV reservoir, protects long-lived cells from persistent infection and may enhance post-treatment control.

Keywords: primary HIV infections, post-treatment controllers, HIV DNA, reservoirs

Introduction

Attempts to eradicate HIV infection are hindered by the persistence of a latent viral reservoir in infected patients.1 Primary HIV-1 infection (PHI) is characterized by exponential viral replication, resulting in intense immune activation2 and massive CD4+ T cell depletion.3–5 HIV reservoirs are believed to be established at the early Fiebig stages I/II, defined by positive HIV RNA, negative HIV western blot and positive HIV ELISA for stage I plus positive antigenemia P24 for stage II,6–8 as early as 10 days after symptoms onset.4 Initiation of suppressive ART during PHI (early ART) has numerous benefits and is now recommended in several guidelines worldwide.9–11 Early ART preserves the immune system both...
quantitatively and qualitatively,\textsuperscript{12,13} notably enhancing the recovery of CD4\textsuperscript{+} T cell numbers and functions.\textsuperscript{14,15} Moreover, early ART also prevents infection of resting CD4\textsuperscript{+} T cells and limits cell-associated HIV DNA levels.\textsuperscript{16} resulting in a more rapid and profound decline in the HIV reservoir size than when ART is started during the chronic phase of infection.\textsuperscript{8,15} the effectiveness of ART is therefore strongly dependent on the timing of its initiation.\textsuperscript{17,18} In addition, early ART impacts rebound of the HIV RNA level during treatment interruption, allowing greater time off therapy.\textsuperscript{19,20} Among long-term-treated patients, central memory CD4\textsuperscript{+} T (TCM) cells are the major cellular reservoirs;\textsuperscript{21} while in HIV controllers TCM cells are protected and contribute minimally to the reservoir, particularly among those carrying protective alleles such as HLA-B*27 and/or B*57.\textsuperscript{22} Our group has previously shown that early therapy may be associated with sustained HIV control after treatment discontinuation in so-called post-treatment controllers (PTCs) from the VISCONTI cohort, who presented remission with an extremely low level of HIV reservoir, particularly in long-lived naive (TN) and TCM cell subpopulations.\textsuperscript{23,24} We also recently reported that, in acute infection, HIV is widely distributed among all CD4 T cell subsets, including TN and TCM cells.\textsuperscript{25,26}

However, the precise impact of ART initiated at the time of the primary HIV infection (early ART) on HIV reservoir size and composition remains unclear. Therefore, we studied 2 years of early initiated ART alters immunovirological outcomes and HIV reservoir characteristics compared with patients who received 6 years of ART and patients who were PTCs.\textsuperscript{23}

**Methods**

**Ethics**

With their written informed consent, peripheral blood and rectal biopsy samples were collected from HIV-1-infected adults (18 years or older) included in research centres participating in the OPTIPRIM ANRS-147 clinical trial (ID RCB: 2009-014742-28) and the VISCONTI ANRS EP47 study. The studies were approved by the French Health Products Safety Agency and complied with the Declaration of Helsinki.

**Study design**

HIV-1-infected subjects with PHI were included in the randomized ANRS-147 OPTIPRIM trial conducted in 33 hospitals throughout France and involving adult participants presenting with PHI. Recruitment began in April 2010 for a planned period of 2 years and was completed in July 2011, with follow-up until December 2013 for the last patient. The primary endpoint was the difference between the two arms in the HIV DNA level per million PBMCs at month 24 (M24). Using data from the ANRS C06 PRIMO cohort of patients with PHI,\textsuperscript{27} we calculated that we would need to enrol 90 patients in order to achieve 80% power to detect a difference in HIV DNA levels of at least 0.42 log\textsubscript{10} copies per 10\textsuperscript{6} PBMCs between the two arms at M24.\textsuperscript{28} The first 12 randomized patients from the OPTIPRIM study who agreed to participate in this HIV reservoir substudy were enrolled immediately (2 year group), receiving either an intensified five-drug regimen (tenofovir/emtricitabine, darunavir/ritonavir 800/100 added to raltegravir 400 mg twice daily and maraviroc 150 mg twice daily; arm 1) or a three-drug regimen (tenofovir/emtricitabine and darunavir/ritonavir 800/100 once daily; arm 2). To be enrolled in the trial, patients had to present with biological criteria of acute infection and/or symptomatic PHI, as previously described.\textsuperscript{29}

