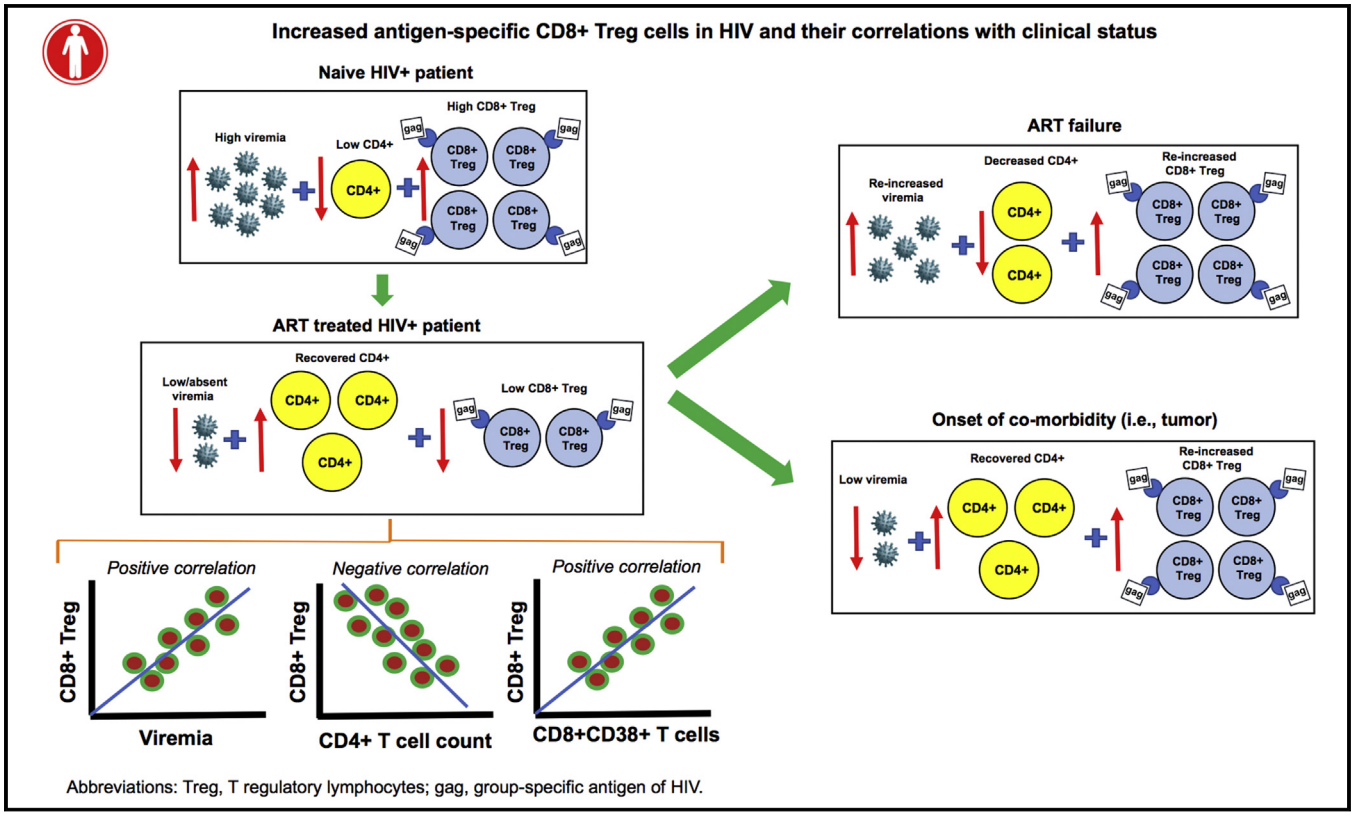
$CD8^+CD28^-CD127^{lo}CD39^+$ regulatory T-cell expansion: A new possible pathogenic mechanism for HIV infection?

