

# Changes in Blood B Cell Phenotypes and Epstein-Barr Virus Load in Chronically Human Immunodeficiency Virus–Infected Patients before and after Antiretroviral Therapy

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**Background.** Switched and nonswitched memory B cells, which usually constitute the main reservoirs of Epstein-Barr virus (EBV), are rapidly depleted in patients with chronic human immunodeficiency virus (HIV) infection. Because the EBV load is frequently increased in these patients, other B cell reservoirs might participate in EBV persistence.

**Methods.** We examined the combined expression of CD27, SigD/G/M, CD38, CD10, CD5, CXCR5, CD62L, CD44, and CXCR3 on B cells from healthy donors ( $n = 30$ ) and from HIV type 1–infected patients ( $n = 23$ ) at diagnosis and after highly active antiretroviral therapy. The plasma HIV load and the DNA EBV load in peripheral blood mononuclear cells were assessed.

**Results.** Increased frequencies of CD38<sup>+</sup>SigD<sup>+</sup>CD10<sup>+</sup> B cells were found in patients with an EBV load  $>10^3$  copies per  $10^6$  peripheral blood mononuclear cells and a strong depletion of memory B cells. This phenotype resembles that of transitional B cell subsets. Elevated percentages of these B cells were still found in 2 patients showing no decrease in EBV load after highly active antiretroviral therapy.

**Conclusions.** Because transitional-like B cells persist concomitantly with high EBV load after highly active antiretroviral therapy, we suggest that this population might be an alternative EBV reservoir in patients with chronic HIV infection who have strongly reduced numbers of memory B cells. The consequences of EBV infection of immature B cells are discussed with regard to B cell maturation and a higher prevalence of B cell lymphoma in HIV-infected patients.

Although CD4 T cells are the major targets of the insult exacted by human immunodeficiency virus (HIV), HIV infection more widely impairs the viability and functions of numerous competent cells and the organization

of mucosal lymphoid tissues [1]. In particular, B cell dysfunction represents a central characteristic of HIV infection and an important pathogenic mechanism [2–7]. In the absence of highly active antiretroviral therapy (HAART), HIV type 1 (HIV-1) infection is associated with a wide range of B cell defects, including polyclonal hypergammaglobulinemia [8], impaired expression of activation and costimulatory markers [2, 9–11], decreased B cell survival [12, 13], and the presence of immature and/or transitional CD10<sup>+</sup> cells [14], exhausted CD27<sup>neg</sup> cells, or terminally differentiated B cells in blood [3, 15–17]. Despite polyclonal activation, the antibody response is strongly impaired [18], resulting in a decreased response to natural or vaccine T cell–independent and T cell–dependent antigens and

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a loss in peripheral memory B cells [5, 13, 19–21]. Most of these defects have been considered the hallmarks of the chronic phase of infection and are frequently correlated with increased HIV load and loss of CD4 T cells [14]. However, patients with primary HIV infection and macaques with acute simian immunodeficiency virus infection also show severe damage to various blood B cell subsets that is not totally reversed after HAART, even though the CD4 T cell count is increasing and the viral load is decreasing [6, 21, 22].

Compared with healthy individuals, patients with chronic HIV infection often show a higher level of Epstein-Barr virus (EBV) in infected cells isolated from lymphoid tissue at diagnosis. These patients have a higher risk of developing non-Hodgkin B cell lymphoma [23–25], with 75% of these AIDS-related B cell lymphomas being EBV positive [26]. In contrast to the situation in transplant recipients, there is no correlation between the absolute EBV load and the occurrence of EBV-positive B cell lymphoma in HIV-infected patients [24, 27, 28]. The progression of the EBV load and the expansion of EBV-infected B cells in tissues result from the combination of HIV-associated chronic immune activation, CD4 T cell depletion, exhaustion of EBV-specific CD8 T cells, and B cell dysfunction [27, 28]. Although EBV persists essentially in switched memory B cells (SIgD<sup>-</sup>CD27<sup>+</sup>) in healthy donors and in nonswitched memory B cells (SIgM<sup>+</sup>SIgD<sup>+</sup>CD27<sup>+</sup>) in patients with X-linked lymphoproliferative disease [29] or acute infectious mononucleosis [30], elevated EBV loads are observed in patients with chronic HIV infection, despite a consistent loss in CD27<sup>+</sup> (switched or nonswitched) memory B cells. This finding suggests that B cell populations other than memory B cells participate in EBV persistence in patients with chronic HIV infection. Therefore, we performed an extensive characterization of peripheral blood B cells and determined the EBV load in 30 healthy individuals and, both before and after HAART, in 23 patients with chronic HIV-1 infection. Our results revealed that all patients had an altered naive B cell phenotype with reduced proportions of CD23<sup>+</sup> B cells. Before HAART, 12 of 23 HIV-infected patients had an EBV load >10<sup>3</sup> DNA copies per 10<sup>6</sup> peripheral blood mononuclear cells (PBMCs) that was associated with higher proportions of SIgD<sup>+</sup>, CD38<sup>+</sup>, CD10<sup>+</sup>, CD5<sup>+</sup>, and CXCR3<sup>+</sup> B cells, compared with healthy individuals and other patients. A high EBV load was maintained in 2 patients who received HAART but still experienced a great decrease in CD4 T cells and memory B cells. These patients had increased proportions of circulating CD38<sup>+</sup>SIgD<sup>+</sup>CD10<sup>+</sup> B cells at diagnosis and after HAART. Taken together, our results suggest that EBV persistence in patients with chronic HIV infection who have reduced proportions of (switched and nonswitched) memory B cells might be associated with CD38<sup>+</sup>SIgD<sup>+</sup>CD10<sup>+</sup>CD27<sup>-</sup> immature and/or transitional B cells.

