Dynamics of CD8 T-Cell Activation After Discontinuation of HIV Treatment Intensification

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Background: Detection of episomal HIV cDNA has been associated with greater levels of CD8 and CD4 T-cell activation in HIV-1-infected highly active antiretroviral therapy (HAART)-suppressed individuals. However, HAART intensification exclusively reduced CD8 T-cell activation.

Methods: We evaluated activation markers 12 weeks after raltegravir withdrawal in a previously described 48-week raltegravir intensification study. The subjects (n = 34) were subgrouped into 2-LTR$^+$ (n = 12) or 2-LTR$^-$ (n = 22) subgroups according to delectability of 2-LTR episomes during the intensification period.

Results: The initial differences in CD8 T-cell activation between subgroups were lost after intensification. Linear mixed models revealed significant reductions in CD8 T-cell activation in both 2-LTR$^-$ and 2-LTR$^+$ subgroups, suggesting that raltegravir impacts subjects irrespective of 2-LTR detection. Remarkably, a partial rebound in CD8 activation markers after raltegravir discontinuation was observed in the 2-LTR$^+$ subgroup. This restored the differences between subgroups observed at study entry, particularly in terms of CD38 expression within CD8 memory T-cells. Conversely, CD4 T-cell activation remained unchanged in both subgroups during the study period, although an early and transient CD45RA$^-$ CD4 T-cell redistribution from tissues was apparent.

Conclusions: CD8 T-cell activation undergoes reversible changes during raltegravir intensification and discontinuation in patients showing detectable 2-LTR circles. The general decrease in CD8 T-cell activation and a transient CD45RA$^-$ CD4 T-cell redistribution in intensified individuals may reflect residual viral replication during apparently suppressive HAART.

Key Words: HIV-1 integrase, HIV-1 eradication, viral Reservoir, CD38

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INTRODUCTION

Chronic activation of the immune system is a hallmark of HIV-1 pathogenicity and leads to increased T-cell turnover and apoptosis,[1,2] resulting in CD4 T-cell depletion in untreated HIV-1-infected subjects. This immune hyperactivation is evidenced by an elevated expression of activation markers, such as CD38 or HLA-DR, on the surface of T-lymphocytes. Indeed, several

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studies have demonstrated that the expression level of CD38 on CD8 T-cells correlates better with disease progression[3,4] than absolute CD4 T-cell counts or plasma viremia.[5] The predictive potential of $CD38^+$ of CD8 T-cell frequency may be further improved by analyzing its coexpression or its expression on the memory subset of T-cells ($CD45RO^+$).[4,6]

Highly active antiretroviral therapy (HAART) reduces the level of T-cell activation. However, the expression of activation markers remains increased in HAART-suppressed subjects as compared with that in healthy individuals.[7] The causes of persistent hyperactivation, and by extension general inflammation, during suppressive HAART are not fully understood. Prevalent coinfections such as cytomegalovirus or hepatitis C virus[8] or translocation of bacterial products from the intestinal lumen[9] might contribute to the increased systemic immune activation. The combination of HAART with therapies aimed at combating coinfections or the effects of microbial translocation have recently been used recently to attempt to reduce CD8 T-cell activation and/or increase absolute CD4 T-cell count.[8,10]

Another contributor to immune hyperactivation during suppressive HAART could be the residual HIV viremia, which can be detected in most treated HIV-infected individuals.[11] Although the origins of residual viremia have not been defined, it may originate from virus release from long-lived reservoirs of latently infected CD4 T-cells, ongoing cycles of viral replication, or from an as-yet, uncharacterized source.[12,13] The nature of the reservoir that contributes to immune activation can be explored through treatment intensification. If immune activation is driven by virus released from the latent reservoir, HAART intensification, through the addition of another antiretroviral agent should have no impact on activation markers.[12,14,15] Alternatively, if immune activation is the result of residual viral replication, ongoing viral replication could be impacted by HAART intensification decreasing immune activation. However, the lack of definitive virological and immunological markers to measure residual viral replication (immune activation, single copy assay, HIV sequence analysis, or episomal forms of HIV DNA), have provided inconclusive results, and the relevance of this issue for the forthcoming HIV-1 cure strategies still drives a strong controversy.

Some intensification protocols using maraviroc or abacavir in HIV-1 infected subjects on stable HAART have shown benefits, such as a reduction in CD8 T-cell activation[14–16] and an increase of CD4 T-cell counts.[10,15,17–19] Raltegravir, a first-in-class integrase strand transfer inhibitor,[16,20] is also an attractive agent to study the effects of treatment intensification due to its novel antiviral mechanism of action and its antiviral potency. However, the effects of raltegravir intensification remain controversial. Short treatment raltegravir intensification studies (12 or 24 weeks) in HAART-suppressed subjects have not demonstrated any significant reduction in residual plasma viremia, decay of CD8 T-cell activation and/or CD4 T-cell increases in peripheral blood.[10,17–19,21–23] In contrast, a trend toward a decrease in CD8 T-cell activation and an increase of CD4 T-cell counts in the ileum after 12 weeks of HAART intensification with raltegravir has been reported.[16,20] Significant reductions in peripheral CD8 T-cell activation seem to require longer intensification periods (48 weeks).[21–24] When viral cDNA integration is blocked, linear viral genomes are captured by the DNA repair machinery and converted to episomal cDNA forms. This leads to an increase in episomal cDNA copy number in the presence of integrase inhibitors.[16,25,26] Therefore, episomal viral cDNA forms containing two or one long terminal repeat (LTR) sequences are surrogate markers of recent infection events as opposed to proviruses that are predominantly archival.[22,24,25,27] This unique relationship between integrase inhibitors such as raltegravir and episome dynamics has been exploited in several intensification clinical trials by us and others to gauge the extent of cryptic viral replication in subjects on HAART.[17,18,21,25,26,28,29] Only 2 studies on HAART-treated subjects were able to detect markers of persistent viral replication (2-LTR circles and unspliced RNA in $CD4^+$ T-cells), concomitantly with a reduction in immune activation.[22,25,27] However, other studies with different experimental design did not confirm these results.[17,18,21,22,28,29] We have previously reported that 24-week raltegravir treatment intensification period decreased CD8 T-cell activation markers in subjects with detectable 2-LTR circles (referred as the 2-LTR^+ subgroup).[22,25] Subsequently, data from the complete 48-week study revealed a decrease in CD8 T-cell activation markers in the entire raltegravir intensification group.[22,25]