Daniela Fenoglio, PhD,[a,b,c]* Chiara Dentone, MD,[a,d]* Alessio Signori, PhD,[e] Antonio Di Biagio, MD,[f] Alessia Parodi, PhD,[a] Francesca Kalli, PhD,[a] Giorgia Nasi, PhD,[a] Monica Curto, PhD,[a] Giovanni Cenderello, MD,[g] Pasqualina De Leo, MD,[h] Valentina Bartolacci, MD,[i] Giancarlo Orofino, MD,[j] Laura Ambra Nicolini, MD,[e,f] Lucia Taramasso, MD,[f] Edoardo Fiorillo, PhD,[k] Valeria Orrù, PhD,[k] Paolo Traverso, MD,[a,c,l] Bianca Bruzzone, MD,[m] Federico Ivaldi, PhD,[a] Eugenio Mantia, MD,[n] Michele Guerra, MD,[o] Simone Negrini, MD,[a,b,c] Mauro Giacomini,[a,p] Sanjay Bhagani, MD, FRCP,[q] and Gilberto Filaci, MD[a,b,c]

Genoa, Imperia, Savona, Albenga, Turin, Lanusei, Alessandria, and La Spezia, Italy, and London, United Kingdom

GRAPHICAL ABSTRACT



From [a]the Centre of Excellence for Biomedical Research, [b]the Department of Internal Medicine, [c]the Department of Health Sciences, [l]the Department of Surgical Science and Integrated Diagnostics, and [p]the Department of Informatics, Bioengineering, Robotic and System Engineering, University of Genoa; [c]IRCCS Azienda Ospedaliero Universitaria San Martino, [f]the Infectious Disease Unit, IRCCS Azienda Ospedaliero Universitaria San Martino, and [m]the Hygiene Unit, Infectious Disease Unit, IRCCS Azienda Ospedaliero Universitaria San Martino, IST–Istituto Nazionale per la Ricerca sul Cancro, Genoa; [d]the Infectious Diseases Department, Sanremo Hospital, Imperia; [g]the Infectious Diseases Department, Galliera Hospital, Genoa; [h]the Infectious Diseases Department, San Paolo Hospital, Savona; [i]S.M. Misericordia Hospital, Albenga; [j]the Infectious Diseases Department, Amedeo di Savoia Hospital, Turin; [k]Istituto di Ricerca Genetica e Biomedica, Consiglio Nazionale delle Ricerche (CNR), Sede Secondaria IRGB, Lanusei; [n]the Infectious Diseases Department, SS Antonio, Biagio, Cesare Arrigo Hospital, Alessandria; [o]the Infectious Diseases Department, Sant'Andrea Hospital, La Spezia; and [q]the Department of Infectious Diseases/HIV Medicine, Royal Free Hospital, National Health Service, London.

*These authors contributed equally to this work.

Supported by a grant from Ministero dell'Istruzione, dell'Università e della Ricerca, PRIN no. 2008WXF7KK entitled "Immunoterapia anti-tumorale operata attraverso l'inibizione dei circuiti regolatori citochino-dipendenti."

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

Received for publication March 10, 2017; revised July 10, 2017; accepted for publication August 28, 2017.

Corresponding author: Gilberto Filaci, MD, Centre of Excellence for Biomedical Research (CEBR), University of Genoa, Viale Benedetto XV no. 7, 16132 Genoa, Italy. E-mail: gfilaci@unige.it.