## MATERIALS AND METHODS

**Controls and patient groups.** For the present study, blood specimens were collected from 23 patients with chronic HIV-1 infection who were 20–52 years of age (mean age ± standard deviation [SD], 33.8 ± 8.8 years; 9 men and 14 women) and 30 healthy subjects (17 men and 13 women; mean age ± SD, 52.1 ± 16.4 years). The HIV-1-infected patients had disease at clinical stages A1 (2 patients), A2 (16), A3 (1), B2 (2), or C3 (2), according to the 1993 Centers for Disease Control and Prevention classification. All patients were drug naive at enrollment and subsequently received classic HAART. Only 12 patients still participated in the study after 9 months of receiving HAART. This study was conducted in accordance with the principles expressed in the Declaration of Helsinki and was approved by the regional ethics committee of Jean Verdier and Avicenne Hospitals. All healthy donors and patients provided written informed consent for the collection of samples and subsequent analysis. At every blood sample collection, before and after HAART, the blood sample was divided into 3 aliquots: 1 for plasma evaluation (HIV load), 1 for flow cytometer analysis, and 1 for PBMC isolation (EBV load).

**Determination of HIV plasma viremia.** The HIV RNA load in plasma was measured using a branched-DNA signal amplification method (Quantiplex HIV-RNA 2.0; Bayer Diagnostics) with a lower detection limit of 50 copies/mL.

**Flow cytometry.** Absolute numbers of leukocytes (expressed as the number of cells per microliter) were determined using analysis of 30 μL of whole blood collected on ethylenediaminetetraacetic acid in MicroDiff II (Beckman Coulter). Lymphocyte subsets were determined using 4-color flow cytometry analysis performed with the use of whole blood that had been briefly treated with ImmunoPrep Reagent (Beckman Coulter). Cells were stained with 2 combinations of antibodies: CD45-FITC/CD4-PE/CD8-ECD/CD3-PC5 and CD45-FITC/CD3-PC5/CD56-PE<sup>+</sup>CD16-PE/CD19-ECD (all from Beckman Coulter). Extended B cell phenotyping was performed using 3-color flow cytometry analysis after staining with various combinations of antibodies: CD20-PC5, CD10-PE, CD27-PE, CD44-FITC, CD5-FITC, CD95-FITC, and CD62L-FITC (Beckman Coulter); CD23-FITC, anti-human IgM-FITC, and anti-human IgG-FITC (Dako); CXCR5-PE, CCR6-PE, and CXCR3-PE (R&D System); or CD38-PE (BD Biosciences). Appropriate isotype controls were used to define the positive populations. An Epics XL-MCL flow cytometer (Beckman Coulter) was used for data acquisition and analysis.

**Quantification of EBV DNA.** PBMCs were isolated from blood samples with use of Ficoll-Paque (Pharmacia Biotech), and pellets corresponding to 10<sup>6</sup> cells were frozen at -80°C. The DNA was extracted from PBMCs by use of a QIAmp blood kit (Qiagen), in accordance with the manufacturer's instructions. The EBV load in PBMCs was measured using real-time

quantitative polymerase chain reaction, as described by Dehee et al [24], with a lower limit detection of 10 DNA copies/10<sup>6</sup> PBMCs. The human albumin DNA quantification was used to control the amount of input cellular DNA in each sample, as described elsewhere [24].

**Determination of soluble CD27 levels.** Plasma levels of soluble CD27 (sCD27) were measured using a commercial enzyme-linked immunosorbent assay kit (Pelikine compact human sCD27 enzyme-linked immunosorbent assay kit; Tebu-Bio) in accordance with the manufacturer's instructions.

**Statistical analysis.** Statistical analysis was performed using Statview (version 5; SAS Institute). The significance of differences between HIV-infected patients and healthy donors was compared using the 2-tailed Mann-Whitney *U* test. The Spearman rank test was used to test correlation between 2 parameters.

## RESULTS

**Phenotypic changes in the B cell compartment of drug-naïve HIV-1-infected patients.** Consistent with previous data [24, 28], the median EBV load was significantly increased in HIV-infected patients, compared with healthy donors. The EBV load at baseline varied greatly from one patient to another, with a median viral load of 1763 copies/10<sup>6</sup> PBMCs (range, <10 to 38,827 copies/10<sup>6</sup> PBMCs) (Table 1) in drug-naïve HIV-infected patients. The plasma EBV load was high (5210 copies/mL) in only 1 of the 23 patients (data not shown); this patient had the highest EBV load in PBMCs (38,827 copies/10<sup>6</sup> PBMCs). The increase in the EBV load was accompanied by a 3.4-fold decrease in the CD4 T cell count, a 9-fold decrease in the CD4/CD8 T cell ratio, and a 1.6-fold total decrease in the B cell count. However, no correlation was found between absolute EBV load and HIV load and the CD4 T cell count or total and memory B cell counts, although a significant correlation was found between HIV load and CD4 T cell or B cell counts in HIV-infected patients (Figure 1). In these patients, a correlation between CD4 T cell count and total or memory B cell count was also statistically significant.

To identify new parameters associated with high EBV load, we divided the HIV-1-infected patients into 2 groups (group 1 [*n* = 11] and group 2 [*n* = 12]) that were characterized by an EBV load lower and higher than 1000 DNA copies per 10<sup>6</sup> PBMCs, respectively (Table 1). The median EBV load was 133 copies/10<sup>6</sup> PBMCs (range, <10 to 867 copies/10<sup>6</sup> PBMCs) in group 1 and 3843 copies/10<sup>6</sup> PBMCs (range, 1763–38,827 copies/10<sup>6</sup> PBMCs) in group 2. A higher (2.9-fold) HIV load combined with a lower CD4 T cell count and lower (2-fold) CD4/CD8 ratio reflected more-active disease in patients in group 2 than in patients in group 1. Compared with healthy donors, the total B cell count was reduced by 1.6- and 1.4-fold in groups 1 and 2, respectively.

In healthy donors, 20.7% and 67.6% of circulating B cells were memory (CD20<sup>+</sup>CD27<sup>+</sup>) and naïve (CD20<sup>+</sup>SIgD<sup>+</sup>) B cells, respectively. The median percentage of memory B cells was only 14.7% in HIV-1-infected patients, and these patients had fewer memory B cells expressing SIgM<sup>-</sup> (5.7% vs. 9.9%; a 2-fold decrease) or SIgG (2.3% vs. 8.1%; a 4-fold decrease) than did healthy donors. Only the decrease in memory B cells expressing SIgG was significant in HIV-infected patients, compared with healthy donors (Table 1). Memory B cell counts were significantly correlated with HIV load and CD4 T cell counts but not with EBV load (Figure 1). A significant decrease in the percentage of SIgG<sup>+</sup> memory B cells was observed in both patient groups, whereas the percentage of SIgM<sup>+</sup> memory B cells was significantly decreased in group 1 only (Table 1).