To further investigate the link between CD8 T-cell activation markers and residual viral replication, we have examined immune activation parameters after raltegravir discontinuation in HIV-1-infected subjects on suppressive HAART who had detectable 2-LTRs during a 48-week intensification period. Our results demonstrate a specific rebound in CD8 T-cell activation markers in $2LTR^+$ individuals, suggesting that abnormal immune activation in the face of HAART may be a consequence of incomplete viral suppression.

METHODS

Study Design and Subjects

The Integral Study has previously been described.[22,25] This is a prospective, controlled, open-label study that includes 69 HIV-1-infected subjects on suppressive HAART for at least 1 year (IntegRal Study, ClinicalTrials.gov number NCT00554398). This study was approved by the Review Boards of Hospital Germans Trias i Pujol, Hospital Clinic de Barcelona and Hospital Santa Creu i Sant Pau. The subjects were randomly assigned 2:1 to intensify their current HAART with raltegravir (Merck Sharp & Dohme, Whitehouse Station, NJ; 400 mg BID) for 48 weeks or to continue their HAART. None of the enrolled subjects had previously been exposed to integrase inhibitors. For the immunological substudy, we enrolled 51 subjects with available immunological data throughout the study period. In these patients, immune parameters were monitored 12 weeks after raltegravir discontinuation. The patient cohort in this study is the same as previously reported for interim analysis at week 24 and study description at week 48.[22,25] Intensified patients were subgrouped in a post hoc analysis according to whether 2-LTRs were detectable in peripheral blood mononuclear cells (PBMCs). Individuals with at least 1 positive sample during the intensification period were referred to as 2-LTR^+ subgroup (n = 12), while patients showing undetectable levels of episomal circles were referred to as 2-LTR^- subgroup (n = 22).

Immunophenotype

Immune activation markers were assessed in fresh blood samples at weeks 0, 2, 4, 12, 24, and 48, and an additional sample was collected 12 weeks after raltegravir withdrawal (week 60). Lymphocyte subsets and immune activation analysis were evaluated in fresh blood samples by 6-color flow cytometry (LSR-II, BD Bioscience) using the following antibody combinations: tube 1—HLA-DR-FITC, CD38-PERCP-Cy5.5, CD45RO-APC, CD3-APC-Cy7, and CD8-PE-Cy7, and tube 2—CD45RA-FITC, CD31-PE, CD38-PERCP-Cy5.5, CD4-APC, CD3-APC-Cy7, and CD8-PE-Cy7. Tube 1 was designed to characterize the activation state (assessed by HLA-DR and CD38 markers) of total CD8 T-cells and, the specific activation of memory compartment ($CD8^+CD45RO^+$). Tube 2 was used to evaluate naive and memory subsets, recent thymic emigrants, and the activation state of CD4 and CD8 T-cells. Because both antibody combinations included CD38 antibody, we were able to evaluate independently the percentage of $CD38^+$ cells in the memory compartment (defined in tube 1 as $CD45RO^+$ and in tube 2 as $CD45RA^-$, see **Figure S1**, **Supplemental Digital Content,** http://links.lww.com/QAI/A392). All antibodies were from BD Biosciences.

Statistics

We analyzed the differences in proportions between the 2-LTR^+ and 2-LTR^- subgroups through the Pearson χ^2 test. We used the Mann–Whitney *U* test to compare medians between subgroups. To compare longitudinal changes (baseline–week 48 and week 48–week 60) within subgroups the signed-rank test (paired test) was computed. Moreover, to quantify the mean change per week (slope coefficient) from baseline up to week 48, linear mixed models were considered. We performed statistical analyses with SAS 9.1 and the R package. We generated graphics with GraphPad Prism 5.0 software.

RESULTS

Patient Characteristics

A total of 51 subjects from the Integral study were included in the immunological substudy; of these, 34 intensified their current HAART with raltegravir (Intensification group) for 48 weeks, whereas 17 continued their HAART regimen (Control group). Furthermore, intensified individuals were stratified in a post hoc analysis according to 2-LTR circles positivity (2-LTR^+, n = 12) or negativity (2-LTR^-, n = 22) in PBMCs during the intensification period.[25,30] Only 3 individuals in the Control groups were 2-LTR^+ at a unique time point. The separate analysis of these individuals did not yield consistent differences with the 2-LTR^- individuals in the control arm; therefore, the Control group was analyzed as a whole. Baseline subject characteristics are shown in Table 1. All baseline demographic, clinical, and virological parameters, and absolute CD4 and CD8 T-cell counts were similar between 2-LTR^- and 2-LTR^+ subgroups. There was a significant trend toward a higher frequency of 2-LTR^+ patients for those on a protease inhibitor (PI)-containing regimen at baseline and a trend toward higher values of soluble CD14 in the 2-LTR^+ subgroup (Table 1).

