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HIV-1 replication and immune dynamics are affected by raltegravir intensification of HAART-suppressed subjects

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Highly active antiretroviral therapy (HAART) results in potent and durable suppression of HIV-1 viremia. However, HIV-1 replication resumes if therapy is interrupted[1,2]. Although it is generally believed that active replication has been halted in individuals on HAART, immune activation and inflammation continue at abnormal levels[3], suggesting continued, low-level viral replication. To assess whether active replication might be driving immune activation in HAART, we examined the impact of treatment intensification with the integrase inhibitor raltegravir on viral complementary DNA and immune activation parameters. In the presence of raltegravir, linear HIV-1 cDNA is prevented from integrating into chromatin and is subsequently converted to episomal cDNAs[4,5]. Raltegravir intensification of a three-drug suppressive HAART regimen resulted in a specific and transient increase in episomal DNAs in a large percentage of HAART-suppressed subjects. Furthermore, in subjects with these episomal DNAs, immune activation was higher at baseline and was subsequently normalized after raltegravir intensification. These results suggest that, despite suppressive HAART, active replication persists in some infected individuals and drives immune activation. The ability of raltegravir intensification to perturb the reservoir that supports active replication has implications for therapeutic strategies aimed at achieving viral eradication.

HAART is able to sustain suppression of plasma viremia below the limit of detection of standard assays[6]. However, viremia rapidly resumes if therapy is interrupted, suggesting that viral reservoirs persist in the face of HAART. HIV-1 persistence in HAART has been attributed to the presence of a long-lived reservoir of latently infected, memory $CD4^+$ T cells. This model is supported by the presence of replication-competent virus in peripheral blood lymphocytes and by the lack of evolution in viral cDNA[7–11]. In fact, therapy intensification should have no impact on the reservoir that persists in HAART[12]. Although it is generally believed that HAART stops active infection, increased immune activation has been shown to persist in HAART-suppressed individuals[3]. This, and additional studies[13–22], suggest that low-level, active or 'cryptic' (undetectable by standard assays) replication may persist during suppressive HAART. Residual low-level viremia has been detected with ultrasensitive assays measuring down to one copy of HIV-1 RNA per milliliter of plasma[23,24]. Whether residual viremia reflects viral replication or the production of virus from stable reservoirs without additional cycles of replication is unknown.

New classes of antiretroviral agents against viral integrase and co-receptors increase the treatment options for HIV-1–infected individuals and provide new tools to assess the viral reservoirs that persist in HAART-suppressed patients. Raltegravir is a first-in-class integrase inhibitor approved for the treatment of HIV-1 infection. It blocks integration of linear viral cDNA that is subsequently circularized by host DNA repair enzymes to form episomes containing two copies of the viral long-terminal repeat (2-LTR circles) or undergoes recombination to form a 1-LTR circle. Therefore, an increase in episomal cDNA occurs when active replication is inhibited by integrase inhibitors[4,5] (**Fig. 1a**). We exploited this unique relationship between episomes and raltegravir to reveal active replication in subjects on HAART and specifically to determine whether raltegravir intensification affects viral cDNA intermediates and immune activation parameters in HAART-suppressed subjects.

We randomly assigned 69 subjects with undetectable plasma viremia by standard assays (<50 HIV-1 RNA copies per ml) for more than 1 year (median of 5 years) to treatment groups in which they would either intensify their HAART with raltegravir ($n = 45$) or continue their HAART ($n = 24$) for 48 weeks (**Table 1**). Here we present data from 24 weeks. We excluded two individuals in the control group from further analysis (see Online Methods).

In the formation of 2-LTR circles, U3 and U5 sequences within the 5′ and 3′ LTRs ligate to form a unique U3-U5 circle junction that is not represented in other forms of viral cDNA (**Fig. 1a**). Therefore, we measured 2-LTR circles by real-time PCR with primers flanking the