The median percentage of naïve B cells was comparable in HIV-infected patients and healthy donors (67.6% and 70.2%, respectively). However, the proportion of CD23<sup>+</sup> B cells among total naïve B cells was greatly decreased in HIV-1-infected patients (29%), compared with healthy donors (71%) (Table 1). Collectively, these results show that HIV-1 infection not only induces a strong depletion in memory B cells but also is associated with defects in the naïve B cell subset.

**B cell expression of chemokine receptors and activation and/or maturation markers in HIV-infected patients.** Compared with healthy donors, a 4-fold increase in the plasma sCD27 level was consistent with sustained immune activation in HIV-infected patients (Table 1), as described elsewhere [31, 32]. Although the plasma sCD27 level was similarly increased in both groups of patients, it significantly correlated with the EBV load in group 2 only (*P* < .03;  $\rho$  = 0.709) (data not shown).

In healthy individuals, most circulating B cells (naïve and memory B cells) strongly expressed L-selectin (CD62L), CD44, CXCR5, and CCR6, whereas activated and germinal center B cells are mostly negative for SIgD, CCR6, CD44, and CD62L but express CD10, CD38, CD95, and lower levels of CXCR5. Consistent with their expression by all naïve and memory B cells, CD44 and CXCR5 were present on most B cells from healthy donors (Figure 2). Their expression was significantly decreased in both groups of HIV-infected patients, compared with healthy donors. The percentage of CD44<sup>+</sup> B cells correlated with the CD4 T cell count (*P* < .05;  $\rho$  = 0.442) but not with EBV or HIV load. In contrast, the percentage of CXCR5<sup>+</sup> B cells correlated with total (*P* < .03;  $\rho$  = 0.489) and memory (*P* < .006;  $\rho$  = 0.594) B cell counts. Whereas CD62L expression was similarly decreased in groups 1 and 2 (44.7% and 53.5%, respectively), compared with healthy donors (74.6%), a statistical significance was reached only for group 2 (*P* < .02). HIV load correlated significantly with the percentages of CD62L<sup>+</sup> B cells (*P* < .03;  $\rho$  = 0.537) but not with that of CXCR5<sup>+</sup> or CD44<sup>+</sup> B cells.

Median CD5, CD10, and CD95 percentages were similarly

**Table 1. B Cell Phenotype and Viral Characteristics in Human Immunodeficiency Virus (HIV)-Infected Patients at Diagnosis**

Characteristic	HIV <sup>-</sup> subjects (n = 30)	HIV <sup>+</sup> subjects (n = 23)	<i>P</i> <sup>a</sup> for HIV <sup>+</sup> vs. HIV <sup>-</sup> subjects			<i>P</i> <sup>a</sup> for group 1 vs. HIV <sup>-</sup> subjects (n = 11)	Group 2 <sup>c</sup> (n = 12)	<i>P</i> <sup>a</sup> for group 2 vs. HIV <sup>-</sup> subjects	<i>P</i> <sup>a</sup> for group 1 vs. group 2
			HIV <sup>-</sup> subjects (n = 30)	HIV <sup>+</sup> subjects (n = 23)	<i>P</i> <sup>a</sup> for group 1 vs. HIV <sup>-</sup> subjects (n = 11)				
EBV load <sup>d</sup>	21 (15)	1763 (3735)	<0.09	<0.09	<0.09	3843 (7710)	<0.09	<0.09	<0.09
Plasma HIV load <sup>e</sup>	NT	70,000 (127,150)	<0.09	<0.09	<0.09	103,200 (151,100)	<0.09	<0.09	NS
CD4 T cell count <sup>f</sup>	662 (454)	195 (279)	<0.09	<0.09	<0.09	159 (162)	<0.09	<0.09	NS
CD4/CD8 ratio	1.9 (1.2)	0.21 (0.33)	<0.09	<0.09	<0.09	0.18 (0.2)	<0.09	<0.09	NS
CD19 cell count <sup>f</sup>	184 (83)	115 (125)	NS	NS	NS	128 (129)	NS	NS	NS
CD19 <sup>g</sup>	12.7 (6)	8 (6.6)	NS	NS	NS	9.1 (7.4)	NS	NS	NS
CD27 cell count <sup>f</sup>	29 (34)	25 (30)	NS	NS	NS	22 (22)	NS	NS	NS
CD27 <sup>h</sup>	20.7 (16)	14.7 (24.4)	NS	NS	NS	14.2 (29.4)	NS	NS	NS
CD27 <sup>+</sup> SigM <sup>h</sup>	9.9 (5.5)	5.7 (8.4)	NS	NS	<0.04	5.7 (21.3)	NS	NS	NS
CD27 SigG <sup>h</sup>	8.1 (9.2)	2.3 (4.8)	<0.02	<0.02	<0.02	2.2 (3.3)	<0.01	<0.01	NS
SigD cell count <sup>f</sup>	112 (98)	72 (86)	NS	NS	NS	83 (76)	NS	NS	NS
SigD <sup>i</sup>	67.6 (29.3)	70.2 (29.2)	NS	NS	NS	70.2 (41.5)	NS	NS	NS
CD23 <sup>+</sup> SigD <sup>+</sup> <sup>j</sup>	71.4 (21.3)	29.4 (26.3)	<0.09	<0.09	<0.09	29.2 (30.9)	<0.09	<0.09	NS
sCD27 <sup>j</sup>	192 (78)	767 (426)	>0.09	>0.09	>0.09	780 (560)	>0.09	>0.09	NS

**NOTE.** Data are the median value (interquartile value). EBV, Epstein-Barr virus; HIV<sup>-</sup>, HIV negative; HIV<sup>+</sup>, HIV positive; NS, not significant; NT, not tested.

<sup>a</sup> Differences between groups were tested for statistical significance by use of the Mann-Whitney *U* test.

<sup>b</sup> Subjects in group 1 were HIV positive and had an EBV load <1000 copies per 10<sup>6</sup> peripheral blood mononuclear cells (PBMCs).