To determine whether post-treatment control could be observed within a randomized clinical trial, treatment interruption was scheduled at month 24, when patients had reached a viral load of <50 copies/mL and >500 CD4\textsuperscript{+} T cells/mm\textsuperscript{3}. Treatment resumption was recommended when the HIV RNA level rose above 50 000 copies/ml or the CD4\textsuperscript{+} cell count fell below 500 cells/mm\textsuperscript{3} or 30%. Co-enrolment in the ANRS PRIMO cohort was proposed to all patients in order to organize post-trial follow-up. Blood samples, socio-demographic and clinical data were collected before and after 2 years of treatment, and during treatment interruption (month 24 to month 30).

The objective of the substudy was to describe the reservoir at the time of the PHI and the impact of two different treatment arms on the CD4\textsuperscript{+} T cell subset reservoir. The second objective was to compare in a cross-sectional study the HIV DNA levels measured after 2 years of treatment with the levels in the VISCONTI groups.

The VISCONTI study included two patient groups, both of whom were treated within 10 weeks of infection with a classical three-drug regimen. The first group consisted of 10 subjects treated continuously for >6 years (6 year group) [median 79 months (IQR 38–142)]. The second group consisted of 11 PTCs who controlled the infection for a median of 101 months (IQR 82–107) after interrupting treatment maintained for a median of 31 months (IQR 17–56), as previously reported.\textsuperscript{23,24}

**CD4\textsuperscript{+} T cell analysis and sorting**

PBMCs preserved in liquid nitrogen, with >80% viability after thawing, were sorted into live activated and resting CD3\textsuperscript{+}CD4\textsuperscript{+} T cells on a five-laser FACSAria flow cytometer (Becton Dickinson) on the CyPS platform as previously described.\textsuperscript{25} Resting CD4\textsuperscript{+} T cells (CD25–CD69–HLADR–) were further sorted into naïve (TN, CD45RA\textsuperscript{+}CCR7\textsuperscript{+}CD27\textsuperscript{+}), transitional memory (TTM, CD45RA\textsuperscript{–}CCR7\textsuperscript{–}CD27\textsuperscript{+}), and effector memory cells (TEM, CD45RA\textsuperscript{–}CCR7\textsuperscript{–}CD27\textsuperscript{–}) (Figure S1, available as Supplementary data at JAC Online). Of note, antigen expression on thawed cells was not significantly different from that on fresh cells, and activation status was evaluated on fresh cells. The number of cells collected ranged from 0.01 million to 2 million per subset and per subject, and the purity of sorted subsets was >98%. Flowjo software (Treestar) was used to analyse the cyto metric data.

**Ultrasensitive total HIV DNA and HIV RNA quantification**

Total HIV DNA was quantified by ultrasensitive real-time PCR in PBMCs, and in activated and resting CD4\textsuperscript{+} T cell subsets using the Generic HIV DNA assay from Biocentric (Bancol, France), with a detection limit of 5 HIV DNA copies per PCR, as previously described.\textsuperscript{26} The entire HIV DNA extract was tested in two to four replicates. Results were recorded as either the number of HIV DNA copies per million cells or as half the detection limit was evaluated on fresh cells. The number of collected cells ranged from 0.01 million to 2 million per subset and per subject, and the purity of sorted subsets was >98%. Flowjo software (Treestar) was used to analyse the cytomeric data.

**HIV reactivation assay**

Variable numbers (0.05 million to 2 million) of sorted peripheral resting CD4\textsuperscript{+} T cells were cultured in 10% FCS-supplemented RPMI 1640 medium for 13 days after stimulation on day 0 with anti-CD3/anti-CD28 (Roche, 5 m\textsuperscript{g}/m\textsuperscript{l}) and IL-2 (Roche, 5 m\textsuperscript{g}/m\textsuperscript{l}) for 14 days. Each CD4\textsuperscript{+}CD27\textsuperscript{+}HLADR\textsuperscript{2} sorted into live activated and resting CD3\textsuperscript{+}CD4\textsuperscript{+} T cells on a five-laser FACSAria flow cytometer (Becton Dickinson) on the CyPS platform as previously described.\textsuperscript{25} Resting CD4\textsuperscript{+} T cells (CD25–CD69–HLADR–) were further sorted into naïve (TN, CD45RA\textsuperscript{+}CCR7\textsuperscript{+}CD27\textsuperscript{+}), transitional memory (TTM, CD45RA\textsuperscript{–}CCR7\textsuperscript{–}CD27\textsuperscript{+}), and effector memory cells (TEM, CD45RA\textsuperscript{–}CCR7\textsuperscript{–}CD27\textsuperscript{–}) (Figure S1, available as Supplementary data at JAC Online). Of note, antigen expression on thawed cells was not significantly different from that on fresh cells, and activation status was evaluated on fresh cells. The number of collected cells ranged from 0.01 million to 2 million per subset and per subject, and the purity of sorted subsets was >98%. Flowjo software (Treestar) was used to analyse the cytomeric data.