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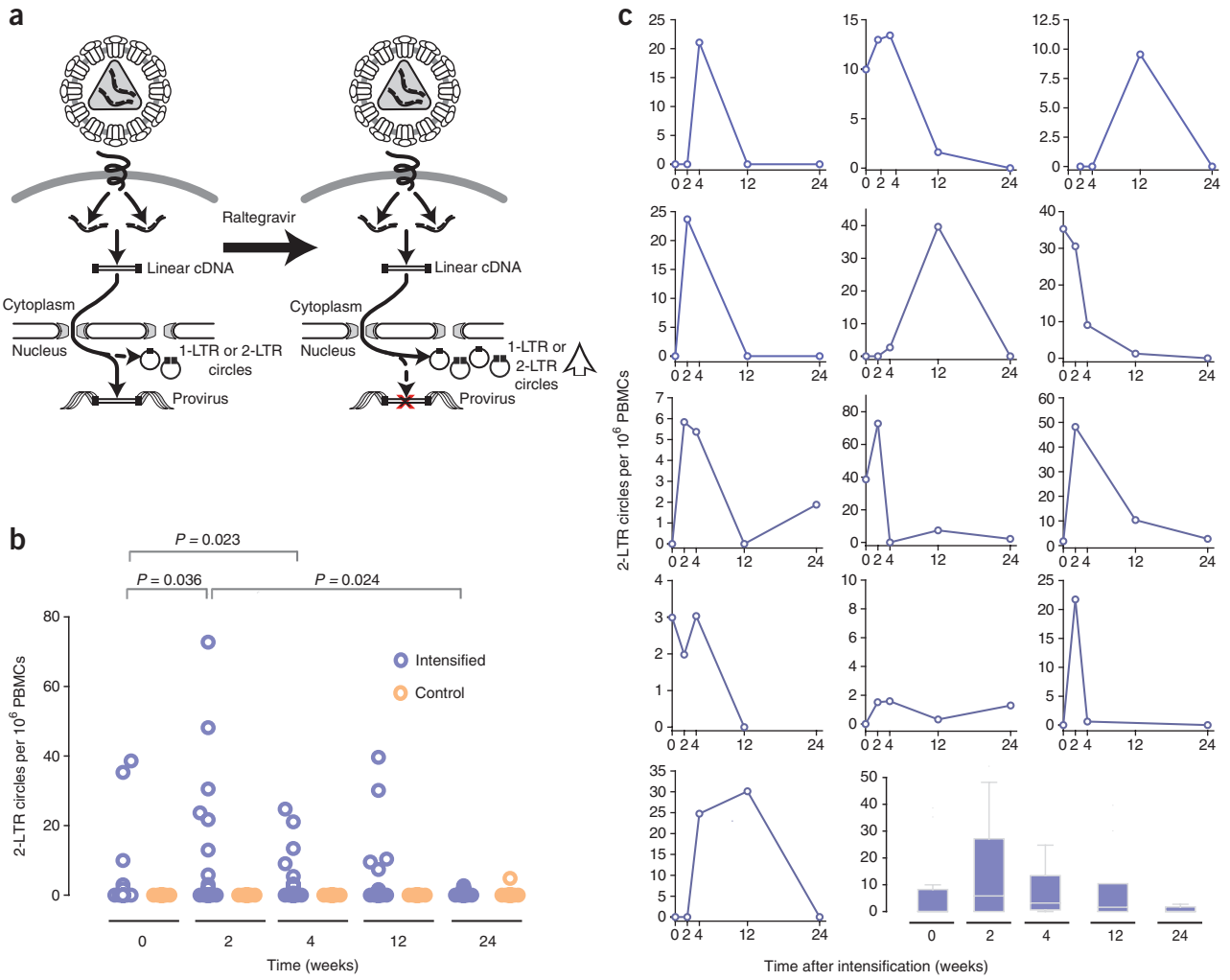

Figure 1 Impact of raltegravir intensification on HIV-1 2-LTR circles in HAART-suppressed subjects. (**a**) Raltegravir blocks integration to promote episome formation. In the presence of raltegravir, integration of linear viral cDNA is blocked, and, subsequently, the DNA is circularized by host DNA repair enzymes to form episomes containing two copies of the viral long terminal repeat (2-LTR circles) or undergoes recombination to form a 1-LTR circle. Therefore, an increase in episomal cDNA occurs when active replication is inhibited by raltegravir. (**b**) Changes in 2-LTR circle levels between intensified groups during the study period. Extrachromosomal DNA was extracted from 6×10^7 PBMCs at weeks 0, 2, 4, 12 and 24 after initiation of the study, and real-time PCR used to measure 2-LTR circles. (**c**) Individual longitudinal changes in 2-LTR circle levels during therapy intensification with raltegravir. The amount of 2-LTR circles was measured at weeks 0, 2, 4, 12 and 24 after initiation of the study. The individual data from the 13 subjects included in the intensified 2-LTR$^+$ subgroup are shown. The bottom right summarizes the overall dynamics of the changes in the amount of detectable 2-LTR circles in the intensified 2-LTR$^+$ subgroup ($n = 13$) during therapy intensification with raltegravir. Data are medians with the 25th and 75th percentiles.