<sup>c</sup> Subjects in group 2 were HIV positive and had an EBV load >1000 copies per 10<sup>6</sup> PBMCs.

<sup>d</sup> EBV loads are expressed as the number of EBV DNA copies per 10<sup>6</sup> PBMCs.

<sup>e</sup> Plasma HIV loads are expressed as the number of RNA copies per milliliter.

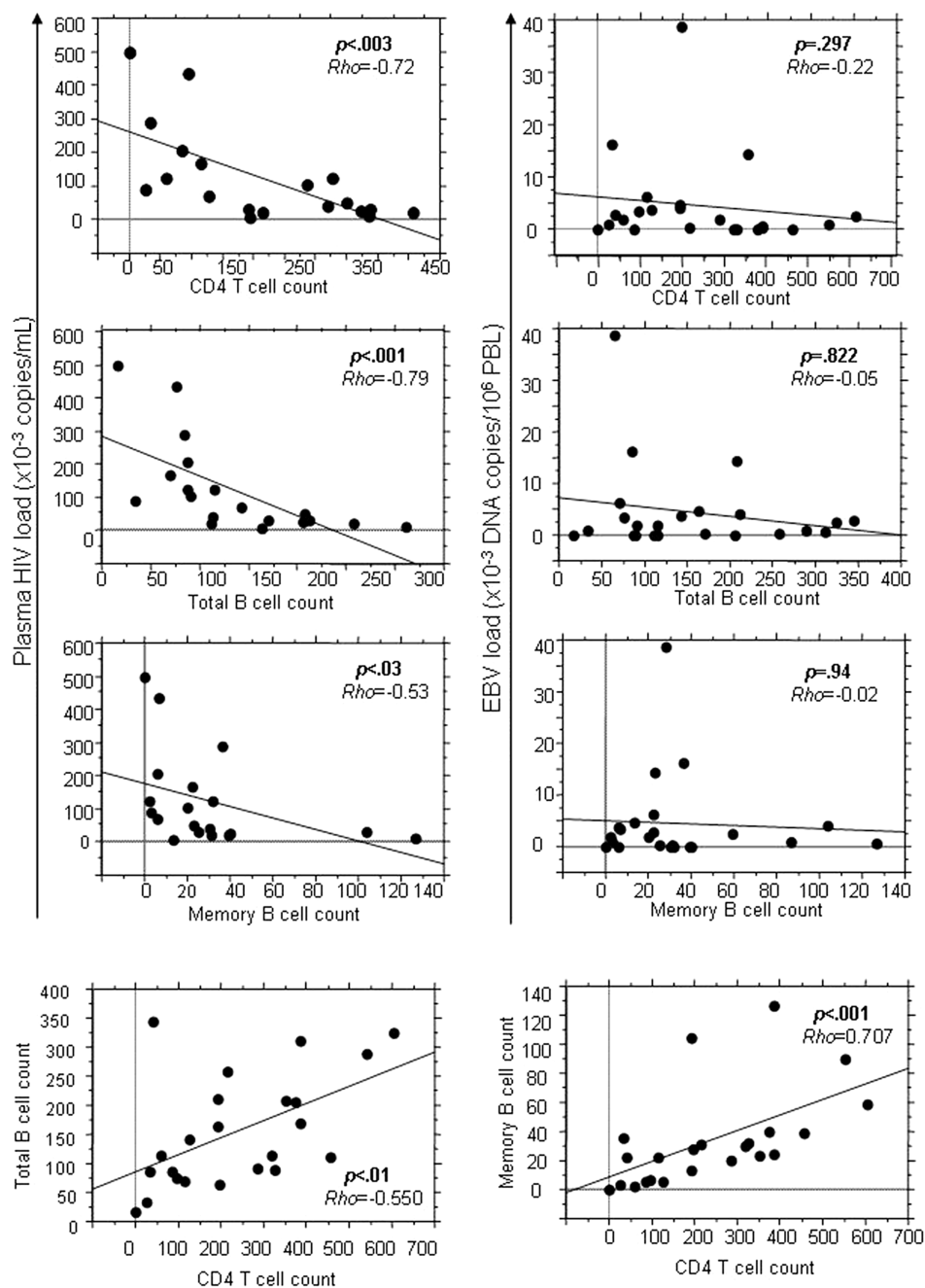
<sup>f</sup> Data are the no. of cells per microliter of blood.

<sup>g</sup> Data are the percentages of CD19 cells within blood.

<sup>h</sup> Data are the percentages within the (CD19) B cell subset.

<sup>i</sup> Data are the percentages within the total naive (SigD<sup>+</sup>) B cells.