**HIV RNA in plasma was quantified by real-time-PCR with the Cobas TaqMan HIV-1 v2.0 assay (Roche Diagnostics) and in culture supernatants by ultrasensitive real-time-PCR using the Generic HIV assay (Biocentric, Bancol, France).**

**HIV DNA genotyping and phylogenetic analysis**

Each CD4\textsuperscript{+} T cell subset was subjected to HIV DNA sequencing and cloning at month 24 as previously described.\textsuperscript{30} The phylogenetic trees include all
clones obtained at baseline and month 24, and were built using Kimura’s two-parameter method.11

All the data were analysed by the same personnel in the same laboratories using the same methods over the 2 year time frame.

Statistical analysis

The two-tailed Wilcoxon matched-pairs signed rank test was used to compare cell subsets, and the Mann–Whitney and Kruskal–Wallis tests were used to compare the different groups of subjects. P < 0.05 was considered to denote a significant difference. All values given in the text are medians and IQRs (25%–75%).

Results

Patient characteristics

Among the subjects, 12 enrolled in the OPTIPRIM clinical trial; 1 dropped out of the study after 17 months because of pregnancy and was excluded from the analysis. For the remaining 11 subjects, diagnosis took place a median of 36 days after infection (IQR 30–41); the virus was predominantly subtype B with CCR5 restriction; most patients were in Fiebig stage III or V, with symptomatic PHI and the average number of antibodies on HIV-1 western blot was 3 (Table 1). At baseline, they had a median of 376 CD4+ T cells/mm3 (IQR 341–516), 5.4 log HIV RNA copies/mL of plasma (IQR 5.0–5.8) and 3.9 log HIV DNA copies/106 PBMCs (IQR 3.48–4.30). No subjects had any of the protective HLA-B*27 or -B*57 alleles, while four had the HLA-B*35 alleles closely associated with disease progression. All 11 patients started ART immediately upon diagnosis and maintained it for 2 years (2 year group), with either an intensified regimen (arm 1, five subjects) or a standard regimen (arm 2, six subjects). After 2 years of ART, the CD4 T cell counts almost doubled, reaching a median of 673 cells/mm3 (IQR 620–791), and HIV DNA levels decreased by more than 1 log, reaching 2.42 log copies/106 PBMCs (IQR 2.20–2.57) and 2.65 log copies/mL (IQR 2.40–2.88) (Table 1). The main characteristics of these 11 subjects were compared with those of 10 subjects in the 6 year group, and those of 11 PTCs from the VISCONTI cohort (Table 2). Baseline characteristics were comparable between groups, with HIV DNA levels 1.3 (IQR 0.7–1.7) log copies/106 PBMCs in the 6 year group and 1.6 (IQR 1.1–2.1) in the PTC group.

Two years of early ART reduces HIV DNA levels in CD4+ TCM cells to the low values found in naive cells

The distribution of the HIV reservoir was first studied in PBMCs from the 11 subjects in the 2 year group, in comparison with their pre-treatment value.12 Total HIV DNA was quantified in PBMCs as well as in sorted total, activated and resting CD4+ T cells, and in TN, TCM, TTM and TEM CD4+ T cells. HIV DNA was detected in all CD4+ T cell subsets after 2 years of early ART, and was significantly lower than at baseline in all cell populations (P = 0.001). However, the decline of HIV reservoir size from baseline values was far more pronounced in all TCM (–1.45 log copies/106 PBMCs), TTM (–1.48) and TEM (–1.37) cells than in TN cells (–0.74) (P < 0.01) (Figure 1 and Figure S2). Interestingly, HIV DNA decay between baseline and 2 years, as well as the overall frequency of cell infection at both timepoints, was similar in activated and resting total CD4+ T cells. After 2 years of treatment, the frequency of infection of the TCM subset did not differ from that of the TN subset, contrasting with the difference observed at baseline. Importantly, the long-lived TN and TCM subsets displayed a significantly lower infection frequency than the more differentiated TTM and TEM subsets after 2 years of ART (P < 0.009), whereas it was similarly elevated in all three memory subsets at baseline (Figure 1).