2-LTR circle junction. We were able to detect 2-LTR circles at one or more time points in 29% (13 of 45) of the subjects given raltegravir but in only 5% (1 of 22) of the controls. Within the treatment intensification group ($n = 45$), there was a significant and transient increase in 2-LTR circles at weeks 2 and 4 compared to baseline ($P = 0.036$ and $P = 0.023$, respectively, Peto-Prentice-Wilcoxon test) and a significant decrease afterward ($P = 0.024$, **Fig. 1b** and **Supplementary Fig. 1**). 2-LTR circle dynamics were consistent in the 13 subjects of the intensification group, with detectable episomes at one or more time points (subsequently referred to as 2-LTR$^+$, **Fig. 1c**). Of the 13 subjects in which 2-LTR circle levels were affected by raltegravir, five were 2-LTR$^+$ at baseline. These subjects did not bias the analysis, as the increases in 2-LTR circle levels at weeks 2 and 4 were still significant when they were excluded (P values of 0.046 and 0.008, respectively, **Supplementary Table 1**).

We amplified total viral DNA (comprising unintegrated and integrated viral cDNA) from all the subjects with internal LTR primers (**Fig. 2**). We did not observe any longitudinal changes in the intensified arm ($P = 0.315$, signed-rank test). We observed an increase in total HIV-1 DNA at week 24 in the controls ($P = 0.010$, **Fig. 2a**), suggesting some loss of viral control. We amplified integrated proviral DNA, using LTR-*Alu* primers, from 87% of the subjects in the intensified treatment group and from 86% of the subjects in the control group ($P = 0.252$, Pearson's chi square). We did not observe any longitudinal changes in any of the groups (control arm: $P = 0.579$; intensification arm: $P = 0.322$, signed-rank test), and we did not observe any differences between the groups at any time interval (**Fig. 2b**). Of note, we found a longitudinal correlation between total and integrated HIV-1 DNA (control arm: rho = 0.45; intensification arm: rho = 0.40, $P < 0.001$), although none of these parameters correlated with 2-LTR

circle levels. Finally, we did not observe any longitudinal changes in either integrated or total HIV-1 DNA within the 2-LTR$^+$ subgroup. Therefore, despite the effect of raltegravir on 2-LTR circles in the intensified treatment group, total and integrated HIV-1 DNA levels remained stable over time (**Fig. 2** and **Supplementary Table 2**). This supports the notion that 2-LTR circles represent a minor fraction of the total cDNA species in the infected cell and that most integrated DNA is archival and nondynamic.

Table 1 Subject characteristics in the control and raltegravir intensification groups