Analysis of CD8 T-Cell Activation During the Intensification Period

To further investigate the basis for the decrease in CD8 T-cell activation that was observed in our previous analyses,[22,25] we independently evaluated the 2-LTR^+ and 2-LTR^- subgroups within the raltegravir-intensified subjects and the

TABLE 1. Baseline Characteristics of Subjects Included in the Integral Immunological substudy

		Intensification (n = 34)		
	Control (n = 17)	2-LTR^- (n = 22)	2-LTR^+ (n = 12)	*P* Value 2-LTR^- Vs. 2-LTR^+ *
Age (yrs), mean ± SD	44 ± 7.7	44 ± 6,4	43 ± 7.3	*0.633*
Females, n (%)†	4 (24)	6 (27)	0 (0)	*0.16*
Antiretroviral therapy				
PI-containing regimen at intensification, n (%)†	6 (35)	5 (23)	8 (67)	***0.016***
Suboptimal antiretroviral therapy before HAART, n (%)†	5 (29)	7 (32)	3 (25)	*0.498*
Time from diagnosis (yrs), mean ± SD	11.8 ± 7.2	12.3 ± 5.1	10.1 ± 6.1	*0.249*
Time with suppressive HAART (yrs), mean ± SD	5.8 ± 3.0	5.2 ± 2.9	3.5 ± 2.5	*0.075*
Viral load (standard assay)	<50	<50	<50	*1*
Total HIV-1 DNA (copies/10^6 PBMCs), median [IQR]	13.4 [4.4–46.4]	10.6 [6.6–65.5]	29.0 [8.4–55.0]	*0.874*
Integrated HIV DNA (copies/10^6 PBMCs), median [IQR]	13.0 [0–43.4]	0 [0–3.1]	0.08 [0–8.9]	*0.989*
CD4 T-cell counts, absolute (cell/μL), median [IQR]	457 [371–600]	533 [448–787]	520 [431–756]	*0.679*
CD8 T-cell counts, absolute (cell/μL), median [IQR]	805 [503–1051]	761 [482–963]	738 [491–943]	*1*
Ultrasensible viral load (SCA), median [IQR]	0.6 [0.5–1.2]	0.5 [0.45–1.1]	1.2 [0.6–3.5]	*0.146*‡
SolubleCD14 (μg/mL), median [IQR]	7.6 [5.9–8.5]	6.8 [5.7–8.2]	8.9 [7.2–9,7]	*0.063*

PI were lopinavir or atazanavir, and nonnucleoside reverse transcriptase inhibitor were efavirenz or nevirapine. Bold text indicates statistically significant difference.
**P* value between groups: Mann–Whitney *U* test.
†Pearson χ^2.
‡*P* value between groups: Peto–Prentice test.
IQR, interquartile ratio; SCA, single copy assay; VIRAL load (copies RNA/mL of plasma).

