Gag-specific CD8 T-cell proliferation is associated with higher peripheral blood levels of TGF-β and gut-homing T cells in youths perinatally infected with HIV-1 – The ANRS- EP38-IMMIP Study

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Gag-Specific CD8 T-Cell Proliferation Is Associated With Higher Peripheral Blood Levels of Transforming Growth Factor-β and Gut-Homing T Cells in Youths Perinatally Infected With Human Immunodeficiency Virus-1: The ANRS-EP38-IMMIP Study

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Background. Gag-specific T lymphocytes play a key role in the control of human immunodeficiency virus (HIV) replication. Their restoration will be important for future reservoir targeting strategies. In this study, we aimed to identify immune correlates of Gag-specific CD8 T-cell proliferation in youths with perinatally acquired HIV-1 infection.

Methods. The ANRS-EP38-IMMIP study included youths of 15 to 24 years of age. Fifty-three were taking combination anti-retroviral therapy and aviremic at the time of the study and had undergone valid 5-6-carboxyfluorescein diacetate succimidyl ester-based flow cytometry T-cell proliferation assays. Plasma analytes were quantified by enzyme-linked immunosorbent assay or multiplex assays. Peripheral blood cells were phenotyped by flow cytometry. Logistic regression was used to study the association between Gag-specific T-cell proliferation and immune markers.

Results. Patients with Gag-specific CD8 T-cell proliferation had higher levels of plasma transforming growth factor (TGF)-β1, a lower proportion of naive cells among regulatory T cells (Tregs), and higher percentages of CD4 and CD8 T cells expressing the $\alpha_4\beta_7$ integrin or CD161 molecule than those without a Gag-specific response. These associations were significant based on analyses including potential confounders.

Conclusions. Preserved Gag-specific CD8 T-cell proliferation was associated with higher TGF-β1 levels and increased percentages of T cells with a gut-homing phenotype at least 15 years after HIV infection during the perinatal period.

Keywords. CD8 T cells; HIV; mucosal immunity; perinatal infection; regulatory T cells.

Subjects infected with human immunodeficiency virus (HIV)-1 in the perinatal period now live with the virus for several decades due to progress in antiretroviral therapy. The immune system of these patients, born at the beginning of the epidemic, have developed in the presence of an immunosuppressive virus and chronic exposure to inflammatory stimuli and antiretroviral drugs. The immune status of perinatally infected patients who reach adulthood has been much less described than that of patients infected as adults. These patients are infected with viruses that were selected to escape CD8 T cells restricted by the human leukocyte antigen (HLA) molecules shared with their mothers [1]. Infants have CD8 T cells with lower antiviral activity during primary infection than adults. Human immunodeficiency virus-specific CD4 and CD8 T cells exert some control over viral replication, even in successfully treated patients, as shown by the acquisition of mutations in CD8 T-cell epitopes [2], and by the evolution of the T-cell receptor Vβ repertoire [3]. Human immunodeficiency virus-specific T cells may play an important role in the destruction of infected cells after the reversal of viral latency in future therapeutic strategies that target the viral reservoir [4, 5].

We initiated the ANRS-EP38-IMMIP study to assess the immune and virological status of adolescents and young adults infected with HIV-1 during the perinatal period [6, 7]. We previously reported a modest frequency of youths with Gag-specific CD8 T-cell proliferation, which was greater in patients of black ethnicity [8]. Youths with Gag-specific CD8 T-cell proliferation who had undetectable plasma HIV-1 ribonucleic acid

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(RNA) at the time of the study (aviremic patients) had more recent exposure to viral replication. In addition to the level of cognate antigen, antiviral T cells are tightly regulated by activating and inhibitory effector cells and molecules, many of which are affected by HIV infection. We aimed to identify immune correlates of preserved HIV-specific T-cell proliferation by analyzing cytokines and T-cell subsets that may regulate Gag-specific CD8 T-cell proliferation in aviremic youths.

PATIENTS AND METHODS

Patients

The ANRS-EP38-IMMIP study included 93 youths between 15 and 24 years of age who had been infected with HIV-1 during the perinatal period [6, 7]. All patients, and their legal guardians for those under 18 years of age, received written information and signed an informed consent form. A single blood sample was collected for immunological evaluations. At the time of the study, 59 patients had a plasma HIV RNA level below the detection level and are referred to as aviremic. The threshold values used in HIV RNA assays depended on the volume of available plasma. The highest threshold used was 80 copies/mL, and this value was selected as the cutoff for HIV RNA detection [6]. Fifty-three combination anti-retroviral therapy (cART)-treated aviremic patients had a valid evaluation of their Gag-specific T-cell proliferation and were included in the present analysis. Their characteristics are presented in Supplementary Table 1. The 6 aviremic patients for whom the T-cell proliferation assay was not performed or not valid were not different from the other 53 patients (data not shown).