	Control (n = 22)[a]	Intensification (n = 45)	P value between groups[b]
Age (years), mean ± s.d.	44.8 ± 8.0	46.1 ± 8.8	0.569
Females, n (%)[c]	6 (27.3)	6 (9.0)	0.187
Antiretroviral therapy			
Protease inhibitor–containing regimen, n (%)[c]	8 (36.4)	14 (31.1)	0.878
Number of antiretroviral drugs previously or currently given to subject, mean ± s.d.	6.7 ± 0.65	7.0 ± 0.38	0.662
Previous treatment failure, n (%)[c]	3 (14)	10 (22)	0.744
Suboptimal antiretroviral therapy before starting HAART, n (%)[c]	7 (32)	13 (29)	1
Time from diagnosis (years), mean ± s.d.	11.0 ± 5.1	11.0 ± 6.5	0.795
Time with suppressive antiretroviral therapy (years), mean ± s.d.	4.5 ± 3.1	5.0 ± 3.0	0.646
Total HIV-1 DNA amounts			
Baseline (copies per million PBMCs), median [IQR]	14.1 [3.1–61.3]	10.3 [4.5–38.3]	0.713
Week 24 (copies per million PBMCs), median [IQR]	41.7 [2.2–124.8]	9.6 [0.75–66.0]	0.183
P value within group (baseline versus week 24)[d]	0.010	0.315	
Integrated HIV-1 DNA amounts			
Baseline (copies per million PBMCs), median [IQR]	1.9 [0–41.7]	0 [0–7.4]	0.229
Week 24 (copies per million PBMCs), median [IQR]	2.3 [0–87.1]	0 [35.6–104.3]	0.097
P value within group (baseline versus week 24)[d]	0.579	0.322	
CD4$^+$ T cell counts			
CD4$^+$ at baseline, absolute (cells per μl), median [IQR]	503 [371–600]	530 [434–786]	0.333
CD4$^+$ at week 24, absolute (cells per μl), median [IQR]	482 [374–636]	614 [486–745]	0.072
P value within group (baseline versus week 24)[d]	0.103	0.138	
CD4$^+$CD45RA$^-$ at baseline (%)[e], median [IQR]	65.9 [63.6–74.2]	68.6 [43.0–80.2]	0.943
CD4$^+$CD45RA$^-$ at week 24 (%)[e], median [IQR]	69.0 [60.7–81.6]	76.1 [59.0–82.4]	0.713
P value within group (baseline versus week 24)[d]	0.787	0.155	
CD4$^+$CD45RA$^-$CD38$^+$ at baseline (%)[e], median [IQR]	26.8 [23.9–31.7]	26.2 [19.9–35.1]	0.910
CD4$^+$CD45RA$^-$CD38$^+$ at week 24 (%)[e], median [IQR]	34.3 [29.7–37.6]	30.4 [23.85–34.7]	0.237
P value within group (baseline versus week 24)[d]	0.008	0.360	
CD8$^+$ T cell counts			
CD8$^+$ at baseline, absolute (cells per μl)[e], median [IQR]	722 [470–1051]	660 [467–961]	0.631
CD8 at week 24, absolute (cells per μl)[e], median [IQR]	830 [590–958]	658 [498–1017]	0.402
P value within group (baseline versus week 24)[d]	0.344	0.163	
CD8$^+$CD45RO$^+$CD38$^+$ at baseline (%)[e], median [IQR]	14.9 [9.9–18.8]	15.6 [10.7–21.0]	0.867
CD8$^+$CD45RO$^+$CD38$^+$ at week 24 (%)[e], median [IQR]	16.7 [12.0–22.9]	11.9 [6.5–15.3]	0.071
P value within group (baseline versus week 24)[d]	1	0.241	
CD8$^+$HLA-DR$^+$CD45RO$^+$ at baseline (%)[e], median [IQR]	19.5 [11.1–25.7]	16.5 [8.9–24.6]	0.502
CD8$^+$HLA-DR$^+$CD45RO$^+$ at week 24 (%)[e], median [IQR]	20.1 [10.4–25.8]	14.7 [8.5–23.8]	0.293
P value within group (baseline versus week 24)[d]	0.898	0.984	
CD8$^+$HLA-DR$^+$CD38$^+$ at baseline (%)[e], median [IQR]	16.1 [11.9–21.1]	11.7 [9.4–27.4]	0.690
CD8$^+$HLA-DR$^+$CD38$^+$ at week 24 (%)[e], median [IQR]	20.4 [18.7–28.7]	14.6 [10.0–25.9]	0.065
P value within group (baseline versus week 24)[d]	0.041	0.641	
Ultrasensitive pVL			
Baseline (copies per ml), median [IQR]	0.5 [0.4–0.6]	0.5 [0.4–0.6]	0.334[f]
Week 12 (copies per ml), median [IQR]	0.4 [0.32–0.5]	0.4 [0.39–0.5]	0.721[f]
P value within group (baseline vs. week 12)[g]	0.102	0.523	
Soluble CD14			
Baseline (μg ml^{-1}), median [IQR]	7.7 [5.9–8.8]	7.8 [6.6–9.2]	0.479
Week 24 (μg ml^{-1}), median [IQR]	7.1 [6.3–8.7]	8.3 [6.9–9.7]	0.136
P value within group (baseline versus week 24)[d]	0.571	0.213	

[a]One individual in the control group was lost to follow-up before study initiation, and a second individual also in this group, who had two consecutive viral load measurements at weeks 12 and 24 (90 and 63 HIV-1 RNA copies per ml, respectively), was excluded from further analysis. [b]P value between groups: Mann Whitney U test. [c]Pearson's chi square. [d]P value within groups: signed-rank test (paired data). [e]Blood samples available for flow cytometry analysis were n = 34 in the intensification group and n = 18 in the control group. [f]P value between groups: Peto-Prentice test. [g]P value within groups: Peto-Prentice-Wilcoxon test. IQR, interquartile range; pVL, plasma viral load (HIV-1 RNA copies per ml of plasma).

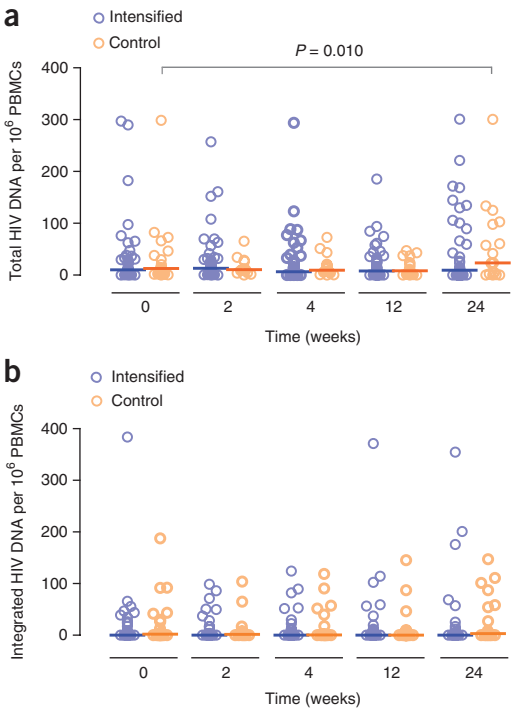

Figure 2 Changes in total and integrated HIV-1 DNA. Chromosomal DNA was extracted from 6×10^7 PBMCs at weeks 0, 2, 4, 12 and 24 after initiation of the study, and real-time PCR was used to measure various forms of HIV-1 cDNA. (**a**) Changes in total HIV-1 DNA during 24 weeks of intensification. (**b**) Changes in integrated HIV-1 DNA during 24 weeks of intensification.

