

# The Genome-wide Methylation Profile of CD4<sup>+</sup> T Cells From Individuals With Human Immunodeficiency Virus (HIV) Identifies Distinct Patterns Associated With Disease Progression

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**Background.** Human genetic variation—mostly in the human leukocyte antigen (HLA) and C-C chemokine receptor type 5 (CCR5) regions—explains 25% of the variability in progression of human immunodeficiency virus (HIV) infection. However, it is also known that viral infections can modify cellular DNA methylation patterns. Therefore, changes in the methylation of cytosine-guanine (CpG) islands might modulate progression of HIV infection.

**Methods.** In total, 85 samples were analyzed: 21 elite controllers (EC), 21 subjects with HIV before combination antiretroviral therapy (cART) (viremic, 93 325 human immunodeficiency virus type 1 [HIV-1] RNA copies/mL) and under suppressive cART (cART, median of 17 months, <50 HIV-1 RNA copies/mL), and 22 HIV-negative donors (HIVneg). We analyzed the methylation pattern of 485 577 CpG in DNA from peripheral CD4<sup>+</sup> T lymphocytes. We selected the most differentially methylated gene (*TNF*) and analyzed its specific methylation, messenger RNA (mRNA) expression, and plasma protein levels in 5 individuals before and after initiation of cART.

**Results.** We observed 129 methylated CpG sites (associated with 43 gene promoters) for which statistically significant differences were recorded in viremic versus HIVneg, 162 CpG sites (55 gene promoters) in viremic versus cART, 441 CpG sites (163 gene promoters) in viremic versus EC, but none in EC versus HIVneg. The *TNF* promoter region was hypermethylated in viremic versus HIVneg, cART, and EC. Moreover, we observed greater plasma levels of TNF in viremic individuals than in EC, cART, and HIVneg.

**Conclusions.** Our study shows that genome methylation patterns vary depending on HIV infection status and progression profile and that these variations might have an impact on controlling HIV infection in the absence of cART.

**Keywords.** genome-wide methylation; HIV-1 infection; HIV-1 disease progression; TNF

Human genetic variation—mostly in the human leukocyte antigen (HLA) and chemokine receptor 5 (CCR5) regions—explains 25% of the variability in progression of human immunodeficiency virus (HIV) infection [1]. Epigenetic chromatin modification regulates cellular gene transcription and expression without changing the DNA sequence [2]. Because viral infections can modify cellular DNA methylation patterns [3],

changes in the methylation of cytosine-guanine (CpG) islands could modulate progression of HIV infection through variations in transcription and expression of relevant genes. During HIV infection, the viral genome integrates into the host cellular genome, altering the chromatin structure and therefore potentially modifying host gene expression. This virus-host interaction produces changes in the epigenetic environment that affect both the virus and the host cell [3, 4]. Regarding DNA methylation, on the one hand, hypermethylation of the HIV 5' long terminal repeat (LTR) may inhibit initiation of viral transcription and thus favor HIV latency [5–8]. Furthermore, recent data suggest that methylation of 5' LTR of HIV is associated with control of viral replication in a subset of individuals with HIV characterized as long-term nonprogressors and/or elite controllers (EC) [9]. On the other hand, cellular DNA methylation may regulate T-lymphocyte gene expression in order to favor the establishment of infection, thus promoting viral replication and

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facilitating viral persistence [8]. Specifically, HIV can induce de novo methylation of host cell genes through the induction of DNA methyltransferase 1 (DNMT1) in vitro and in vivo [4, 10]. In addition, several studies showed differences in genome-wide DNA methylation between individuals with HIV and individuals who do not have HIV [11–13]. However, there are no data comparing genome-wide methylation patterns in CD4<sup>+</sup> T cells from HIV-negative individuals with longitudinal samples from individuals with HIV with different phenotypes of spontaneous viral control and under suppressive combination antiretroviral therapy (cART).

In this context, the present study aimed to evaluate the in vivo genome-wide DNA methylation profile in individuals with HIV with different phenotypes of spontaneous viral control and under suppressive cART. This approach enabled us to determine (i) which changes are preferentially associated with viremia and/or CD4 depletion (only observed in viremic individuals), (ii) which changes are reversible or nonreversible after viral suppression with cART (comparison between viremic and cART samples), and (iii) which changes might be associated with viral control in the absence of cART (comparison between ECs and viremic/cART-treated individuals).