Interestingly, these differences persisted after analysing the absolute number of infected cells in each CD4+ T cell subset per mL of blood (Figure S3A). The decline of HIV DNA levels in all cell subsets did not differ between the standard and intensified antiretroviral regimen groups, as also reported in the 90 patients of the OPTIPRIM clinical trial (Figure S3B).28 Given that an infected resting CD4+ T cell generally contains only one HIV DNA molecule,12 we estimated that ~0.09% of TN cells, 0.10% of TCM cells and up to 0.20% of all memory cells remained infected after 2 years of treatment, as opposed to the 0.30% of infected TN and 3.49% of infected TCM cells at baseline. Importantly, although we could not quantify the number of cells harbouring replication-competent genomes, proviruses in each of the infected resting CD4+ T cell subpopulations remained fully inducible upon in vitro stimulation after 2 (and 6) years of treatment (Figure S4).

We further compared these circulating HIV reservoirs after 2 years of early ART with tissue reservoirs in rectal cells (RCs) obtained from 3 subjects at baseline and from 14 subjects after 2 years of ART (who agreed to the rectosigmoid biopsy procedure). Total HIV DNA was quantified in total RCs as well as in sorted rectal CD3+CD4+ T cells. HIV DNA levels decreased in both PBMCs and RCs, which displayed the same level after 2 years; interestingly, RCs seemed to be more frequently infected than PBMCs (Figure S5).

Resting TTM cells represent the principal HIV reservoir after 2 years of early treatment

Two years of early ART resulted in immune recovery, with a significant increase in both TN and TCM CD4+ T cell frequencies, contrasting with a relative decay of both the TTM and TEM subsets (Figure S6), with an increase in absolute numbers (P < 0.004, data not shown). However, total cell numbers remained low in comparison with uninfected healthy individuals, except for the TCM subset. Expression of at least one of the three cell-surface activation markers (CD69, CD25 and HLA-DR) reached normal values on all cell subsets after 2 years, decreasing solely in the long-lived TN and TCM subsets and increasing significantly in the short-lived differentiated TTM and TEM subsets (Figure 2a). Expression of the intracellular Ki67 molecule diminished significantly in all CD4+ T cell subsets and returned to normal after 2 years (Figure 2b). Also, the proportion of Ki67+ cells among TEM cells was higher than that of CD69+CD25+ and HLA-DR+ cells at baseline; a similar pattern was observed in TTM cells but not in the long-lived subsets. Together, these results suggested that early ART protected those CD69+CD25+HLA-DR+Ki67+ cells that survived during the first weeks of infection despite active HIV replication and viral cytopathogenic effects.

We then estimated the contribution of each resting CD4+ T cell subset to the circulating HIV reservoir by taking into account the frequency and infection level of each cell subset (Figure 3). This calculation resulted in a minor contribution of activated CD4+ T cells to the peripheral HIV reservoir after 2 years of early
<table>
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<tr>
<th>Patient</th>
<th>Sex/age</th>
<th>Symptomatic primary HIV infection</th>
<th>Estimated days from infection</th>
<th>Fiebig stage</th>
<th>Number of anti-HIV-1 antibodies (western blot)</th>
<th>CD4 count (CD4/CD8 ratio)</th>
<th>HIV RNA (log copies/mL)</th>
<th>HIV DNA (log copies/mL)</th>
<th>Viral subtype tropism</th>
<th>HLA</th>
<th>HLA</th>
<th>CD4 count (CD4/CD8 ratio)</th>
<th>HIV DNA (log copies/mL)</th>
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<td>35</td>
<td>51</td>
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<td>44</td>
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<td>35</td>
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M, male; F, female; CD4, CD4+ T lymphocyte; CD8, CD8+ T lymphocyte.
Table 2. Characteristics of the HIV-1-infected study participants for all groups