We analyzed why raltegravir affected 2-LTR circle dynamics in only 29% of the intensified subjects by conducting a *post hoc* analysis to assess whether these 2-LTR$^+$ subjects had different baseline characteristics compared to the 2-LTR$^-$ subjects (**Supplementary Table 2**). We found increases in 2-LTR circles mainly in subjects who were given raltegravir with a protease inhibitor–containing regimen ($P = 0.011$, Fisher's exact test, **Supplementary Table 2**). We performed a single-copy assay of plasma HIV-1 RNA on baseline and week-12 samples[23] and found no significant differences between or within the control and the intensification groups (**Supplementary Table 2**). Similarly, there were no differences between the 2-LTR$^+$ and 2-LTR$^-$ subgroups, or within the 2-LTR$^+$ subgroup, over time (**Supplementary Table 2**). In summary, the virological impact of raltegravir intensification was only revealed by analysis of 2-LTR circle dynamics.

Immune hyperactivation is a hallmark of HIV-1 infection[25], causing increased proliferation and apoptosis. Suppressive HAART reduces immune activation[26] but does not normalize it[27]. However, a causative link between active viral replication and immune activation has not been established. Both the intensified and the control groups had stable CD4$^+$ and CD8$^+$ T cell counts during the study, with only a trend toward an increase in absolute CD4$^+$ T cell counts in the intensification arm ($P = 0.072$, signed-rank test, **Supplementary Fig. 2**) that was not confirmed by the percentage of CD4$^+$ T cells ($P = 0.947$). In addition, both arms showed no major differences in CD4$^+$CD45RA$^-$ or activated CD8$^+$ T cell counts during the study (**Table 1** and **Supplementary Fig. 3**). However, we observed increases in the percentage of CD4$^+$CD45RA$^-$CD38$^+$ ($P = 0.008$) and CD8$^+$HLA-DR$^+$CD38$^+$ cells ($P = 0.041$) in the control arm after 24 weeks (**Table 1**). Although no rebound in plasma viremia or 2-LTR circles in peripheral blood mononuclear cells (PBMCs) was evident in the control group after 24 weeks, it is possible that compartmentalized viral replication in the tissues contributed to the increase in T cell activation and in proviral DNA.

To examine the relationship between immune activation and the observed increases in 2-LTR DNA forms, we compared activation marker expression between the 2-LTR$^+$ and 2-LTR$^-$ subgroups of subjects on intensified treatment in a *post hoc* analysis. At baseline, the 2-LTR$^+$ subgroup showed higher percentages of activated CD8$^+$ T cells (CD8$^+$CD45RO$^+$CD38$^+$ ($P = 0.009$; **Fig. 3a**), CD8$^+$HLA-DR$^+$CD45RO$^+$ ($P = 0.007$; **Fig. 3b**), and CD8$^+$HLA-DR$^+$CD38$^+$ ($P = 0.003$; **Fig. 3c**)) than did 2-LTR$^-$ subjects. Similarly, there was a higher percentage of CD4$^+$CD45RA$^-$CD38$^+$ T cells ($P = 0.019$) and a trend toward a higher concentration of plasma-soluble CD14 ($P = 0.064$) —a surrogate marker of bacterial translocation[28]— in the 2-LTR$^+$ subgroup at baseline (**Supplementary Table 2**). There was no correlation between immune activation and total or integrated HIV-1 DNA at baseline (data not shown).

Longitudinal analysis showed a significant reduction in CD8$^+$ T cell activation markers in the 2-LTR$^+$ subgroup that was particularly evident in CD8$^+$CD45RO$^+$CD38$^+$ T cells ($P = 0.047$, **Fig. 3a**). Additionally, the numbers of CD8$^+$HLA-DR$^+$CD45RO$^+$ and CD8$^+$HLA-DR$^+$CD38$^+$ activated cells in the 2-LTR$^+$ subgroup were reduced over time compared to those in the 2-LTR$^-$ subgroup