## MATERIAL AND METHODS

### Study Design

In total, 42 individuals living with HIV with controlled infection (EC group,  $n = 21$ , defined as undetectable plasma viral load [pVL] in the absence of cART) or uncontrolled infection (viremic group,  $n = 21$ , defined as detectable pVL  $> 4 \log_{10}$  cop/mL before initiation of cART). Paired with the viremic group, longitudinal samples were also taken after at least 6 months under effective suppression with cART (cART group). Samples from 22 HIV-negative donors (HIVneg group) were used based on the cis-gender and age of the groups with HIV. The clinical characteristics of the discovery cohort at sampling are

detailed in Table 1. The specific methylation analysis was based on a validation cohort of 5 participants with HIV with available samples before cART and at 2 time points after initiation of suppressive cART (1 and 2 years after initiation). The clinical characteristics of the validation cohort at sampling are detailed in Supplementary Table 1. For this retrospective study, cryopreserved peripheral blood mononuclear cell (PBMC) samples from HIV-infected and HIV-negative individuals for the discovery and the validation cohorts were selected from the IrsiCaixa collection. All samples met legal requirements, including written informed consent from participants and donors for the use of biological samples, and complied with the stipulations of the Institutional Review Board (PI-13-024).

### Procedures

CD4<sup>+</sup> T cells purified from 85 cryopreserved PBMC samples were analyzed for genome-wide methylation quantification on an Infinium HumanMethylation450 BeadChip following the Illumina Infinium HD Methylation Assay Protocol. Bisulfite genomic sequencing of multiple clones, methylation-specific polymerase chain reaction (PCR), messenger RNA (mRNA) expression, and plasma levels of the selected tumor necrosis factor (TNF) gene were performed in samples of the validation cohort. The candidate CpGs contained in the methylation array were included in the PCR amplicon (Supplementary Table 2). The primer sequences are listed in Supplementary Table 3. More detailed procedures are described in Supplementary Materials.

### Statistical Analyses

Methylation raw data normalization was performed using minfi, lumi, and associated R packages following Quantile normalization. In the differential methylation analysis between cohorts of each CpG, a *t* test was used to compare  $\beta$ -value means between subject groups through pairwise comparisons, correcting by multiple testing (false discovery rate). The CpGs selected were those with adjusted values of

**Table 1. Characteristics of the Discovery Cohort at the Sampling Time Point, by Group**

	HIV Positive			
	Viremic (n = 21)	cART (n = 21)	EC (n = 21)	HIV Negative (n = 22)
Age (years) <sup>a</sup>	32 (28–35)	34 (29–37)	44 (40–50)	31 (29–42)
Sex (n, %)				
Men	21 (100)	21 (100)	8 (38)	4 (18)
Women	0	0	12 (57)	9 (41)
Missing	0	0	1 (5)	9 (41)
CD4 <sup>+</sup> T-cell counts (cells/ $\mu$ L) <sup>a</sup>	444 (350–565)	734 (578–808)	645 (505–1001)	ND
CD4/CD8 ratio <sup>a</sup>	0.5 (0.3–0.6)	1 (0.7–1.1)	ND	ND
Plasma viremia (HIV RNA copies/mL)	$9.4 \times 10^4$ (0.4–1.4 $\times 10^5$ )	UD	UD	NA
Time since diagnosis (years) <sup>a</sup>	1 (0.8–1.3)	2.8 (2.0–3.1)	17 (8–20)	NA
Time since initiation of cART (years) <sup>a</sup>	0	1.4 (1.2–1.5)	0	NA

Abbreviations: cART, combination antiretroviral therapy; EC, elite controller; HIV, human immunodeficiency virus; NA, not applicable; ND, no data; UD, undetectable (either with 25, 40, or 50 HIV-1 RNA copies/mL threshold).

<sup>a</sup>Median (interquartile range (IQR)).

$P < .05$  and an absolute methylation differential value of  $>0.05$  ( $>5\%$ ). Hypergeometric test was used to determine differentially methylated gene promoter regions by comparing the number of differentially methylated CpGs and the number of CpGs tested in each region. KEGG pathway enrichment analysis was performed taking into account only genes with a differentially methylated promoter region. Wilcoxon signed rank test (paired comparisons: viremic vs cART) or Mann-Whitney test (nonpaired comparisons: viremic vs EC and HIVneg, and EC vs HIVneg) were used to analyzed tumor necrosis factor (TNF) specific methylation, mRNA expression, and plasma levels using GraphPad Prism (v5.0).