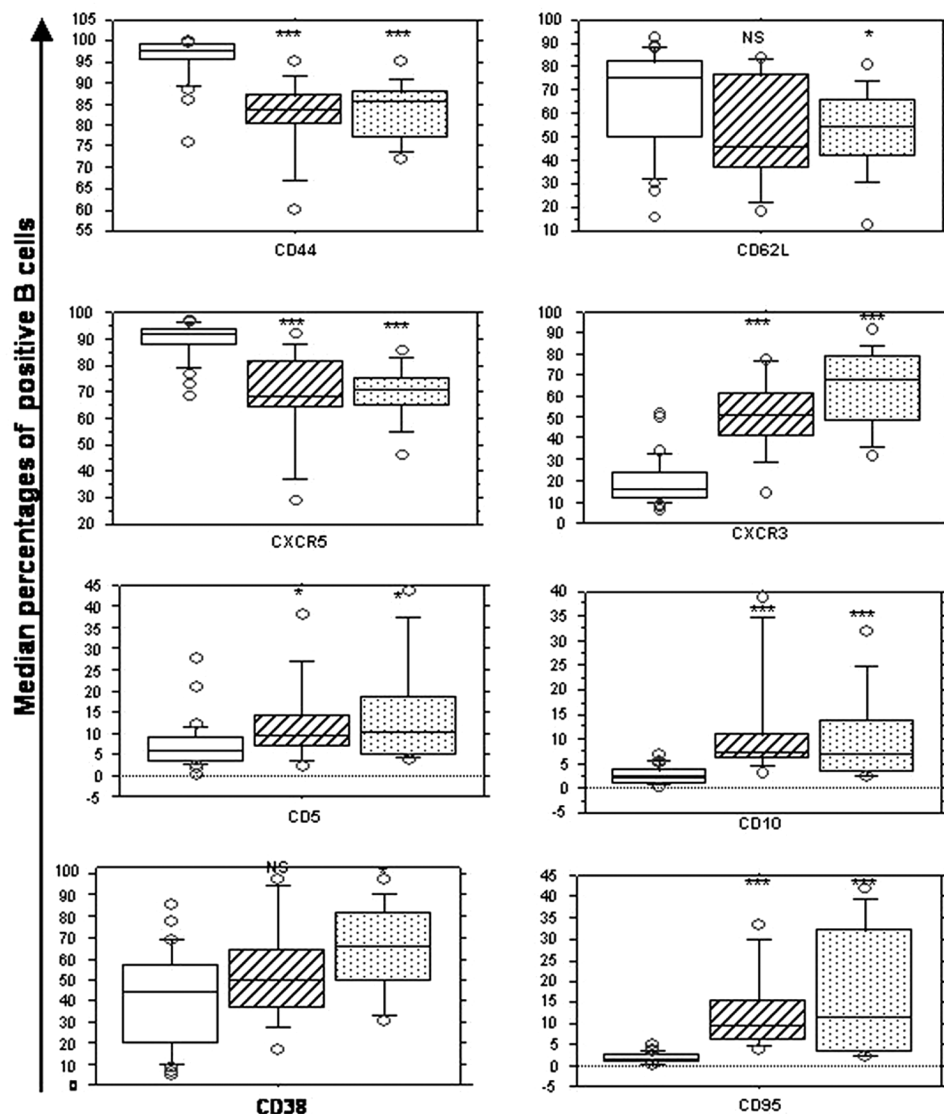
<sup>j</sup> Data are expressed as nanograms per milliliter.



**Figure 1.** Scatter plots showing associations between CD4 T cell count or B cell count and human immunodeficiency virus (HIV) load or Epstein-Barr virus (EBV) load in HIV-infected patients. CD4 T cell count correlates with total and memory B cell counts. Results of the Spearman rank correlation tests are indicated for each plot. PBL, peripheral blood lymphocytes.

increased in groups 1 and 2, whereas CD38 expression was mainly increased in group 2 (44.5%, 49.9%, and 66.3% in healthy donors, group 1, and group 2, respectively) (Figure 2). HIV load and CD4 T cell count significantly correlated with the percentages of CD10<sup>+</sup> and CD38<sup>+</sup> B cells (Figure 3), whereas the percentage of CD5<sup>+</sup> B cells correlated with HIV load only ( $P < .04$ ;  $\rho = 0.489$ ). The percentages of CD10<sup>+</sup> and CD38<sup>+</sup> B

cells significantly correlated with the percentage of CD5<sup>+</sup> B cells ( $P < .002$  [ $\rho = 0.675$ ] and  $P < .002$  [ $\rho = 0.705$ ], respectively) and inversely with the number of total and memory B cells (Figure 3). Thus, increased frequency of circulating CD10<sup>+</sup>, CD38<sup>+</sup>, and CD5<sup>+</sup> B cells may be the consequence of the HIV-induced depletion in CD4 T cells and in total and memory B cells. CD95 expression was highly heterogeneous in group 2,