Biological Investigations

Gag-specific T-cell proliferation was assessed against peptide pools with a 5-6-carboxyfluorescein diacetate succimidyl ester (CFSE)-based proliferation assay [8]. Peripheral blood mononuclear cells (PBMCs) were isolated from blood by density centrifugation, immediately labeled with CFSE, and stimulated with a pool of Gag peptides (15-mer peptides covering the clade B consensus sequence; NIH AIDS Reagent program, Catalog number#8117). Peptide diluent (0.034% dimethyl sulfoxide) was used as a negative control, and enterotoxin B of *Staphylococcus aureus* ([SEB] 500 ng/mL) was used as a positive control. Forty-eight hours after stimulation, 25 µL of supernatant was collected from each P96 well and stored frozen at −20°C before quantification of the 25 cytokines by the Cytokine Human 25-Plex Panel (LHC0009; Invitrogen). On day 6, PBMCs were labeled with a combination of anti-CD3-ECD, anti-CD4-PC7, and anti-CD8β-PC5 antibodies (Beckman Coulter, Villepinte, France). The cells were analyzed on a FC500 flow cytometer (Beckman Coulter). The differences in the percentage of $CFSE^{low}CD8^{+}$ T cells between antigen-stimulated and mock-stimulated cells and the ratio of these percentages (stimulation index) were calculated. The PBMCs from 8 uninfected donors were tested with Gag peptides. The mean + 2 standard deviation antigen-specific T-cell proliferation for these control subjects was 0.8% for the difference and 4 for the ratio. These values were used as a dual cutoff criterion to define a positive response in the T-cell proliferation assay. Patients with positive/negative results in the Gag-specific CD8 T-cell assay are referred to as CD8 responders (CD8Rs) and CD8 nonresponders (CD8NRs).

Plasma samples were stored at −80°C and interferon (IFN)-γ, interleukin (IL)-1β, IL-1Rα, IL-10, IL-12p70, IL-17A, IL-18, IL-18BPA, IL-2, IL-21, IL-23, IL-6, transforming growth factor (TGF)-β1, and tumor necrosis factor (TNF)-α levels were quantified by enzyme-linked immunosorbent assay or Luminex technology-based assay according to the manufacturer's instructions for each kit. The detection kits and quantification thresholds are presented in Supplementary Table 2. We used flow cytometry for the phenotypic study of frozen PBMCs and quantified CD4 regulatory T cells (Tregs), gut-homing CD4 and CD8 T cells, and activated and exhausted memory CD8 T cells. Supplementary Table 3 presents the combinations of antibodies used, and Supplementary Table 4 shows the phenotypic definitions of the lymphocyte subsets. Data were collected on an LSR II cytometer (BD Biosciences) and analyzed using FlowJo software (Treestar, Ashland, OR). The gating strategies are shown in Supplementary Figures 1 to 6.

Statistical Analysis

Logistic regressions were performed to study the relationships between immune parameters and Gag-specific CD8 T-cell proliferation. Before carrying out multivariate analyses, we determined whether there were interactions between ethnicity and Gag-specific T-cell proliferation. We carried out an analysis of variance for continuous variables and assessed the variation of the odds ratio across the strata for categorical variables (data not shown and Supplementary Table 5). For multivariate analyses, ethnicity and duration of plasma HIV RNA <500 copies/mL were included in the model, because these variables were significantly associated with Gag-specific T-cell proliferation [8]. Other variables were included if associated with Gag-specific T-cell proliferation, with a P value ≤.10 in univariate analysis of the whole group (Table 1) or in at least one of the ethnic groups (Supplementary Table 5). We did not build a model with all immunological parameters, because these were not quantified for all patients, due to the lack of available samples for some. Mann-Whitney U and Fisher's exact test were used to compare cytokine production by cell culture from CD8Rs and CD8NRs. Analyses were conducted using STATA software (version 12.1). A P value of <.05 was considered to indicate statistical significance.

RESULTS

Association of Gag-Specific CD8 T-Cell Proliferation With Higher Levels of Transforming Growth Factor-β1 in Plasma

Twenty-two (42%) of 53 aviremic youths had detectable Gag-specific CD8 T-cell proliferation. The immune characteristics of CD8Rs were compared with those of CD8NRs by logistic regression. We searched for immune factors that

Table 1. Univariate Analysis of Immune Parameters Associated With Gag-Specific CD8 T-Cell Proliferation