## RESULTS

### Genome-wide Differences in Methylation Between Viremic, cART-Suppressed, and EC Individuals With HIV and HIVneg Individuals

In order to determine whether HIV infection affects host cellular genome-wide methylation, and the steadiness of such potential modifications, we compared the methylation level of 485 577 CpG sites in CD4<sup>+</sup> T cells from viremic and paired cART-suppressed, EC, and HIV-negative individuals. Regarding methylated CpG sites for which statistically significant differences were recorded, we observed 129 differentially methylated CpGs between viremic and HIVneg individuals (median [min, max] methylation difference: 7.3 [ $-15.6$ , 16.6%]), 162 between viremic and cART-suppressed individuals (6.5 [5.0, 12.8%]), and 441 between viremic and EC individuals (6.3 [ $-16.4$ , 13.8%]). However, we did not observe statistically significant differences in methylated CpG sites between EC and HIVneg individuals (Figure 1A-D).

To characterize the genes associated with these differentially methylated CpG sites, we analyzed the CpG located in gene regulatory regions (5' UTR, TSS, and the first exon were included) and with a methylation difference  $>5\%$  (Figure 2A). Specifically, we detected statistically significant differences in methylation between viremic and HIVneg individuals in 43 genes (Supplementary Table 4), between viremic and cART-suppressed individuals in 55 genes (Supplementary Table 5), and between viremic and EC individuals in 163 genes (Supplementary Table 6).

Within the top 15 genes per comparison, we observed that TNF was the most differentially methylated gene when comparing viremic versus HIVneg and viremic versus EC ( $p_{adj} < 1 \times 10^{-25}$ ), with hypermethylation observed in the viremic individuals (Figure 2B). We also observed hypermethylation in TNF promoter when comparing longitudinal samples from individuals with HIV before and after viral suppression with cART ( $p_{adj} < 0.001$ ), thus indicating that viremia-driven TNF hypermethylation was reverted. However, when we compared viremic versus cART ( $p_{adj} = 1.68 \times 10^{-8}$ ), the greatest difference in methylation was in BCL9 transcription coactivator (BCL9) with