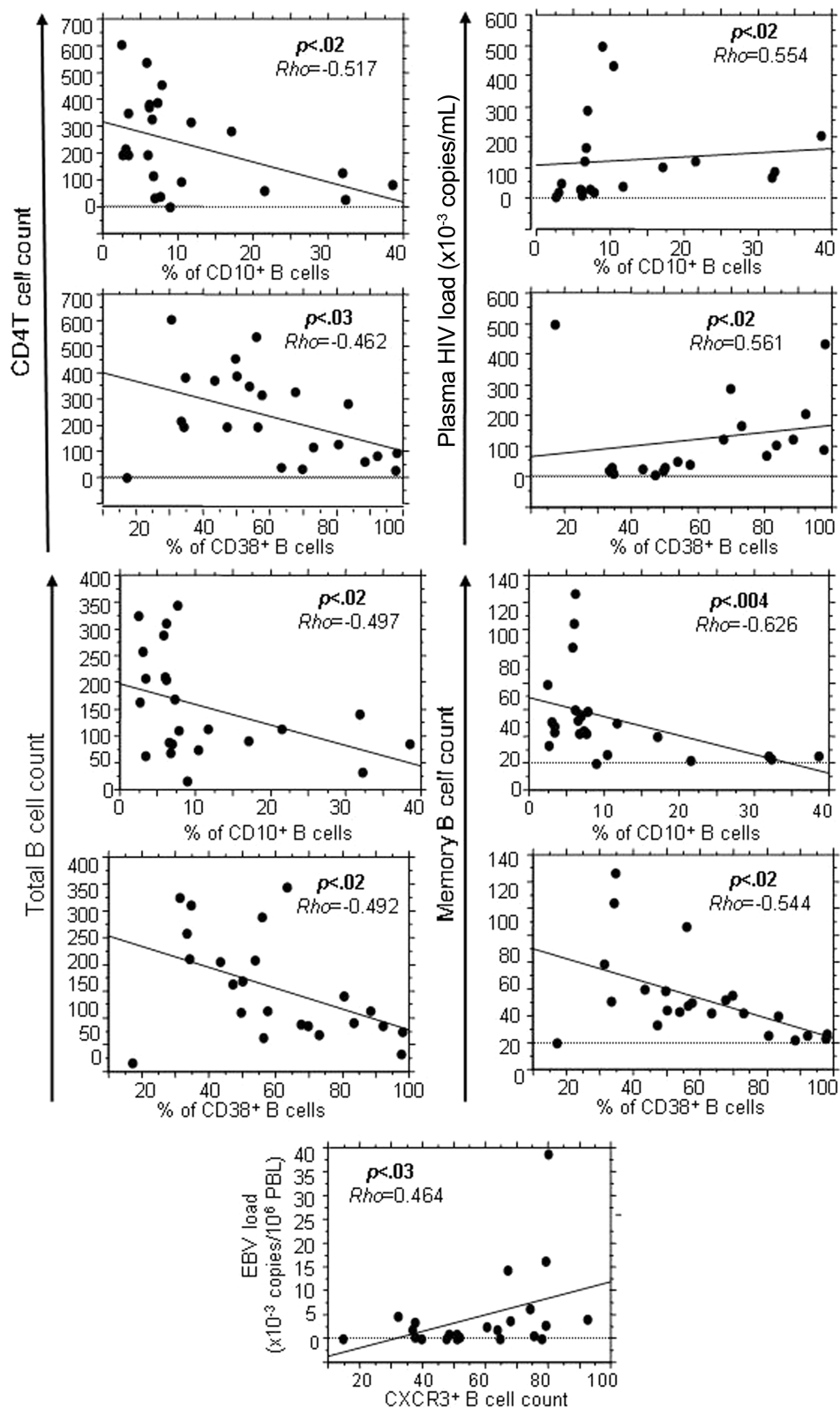


**Figure 2.** Differential B cell expression of activation markers CD44, CXCR5, and CD62L between healthy donors and patients infected with human immunodeficiency virus (HIV). The phenotype of peripheral blood mononuclear cells isolated from healthy donors and HIV-infected patients was determined using flow cytometry. Results are expressed as median percentage and interquartile ranges. Differences between values in healthy donors (*open area*) and HIV-infected patients of either group 1 (*hatched area*) or group 2 (*dotted area*) were tested for statistical significance with use of the Mann Whitney *U* test. NS, not statistically significant. \* $P < .05$  and  $P > .01$ . \*\* $P < .001$ . \*\*\* $P < .009$ .

and its percentage did not correlate with the expression of other activation markers, HIV load, or CD4 T cell count. The expression of CXCR3 was increased in both groups of patients; however, the increase was greater in group 2 (67.4%, 50.7%, and 15.8% in group 2, group 1, and healthy donors, respectively) (Figure 2). Only the CXCR3 B cell count significantly correlated with EBV load in HIV-infected patients (Figure 3).

**Changes in EBV load and B cell phenotypes in treated patients.** Only 12 of the 23 drug-naïve patients initially included in the study (52%) still participated after 9 months of HAART. At enrollment, 6 of these 12 patients were in group 1, and 6 were in group 2. The viral and immunological char-

acteristics of these patients before and after HAART are shown in Table 2. HAART was associated with a decrease of >95% in the HIV load in both groups but resulted in a stronger increase in the CD4 T cell (3.1-fold vs. 1.5-fold) and B cell (1.5-fold vs. 1.2-fold) counts in group 2, compared with group 1. In both groups, the percentages of CD44 and CXCR5 increased toward normal values, but the expression of CD62L remained low in both groups, compared with healthy individuals. The expression of CXCR3 and sCD27, although decreasing, remained 2-fold higher in patients than in healthy individuals, whereas the mean expression of CD5, CD10, and CD95 returned to baseline values after HAART.



**Figure 3.** Linear regression plots showing the correlation of frequency of CD10<sup>+</sup> or CD38<sup>+</sup> B cells and CD4 T cell count and that of the total and memory B cell counts and human immunodeficiency virus (HIV) load. Epstein-Barr virus (EBV) load correlated only with CXCR3<sup>+</sup> B cell count. Results of the Spearman rank correlation tests are indicated for each plot. PBL, peripheral blood lymphocytes.

**Table 2. Changes in the B Cell Phenotype and Viral Loads after Highly Active Antiretroviral Therapy (HAART)**

HAART	HIV-negative subjects	Group 1 <sup>a</sup>		Group 2 <sup>b</sup>	
		Before	After	Before	After
EBV load <sup>c</sup>	21 (15)	72 (173)	<10	3509 (2009)	286 (2120)
Plasma HIV load <sup>d</sup>	NT	25,400 (187,860)	<50 (100)	114,400 (221,000)	198 (2347)
CD4 T cell count <sup>e</sup>	662 (454)	294 (299)	454 (205)	111 (132)	348 (267)
CD4/CD8 ratio	1.9 (1.2)	0.31 (0.37)	0.54 (0.48)	0.18 (0.12)	0.38 (0.18)
CD19 cell count <sup>e</sup>	184 (83)	159 (170)	194 (65)	103 (57)	150 (97)
CD19 <sup>f</sup>	12.7 (6)	9 (5.4)	12.3 (2.8)	7.6 (3.8)	9 (3.5)
CD27 cell count <sup>e</sup>	29 (34)	35 (34)	34 (19.5)	13.5 (30)	19.5 (10)
CD27 SIgD <sup>g</sup>	20.7 (16)	16 (28.8)	13.2 (9)	14.5 (37.4)	13.5 (12.8)
SIgD cell count <sup>e</sup>	112 (98)	90 (81)	125 (29)	67 (33)	124.5 (112)
SIgD <sup>g</sup>	67.6 (29.3)	64 (31.3)	69.4 (21.3)	80.2 (36.5)	73.9 (35.1)
CD23 <sup>+</sup> SIgD <sup>h</sup>	71.4 (21.3)	26.7 (38.1)	31.4 (16.9)	32.1 (23.7)	31 (28.8)
CD44 <sup>g</sup>	97.3 (3.6)	81.3 (12.2)	91.8 (7.4)	85.6 (14.5)	93.1 (2.1)
CXCR5 <sup>g</sup>	92.5 (5.2)	69.6 (20.7)	82.5 (10.1)	73.6 (6.1)	85.3 (16.1)
CD62L <sup>g</sup>	74.6 (32.5)	52.1 (35.7)	47 (45.7)	62.7 (13.6)	51.5 (4.6)
CXCR3 <sup>g</sup>	15.8 (12)	49.2 (26.9)	35.7 (13.4)	65.8 (41.6)	37.6 (18.4)
CD5 <sup>g</sup>	5.9 (5.7)	9.4 (7.7)	6.7 (7.8)	16.9 (6.6)	6.5 (9.2)
CD10 <sup>g</sup>	2.3 (2.7)	7 (2.7)	2.1 (2.6)	13.7 (14.7)	2.0 (2.7)
CD38 <sup>g</sup>	44.5 (36.7)	38.9 (16.4)	37.6 (10.4)	81.8 (19)	52.3 (54.6)
CD95 <sup>g</sup>	1.3 (1.4)	7.7 (4.9)	1.7 (3.9)	10.9 (30.9)	1.4 (2.5)
sCD27 <sup>i</sup>	192 (78)	723 (242)	374 (211)	780 (394)	369 (327)

**NOTE.** Data are the median value (interquartile value). EBV, Epstein Barr virus; HIV, human immunodeficiency virus.

<sup>a</sup> Subjects in group 1 were HIV positive and had an EBV load <1000 copies/10<sup>6</sup> peripheral blood mononuclear cells (PBMCs) at diagnosis (or before HAART).