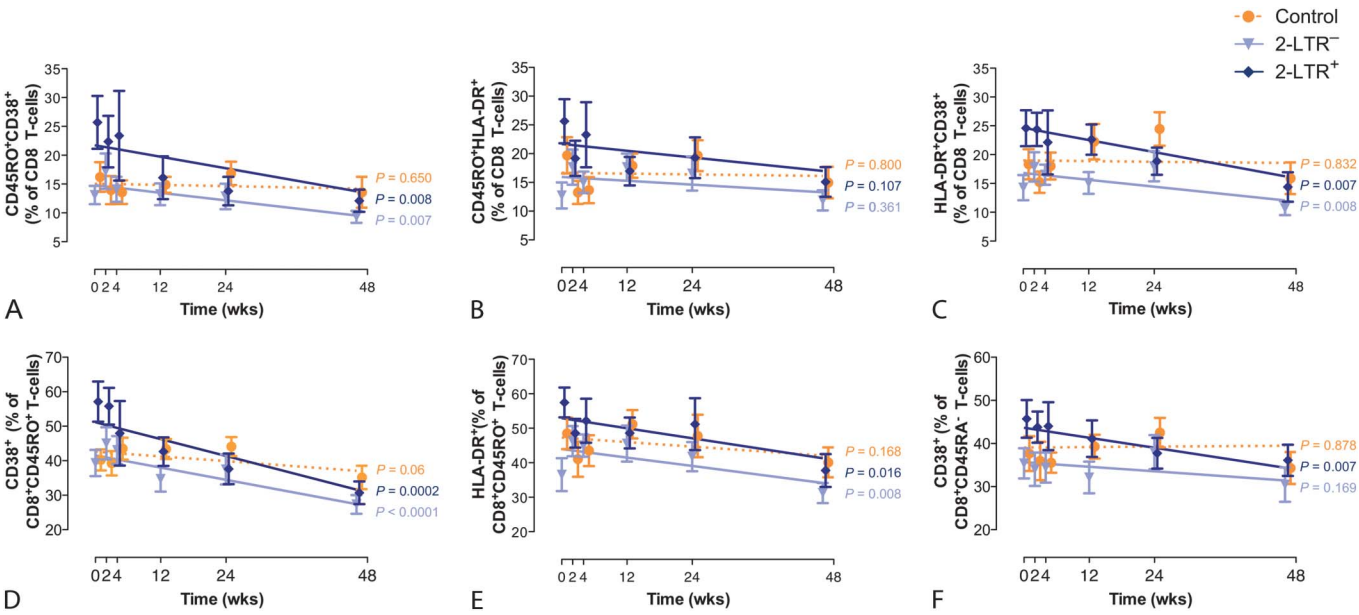

FIGURE 1. Time course of CD8 T-cell activation markers during the intensification phase. CD8 T-cell activation was measured as the percentage of $CD8^+CD45RO^+CD38^+$ (A), $CD8^+CD45RO^+HLA\text{-}DR^+$ (B), $CD8^+HLA\text{-}DR^+CD38^+$(C) cells, the frequency of $CD38^+$ (D) or $HLA\text{-}DR^+$ (E) cells in the $CD8^+CD45RO^+$ subset and the frequency of $CD38^+$ (F) cells in the $CD45RA^-CD8^+$ subset. Fitted results from linear mixed models analysis are overlaid on the mean [±standard error (SE)] of the control group (orange circles), $2\text{-}LTR^-$ (light blue triangles) and $2\text{-}LTR^+$ (dark blue diamonds) intensified subgroups. *P* values of longitudinal changes within groups are indicated.

compared both with the control group. Longitudinal mixed models depicted in Figure 1 and Table 2 show the decay slopes for different CD8 T-cell activation markers. We evaluated the coexpression of CD38 and HLA-DR as a marker of general CD8 T-cell activation. In addition, because both markers are differently modulated by immune responses,[22,25,30] we independently analyzed their expression in the memory or naive compartments. All CD8 activation markers remained constant in the control group throughout the study period although there was a decreasing trend in CD38 expression in the memory ($CD45RO^+$) CD8 T-cell subset ($P = 0.06$, Fig. 1D). In contrast, both intensified subgroups showed significant decreases in most CD8 T-cell activation markers as measured by $CD45RO^+CD38^+$ or $HLA\text{-}DR^+CD38^+$ in the whole CD8 T-cell population or by $CD38^+$ or $HLA\text{-}DR^+$ in the memory ($CD45RO^+$) CD8 T-cell compartment. As reported previously,[25,31] baseline CD8 T-cell activation markers were significantly higher in the $2\text{-}LTR^+$ subgroup compared to the $2LTR^-$ subgroup (Fig. 2); however, slope coefficients obtained from longitudinal mixed models of both subgroups did not show significant differences (Table 2). This result indicated that raltegravir intensification similarly impacted both the $2\text{-}LTR^+$ and $2\text{-}LTR^-$ subgroups. Interestingly, the CD38 marker was, in general, more sensitive than HLA-DR in revealing the decay in the levels of CD8 T-cell activation. Indeed, linear mixed models of $CD8^+CD45RO^+HLA\text{-}DR^+$ did not show a significant decay in any subgroup, whereas a significant decrease was noticed for $CD8^+CD45RO^+CD38^+$

TABLE 2. Linear Mixed Models From Baseline to Week 48 Comparing Activation Markers

	Control		Intensification				
			2LTR⁻		2-LTR⁺		
	Coefficient (SE)*	*P* Value†	Coefficient (SE)*	*P* Value†	Coefficient (SE)*	*P* Value†	*P* Value‡ 2-LTR⁻ Vs, 2-LTR⁺
$CD45RO^+CD38^+$ (% of $CD8^+$ T-cells)	−0.02 (0.04)	*0.650*	−0.11 (0.04)	***0.007***	−0.17 (0.06)	***0.008***	0.325
$CD45RO^+HLA\text{-}DR+$ (% of $CD8^+$ T-cells)	−0.01 (0.04)	*0.800*	−0.06 (0.04)	*0.361*	−0.10 (0.06)	*0.107*	0.548
$HLA\text{-}DR^+CD38^+$ (% of $CD8^+$ T-cells)	−0.009 (0.04)	*0.832*	−0.1 (0.04)	***0.008***	−0.18 (0.06)	***0.007***	0.274
$CD38^+$ (% of $CD8^+CD45RO^+$ T-cells)	−0.11 (0.06)	*0.060*	−0.29 (0.06)	***<0.0001***	−0.41 (0.10)	***0.0002***	0.361
$HLA\text{-}DR^+$ (% of $CD8^+CD45RO^+$ T-cells)	−0.10 (0.08)	*0.189*	−0.20 (0.08)	***0.008***	−0.24 (0.09)	***0.016***	0.765
$CD38^+$ (% of $CD8^+CD45RA^-$ T-cells)	0.03 (0.04)	*0.878*	−0.09 (0.06)	*0.169*	−0.19 (0.07)	***0.007***	0.622

*Linear mixed model: regression coefficient (mean change per week) and SE of the regression coefficient.
†*P* value of the regression coefficient.
‡*P* value of the interaction term of the linear mixed model: response = intercept + group + week + group × week.
SE, standard error.