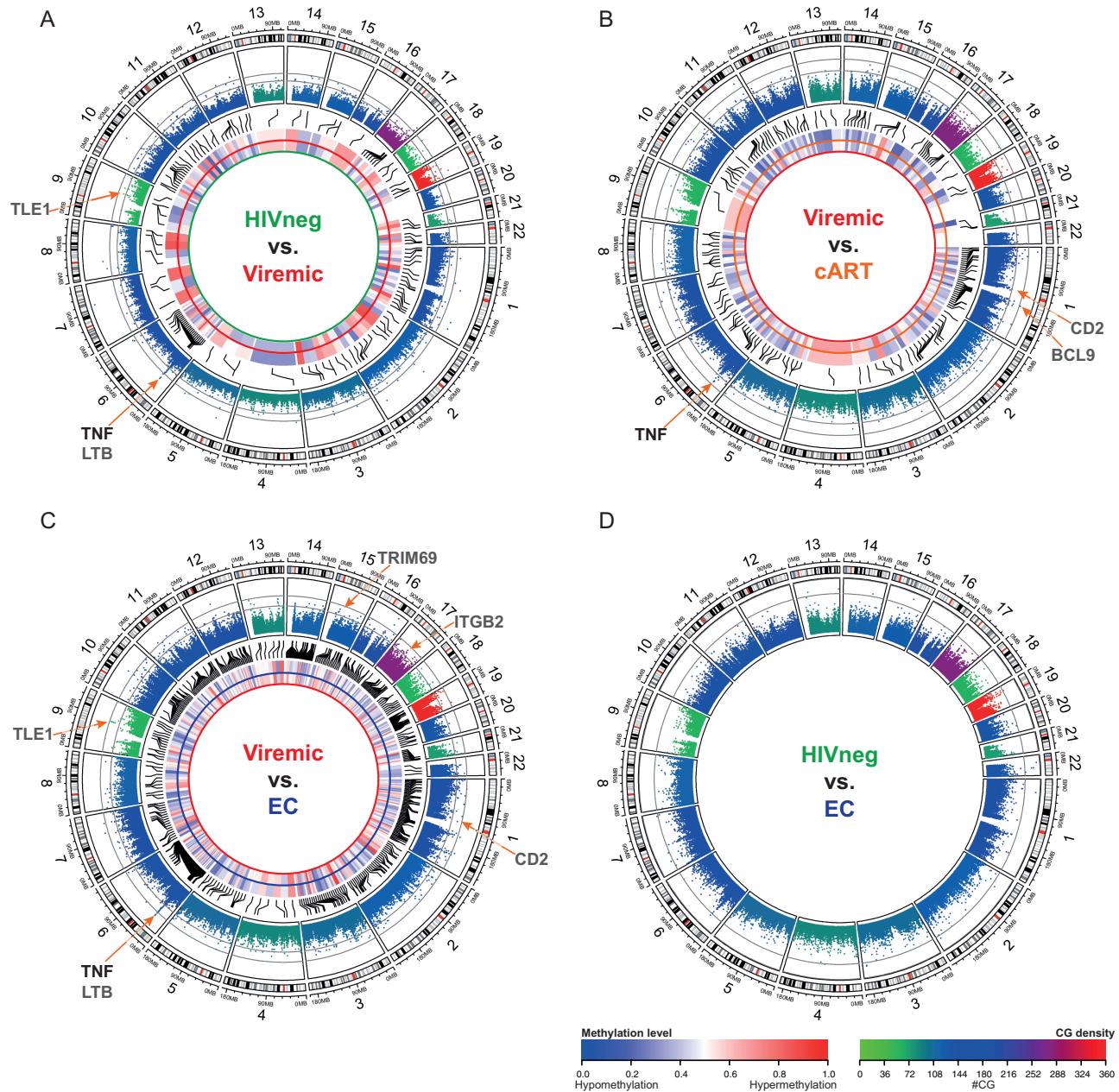
hypomethylation being observed only under suppressive cART, thus suggesting that BCL9 methylation might be affected by administration of cART. Furthermore, we observed hypomethylation of tripartite motif containing 69 (TRIM69) and integrin subunit beta 2 (ITGB2) (all  $p_{adj} < 1 \times 10^{-7}$ ) in EC when compared with viremic individuals, suggesting that the expression of these genes might be upregulated (hypomethylated) in EC and, therefore, have an impact on control of HIV infection. We also observed hypermethylation of lymphotoxin beta (LTB) ( $p_{adj} < 1 \times 10^{-7}$ ) and hypomethylation of transducing-like enhancer family member 1 (TLE1) ( $p_{adj} = 1.19 \times 10^{-7}$ ) when we compared viremic vs HIVneg and EC, thus indicating a viremia-driven methylation pattern that was not reverted under suppressive cART. Moreover, we observed that cluster of differentiation 2 (CD2) was hypomethylated both in EC ( $p_{adj} = 3.25 \times 10^{-4}$ ) and in cART ( $p_{adj} = 2.98 \times 10^{-5}$ ) compared with viremic individuals, suggesting upregulation of CD2 in virally suppressed CD4<sup>+</sup> T cells and therefore an association between CD2 expression with HIV latent infection.

Regarding the association between these 7 genes of interest and HIV, we analyzed the STRING protein interaction and previous bibliography related to HIV (Figure 2C). Interaction with HIV was previously reported for TNF, LTB, ITGB2, CD2, TLE1, and BCL9. Furthermore, we observed that TNF, LTB, ITGB2, and CD2 interact with each other and are associated with the immune response, whereas TLE1 and BCL9 interact with each other and are associated with gene expression, thus indicating that HIV infection might affect the methylation pattern of immune response-related genes and transcription factors. Interestingly, no interaction has been reported between HIV and TRIM69, which is involved in induction of apoptosis and, in this study, was hypomethylated in EC, thus suggesting that its expression might be associated with control of HIV in the absence of cART.

Overall, these data suggest that HIV infection modifies levels of cellular host genome methylation and that methylation levels might be associated with progression of HIV infection.

### The T-Cell Receptor Signaling Pathway Is Enriched in Differentially Methylated CpG Sites in Viremic Individuals

To determine the cellular pathways associated with statistically significant differences in methylation at CpG sites located in a promotor region and with a fold-change  $>5\%$ , we performed a KEGG pathway enrichment analysis using the previously listed genes related to these CpG sites. We observed 58 differentially enriched cellular pathways (Supplementary Table 7), from which 5 were enriched in the comparisons of viremic versus cART-suppressed, versus EC individuals, and versus HIVneg; the "T-cell receptor signaling pathway" was the most significantly enriched KEGG pathway (Supplementary Table 7).