<sup>b</sup> Subjects in group 2 were HIV positive and had an EBV load >1000 copies/10<sup>6</sup> PBMCs at diagnosis (or before HAART).

<sup>c</sup> EBV loads are expressed as the number of EBV DNA copies/10<sup>6</sup> PBMCs.

<sup>d</sup> Plasma HIV loads are expressed as the number of RNA copies per milliliter.

<sup>e</sup> Data are the no. of cells per microliter of blood.

<sup>f</sup> Data are the percentage of CD19 cells within blood.

<sup>g</sup> Data are the percentages within the (CD19) B cell subset.

<sup>h</sup> Data are the percentages within the total naive (SIgD<sup>+</sup>) B cells.

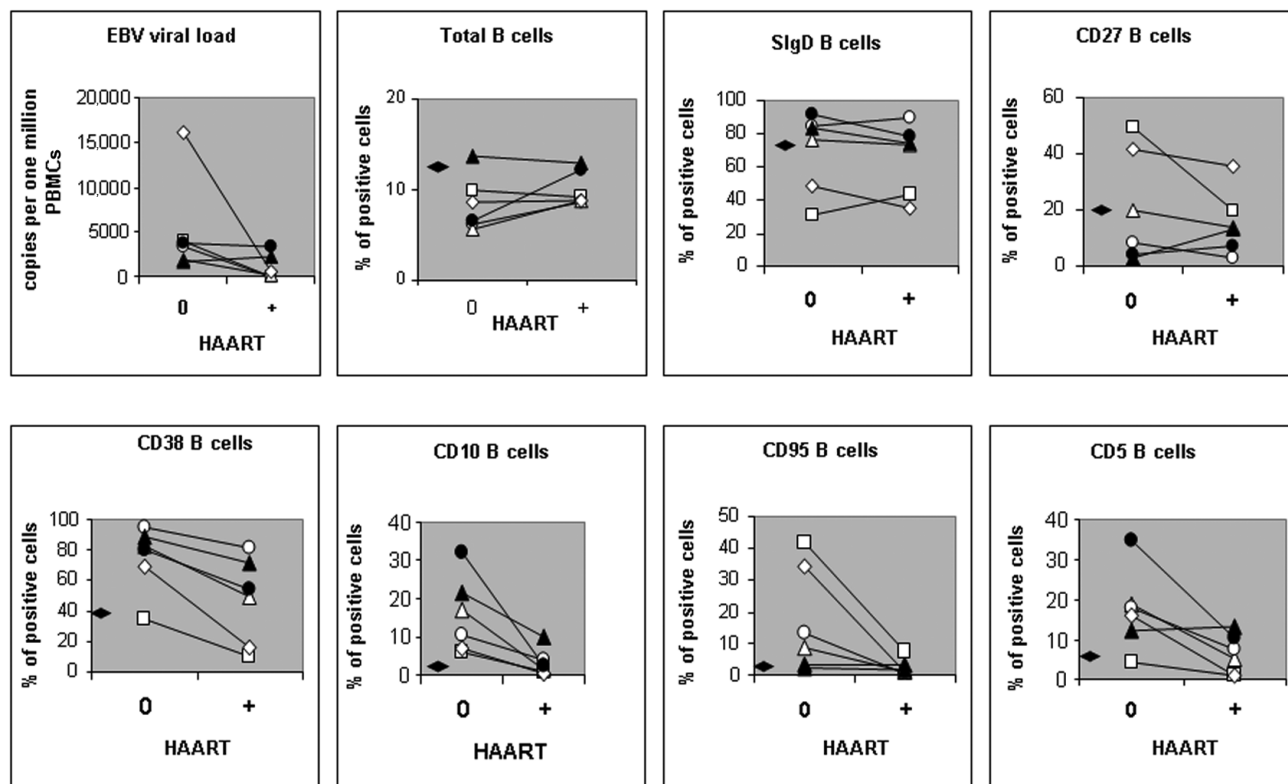
<sup>i</sup> Data are expressed as nanograms per milliliter.

In group 2, a total of 4 patients had particularly high percentages of SIgD<sup>+</sup> (>75%) and CD38<sup>+</sup> B cells (>80%) before HAART and remained strongly positive for these 2 markers after HAART (Figure 4). Two of these patients had <5% memory B cells or CD95<sup>+</sup> B cells and >20% CD10<sup>+</sup> B cells before HAART. In these 2 patients, after HAART, memory B cell percentages were still below the normal value, and >10% of B cells still expressed CD5 and (for only one of these patients) CD10 cells. Moreover, HAART failed to result in a decrease in the EBV load in these 2 patients, who had 1763 and 3763 EBV copies/10<sup>6</sup> PBMCs, respectively, both before and after HAART. Similar conclusions were obtained when B cell counts were considered instead of percentages (data not shown). The 2 patients with steady EBV loads had higher CD38<sup>+</sup> and CD10<sup>+</sup> B cell counts than did other patients in group 2 both before and after HAART, concomitantly with high SIgD<sup>+</sup> and very low CD27<sup>+</sup> B cell counts.