	CD8NRs[a]	CD8Rs	Logistic Regression[b]	
Immune Parameters	Median (IQR) % (n)	Median (IQR) % (n)	OR [95% CI][c]	*P* Value
IL-12p70 (pg/mL)	2.7 (0.3–8.5)	4.6 (1.2–10.8)	1.14 [0.91–1.43]	.25
IL-18 (pg/mL)	252 (158–133)	133 (91–277)	**0.60 [0.37–0.99]**	**.05**
IL-18BPA (ng/mL)	0.66 (0.55–0.99)	0.54 (0.38–0.75)	0.19 [0.03–1.48]	.11
IFN-γ				
<0.64 pg/mL	60.0 (15)	40.0 (10)	1.03 [0.34–3.13]	.96
≥0.64 pg/mL	59.3 (16)	40.7 (11)		
TNF-α (pg/mL)	13.4 (8.6–20.4)	18.1 (11.5–23.4)	1.21 [0.77–1.90]	.40
IL-10 (pg/mL)	10.9 (1.5–35.1)	11.8 (4.1–20.9)	1.04 [0.95–1.14]	.41
TGF-β1 (ng/mL)	5.6 (4.0–7.3)	9.1 (5.1–10.7)	**1.37 [1.10–1.71]**	**.005**
IL-2				
<0.64 pg/mL	62.5 (15)	37.5 (9)	1.25 [0.41–3.81]	.70
≥0.64 pg/mL	57.1 (16)	42.9 (12)		
IL-1Rα (pg/mL)	256 (174–305)	211 (141–264)	0.97 [0.93–1.02]	.24
IL-1β (pg/mL)	3.6 (0.3–8.9)	6.9 (2.6–13.4)	1.99 [0.87–4.57]	.10
IL-6 (pg/mL)	1.1 (0.8–1.9)	0.9 (0.7–1.4)	0.92 [0.73–1.15]	.46
IL-21 (pg/mL)	79 (59–151)	112 (59–137)	1.02 [0.95–1.10]	.61
IL-23 (pg/mL)	40.3 (8.0–135.9)	60.9 (25.5–155.0)	0.82 [0.52–1.31]	.41
IL-17A (pg/mL)	1.3 (0.6–6.2)	3.3 (1.3–11.7)	1.37 [0.90–2.10]	.14
CD4 Tregs (%)[d]	5.0 (4.5–5.5)	5.6 (4.8–7.0)	1.94 [0.98–3.82]	.06
Naive Tregs (%)[e]	57.8 (48.9–67.0)	48.7 (47.1–51.3)	0.95 [0.89–1.01]	.10
Central memory Tregs (%)[e]	13.9 (9.3–19.9)	22.1 (9.4–27.2)	1.03 [0.97–1.10]	.30
Effector memory Tregs (%)[e]	16.8 (10.2–28.0)	17.4 (14.4–22.7)	1.00 [0.94–1.07]	.89
$CD161^+CD4$ T cells (%)[d]	8.1 (4.9–11.6)	14.1 (7.6–18.3)	**1.15 [1.00–1.33]**	**.05**
$CD161^+CD8\alpha\beta$ T cells (%)[f]	3.9 (1.7–5.9)	4.8 (2.5–6.2)	1.04 [0.86–1.26]	.69
$CD161^+CD8\alpha\alpha$ T cells (%)[f]	1.4 (0.7–2.0)	2.3 (1.2–4.1)	1.29 [0.87–1.92]	.20
$\alpha_4^+\beta_7^+CD4$ T cells (%)[d]	6.0 (3.9–8.2)	9.8 (6.2–11.5)	**1.65 [1.09–2.48]**	**.02**
$\alpha_4^+\beta_7^+CD8$ T cells (%)[g]	8.7 (7.9–13.0)	18.4 (10.9–26.0)	**1.27 [1.05–1.54]**	**.01**
$HLA\text{-}DR^+CD38^+$ memory CD8 T cells (%)[h]	8.3 (4.6–14.0)	12.4 (8.9–15.2)	1.09 [0.97–1.24]	.16
$CD279^+$ memory CD8 T cells (%)[h]	29.5 (24.9–33.7)	41.0 (26.9–51.4)	1.03 [0.97–1.09]	.34
$CD57^+CD28^-$ effector memory CD8 T cells (%)[i]	23.6 (19.9–31.4)	20.0 (12.4–35.7)	0.98 [0.93–1.03]	.42
$CD57^+CD28^-$ effector CD8 T cells (%)[i]	22.6 (13.0–37.2)	20.4 (9.5–30.9)	1.00 [0.96–1.04]	.90

Abbreviations: CD8NRs, CD8 nonresponders in the Gag-specific T-cell proliferation assay; CD8Rs, CD8 responders in the Gag-specific T-cell proliferation assay; CI, confidence interval; IFN, interferon; IL, interleukin; IQR, interquartile range; OR, odds ratio; TGF, transforming growth factor; TNF, tumor necrosis factor; Tregs, regulatory T cells.

[a]Medians and IQR are reported for quantitative variables; percentages and numbers are presented for qualitative variables.

[b]Crude ORs, 95% CIs, and *P* values were obtained with univariate logistic regression, significant associations are indicated in bold characters.

[c]ORs were calculated per 10 pg/mL of IL-12p70, TNF-α, IL-10, IL-1Rα, IL-1β, IL-6, IL-21, and IL-17A; per 100 pg/mL of IL-18 and IL-23; per ng of TGF-β1; per 10 ng of IL-18BPA; per 1% of each T cell subset.

[d]Percentages among CD4 T cells.

[e]Percentages among CD4 Tregs.

[f]Percentages among CD8αβ or CD8αα T cells.

[g]Percentages among CD8 T cells.

[h]Percentages among memory CD8 T cells.

[i]Percentages among $CD28^-$ effector memory or effector CD8 T cells.

may influence Gag-specific CD8 T-cell proliferation by first measuring levels of plasma cytokines associated with type 1 T-cell priming (IL-12p70, IL-18, IL-18BPa), effector function (IFN-γ and TNF-α), or suppressive activity (IL-10 and TGF-β1). The CD8Rs had significantly lower IL-18 levels and higher TGF-β1 levels than CD8NRs (Figure 1, Table 1). Plasma levels of IL-12p70, IL-18PBa, IFN-γ, TNF-α, and IL-10 were similar in CD8Rs and CD8NRs. We stratified the analyses on the basis of ethnicity because proliferative responses were more frequent in patients of black ethnicity than in those of other ethnicities [8]. The CD8Rs had lower IL-18 and higher TGF-β1 levels than CD8NRs in all ethnic groups (Supplementary Table 5).