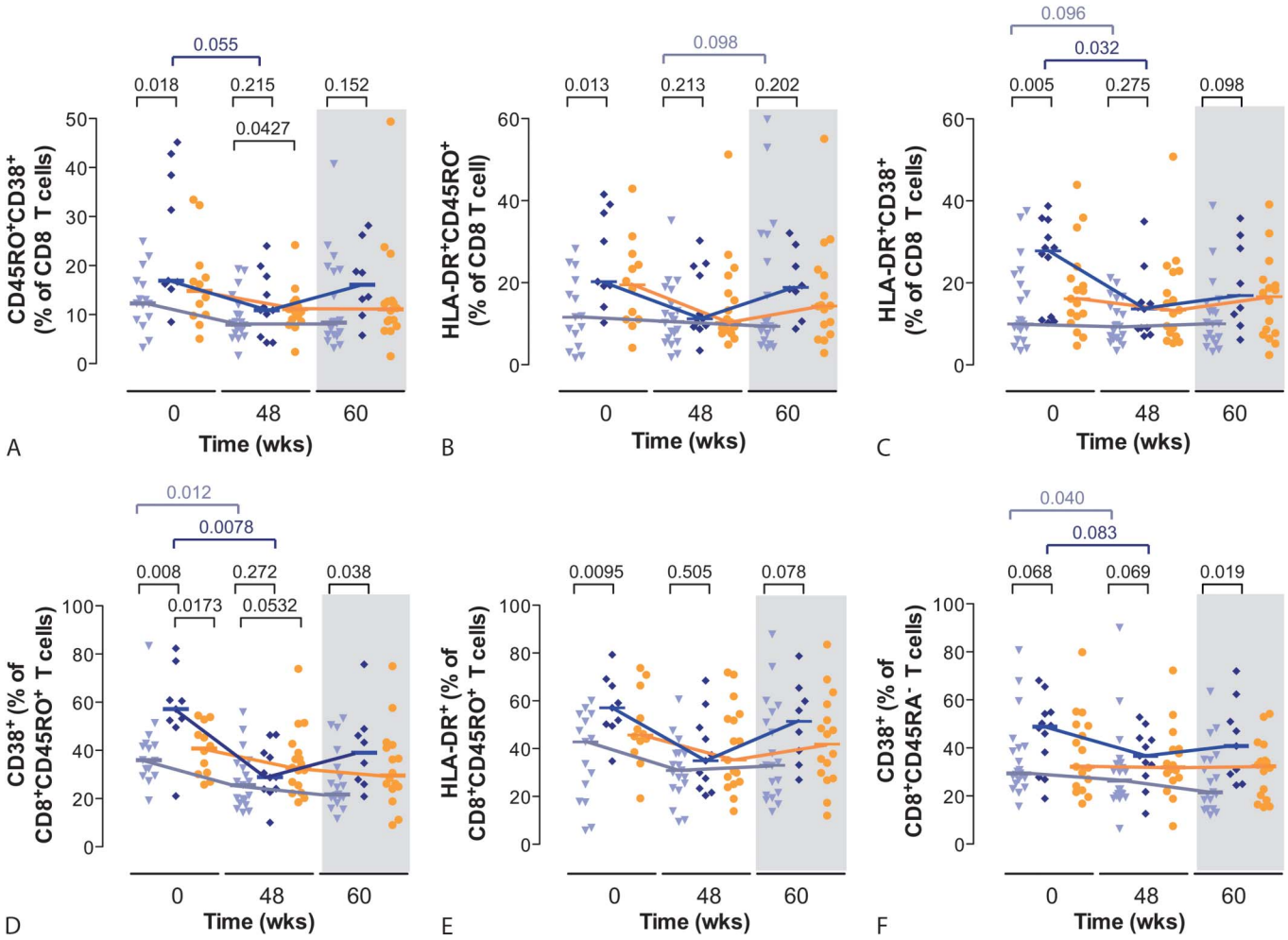

FIGURE 2. Effect of raltegravir discontinuation on CD8 T-cell activation. Comparison of CD8 T-cell activation markers between 2--LTR$^-$ (light blue triangles), 2-LTR$^+$ (dark blue diamonds) subgroups, and control group (orange circles) at baseline, 48 weeks after intensification and 12 weeks after raltegravir withdrawal (week 60). The percentage of CD8$^+$CD45RO$^+$CD38$^+$ (A), CD8$^+$CD45RO$^+$HLA-DR$^+$ (B), CD8$^+$HLA-DR$^+$CD38$^+$ (C) cells, the frequency of CD38$^+$ (D) or HLA-DR$^+$ (E) cells in the CD45RO$^+$CD8$^+$ subset and the frequency of CD38$^+$ (F) cells in the CD45RA$^-$CD8$^+$ subset are plotted. Individual and median values are shown. Two-sided *P* values (Mann–Whitney *U* test between groups, and signed-rank test, paired data, within groups) are indicated.

(Figs. 1A, B). The analysis of the memory (CD45RO$^+$) compartment was better to reveal a decay in activation in both CD38 and HLA-DR, with steeper decay slopes, being more evident in CD38 expression as compared with HLA-DR expression (Figs. 1D, E and Table 2). Changes in CD38 expression in the memory subset were confirmed using a different combination of antibodies with independent staining and analysis (CD38$^+$ on CD8$^+$CD45RA$^-$ T-cells, Fig. 1F). Conversely, the analysis of activation markers in naive subset showed similar levels of expression of the activation marker HLA-DR in both the control and the 2-LTR$^+$ and the 2-LTR$^-$ subgroups (data not shown).

Partial Rebound in CD8 T-Cell Activation upon Raltegravir Discontinuation

We extended the analysis of activation markers to week 60, which was 12 weeks after raltegravir withdrawal. Figure 2 summarizes data for the different activation markers in the whole CD8 T-cell or CD45RO$^+$ CD8 T-cell compartment at baseline, week 48, and week 60. All activation markers showed similar V-shaped decreases and increases in the 2LTR$^+$ subgroup, whereas a smoother trend was observed in the 2LTR subgroup. Once again, the most significant decay trends between baseline and week 48 in both subgroups were detected by analyzing CD38 expression. Interestingly, some of the initial significant differences in activation levels between both subgroups, which were normalized by the end of the intensification period, were partially restored upon raltegravir withdrawal. A trend was observed for the frequency of HLA-DR$^+$CD38$^+$ in CD8 T-cells and HLA-DR$^+$ in the memory (CD45RO$^+$) subset, whereas significant differences were evident for CD38 expression on memory cells (Fig. 2D). In addition, the change in the latter subset was again confirmed by an independent evaluation of CD38 expression in the CD45RA$^-$ subset using a different antibody combination

(Fig. 2F). Thus, in the 2-LTR$^+$ subgroup, 12-week raltegravir discontinuation interval reversed the impact of the initial 48 weeks of raltegravir intensification on the CD8 activation levels (week 60 values were comparable with baseline for all activation makers). Conversely, upon raltegravir discontinuation in the 2-LTR$^-$ subgroup, there was a more gradual trend toward a baseline activation (CD38) levels in both the CD45RO$^+$ and the CD45RA$^-$ subsets. No changes were observed in the control group (data not shown).