## DISCUSSION

Although patients with HIV infection are at higher risk of developing EBV-associated B cell lymphoma, the absolute EBV load in PBMCs is not predictive of the occurrence of HIV-related B cell lymphoma [27, 28, 33]. Because switched and nonswitched memory B cells, which constitute the main reservoirs of EBV in healthy individuals, are rapidly depleted in HIV-infected patients, other B cell reservoirs might participate in the persistence of EBV. Therefore, we compared peripheral B cell subsets present in healthy donors and in patients with chronic HIV infection before and after HAART. In agreement with previous data [24, 27], the median EBV load at diagnosis was higher in HIV-infected patients than in healthy individuals, with approximately one-half (12 of 23) of the patients having an EBV load >1000 copies/10<sup>6</sup> PBMCs (group 2). EBV load did not correlate with HIV load, CD4 T cell count, or total or





**Figure 4.** Epstein-Barr virus (EBV) load and percentages of positive cells for various markers before (0) and after (+) highly activated antiretroviral therapy (HAART), for each patient in group 2. Each symbol identifies a particular patient. Black symbols (*circle* and *triangle*) denote the 2 patients with a steady EBV load after HAART. Median values for healthy control subjects are denoted by a black lozenge in every panel. PBMCs, peripheral blood mononuclear cells.

memory B cell counts. However, a higher HIV load and a more pronounced decrease in CD4 T cell counts were observed in patients in group 2, consistent with a more active disease. At diagnosis, EBV load correlated only with the percentage of CXCR3<sup>+</sup> B cells. According to the privileged CXCR3 expression on circulating B cells from patients with B cell chronic lymphocytic leukemia and extranodal marginal-zone B cell lymphoma [34–36], CXCR3 expression in HIV-infected patients might be associated with trafficking of particular EBV-positive B cell subsets from the spleen marginal zone or the gut-associated mucosa.

Independently of EBV load, we observed an altered phenotype of total B cells with a decreased expression of CXCR5, CD62L, and CD44 in all patients. Decreased expression of CXCR5 on blood B cells from HIV-infected patients has been described elsewhere [37, 38] and was recently associated with abnormal CXCL13 production by peripheral blood B cells and increased B cell responsiveness to CXCL13 in HIV-1-infected patients with low CD4<sup>+</sup> T cells counts [39]. Although EBV also down-regulates the expression of CXCR5 in EBV-transformed lymphoblastoid cell lines [40], it is unlikely that EBV acts similarly in primary B cells in HIV-infected patients. Indeed, the

percentage of CXCR5<sup>+</sup> B cells did not correlate with EBV load, and CXCR5 expression increased toward normal values in patients in group 2 after HAART, independently of changes in EBV load (Table 2). Dysfunctions of the CXCR5/CXCL13 pair, which is essential for the entry of B cells into follicles [41, 42], may contribute to abnormal B cell responses during the course of HIV-1 infection through impaired B cell trafficking. The homing of circulating naive and memory B cells to lymph nodes [43] is also probably impaired in HIV-infected patients who express low levels of CD62L. The membrane expression of CD62L is tightly regulated through its cleavage by metallo-proteinase-dependent mechanisms, particularly after HIV-induced CXCR4 triggering [44, 45].

Although the percentage of naive B cells was comparable in HIV-infected patients at diagnosis and in healthy donors, these B cells exhibited an altered expression of CD23 in HIV-infected patients. The proportions of CD23<sup>+</sup>-naive B cells were still low after HAART, despite the increase in the total and naive B cell counts and a strongly reduced HIV load. These findings extend previous data showing phenotypic alterations of naive B cells in drug-naive HIV-infected patients [6, 37]. In accordance with these findings, chronic B cell activation and overproduction of

cytokines associated with rare T cell–B cell interactions might contribute to these changes.

Three populations of B cells can be discriminated in HIV-infected patients on the basis of the relative distribution of CD27, SIgD, SIgM/G, CD23, CD10, CD38, and CD5: the naive and switched memory B cell populations and a CD38<sup>+</sup>SIgD<sup>+</sup>CD10<sup>+</sup>CD5<sup>+/−</sup>CD27<sup>−</sup>CD95<sup>−</sup> population that was particularly expanded in patients in group 2. This phenotype is reminiscent of circulating immature and/or transitional and intermediate B cells characterized by several groups in healthy individuals [46–48] or patients with X-linked lymphoproliferative disease [49]. The comparison between our 2 groups of HIV-infected patients suggests that these particular immature and/or transitional B cell populations might constitute an alternative EBV reservoir in patients with very low CD4 T cell and memory B cell counts. According to more recent data [48], a continuum in surface marker expression occurs between transitional type 1 and mature naive B cells during which CD21 expression increases but CD10 and CD5 expression is progressively lost. In accordance with these data, transitional type 2 and/or 3 B cells express CD21 and thus could be preferentially targeted by EBV in lymphopenic HIV-infected patients. Although the presence of circulating immature and/or transitional CD10<sup>+</sup> B cells in HIV-infected patients was previously reported as a consequence of strong B cell lymphopenia [14, 49], its relationship with EBV persistence was not investigated. Our present results deserve to be confirmed by the quantification of EBV load in isolated blood B cell subsets (CD38<sup>+</sup>SIgD<sup>+</sup>CD27<sup>−</sup> B cells expressing or not expressing CD10 and/or CD5, compared with naive and memory B cells) from patients with chronic HIV infection who had very low memory B cell counts and EBV loads >1000 copies per 10<sup>6</sup> PBMCs at diagnosis and after HAART. A particular set of EBV genes could be expressed in these discrete transitional-like B cell subsets, which could impair their further maturation and selection process, leading to their progression into autoreactive or pretumoral B cells, as suggested by adoptive transfer experiments in mice [50]. Data in other immunodeficient states suggest that this population can fill the space of memory B cells and participate in the impaired humoral response observed in HIV-infected patients. Additional work will determine to what extent these EBV-infected immature and/or transitional B cells participate in the occurrence of AIDS-related lymphoma.

In conclusion, our data suggest that different B cell subsets participate in the high EBV load observed in patients with chronic HIV infection at different stages characterized by the intensity of HIV-induced lymphopenia and B cell dysfunction: primarily the switched and nonswitched memory B cells when they are still present and, then, the CD38<sup>+</sup>SIgD<sup>+</sup>CD10<sup>+</sup>CD95<sup>−</sup>CD27<sup>−</sup> immature and/or transitional B cells during later stages. Because this population may persist and participate in the maintenance

of increased EBV load after HAART, we suggest that an increased frequency of this population in PBMCs might be associated with a higher risk of B cell lymphoma.

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