Transforming growth factor-β1 is a pleiotropic cytokine. Higher levels of TGF-β1 in patients with Gag-specific CD8 T-cell proliferation are not consistent with the direct suppressive action of this cytokine on responding T cells. The action of TGF-β1 is modulated by the other locally produced cytokines during T-cell activation to regulate the Treg/Th17 differentiation pathway. We quantified 6 of these cytokines. The combination

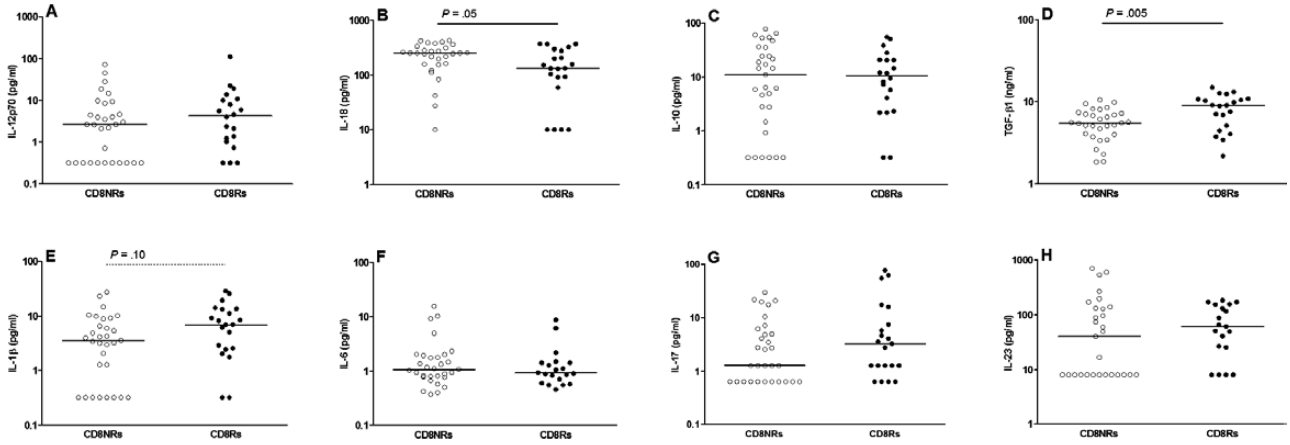

Figure 1. Gag-specific CD8 T-cell proliferation and plasma levels of cytokines. The levels of immune parameters are presented as a function of Gag-specific CD8 T-cell proliferation. Open and closed symbols represent CD8 non-responders (CD8NRs) and CD8 responders (CD8Rs) in the Gag-specific T-cell proliferation assay. Lines represent median values. A: plasma IL-12p70 (pg/mL), B: plasma IL-18 (pg/mL), C: plasma IL-10 (pg/mL), D: plasma TGF-β1 (ng/mL), E: plasma IL-1β (pg/mL), F: plasma IL-6 (pg/mL), G: plasma IL-17 (pg/mL), and H: plasma IL-23 (pg/mL).

of IL-2 and TGF-β1 promotes the differentiation of naive CD4 T cells into CD4 Tregs. However, plasma IL-2 was detectable in a similar proportion of CD8Rs and CD8NRs (43% vs 57%, $P = .78$; Table 1). Transforming growth factor-β1 promotes the differentiation and/or expansion of Th17 cells in combination with IL-1β, IL-6, IL-21, and IL-23 [9]. Plasma IL-1β levels tended to be higher in CD8Rs than in CD8NRs (Figure 1). The IL-17 and IL-23 levels were higher in CD8Rs than in CD8NRs, but the differences were not significant. The CD8Rs and CD8NRs had similar plasma levels of IL-1Rα, IL-6, and IL-21. Overall, IL-18 and TGF-β1 are the only 2 plasma cytokines for which the levels were significantly different between CD8Rs and CD8NRs.

Association of Gag-Specific CD8 T-Cell Proliferation With Higher Regulatory T, T Helper 17, and T Cytotoxic 17 Cell Levels

We next performed a phenotypic study of Treg, Th17, and Tc17 cells, which share a TGF-β1-dependent differentiation pathway for the 35 patients with available frozen PBMCs. The CD8Rs tended to have higher percentages of CD4 Treg and lower percentages of naive cells among Tregs than CD8NRs (Figure 2). The percentage of central memory Tregs was higher in CD8Rs than in CD8NRs, but the differences were not statistically significant.

We used several markers to define T-cell populations that comprise IL-17-producing cells. We assessed CD161 expression on CD4, CD8αβ, and CD8αα T cells. The CD8Rs had higher percentages of $CD161^+CD4$ and $CD161^+CD8αα$ T-cell subsets than CD8NRs (Figure 2E and 2F). The levels of memory T cells expressing the gut-homing $\alpha_4\beta_7$ integrin were significantly higher in CD8Rs than in CD8NRs (Figure 2G and 2D). We also used CXCR3, CCR4, and CCR6 expression on memory CD4 T cells to study T-cell polarization. The CD8Rs had higher levels of Th17 ($CXCR3^-CCR4^-CCR6^+$) and Th1/17 ($CXCR3^+CCR4^-CCR6^+$) cells than CD8NRs, and both groups had similar levels of Th1 and Th2 cells (data not shown). Thus, CD8Rs have higher levels of T cells with a type 17/gut-homing phenotype.