<table>
<thead>
<tr>
<th>Characteristics of study participants</th>
<th>2 years</th>
<th>6 years</th>
<th>PTC</th>
<th>P value (Kruskal-Wallis test)</th>
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<td>baseline</td>
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<td>6 years</td>
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<tr>
<td>Number of participants</td>
<td>11</td>
<td>10</td>
<td>11</td>
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<td>Frequency of male subjects (%)</td>
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<td>100</td>
<td>73</td>
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<td>Age (years), median (IQR)</td>
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<td>46 (41–54)</td>
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<td>Infection mode</td>
<td>sexual contacts</td>
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<td>cART, median (IQR)</td>
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<td>&lt;10 weeks</td>
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<tr>
<td>time under cART (months)</td>
<td>24</td>
<td>79 (38–142)</td>
<td>—</td>
<td>31 (17–56)</td>
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<td>interruption of cART (months)</td>
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<td>—</td>
<td>—</td>
<td>101 (82–107)</td>
</tr>
<tr>
<td>CD4+ T cell count (cells/mm³)</td>
<td>376 (341–516)</td>
<td>673 (620–791)</td>
<td>592 (563–612)</td>
<td>804 (657–983)</td>
</tr>
<tr>
<td>viral load (log copies/mL)</td>
<td>5.4 (5.0–5.8)</td>
<td>1.4 (1.3–1.6)</td>
<td>4.9 (4.6–6.0)</td>
<td>0.4 (0.2–0.6)</td>
</tr>
<tr>
<td>HIV DNA load (log copies/million PBMCs)</td>
<td>3.9 (3.5–4.3)</td>
<td>2.42 (2.20–2.57)</td>
<td>—</td>
<td>1.3 (0.7–1.7)</td>
</tr>
</tbody>
</table>

cART, combined ART; 2 years, early cART for 2 years; 6 years, early cART for 6 years.
Statistical analyses were conducted strictly between the 2 year, 6 year and PTC groups.
*6 year group significantly different from both the 2 year and PTC groups.
**2 year group significantly different from both the 6 year and PTC groups.
***2 year group significantly different from the 6 year group.
Treatment in PHI protects central memory CD4+ T cells

Among the resting CD4+ T cell subsets, the main contributor to the viral reservoir was the TTM subset ($P = 0.019$), as observed at baseline. The long-lived TCM subset contributed less to the reservoir than TTM cells, although its contribution was larger at 2 years than at baseline, probably reflecting the reconstitution of the TCM subset. The only difference between baseline and 2 years was an increase in the relative contribution of the TN subset to the reservoir, from a median of 2% at baseline to

![Graph showing decrease in the HIV reservoir after 2 years of early combined ART.](https://academic.oup.com/jac/article-abstract/70/7/2108/778253)

**Figure 1.** Decrease in the HIV reservoir after 2 years of early combined ART. The total HIV DNA content was evaluated in total CD4+ T cells (CD4 TLy), TN, TCM, TTM and TEM CD4 cell subsets. Measurements were made during the acute infection (D0, baseline, black symbols) and after 2 years of early combined ART (M24, early combined ART for 2 years, grey symbols). Only significant $P$ values are shown. Results are expressed as log10 HIV DNA copies/million PBMCs and black lines represent the medians. Open symbols represent values below the detection limit.

![Graph showing CD4 T cell activation status (2 year group).](https://academic.oup.com/jac/article-abstract/70/7/2108/778253)

**Figure 2.** CD4 T cell activation status (2 year group). Activation status was evaluated in total CD4+ T cells (CD4 TLy), resting TN cells and TCM, TTM and TEM CD4+ cells. Activation levels were determined by measuring either the expression of CD25, CD69 or HLA-DR (a) or that of Ki67 (b). Results are expressed as the percentage of cells expressing these molecules, and were compared between patients with acute infection (D0), patients treated early for 2 years (M24, early combined ART for 2 years) and uninfected individuals. Each symbol represents one individual and horizontal lines are medians. Black circles represent baseline values, grey squares 2 year values and black triangles uninfected individuals. NS, $P$ value for comparison not significant.
17% after 2 years ($P = 0.001$), due to a modest decline in their infection frequency rather than an increase in their overall number.