CD4 T-Cell Activation

Our previous data suggested that the CD4 T-cell compartment was much more refractory to changes induced by raltegravir intensification.[22,25,32] Indeed, no changes in activation markers on CD4 T-cells were observed either at 24 or 48 weeks in the control or the intensification groups. To further explore possible changes in CD4 T-cells, we performed a similar post hoc analysis to that described for CD8 T-cells (Fig. 3). The 2-LTR$^+$ subgroup showed higher levels of CD4 T-cell activation compared with that of the 2-LTR$^-$ subgroup at baseline (measured as CD38$^+$CD45RA$^-$ of CD4 T-cells or CD38$^+$ in the CD4$^+$CD45RA$^-$ T-cell subset). Although these initial differences between subgroups were reduced at week 48 (Fig. 3A), there were no changes over time as measured either by linear mixed models (Fig. 3B) or by comparing baseline and week 48 values (Fig. 3A) in 2-LTR$^+$ or 2-LTR$^-$ subgroups. No effect of raltegravir withdrawal was noticed for any subgroup (Fig. 3A) even though a trend toward a difference in CD45RA$^-$CD38$^+$ between 2-LTR$^+$ and 2LTR$^-$ subgroups was observed at week 60.

CD4 T-Cell Dynamics

Recent studies have suggested that switching HAART from protease to integrase inhibitors is associated with an