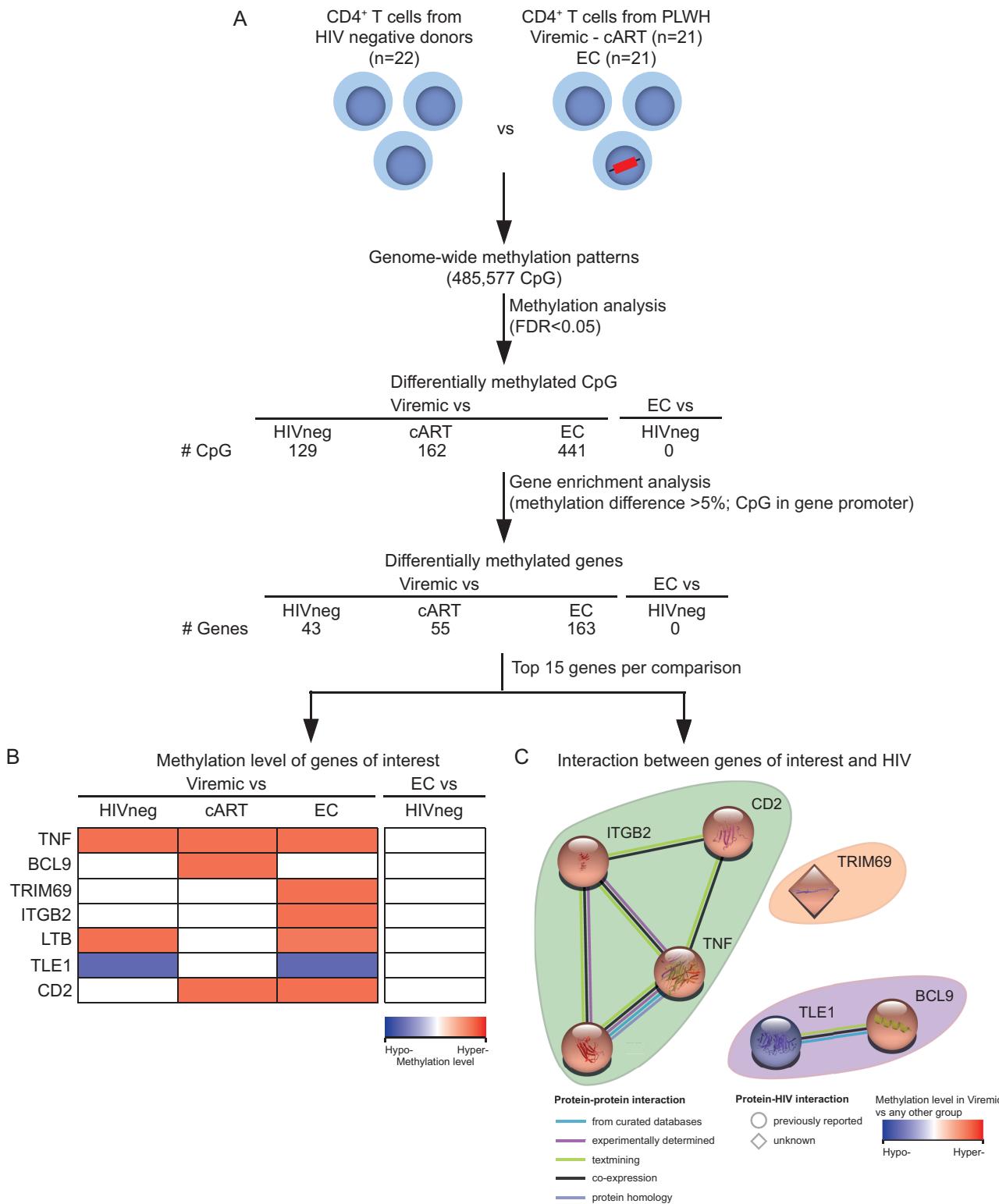


**Figure 1.** Genome-wide methylation levels by CpG site. (A) HIV-negative (HIVneg) vs viremic, (B) viremic vs cART, (C) viremic vs EC, and (D) HIVneg vs EC. From inside to outside: (i) heatmap of CpGs with significant adjusted  $P$ value and differential methylation over 5%, following the order of the group comparison (no significant differences in methylation of CpG sites were detected in HIVneg vs EC, D); (ii) Manhattan plot of  $-\log_{10}(P\text{-value})$  for CpG sites with  $P$ -value thresholds at  $10^{-5}$  and  $10^{-7}$ , and (iii) chromosomes. CpG sites for which differences in methylation were statistically significant are shown in blue (hypomethylated) and red (hypermethylated). The density of CpG sites per chromosome is color-coded. Abbreviations: cART, combination antiretroviral therapy; CpG, cytosine-guanine; EC, elite controllers; HIV, human immunodeficiency virus; LTB, lymphotoxin beta; TNF, tumor necrosis factor.