**Two years of early ART restricts viral diversity**

The evolution of viral diversity was examined between baseline and 2 years of early ART by sequencing HIV DNA and HIV RNA in the Env C2V5 region of the gp120 gene in T cell subsets, in 6/11 patients for whom sufficient cell numbers were available (Figure 4). Cloning by the limiting dilution approach was not possible for some subsets in some patients because of insufficient yields. Viral tropism was CCR5 restricted for all 800 clones isolated at baseline and after 2 years. The topography of phylogenetic trees showed that the viral quasispecies in plasma, PBMCs, RCs and purified peripheral and rectal CD4$^+$ T cell subsets all belonged to the same viral cluster in a given patient. The limited quasispecies diversity observed in the acute phase of infection was maintained after 2 years of early ART. In one patient (Patient 7), the same limited viral diversity observed at baseline was found after 2 years of treatment. We identified three genetic mutations both at baseline and after 2 years, differentiating a cluster exclusively found in plasma from another cluster found in plasma, total activated CD4$^+$ T cells, resting TTM and TEM cells. The first was the silent C331C mutation, while the other two were coding mutations. The viral cluster found exclusively in the plasma compartment at baseline was also found after 2 years of ART in TN cells, activated CD4$^+$ T cells, TTM cells and RCs and harboured the R335G and N355K coding mutations. The other cluster isolated from CD4$^+$ T cell subsets both at baseline and after 2 years and from plasma at baseline harboured the coding mutations R335R and N355E (Figure 4).

**Two years of early ART can induce HIV remission**

Two patients in the OPTIPRIM trial (Patients 4 and 8) managed to maintain viral control while off treatment, and were included in this substudy. Patient 4 had received a standard tritherapy, presented the HLA-B*35 allele and had a high viral load at baseline (5.04 log HIV RNA copies/mL) with a severe CD4$^+$ T cell depletion (185 cells/mm$^3$). HIV RNA in this patient declined rapidly after 3 months of early ART; it remained $<20$ copies/mL at all time-points during treatment and for 24 months after treatment interruption. Patient 8 was treated with intensified pentatherapy, presented the HLA-B*08 allele and had milder acute infection (3.2 log HIV RNA copies/mL and 530 CD4$^+$ T cells/mm$^3$ at baseline). His viral load was rapidly controlled after treatment uptake ($<20$ copies/mL at month 1), and he experienced a transient loss of viral control 2 months after treatment discontinuation (with a viral load peak up to 3.4 log HIV RNA copies/mL), but control was regained spontaneously after 6 months off therapy. This subject’s viral load was 530 copies/mL 18 months after treatment discontinuation.

The decay in HIV DNA levels from baseline to 2 years was $-1.64$ and $-1.96$ log copies/mL in Patients 4 and 8, respectively, and did not differ markedly from that observed in other patients in the study, whatever the treatment regimen. Of note, in these two patients the HIV DNA burdens were among the lowest observed in
Figure 4. Genetic diversity of the HIV reservoir (2 year group). HIV-1 diversity was evaluated in blood and rectal tissue (rectal cells, RC) during the acute infection and after 2 years of early combined ART. Each HIV-1 clone was compared with the sequence of the FR-HXB2 reference, appearing at the top of each maximum likelihood tree as the root. All clusters identified with the maximum likelihood approach were confirmed by neighbour-joining analysis. The numbers near the nodes indicate the percentage of bootstrap replicates (the reliability of each tree topology was estimated from 1000 bootstrap replicates). The ENV gene was cloned from HIV RNA at day 0 [plasma (blue circles)]. The ENV gene was cloned from HIV DNA at day 0 [resting CD4 cells (red inverted triangles), activated CD4 cells (red open diamonds), resting TN cells (red diamonds), resting TCM cells (red squares), resting TTM cells (red circles), resting TEM cells (red triangles), total RCs (blue squares) and isolated rectal CD4 cells (blue open squares)] and month 24 [resting CD4 cells (black inverted triangles), activated CD4 cells (black open diamonds), resting TN cells (black diamonds), resting TCM cells (black squares), resting TTM cells (black circles), resting TEM cells (black triangle) and total RCs (green squares)].
These responses were unable to suppress HIV-1 infection of all 11 patients in the 2 year group (data not shown). Moreover, memory CD4 T cell subsets, both at treatment interruption and 6 months later, confirmed that both patients carried replication-competent viruses (Figure 5).