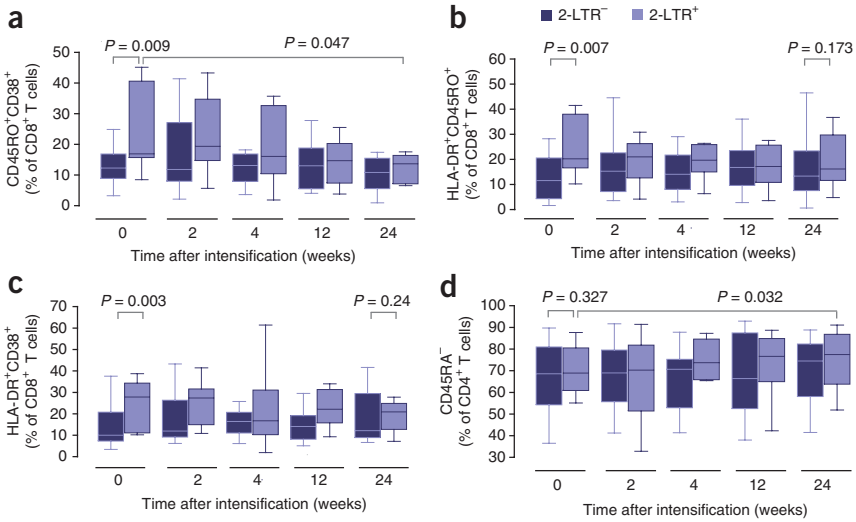

Figure 3 Phenotypic analysis of lymphocyte subsets in subjects treated with raltegravir after stratification on the basis of 2-LTR positivity (2-LTR$^+$) and negativity (2-LTR$^-$), as assessed in fresh blood samples by multicolor flow cytometry at weeks 0, 2, 4, 12 and 24. (**a**–**c**) CD8$^+$ T cell activation, measured as the percentage of CD8$^+$CD45RO$^+$CD38$^+$ (**a**), CD8$^+$HLA-DR$^+$CD45RO$^+$ (**b**) and CD8$^+$HLA-DR$^+$CD38$^+$ (**c**) cells. HLA-DR, human leukocyte antigen-DR. (**d**) Time course of the percentage of CD4$^+$CD45RA$^-$ T cells. Data are medians with the 25th and 75th percentiles. 2-LTR$^+$ subgroup, $n = 12$; 2-LTR$^-$ subgroup, $n = 24$. One-sided P values (Mann-Whitney U test between groups; and signed-rank test, paired data, within groups) are indicated.

(**Fig. 3b**,**c**). In contrast, a higher percentage of $CD4^+CD45RA^-CD38^+$ T cells ($P = 0.017$) was maintained in the 2-LTR^+ subgroup at week 24 (data not shown). Notably, changes in $CD8^+$ T cell activation have been linked to changes in HIV-1 replication[25], whereas $CD4^+$ T cell activation seems to be more dependent on homeostatic responses and bacterial translocation[29]. Finally, there was also a trend toward a greater increase in absolute $CD4^+$ T cell counts in the 2-LTR^+ subgroup at week 24 ($P = 0.085$; signed rank test), with a larger increase in the percentage of $CD4^+CD45RA^-$ T cells ($P = 0.032$, **Fig. 3d**). To examine whether the five subjects with 2-LTR circles at baseline might have biased the analysis, we used regression models adjusting for the presence of 2-LTR circles at baseline (**Supplementary Tables 3** and **4**). These results strengthen the statistical conclusions drawn from the main analysis and indicate that raltegravir is more likely to affect immune activation in people showing altered 2-LTR circle dynamics. This supports the conclusion that residual viral replication, as revealed by 2-LTR circle levels, drives immune activation and that raltegravir intensification can reduce the extent of immune activation by suppressing residual viral replication.

In this study, raltegravir intensification revealed the presence of active replication in a large percentage (29%) of subjects on suppressive HAART. Raltegravir specifically prevents the integration of linear viral cDNA to promote an increase in episomal cDNA formation. Because linear cDNA is a product of reverse transcription, the increase in episomal cDNA after raltegravir intensification requires the presence of infectious virus and also requires *de novo* infection and reverse transcription, which strongly suggests that active viral replication persists in HAART. This, together with the observation that, in subjects on suboptimal antiviral regimens, 2-LTR circles undergo rapid evolution[20], indicates that episomes are labile *in vivo* and, as such, are surrogate markers for ongoing infection. The fact that the increase in episomal cDNA was transient further suggests that raltegravir blocked active replication and production of infectious virions (**Supplementary Fig. 4**). However, other factors such as cytotoxic T lymphocyte targeting of cells containing transcriptionally active episomal HIV-1 cDNA, action of cell nucleases or 2-LTR dilution due to cell proliferation might also have a role. Raltegravir intensification did not alter levels of total or integrated viral cDNA. This is consistent with our previous observations that, in contrast to episomal cDNA, most proviral DNA is archival and nondynamic[20].