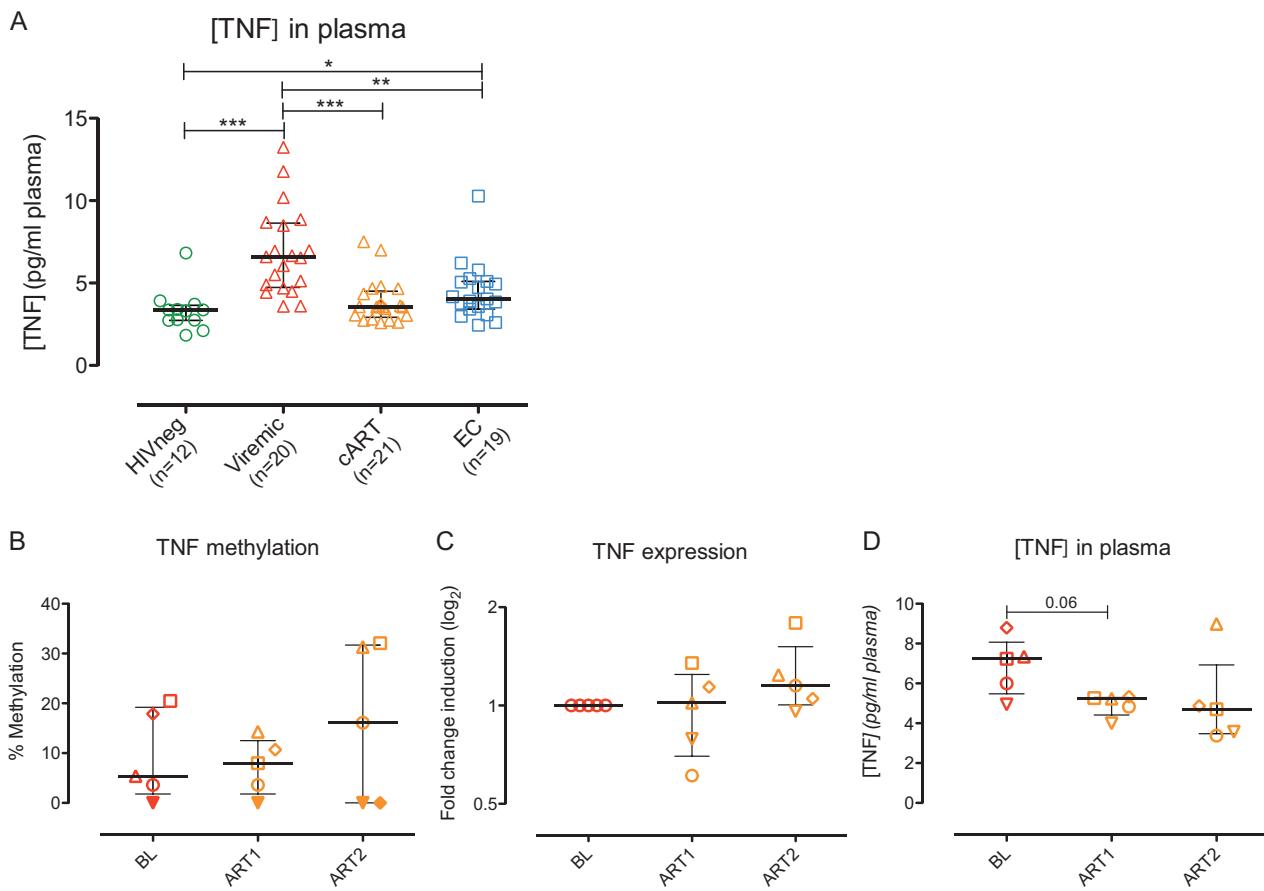
#### Greater Promoter TNF Methylation as a Counteraction of TNF Overexpression in Viremic Individuals

To characterize the effect of HIV viremia on methylation, mRNA expression, and plasma levels of TNF, we quantified the plasma level of TNF in the discovery cohort, and the methylation, mRNA expression, and plasma levels of TNF in longitudinal samples (before and after initiation of cART) from 5 independent individuals with HIV. We observed greater plasma

levels of TNF in viremic individuals than in cART-suppressed, EC, and HIV-negative individuals, as well as in EC versus HIVneg (Figure 3A). These data suggest that hypermethylation of TNF promoter might be a homeostatic mechanism for normalization of TNF production and therefore immune activation due to HIV infection. In the validation cohort, we did not observe significant changes in specific DNA methylation in the TNF promoter region or mRNA expression in TNF in