We also investigated the HIV-specific CD8+ T cell responses in these subsets, which are thought to play an important role in the spontaneous control of infection. These responses were weak in all 11 patients in the 2 year group (data not shown). Moreover, these responses were unable to suppress HIV-1 infection of autologous CD4+ T cells at baseline, 2 years and month 30 in Patients 4 and 8 (data not shown).

**Early treatment maintained for 6 years drives the HIV reservoir down to levels close to those of PTCs**

Finally, we compared the HIV reservoir size and distribution in the 2 year study participants with those observed after longer early treatment (6 year group), as well as with those of PTCs. In all subsets, the prevalence of infection, whether expressed as the frequency (per million PBMCs) or the absolute number (per mL of blood), was significantly higher in the 2 year group than in both the 6 year and PTC groups (P<0.019) (Figure 6a). All naive and memory CD4+ T cell subsets in the 6 year group contained low HIV DNA levels, similar to those of PTCs. HIV DNA was detected in sorted TN samples from 4/9 patients in the 6 year group, as opposed to 11/11 patients in the 2 year group and only 2/11 PTCs (Figure 6a).

The contribution of the different subsets to the HIV reservoir displayed the same pattern in the two early treatment groups (Figure 6b). The main finding was a limited contribution of the TCM subset as opposed to the predominant contribution of the TTM subset to the HIV reservoir (P<0.027). The only noteworthy difference among the three groups was a low frequency of infection but a far stronger contribution of the TN subset in the 2 year group than in the PTCs (P=0.010).

**Discussion**

This study shows that early, prolonged, effective ART has a major impact on the size of the HIV reservoir and on the abundance of the main CD4+ T cell subsets.

ART initiation within 6 weeks after infection resulted, after 2 years of continuous effective therapy, in a massive decrease in the HIV reservoir size among the major CD4+ T cell subsets in blood and in rectosigmoid biopsy specimens, in accordance with previous studies. This decrease may have resulted both from the disappearance of labile unintegrated viral forms stored during acute infection when viral replication was highly active, and from the slow death of infected cells as a result of peripheral homeostasis. Early treatment also impacted the HIV reservoir distribution, affecting predominantly the TCM CD4+ cells, which contributed less to the overall reservoir load after 2 years of ART than prior to treatment and that also contribute more to the reservoir burden in chronic patients. This exquisite effect might reflect several mechanisms relative to the relative homeostasis of the various T cell subsets, the exploration of which was beyond the scope of this study. Indeed, our results suggest that 2 years of ART leads to a more profound reduction of the HIV reservoirs in memory subsets with relatively short half-lives, from a few weeks to several months, but is not enough to purge the virus from long-lived TN cells. The preferential reduction of the TCM reservoirs compared with other memory subsets might also reflect the immune reconstitution process during 2 years of early treatment, which favours replenishment of the TCM compartment by uninfected cells, thereby reducing its infection level relative to its naive counterparts. This was not the case for TTM or TEM cells, however, the relative numbers of which decreased after 2 years of control of viral replication. Two years of early ART also normalized the activation status of all CD4+ T cell subsets, despite differences in the expression of activation markers by TCM, TTM and TEM cells. Indeed, Ki67, an early activation marker associated with cell cycling, displayed an increasing expression gradient from TN to TCM, TTM and TEM cells, returning to normal values after 2 years of early treatment. Unexpectedly, at baseline, the proportions of TTM and TEM cells co-expressing the CD69, CD25 and HLA-DR markers linked to T cell activation and type I interferon expression did not differ from that of TCM cells and were even lower than the proportions of TTM and TEM cells expressing Ki67. Two years of effective ART normalized CD25+CD69+HLA-DR+ and Ki67+ expression on TEM and TTM cells, suggesting that CD25+CD69+HLA-DR+ and Ki67+ cells represent two distinct HIV reservoirs. The most strongly activated (CD25+CD69+HLA-DR+) TEM and TTM cells were probably eliminated early after infection due to active viral replication and cytopathogenic effects. These cells could then be restored during treatment, while cells expressing only Ki67 persist, as treatment may have been started before they were engaged in viral production.
In contrast with 6 years, 2 years of early ART did not reduce the size of the HIV reservoir to the level observed in PTCs, particularly in the long-lived TCM subset. This may be due to the relatively long lifespan of the reservoir, and demonstrates that not only the timing but also the duration of ART is an important determinant of HIV reservoir decay. This challenges a study that showed that levels of cellular HIV DNA correlated with the timing of ART initiation but not with its duration, although this was based on a comparison of 55 patients treated during the chronic phase with only 6 patients treated during the acute phase of the infection.