No Association Between Gag-Specific CD8 T-Cell Proliferation and the Activation or Exhaustion Status of Total CD8 T Cells

We assessed whether CD8Rs had lower levels of activated $HLA\text{-}DR^+CD38^+$, exhausted $CD279/PD\text{-}1^+$, or senescent $CD57^+$ cells among the total memory CD8 T cells, because Gag-specific CD8 T-cell proliferation was associated with increased levels of CD4 Tregs. Both groups had similar levels of $HLA\text{-}DR^+CD38^+$ and $CD279^+$ cells among the memory CD8 T cells as well as similar proportions of $CD57^+$ among $CD28^-$ CD8 T_{EM} and T_E (Figure 2). Furthermore, we observed no associations when considering CD4 T-cell activation or differentiation status (data not shown).

Gag-Specific CD8 T-Cell Proliferation, Cytokine Production, and Other Proliferative Responses

During the proliferation assay, supernatants were collected for the quantification of cytokines and chemokines (Table 2). Of the 4 cytokines and chemokines induced by Gag-peptide stimulation, IFN-γ and CXCL10 were more frequently detected in cultures from CD8Rs than in those from CD8NRs, whereas rates of IL-1β and CCL3 detection were not different between the 2 groups. The levels of several Th1-related cytokines and chemokines produced after SEB stimulation were significantly higher in cultures from CD8Rs than from CD8NRs (Table 2). Neither Th2 nor Th17-related cytokines were detectable after Gag-peptide or SEB stimulation. The production of TGF-β by antigen-specific T cells could not be detected due to high levels of TGF-β in the culture medium. Overall, Gag-specific CD8 T-cell proliferation

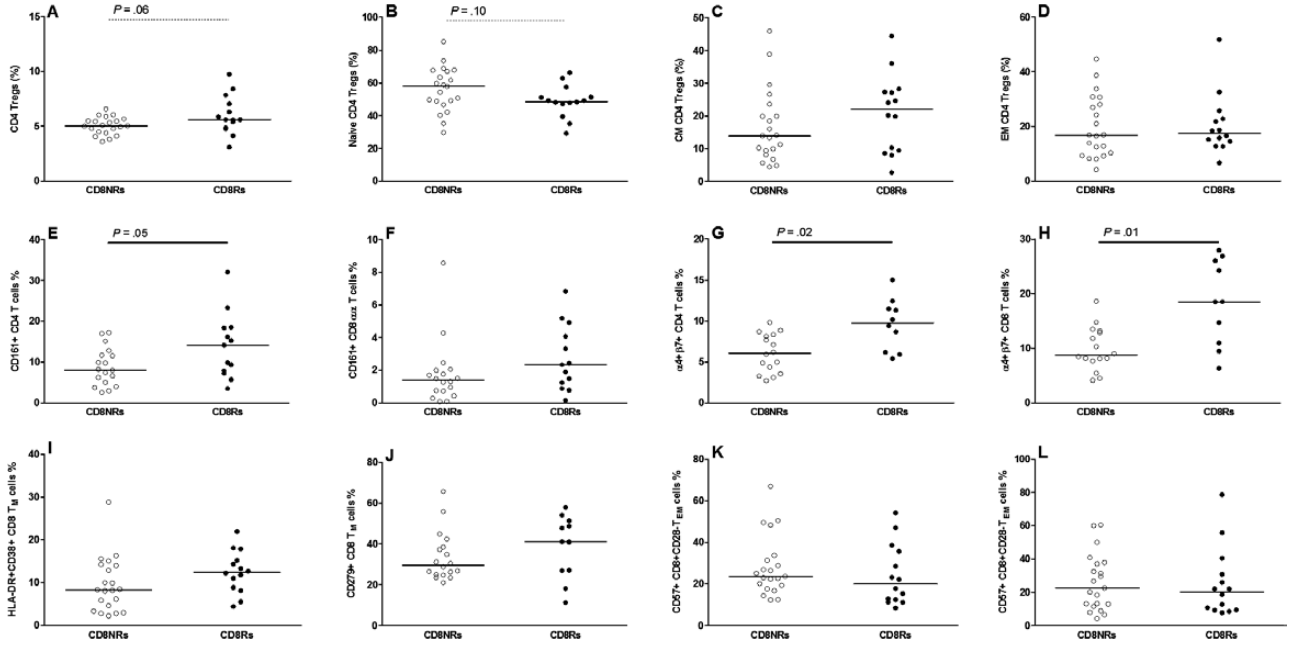

Figure 2. Gag-specific CD8 T-cell proliferation and T cell subsets. Data are presented as described in Figure 1. A: percentages of CD4 Tregs among total CD4 cells, B: percentages of naive cells among CD4 Tregs, C: percentages of central memory cells among CD4 Tregs, D: percentages of effector memory cells among CD4 Tregs, E: percentages of CD161+ CD4 T-cells among total CD4 T cells, F: percentages of CD161+ CD8αα T-cells among total CD8 T cells, G: percentages of α4+β7+ memory CD4 T-cells among total CD4 T cells, H: percentages of α4+β7+ memory CD8 T-cells among total CD8 T cells, I: percentages of HLA-DR+CD38+ cells among memory CD8 T cells, J: percentages of CD279+ cells among memory CD8 T cells, K: percentages of CD57+ T-cells among CD28- effector memory (CD45RA-CD197–) CD8 T cells, L: percentages of CD57+ T-cells among CD28- effector (CD45RA+CD197–) CD8 T cells.

was associated with type 1 cytokines and IL-10 production in response to either Gag peptides or a superantigen.