**Figure 2.** Analysis pipeline overview, methylation level of genes of interest, and interaction between genes of interest and HIV. *A*, Schematic representation of the bioinformatics analysis pipeline overview. *B*, The methylation level of the differentially methylated genes of interest. *C*, The interaction between the genes of interest (using STRING) and HIV (based on previous bibliography). The methylation level is shown in blue (hypomethylated) and red (hypermethylated). Abbreviations: cART, combined antiretroviral therapy; EC, elite controllers; FDR, false discovery rate; HIV, human immunodeficiency virus; LTB, lymphotoxin beta; PLWH, people living with HIV; TNF, tumor necrosis factor.



**Figure 3.** Association between HIV infection, cART, and disease progression on TNF production. *A*, Plasma TNF level in HIV-negative, viremic, cART, and EC individuals from the discovery cohort ( $n = 12$  HIVneg,  $n = 20$  viremic,  $n = 21$  cART, and  $n = 19$  EC). *B*, Levels of TNF-specific methylation, (*C*) mRNA, and (*D*) plasma in the validation cohort ( $n = 5$ , samples before [BL, baseline], and 1 and 2 years after initiation of cART [ART1 and ART2]). Solid symbols represent undetectable values. The statistical analysis was performed using the Wilcoxon signed rank test (paired comparisons: viremic vs cART) or Mann-Whitney test (nonpaired comparisons: viremic vs EC and HIVneg, and EC vs HIVneg) [GraphPad Prism (v5.0)]. Abbreviations: cART, combined antiretroviral therapy; EC, elite controllers; HIV, human immunodeficiency virus; mRNA, messenger RNA; TNF, tumor necrosis factor.

longitudinal samples pre- and post-viral suppression by cART (Figure 3B, C). However, we observed a decrease in the plasma level of TNF after initiation of cART (Figure 3D). These results corroborate that initiation of cART, which suppresses HIV viremia, helps to decrease plasma TNF levels [14] and therefore can partially contribute to the reduction of global immune activation under suppressive cART.

## DISCUSSION

The present study is the first to our knowledge to evaluate the genome-wide DNA methylation profile in vivo in peripheral CD4<sup>+</sup> T cells from HIV-negative individuals and individuals with HIV with different phenotypes of viral control (EC) and degrees of disease progression (viremic and paired cART-suppressed and EC individuals). We observed differential genome-wide patterns depending on spontaneous ability to control HIV replication and on whether or not suppressive cART had been initiated.

Several studies performed in vitro and in vivo have analyzed how HIV affects host epigenetic mechanisms [11, 13, 15, 16]. On the one hand, viral infection alters the expression of cellular genes that modify chromatin structure. On the other hand, the viral genome itself is modified by some of these mechanisms to regulate its level of transcription [5]. Moreover, lifestyle (eg, nutrition, physical activity, working habits, smoking, and alcohol consumption) and the administration of drugs, such as cART, may influence epigenetic mechanisms [17].

In our study, we observed 129 differentially methylated CpG sites in viremic versus HIVneg individuals, 162 sites in viremic versus cART individuals, and 441 sites in viremic versus EC individuals. These results confirm not only that HIV infection modifies the DNA methylation profile of the target cells in vivo [11–13] but also that these epigenetic modifications differ according to HIV disease progression status and control of infection [9].

Regarding specific genes, we found that hypermethylation of the TNF gene was a differential hallmark of viremic

individuals. Several studies have shown that TNF and TNF receptor superfamily members have a role in the pathogenesis of HIV, affecting different stages of the HIV replication cycle and being able to increase HIV transcription through activation of the NF- $\kappa$ B signaling pathway [18]. Moreover, it has been shown that expression of TNF is regulated by DNA methylation and other mechanisms [19], and that TNF acts as a link between T-cell dysfunction and chronic inflammation during chronic viral infection [20]. The hypermethylation of the TNF promoter region and greater plasma levels in viremic individuals we observed might constitute a homeostatic mechanism for downregulation of TNF expression and plasma levels in order to decrease immune activation and HIV expression. On the other hand, TNF produced by other cell types, such as CD8 $^{+}$  T cells, monocytes, and dendritic cells, which we did not include in this study, should be further characterized in future studies.