Figure 6. Two years of early combined ART is not sufficient to reach the low HIV reservoir status of PTCs. Reservoir size and composition were evaluated in TN, TCM, TTM and TEM CD4 cell subsets. Measurements were made in subjects who began treatment early for 2 years (grey circles) or 6 years (black circles), and in the unique control of the infection after interruption of an early treatment that represents the PTC group (black triangles). The grey star represents putative PTC Patient 4 and the grey square represents putative PTC Patient 8, both from the 2 year treatment group. (a) Results are expressed as log10 HIV DNA copies/mL of blood, and values below the detection limit were calculated for each assay according to available cell numbers. (b) The contribution of TN, TCM, TTM and TEM cells to the HIV reservoir was estimated by taking into account the frequency and infection level of each subset. Results are expressed as the percentage of the HIV blood cell reservoir in the different groups of subjects. Values under the detection limit are represented by open symbols.
One limitation of our study is the cross-sectional nature of the comparison of the different patient groups. However, each group was highly homogeneous, all the patients started treatment during primary HIV infection, and all samples were tested with the same methodologies by the same operators in the same laboratories over a 2 year time frame. Another limitation is that only half the patients in the 2 year group received a standard three-drug ART comparable to that of the 6 year group, while the other half received intensified five-drug ART. However, we show that there was no difference between the two regimens in terms of reservoir size and distribution. Also, we consider that there was minimal confounding bias in making these comparisons across these three different cohorts.

These early 2 year ART regimens resulted in spontaneous post-treatment control of viral replication for over 1 year in Patient 4 and in partial control for 18 months in Patient 8. These two subjects shared the same characteristics as PTCs previously reported, notably regarding the absence of protective HLA-B*27 and B*57 alleles with lower CD8+ T cell activation compared with HIV controllers. 23 HIV DNA levels in these two patients’ TCM cells after treatment interruption were similar to those observed in PTCs. In accordance with previous reports, 18-42 our findings suggest that the smaller the HIV reservoir at treatment interruption, the better the post-treatment control. We also show that early treatment tends to lead to low reservoir levels. The decline in the HIV reservoir in the two PTC subjects was not different from that observed in their non-PTC counterparts, suggesting that the decline in HIV DNA during the first 2 years of early ART is also an important factor, necessary but not sufficient to induce PTC.

We chose to quantify the persistent reservoir by measuring total HIV DNA, as it has been shown to be strongly correlated with the frequency of integrated HIV DNA in patients with full viral suppression. 44 This cell-associated HIV DNA was fully inducible upon in vitro stimulation in a 13 day cell culture, confirming that at least a portion of the viruses stored in long-lived resting cell compartments are replication competent. 44 However, given the limited available blood volumes, it was not possible to precisely quantify the proportion of infectious and defective viruses in the different CD4+ T cell subpopulations, as recently reported. 44 The persistence of the same limited viral diversity after 2 years of early ART underlines the importance of early treatment initiation for preventing viral diversification. The similar frequencies of infected cells in blood and rectal tissue after 2 years of early treatment are in line with previous studies on virally suppressed, chronically infected patients. 45,46 This finding, together with the detection of the same viral cluster in both compartments, suggests cross-infection events between blood and the gut-associated lymphoid tissues (GALT), and also hints that CD4+ T cells are the main HIV reservoir at different stages of the infection. 47

In conclusion, our results demonstrate the ability of early initiated ART followed by long-term HIV RNA suppression to reduce the size and alter the distribution of the CD4+ T cell HIV reservoir by preserving long-lived TCM cells. Our findings show that ART during PHI is not only quantitatively but also qualitatively beneficial. They underline the importance of sustained ART in inducing long-term remission and should contribute to the design of novel trials in the HIV cure agenda.

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Author contributions
A. C., B. A. and C. R. supervised the rectal biopsy study. A. C., B. A., C. R., A. S.-C., A. M. and C. B. performed the laboratory investigations. L. M. and G. N. coordinated the data collection and regulatory requirements. A. C., B. A. and C. R. generated the tables and figures, interpreted the data, and wrote the first draft of the manuscript. All authors reviewed, revised and approved the final manuscript.

Supplementary data
Figures S1 to S6 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).
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