Then, we investigated whether the immunoregulatory cytokines and cells were associated with other proliferative responses. We observed trends for associations between plasma cytokine/Tregs levels and Gag-specific CD4 T-cell proliferation (Supplementary Table 6). However, the analysis of CD4 responses was underpowered due to the small number of patients with Gag-specific CD4 T-cell proliferation [10]. The percentage of proliferating CD8 T cells in response to SEB were positively correlated with the levels of $CD161^{+}CD8\alpha\alpha$ T-only (data not shown).

Multivariate Logistic Regression Analysis of Immune Factors Associated With Gag-Specific T-Cell Proliferation

We adjusted for the 2 parameters associated with CD8 T-cell proliferation in our previous study [8], ie, ethnicity and duration of the last period with plasma HIV RNA <500 copies/mL (Figure 3), for each parameter with a *P* value ≤.1 in univariate analysis of either the whole group (Table 1) or at least one of the ethnic groups (Supplementary Table 5). Plasma levels of TGF-β1, the percentages of naive Treg, $CD161^{+}CD4$, $CD161^{+}CD8\alpha\alpha$, and $\alpha_4^{+}\beta_7^{+}CD8$ T cells were significantly associated with Gag-specific CD8 T-cell proliferation. Ethnicity and the duration of viral suppression remained associated with Gag-specific CD8 T-cell proliferation in these multivariate analyses.

DISCUSSION

Our study aimed to identify immune correlates of preserved HIV-specific T-cell proliferation after long-term perinatally acquired HIV-1 infection. Gag-specific CD8 T-cell proliferation in treated youths with viral control was associated with higher levels of plasma TGF-β1 and lower levels of naive Tregs, as well as a higher proportion of CD4 and CD8 T cells with a gut-homing and/or IL-17-producing phenotype. The presence of higher levels of TGF-β1 in youths who maintained Gag-specific CD8 T-cell proliferation appears to be at odds with many previous observations on the immunosuppressive action of this cytokine. Two issues should be considered when interpreting our data. First, we assessed the proliferative capacity of Gag-specific CD8 T cells, not their immediate effector functions. In patients with suppressed viral replication, most of these HIV-specific T cells belong to the long-term memory pool and were in a resting state at the time of in vitro stimulation. Reduced immune activation favors the generation of such long-lived memory cells at the expense of short-lived effector cells [11]. Second, the opposite roles of immunoregulatory effector cells and molecules on HIV disease progression have been well described: they reduce both beneficial antiviral responses and deleterious immune activation [12]. Here, we studied patients whose immune systems had been exposed to HIV replication and antiviral drugs over 15 to 24 years. In this setting, higher levels of immune

Table 2. Cytokines Produced During the Proliferation Assay and Their Association With Gag-Specific CD8 T-Cell Proliferation

Cytokine[a]	CD8NRs[b] Median (IQR)% (n)	CD8Rs Median (IQR)% (n)	*P* Value[c]
Gag-Specific			
IL-1β			
<10 pg/mL	84.0 (21)	16.0 (4)	.39
≥10 pg/mL	74.1 (20)	25.9 (7)	
IFN-γ			
<10 pg/mL	84.2 (16)	38.9 (7)	.007
≥10 pg/mL	15.8 (3)	61.1 (11)	
CCL3			
<50 pg/mL	70.6 (12)	58.8 (10)	.72
≥50 pg/mL	29.4 (5)	41.2 (7)	
CXCL10			
<12 pg/mL	88.2 (15)	36.4 (4)	.01
≥12 pg/mL	11.8 (2)	63.6 (7)	
SEB-Specific			
IL-1β			
<10 pg/mL	88.9 (16)	86.7 (13)	1.00
≥10 pg/mL	11.1 (2)	13.3 (2)	
IL-2 pg/mL	103 (33–172)	205 (152– 292)	.003
sIL-2Rα			
<250 pg/mL	83.3 (15)	53.3 (8)	.13
≥250 pg/mL	16.7 (3)	46.7 (7)	
IL-10			
<10 pg/mL	84.2 (16)	22.2 (4)	<.0001
≥10 pg/mL	15.8 (3)	77.8 (14)	
IL-12			
<20 pg/mL	81.3 (13)	66.7 (10)	.43
≥20 pg/mL	18.8 (3)	33.3 (5)	
IFN-γ pg/mL	132 (37– 467)	984 (420–1384)	.001
GM-CSF			
<25 pg/mL	84.2 (16)	50.0 (9)	.04
≥25 pg/mL	15.8 (3)	50.0 (9)	
CCL3			
<50 pg/mL	64.7 (11)	41.2 (7)	.30
≥50 pg/mL	35.3 (6)	58.8 (10)	
CCL4			
<50 pg/mL	23.5 (4)	16.7 (3)	.69
≥50 pg/mL	76.5 (13)	83.3 (15)	
CXCL9			
<80 pg/mL	73.7 (14)	11.1 (2)	<.0001
≥80 pg/mL	26.3 (5)	88.9 (16)	
CXCL10 pg/mL	120 (34–218)	275 (166–358)	.04

Abbreviations: GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; IQR, interquartile range; OR, odds ratio; s, soluble; SEB, enterotoxin B of *Staphylococcus aureus*; TGF, transforming growth factor; TNF, tumor necrosis factor.