Although all subjects had undetectable plasma viremia by standard assays, we observed the increase in 2-LTR circles mainly in those raltegravir-treated subjects on protease inhibitor–containing regimens. It is possible that the presence of three reverse transcriptase inhibitors in protease inhibitor–sparing regimens lowers the probability of formation of the linear cDNA precursor to episomal cDNA. It is also possible that active replication occurs in anatomic compartments that are less accessible to protease inhibitors or that pharmacodynamic variability of protease inhibitors contributes to this observation. Furthermore, our inability to detect episomal cDNA in about 70% of the subjects in this study suggests that current HAART can suppress active replication in most individuals. This agrees with previous studies where there was no evidence for viral evolution in subjects on HAART[7,9,10].

Our study also reveals a causal relationship between active replication and immune activation in $CD8^+$ T cells. Our observed normalization of immune activation in subjects showing detectable increases in 2-LTR circles after raltegravir intensification suggests that, in HAART, active replication is a cause rather than a consequence of aberrant immune activation. The extent of immune activation and plasma lipopolysaccharide concentrations are predictive of $CD4^+$ T cell evolution in HIV infection[3,30,31]. Therefore, full suppression of viral replication may facilitate immune reconstitution[27] by reducing $CD8^+$ T cell activation and increasing $CD4^+$ T cell survival. Our observation that raltegravir can affect both 2-LTR circle dynamics and immune activation indicates that these parameters could prove valuable in assessing the extent to which residual replication occurs in aviremic subjects on HAART. However, we cannot exclude the possibility that there is a spectrum of residual replication that, at the lower range, might include even individuals with undetectable 2-LTR circles.

Our study leads us to ask to what extent active replication contributes to viral persistence in HAART. For example, the longevity of the latent reservoir may be partly attributable to continual replenishment by virus produced by active replication. It could be argued that, in the presence of HAART, there is not a complete life cycle within individual infected cells (that is, a cell gets infected but does not make particles) and that the infectious particles are being made by a chronically infected cell that is simply manufacturing virions. However, even in this scenario, conditions would exist for sequence evolution and for viral reservoir replenishment. Therefore, intensification regimens that prevent active replication may truncate this replenishment and accelerate the decay of the reservoirs that persist in HAART.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

M.J.B. and M.M. designed, executed and interpreted most experiments and prepared the manuscript. J.M.L. designed the study and participated in subject recruitment and clinical care. A.E. performed statistical analyses. V.D. executed single-copy assays. M.C.P. helped with real-time PCR experiments. J.M.G. and P.D. participated in subject recruitment and clinical care. R.P. contributed in experimental design. M. Sharkey helped in experimental setup and provided reagents. S.P. executed single-copy assays. M. Stevenson provided interpretation of the data and helped with the manuscript preparation. B.C. designed the study, participated in subject recruitment and clinical care and provided interpretation of the data. J.B. supervised immunological experiments, data interpretation and participated in manuscript preparation. J.M.-P. supervised all aspects of this study including study design, execution and interpretation and manuscript preparation.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemedicine/.

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ONLINE METHODS

Study subjects. We enrolled 69 HIV-seropositive subjects on suppressive HAART for at least one year in this open-label and not placebo-controlled study. All participants provided informed consent approved by the Germans Trias i Pujol, the Clinic de Barcelona and the Santa Creu i Sant Pau Hospital Review Boards. HAART regimens included two nucleoside reverse transcriptase inhibitors and either a protease inhibitor (lopinavir (Abott Laboratories) or atazanavir (Bristol-Myers Squibb)) or a non-nucleoside reverse transcriptase inhibitor (efavirenz (Bristol-Myers Squibb) or nevirapine (Boehringer Ingelheim)). We randomly assigned 45 subjects to a treatment group intensifying HAART with raltegravir (Merck Sharp & Dohme) for 48 weeks and 24 subjects to a control arm (**Table 1**). One individual in the control group was lost to follow-up before study initiation. A second individual also in this group, with two positive viral load measurements, was excluded for further analysis. We stratified raltegravir-treated subjects in a *post hoc* analysis into 2-LTR$^+$ and 2-LTR$^-$ groups according to the detection of episomal cDNA (**Supplementary Table 2**).

Subjects in the intensified treatment group had been exposed to a median of seven antiretroviral drugs with a median of 4.4 nucleoside reverse transcriptase inhibitors, 1.1 NNRTI and 1.5 protease inhibitors. Nonintensified subjects had been exposed to a median of 6.7 antiretroviral drugs with a median of 4.2 nucleoside reverse transcriptase inhibitors, 1.0 non-nucleoside reverse transcriptase inhibitors and 1.4 protease inhibitors. None of the enrolled subjects had previously been exposed to integrase inhibitors.