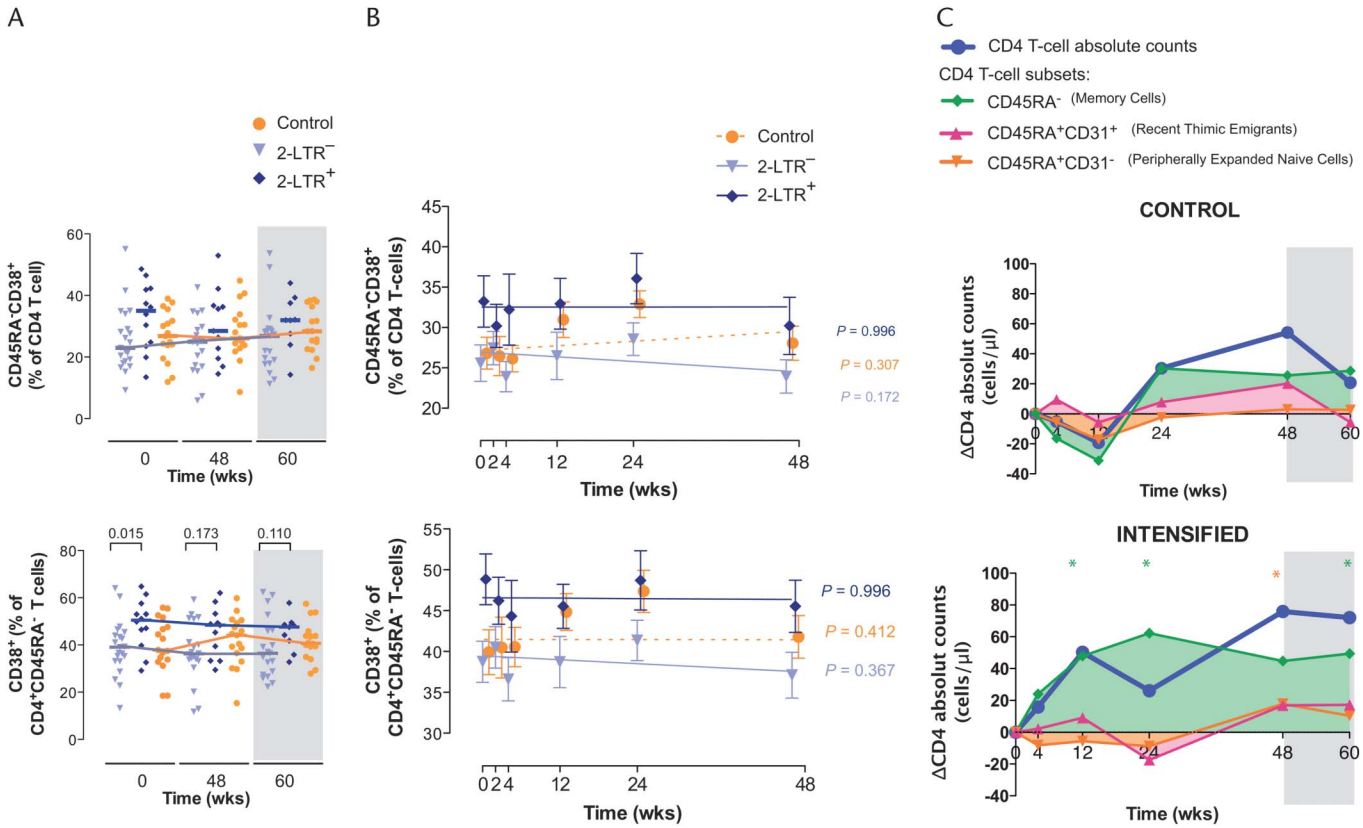

FIGURE 3. CD4 T-cell changes during intensification and discontinuation phases. A, Time course of CD4 T-cell activation markers measured as the percentage of CD4$^+$CD45RA$^-$CD38$^+$ cells and the frequency of CD38$^+$ cells in the CD45RA$^-$ CD4$^+$ subset. Fitted results from linear mixed models analysis are overlaid on the mean (±SE) of control group (orange circles), 2-LTR$^-$ (light blue triangles) and 2-LTR$^+$ (dark blue diamonds) intensified subgroups. B, Comparison of CD4 T-cell activation markers between 2-LTR$^-$ (light blue triangles), 2-LTR$^+$ (dark blue diamonds) subgroups and control group (orange circles) at baseline, 48 weeks after intensification and 12 weeks after raltegravir withdrawal (week 60). The percentage of CD4$^+$CD45RA$^-$CD38$^+$ and the frequency of CD38$^+$ cells in the CD45RA$^-$CD4$^+$ subset are plotted. Individual and median values are shown. Two-sided *P* values (Mann–Whitney *U* test between groups) are indicated. C, Absolute counts of CD4 T-cell subsets in control (upper) and intensified groups (lower) were analyzed during the study period and 12 weeks after raltegravir withdrawal (week 60). Mean values of absolute increases from baseline observed in total CD4 T-cells (blue) and CD45RA$^-$ (green), CD45RA$^+$CD31$^+$ (pink), and CD45RA$^+$CD31$^-$ (orange) cell subsets are shown. Color-coded asterisks denote significant differences from baseline (*$P < 0.05$) assessed by the signed-rank test for paired data.

increase of thymic production.[22,31] Therefore, although raltegravir intensification did not seem to reduce CD4 T-cell activation, we investigated whether there were changes in recent thymic emigrants (defined as $CD4^+CD45RA^+CD31^+$ [25,32]), peripherally expanded naive cells ($CD4^+CD45RA^+CD31^-$), and memory CD4 T-cells ($CD4^+CD45RA^-$) in patients enrolled in the IntegRal immunological substudy (n = 17 and n = 34 for control and intensified group, respectively). The whole IntegRal study group (n = 22 and n = 45 for control and intensified, group, respectively) showed significant increases of CD4 T-cell counts in the control and intensified groups at week 48[4,6,15,22,33] and a significant transient increase in the intensified group at week 12 ($P = 0.0285$). However, intensified patients included in the immunological substudy showed only a trend toward an increase in absolute CD4 T-cell counts at weeks 12 and 48 ($P = 0.0808$ and $P = 0.0850$, respectively), whereas no significant changes were observed in the control arm throughout the study (Fig. 3C). Changes in the intensified arm were associated with a significant increase of absolute numbers of memory ($CD45RA^-$) CD4 T-cells at weeks 12 and 24 ($P = 0.035$ and $P = 0.012$, respectively). This increase remained significant compared with baseline 12 weeks after raltegravir withdrawal ($P = 0.040$, Fig. 3C). Furthermore, the intensified group showed no significant changes in absolute numbers of naive subsets ($CD45RA^+CD31^+$ and $CD45RA^+CD31^-$ CD4 T-cells), except for peripherally expanded naive cells, which showed a significant increase at week 48 ($P = 0.012$) that was not sustained after raltegravir withdrawal (Fig. 3 C).

Post hoc analysis of the intensified arm revealed no significant differences between 2-LTR^- and 2-LTR^+ subgroups. Both showed trends toward an increase in absolute $CD45RA^-$ CD4 T-cells at week 24 ($P = 0.0987$ and $P = 0.084$ for 2-LTR^- and 2-LTR^+ subgroups, respectively, data not shown). Overall, these data suggest that, rather than changes in thymic output, raltegravir may induce a redistribution of memory cells, which seems to be independent of the 2-LTR status.

DISCUSSION

We have previously demonstrated that HIV-1-infected HAART-suppressed subjects who exhibited increases in 2-LTR circles upon raltegravir intensification had greater levels of immune activation at baseline.[23,25] In this study, we show that CD8 activation levels were reduced to similar levels in both the 2-LTR^- and 2-LTR^+ subgroups after 48 weeks of raltegravir intensification. Remarkably, after 12 weeks of raltegravir discontinuation, CD8 activation levels reverted to pre-intensification values in the 2-LTR^+ subgroup. This suggests that immune activation is driven, at least in part, by residual viral infection as a result of incompletely suppressive HAART and raltegravir may reduce immune activation by increasing the level of viral suppression.