[a]Forty-eight hours after stimulation, 25 µL of supernatant were collected from each P96 well and stored frozen at −20°C before quantification of the 25 cytokines by the Cytokine Human 25-Plex Panel (LHC0009; Invitrogen): IL-1RA, IL-1β, IL-2, sIL-2Rα, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12(p40/p70), IL-13, IL-15, IL-17, IFN-α, IFN-γ, GM-CSF, TNF-α, CCL2, CCL3, CCL4, CCL11, CCL5, CXCL9, and CXCL10. We detected Gag-specific production of IL-1β, IFN-γ, CCL3, CXCL10 and SEB-specific production of IL-1β, IL-2, sIL-2Rα, IL-10, IL-12, IFN-γ, GM-CSF, CCL3, CCL4, CXCL9, and CXCL10. For cytokines in which <80% of data were above the limit of detection, data were dichotomized to above or below the detection threshold.

[b]Medians and IQRs are reported for quantitative variables, and percentages and numbers are presented for qualitative variables.

[c]*P* values were obtained from Fisher's exact test for qualitative variables and from the Mann-Whitney *U* test for quantitative variables.

suppression may have limited the exhaustion and/or deletion of HIV-specific T cells, resulting in the long-term maintenance of these virus-specific immune cells.

The CD8Rs had higher plasma levels of TGF-β1 than CD8NRs. In vivo, TGF-β1 may have favored the development of long-lived memory T cells, resulting in higher in vitro HIV-specific T-cell proliferation [13]. In murine models, TGF-β1 increases expression of antiapoptotic molecules in memory T cells after clearance of the antigen [14] and the prosurvival action of TGF-β1 is specific for memory cells [15]. Transforming

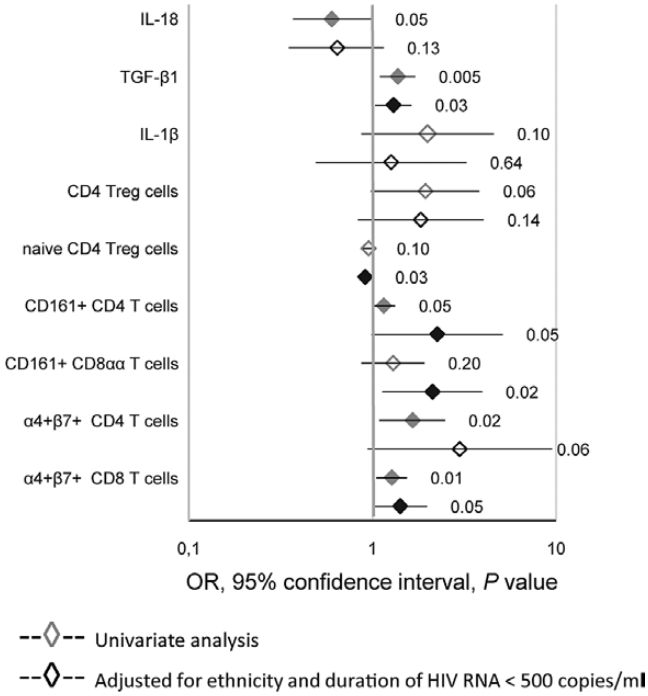

Figure 3. Logistic regression analysis of the association between Gag-specific CD8 T-cell proliferation and immune parameters. Results from univariate and multivariate logistic regression are presented as ORs and 95% confidence intervals. P values are indicated and black symbols correspond to P values < .05. Multivariate analysis included ethnicity and the duration of plasma HIV RNA suppression as covariables. The ORs are given per 100 pg/mL of IL-18, per ng of TGF-β1, per 10 pg/mL of IL-1β, and per 1% of each T-cell subset.

growth factor-β1 also downregulates KLRG1 expression, a marker of terminally differentiated CD8 T cells, on both human and murine cells [16]. In addition to its T-cell intrinsic effects, TGF-β1 may act through T-cell extrinsic pathways involving antigen-presenting cells [13].

The CD8Rs had higher levels of Tregs and of T cells with a gut-homing phenotype than CD8NRs. Associations between Gag-specific CD8 T-cell proliferation and T-cell subsets were restricted to the Tregs and Th17 subsets. There was no difference between the T-cell differentiation, activation, or exhaustion in CD8Rs and CD8NRs ([8] and this study), and we also observed no differences in Th1 and Th2 cell levels (data not shown). The observed differences are consistent with elevated plasma TGF-β1 levels because this cytokine stimulates the differentiation of both Tregs and type 17 T-cell subsets [13]. Furthermore, TGF-β1 mediates the conversion of conventional T cells into Tregs in HIV-infected patients, leading to the selective accumulation of Tregs [17]. T-regulatory cells suppress HIV-specific T-cell activation both directly [18, 19] and indirectly by dampening viral replication. In adults, the suppressive activity of Tregs is associated with lower viral load and higher CD4/CD8 ratios [18].

Our results support that preservation of the proliferative capacity of HIV-specific T cells may result from their reduced exposure to inflammatory molecules through better immune control at mucosal sites by Th17 cells. The Th17 and Tc17 cells play a key role in the defense against fungal and bacterial infections. Mucosal CD4 T-cell depletion and the reduction of both Th17 and Tc17 cells in the gut of HIV-infected patients leads to decreased antimicrobial function and the persistence of increased immune activation. Combination therapy partially restores these populations [20–22]. Our data are consistent with previous reports in adults. During primary infection, higher Th17 levels are associated with increased functionality of HIV-specific T cells [23]. After long-term highly-active antiretroviral therapy, efficient gut-associated lymphoid tissue (GALT) CD4 T restoration is associated with both higher polyfunctionality of HIV-specific CD4 and CD8 T cells and an increased number of GALT CD4 T cells producing IL-17 in response to mitogens [24]. In simian immunodeficiency virus (SIV)-infected macaques, enhanced SIV-specific T-cell responses and higher levels of Th17 cells were concomitantly observed [25, 26].