With respect to differences in methylation between viremic and EC individuals, we observed that the genes TRIM69, and ITGB2, were hypomethylated in EC. These results suggest that the expression of these genes might be upregulated and therefore play a role in the control of HIV replication in the absence of cART. To our knowledge, TRIM69, which encodes a member of the RING-B-box-coiled-coil family and which, when overexpressed, induces apoptosis, and although its DNA methylation has been associated with HIV frailty [21], it has never been related to HIV replication or pathogenesis. Its hypomethylation in EC may increase its expression in CD4 $^{+}$  T cells and therefore in HIV-infected cells, causing specific apoptosis of these cells and potentially leading to better control of HIV infection. Interactions between the HIV replication cycle and the LTB, ITGB2, CD2, and TLE1 genes have already been described [22–35]. ITGB2 encodes an integrin beta chain, which plays an important role in the immune response by helping leukocytes gather at sites of infection or injury, where they are needed to contribute to the immune response. Hypomethylation of ITGB2 in EC may upregulate their expression, thus enhancing the immune response against HIV and potentially helping to control HIV infection in the absence of cART.

The methylation pattern found when we compared viremic versus HIVneg and EC, and consisting of LTB hypermethylation and TLE1 hypomethylation, defines a hallmark of pathogenic HIV infection not reverted by cART. LTB is a type II membrane protein of the TNF family and an inducer of the inflammatory response system and is involved in the development of lymphoid tissue. TLE1 is a transcription factor that inhibits NF- $\kappa$ B and Wnt signaling pathways, which are involved in activation of HIV transcription [36]. Conversely, the lack of hypomethylation of TLE1 in EC may prevent increased expression, thus decreasing its impact on the NF- $\kappa$ B and Wnt signaling pathways, enhancing HIV antigen expression, and

therefore helping to detect and eliminate HIV-infected cells, which could contribute to the lower reservoir levels described in EC. Interestingly, none of the abovementioned genes has ever been related to control of HIV infection or disease progression. Therefore, further investigation of these genes and their role in HIV infection could prove helpful for deciphering the mechanisms that may regulate HIV control in EC, controllers, and long-term nonprogressors.

BCL9, which is involved in WNT pathway and required for HIV transcription [37], was hypermethylated in the viremic samples compared with cART-suppressed samples, whereas its methylation pattern in viremic individuals was similar to that of HIVneg donors, suggesting that its expression might be lower in viremic individuals as a mechanism for reducing replication of HIV or as a direct effect of cART. CD2 is a surface antigen that interacts with LFA3 (CD58) on antigen-presenting cells to optimize immune recognition, and its expression in resting memory CD4 $^{+}$  T cells has been related to latently HIV-infected cells [34]. In contrast, CD2 has been positively associated with the expression of restriction factors [32], and its hypermethylation in viremic individuals was downregulated by cART, suggesting that its increased expression might favor HIV replication.

The limitations of the study should be acknowledged. First, a low number of individuals was analyzed in both the discovery and the validation cohorts. This may explain why we did not observe statistically significant differences in the methylation of CpG sites when comparing EC with HIVneg. Second, although cART may have an impact on epigenetics, the low number of individuals and the different cART regimens they were taking prevented us from determining changes in DNA methylation related to the specific antiretroviral drugs used. Third, although age and sex bias are undesirable, there is no evidence of a strong influence of either variable on the methylation level of our candidate genes. Moreover, most aging-related studies focus on epigenetic reprogramming during early development [38] or on cohorts with considerable variation in age that exceed the 10 years found among some of our study groups. As for the sex composition of our cohorts, we excluded all CpG located in the X chromosome and in imprinted genes [39]. In this sense, little is known about sex-specific variations in methylation patterns for sequences of coding genes in autosomal chromosomes. Finally, as latently infected cells cannot be differentiated from uninfected cells and represent 1 in 1000 total CD4 T cells [40], we were not able to specifically analyze the infected peripheral CD4 $^{+}$  T-cell subset. Thus, noninfected cells might dilute the changes in host DNA methylation. However, by analyzing total CD4 $^{+}$  T cells, we can assume that the changes observed in the DNA methylation patterns may be due to HIV infection itself and to bystander effects in noninfected cells.

Data on changes in host genes after *in vivo* infection are limited, thus highlighting the need for more comprehensive global studies. In this regard, single-cell analysis techniques might

help to isolate the several types of cells representing the immune system and interacting with the virus and to compare changes in gene and protein expression, as well as epigenetic modifications.

Our findings confirm that HIV infection modifies host genome methylation patterns in vivo and that these patterns differ and may play a role in progression of HIV infection.

## Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

## Notes

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