Nucleic acid purification. We purified a median of 6×10^7 PBMCs at weeks 0, 2, 4, 12 and 24 by Ficoll centrifugation and resuspended cell pellets in 350 µl of P1 buffer (Qiaprep miniprep kit, Qiagen). Then we used 250 µl of cell resuspensions for extrachromosomal HIV-1 DNA extraction (Qiaprep miniprep kit, Qiagen) using the modification for the isolation of low-copy-number plasmids, and we purified total cellular DNA from 100 µl of cell resuspensions with a standard protocol (QIAamp DNA Blood Kit, Qiagen).

Quantification of HIV-1 2–long terminal repeat circles. We used a single-step, real-time PCR to quantify 2-LTR circles in a 50-µl PCR reaction mix containing 25 µl of TaqMan Universal PCR Master Mix (Applied Biosystems), 20 µl of extrachromosomal HIV-1 DNA, primers (forward: 5′-CTAACTAGGGAACCCAC TGCT-3′; reverse: 5′-GTAGTTCTGCCAATCAGGGAAG-3′) and a fluorescence Taqman probe (5′-AGCCTCAATAAAGCTTGCCTTGAGTGC-3′). We performed amplification reactions spanning the 2-LTR circle junction with an Applied Biosystems 7000 real-time PCR system. We extrapolated copy numbers of 2-LTR circles from a standard curve generated with a plasmid that harbors the sequence of the 2-LTR junction and the *CCR5* gene. We normalized the 2-LTR values relative to *CCR5* gene copy number as determined from the chromosomal fraction.

Quantification of total and integrated HIV-1 DNA. We used a single-step real-time PCR to quantify total HIV-1 DNA in 50 µl of PCR reaction mix containing 25 µl of TaqMan Universal PCR Master Mix, 20 µl of chromosomal HIV-1 DNA and primers and probe as previously described[32]. We used a two-step real-time PCR to measure integrated HIV-1 DNA with *Alu*-LTR primers as previously described[32]. We performed amplification reactions with an Applied Biosystems 7000 Real-time PCR system. We calculated copy number as we did for 2-LTR DNA.

Single-copy assay for HIV-1 RNA concentrations in plasma. We used a real-time reverse transcriptase–initiated PCR assay that quantifies HIV-1 RNA concentrations down to one copy per ml of plasma on all samples at baseline and at week 12[23].

Analysis of lymphocyte subsets and immune activation. Thirty-four subjects in the intensification group (12 in the 2-LTR$^+$ subgroup and 22 in the 2-LTR$^-$ subgroup) and 18 subjects in the control group had fresh blood samples available for flow cytometry analysis through the study period. We used two combinations of antibodies to characterize the various lymphocyte subsets; one combination evaluated naive or memory subsets and contained antibodies to CD45RA (clone HI100), CD31 (clone WM59), CD38 (clone HIT2), CD3 (clone SK7), CD4 (clone RPA-T4) and CD8 (clone SK1) (all Becton Dickinson), and the other evaluated the activation of CD8$^+$ T cells and contained antibodies to HLA-DR (clone L243), CD38 (clone HIT2), CD45RO (clone UCHL-1), CD3 (clone SK7), CD4 (clone RPA-T4) and CD8 (clone SK1) (all Becton Dickinson). We incubated fresh whole blood with antibodies for 15 min at 21 °C, lysed cells with the BD FACS lysing solution (Becton Dickinson), washed the cells and acquired data on an LSRII flow cytometer (Becton Dickinson). We performed analyses with FlowJo software (Tree Star Inc.).

Soluble CD14. We measured soluble CD14 in duplicate by ELISA (Diaclone) with 1 in 50 plasma dilutions at baseline and at week 24.

Statistical Analysis. We used the Mann-Whitney *U* test to compare medians between the control and intensification arm (or between the 2-LTR$^+$ and 2-LTR$^-$ subgroup) and the signed rank test (paired test) to compare longitudinal changes. To adjust for the presence of 2-LTR circles at baseline, we also used analysis of variance models for comparisons between groups and linear mixed models to study longitudinal changes within groups. Because the ultrasensitive viral load and 2-LTR circles presented either multiple detection limits or a high percentage of values below them, we used regression order statistics methods to calculate summary statistics, and we performed comparisons between groups by the Peto-Prentice test. We analyzed differences in proportions between groups through the Pearson's chi square, considering the continuity correction or the Fisher's exact test, as appropriate. We analyzed longitudinal differences in 2-LTR circles and ultrasensitive viral load within groups by the Peto-Prentice-Wilcoxon test for paired data. We computed correlation between total or integrated HIV-1 DNA and immune activation parameters with the Spearman's rho coefficient. We performed statistical analyses with SAS 9.1 and the R package. We generated graphics with GraphPad Prism 5.0 software.

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