Our work raises the question of whether the observed pattern of immune restoration is specific to infection during the perinatal period. Indeed, infants differ from adults by having higher levels of Tregs and lower mucosal immunity [1]. A seminal study showed the ability of cord blood Tregs to suppress HIV-specific T-cell responses [27]. In SIV-infected macaques, Tregs are present at a higher frequency and possess higher in vitro suppressive activity in infants than in adults [28]. Acute SIV infection leads to the rapid loss of Tregs and increases immune activation in neonatal intestinal mucosa [29]. In infected children, the proportion of Tregs increases with immune suppression [30, 31]. To the best of our knowledge, only a single study has stated that the frequency of IL-17-producing cells was strongly reduced in the peripheral blood of HIV-infected pediatric patients, with a stronger reduction in those with active replication than in those who were successfully treated [32]. Further studies are clearly needed to describe and understand the Th17 compartment in pediatric HIV infection.

The patients we studied experienced long-term chronic HIV infection, because cART became available when they were between 3 and 10 years of age. Moreover, most received bitherapy with nucleoside reverse-transcriptase inhibitor and could have had adherence problems over the first years of infection. Most of them experienced severe immune suppression followed by treatment-induced viral control and immune restoration [6]. For patients with such disease histories, the concomitant increase of both Th17 and Treg cells is a relevant parameter of functional restoration of Gag-specific T-cell proliferation. Indeed, Th17 and Treg percentages were positively correlated, and their ratio was not associated with Gag-specific T-cell proliferation (data not shown). This association is different from the loss of the Th17/Treg balance seen in untreated adults during primary infection [33, 34]. These differences may be related to the evolution of the immune equilibrium from acute to

chronic HIV infection. T-regulatory cell frequencies are indeed lower in primary than in chronic infection in adults [35, 36]. In nonhuman primates, non-pathogenic SIV infections are characterized by early and transient anti-inflammatory responses, including the elevation of TGF-β1, whereas delayed and persistent elevation of this cytokine is associated with pathogenic infection [37].

The strength of our study was in performing an exploratory analysis on a large number of immune parameters. Nevertheless, we were limited by the number of patients with available samples for T-cell phenotyping. The proliferation assay was carried out using PBMCs, and T-cell proliferation depends on the functional status of both responding and bystander cells. We indeed observed an association between blood dendritic cell levels and HIV-specific CD4 T-cell proliferation [11]. Based on the observed associations between Gag-specific CD8 T cells and peripheral blood levels of Tregs, Th17, and Tc17, it would have been of interest to perform new assays to compare the proliferation of purified CD8 T cells to that of PBMCs. Assays combining functional (proliferation) and physical (tetramer) detection would have provided information about the physical absence versus the inhibition of proliferation of Gag-specific T cells in samples from CD8NRs. However, we lacked samples for these analyses. Finally, the reported associations may not reflect direct interactions between the immune factors but instead their common temporal or mechanistic pathways of restoration under suppressive cART.

CONCLUSIONS

Our work has demonstrated the association between the presence of Gag-specific CD8 T cells that have maintained their proliferative capacity, higher levels of suppressive T cells and cytokines, and better preservation of T cells involved in protective mucosal immunity. These results represent a step ahead in our understanding of the dynamics of these antiviral cells that may help in the design of future therapeutic strategies. Our results are consistent with the increasingly recognized deleterious impact of persistent immune activation on immune restoration.

Supplementary Data

Supplementary material is available at *Open Forum Infectious Diseases* online.

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APPENDIX

This study was approved by the Comité de Protection des Personnes Ile-de-France II" (registration number 06-09-08), authorized by the "Direction Générale de la Santé (authorization number 2006-AOO142-49), and registered as an observational study at www.clinicaltrials.gov under the identifier NCT01055873. The institutions and investigators of the ANRS-EP38-IMMIP Study were as follows: Pédiatrie-Néonatologie, Hôpital Louis Mourier, Colombes (Corinne Floch-Tudal); Gynécologie-Obstétrique, Groupe Hospitalier Cochin Tarnier-Port-Royal, Paris (Ghislaine Firtion); Pédiatrie-Centre Hospitalier Intercommunal, Créteil (Sophie Lemerle); Pédiatrie-Centre Hospitalier Intercommunal, Villeneuve Saint-Georges (Anne Chace); Immuno-Hématologie Pédiatrique, Groupe Hospitalier Necker-Enfants Malades, Paris (Stéphane Blanche, Florence Veber); Pédiatrie, Centre Hospitalier Sud-Francilien, Evry (Adrien May); Maladies Infectieuses, Hôpital Jean Verdier, Bondy (Vincent Jeantils); Onco-Hématologie Pédiatrique Hôpital Trousseau, Paris (Catherine Dollfus); Pédiatrie-Hôpital Robert Debré, Paris (Martine Levine, Albert Faye); Centre de Diagnostic et de Thérapeutique, Hôpital de l'Hôtel-Dieu, Paris (Jean-Paul Viard).