Interestingly, decreases in CD8 activation were more evident for those combinations assessing CD38 expression in memory cells (steeper decay slopes). In fact, the frequency of $CD45RO^+HLA\text{-}DR^+$ CD8 T-cells remained unchanged over the study, although a significant decay was observed in the frequency of $HLA\text{-}DR^+$ cells in $CD45RO^+$ CD8 T-cells in both intensified 2-LTR^- and 2-LTR^+ subgroups. Conversely, the level of CD38 expression, particularly in the memory ($CD45RO^+$) subset, when assessed by different staining procedures, consistently decreased upon intensification. These data underscore the previously reported utility of the CD38 marker in the analysis of immune activation in HIV-1 infection[4,6,9,15,33,34] and its potential link to HIV replication under HAART.[8,23] Therefore, this marker could prove useful for gauging the impact of treatment intensification on immune activation parameters. However, other mechanisms are likely to contribute to immune activation in HIV-1 infected individuals. CD38 expression can be modulated by coinfections or microbial translocation in HIV-infected individuals.[9,10,34] For example, inhibition of asymptomatic cytomegalovirus replication by valganciclovir in HIV-1–infected individual on HAART resulted in a rapid reduction in CD8 T-cell activation.[8,35] No such effects were found using an antilipopolysaccharide immunoglobulin.[10,30,36] Therefore, the analysis of CD38 expression on CD8 T-cells together with analysis of episome dynamics are likely required to provide a specific insight into the contribution of residual HIV replication to immune activation. Surprisingly, our data show that raltegravir reduced CD38 activation markers not only in the 2-LTR^+ subgroup but also in the 2-LTR^- subgroup. However, 2-LTRs are a minor unintegrated cDNA species and fall below the limits of detection in some patients. Therefore, there may have been low levels of ongoing infection in the 2-LTR^- that was below the limit of detection of the 2-LTR assay. As such, raltegravir likely impacted immune activation in both the 2-LTR^+ and 2-LTR^- subgroups by curtailing residual viral replication.

HIV infection generates an inflammatory environment associated with type I interferon,[35,37] which induces CD38 upregulation mainly in memory CD8 T-cells in vitro and in vivo.[30,36,38] HAART is able to reduce immune activation but does not normalize it.[25,37] Persistent residual HIV infection in lymphoid tissues, or anatomical sanctuaries with low drug penetration, would therefore sustain upregulated levels of CD38 in memory CD8 T-cells (as observed in the 2-LTR^+ subgroup). Recent studies using deep sequencing of episomal and integrated viral cDNA from these patients have suggested that there are limited rounds of viral replication virus activity. This could be a result of stochastic production of virions from a chronic virus source (perhaps reactivated, latently infected cells) and limited rounds of de novo infection.[23,38] Raltegravir efficacy in the patients studied here might be explained by an increased ability of raltegravir to sequester within lymphoid tissue, which is the predominant viral reservoir.[39] This possibility is currently under investigation. Of note, limited rounds of de novo infection may explain the gradual changes in CD8 T-cell activation induced by raltegravir. Significant changes required 24 weeks of intensification in the 2-LTR^+ subgroup[17–19,25] and 48 weeks in the 2-LTR^- subgroup, whereas significant increases in CD8 T-cell activation were observed after 12 weeks of raltegravir discontinuation in the 2-LTR^+ subgroup. Consistently, we also found slow (24–48 weeks) decreases in CD8 T-cell activation in HAART-treated subjects with poor CD4 T-cell recovery upon raltegravir intensification,[20,23] and other studies have shown that shorter raltegravir interventions did not have significant effect on peripheral CD8 T-cell activation,[14,17–19] except

for Yukl et al[20] that found a fast trend toward a decrease of CD8 T-cell activation in gut sites and PBMCs after 12-weeks raltegravir intensification.[15] As gut-associated lymphoid tissue is the principle site of viral replication, it is also likely to be the principle site for cryptic viral replication in patients on HAART. Therefore, the impact of raltegravir on CD8 T-cell activation may be more rapid in the gut-associated lymphoid tissue relative to the periphery. However, other intensification strategies adding maraviroc[14,40,41] or abacavir[15,20] to stable HAART reported faster CD8 T-cell activation decays after 12 or 24 weeks of intensification, respectively.

In contrast to the observation with CD8 T-cells, linear mixed models did not show changes in CD4 T-cell activation markers during the intensification period or after raltegravir withdrawal. The relative influence of the homeostatic response to CD4 T-cell depletion and viral forces on the activation of CD4 and CD8 T-cells in HIV infection might explain the different dynamics observed.[18,40,41] Surprisingly, we observed a rapid and transient increase in absolute CD4 T-cell levels at week 12 in the intensified group, although both arms showed similar absolute CD4 T-cell gains at week 48. Comparable trends in CD4 T-cell increases were found at week 12 in other raltegravir intensification studies in the ileum[20,42,43] and PBMCs.[18,31] Unfortunately, no long (48 weeks) intensification effects were assessed in those studies. Our data support an early outburst of memory ($CD45RA^-$) CD4 T-cells from tissues to the periphery in both intensified subgroups (independent of episomal cDNA detection). Memory CD4 T-cells might be retained in tissues where cryptic replication might occur and, after raltegravir intensification and reduction in residual replication, the cells could be mobilized and recirculate. This effect may be similar to the memory T-cell redistribution from secondary lymphoid compartments observed in HIV-infected patients after HAART initiation,[42–44] and the associated decrease in tissue inflammation. However, this result contrasts with those of a recent study that found an increased production of naive CD4 T-cells after a HAART switch from protease inhibitors to raltegravir.[31] Different therapeutic strategies (switch vs. intensification) and baseline patient characteristics showing different viral suppression periods that may affect the rate of naive CD4 T-cells production[44] could explain these differences.

In summary, this study indicates that CD8 T-cell activation, specifically CD38 expression on memory cells, is modulated by raltegravir intensification and discontinuation. Conversely, CD4 T-cell activation remains unchanged, although $CD45RA^-$ CD4 T-cells seem to redistribute from tissues in intensified subjects. Both observations suggest that a continued de novo infection may persist in some HAART-suppressed subjects and that the effect of drug intensification is reversible. Although our data suggest that the measure of immune activation may help to identify incomplete viral suppression, direct analysis of tissue samples and more robust technical approaches will be needed to understand the contribution of residual HIV replication to immune activation.

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