0091-6749

https://doi.org/10.1016/j.jaci.2017.08.021

Background: HIV-associated immunodeficiency is related to loss of $CD4^+$ T cells. This mechanism does not explain certain manifestations of HIV disease, such as immunodeficiency events in patients with greater than 500 $CD4^+$ T cells/μL. $CD8^+CD28^-CD127^{lo}CD39^+$ T cells are regulatory T (Treg) lymphocytes that are highly concentrated within the tumor microenvironment and never analyzed in the circulation of HIV-infected patients.
Objectives: We sought to analyze the frequency of $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells in the circulation of HIV-infected patients.
Methods: The frequency of circulating $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells was analyzed and correlated with viral load and $CD4^+$ T-cell counts/percentages in 93 HIV-1–infected patients subdivided as follows: naive (n = 63), elite controllers (n = 19), long-term nonprogressors (n = 7), and HIV-infected patients affected by tumor (n = 4). The same analyses were performed in HIV-negative patients with cancer (n = 53), hepatitis C virus–infected patients (n = 17), and healthy donors (n = 173).
Results: HIV-infected patients had increased circulating levels of functional $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells. These cells showed antigen specificity against HIV proteins. Their frequency after antiretroviral therapy (ART) correlated with HIV viremia, $CD4^+$ T-cell counts, and immune activation markers, suggesting their pathogenic involvement in AIDS- or non–AIDS-related complications. Their increase after initiation of ART heralded a lack of virologic or clinical response, and hence their monitoring is clinically relevant.
Conclusion: HIV infection induces remarkable expansion of $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells, the frequency of which correlates with both clinical disease and signs of chronic immune cell activation. Monitoring their frequency in the circulation is a new marker of response to ART when effects on viremia and clinical response are not met. (J Allergy Clin Immunol 2017;■■■:■■■-■■■.)

Key words: *HIV, $CD8^+CD28^-CD127^{lo}CD39^+$ regulatory T cell, antiretroviral therapy*

Abbreviations used
APC: Allophycocyanin
ART: Antiretroviral therapy
BV: Brilliant Violet
EC: Elite controller
FITC: Fluorescein isothiocyanate
HCV: Hepatitis C virus
LTNP: Long-term nonprogressor
PE: Phycoerythrin
PerCP-Cy5.5: Peridinin chlorophyll protein complex–cyanin 5.5
Treg: Regulatory T

The pathogenesis of immunodeficiency during HIV infection is mainly related to the cytopathic effects exerted by the virus against infected $CD4^+$ T cells.[1,2] However, this mechanism does not satisfactorily explain certain aspects of the disease. For instance, in the recent Strategic Timing of Antiretroviral Treatment study absolute $CD4^+$ T-cell counts were not predictive of AIDS and non-AIDS events (including non-AIDS tumors) because a number of these events were noted in both immediate versus delayed initiation of antiretroviral therapy (ART) at absolute CD4 counts of greater than 500 cells/μL.[3] Moreover, some HIV-infected patients might have reduced $CD4^+$ T-cell counts for several years without having immunodeficiency.[4] Conversely, HIV-infected patients with normal $CD4^+$ T-cell counts after ART initiation can present with severe opportunistic infections.[5,6] Hence it is possible that other pathogenic mechanisms causing immunodeficiency might be at play during HIV infection. Regulatory T (Treg) lymphocytes mediate immune-suppressive functions contributing to immune homeostasis and regulation under physiologic conditions.[7,8] Little is known about the role of these cells in HIV immunodeficiency pathogenesis.[9,10] Both increased or reduced Treg cell activity could be deleterious for HIV-infected patients. An excessive immune-suppressive function would worsen the immunologic and clinical scenario.[11,12] Conversely, impaired regulatory processes would lead to increased activation and proliferation of residual $CD4^+$ T lymphocytes, thus favoring viral infection and spread, and chronic inflammation.[13-15] To date, studies on alterations of $CD4^+$ Treg cells in HIV-infected patients led to controversial results, so that the precise role played by Treg cells in HIV pathogenesis is still unclear.[10-19]

In addition to CD4 T cells, the Treg cell compartment includes cells belonging to the $CD8^+$ T-cell lineage.[20] Among the various $CD8^+$ Treg cell subsets, a subgroup characterized by the $CD8^+CD28^-CD127^{lo}CD39^+$ phenotype has been found to be highly concentrated within the tumor microenvironment. There, these cells inhibit lymphocyte proliferative and cytotoxic functions, thus favoring tumor immune escape.[21-23] Interestingly, similarities in composition and function of Treg cell subsets between patients with tumors and those with HIV infection have been highlighted.[19] Here we analyzed $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells in HIV-infected patients, seeking to understand whether their frequency in the circulation might correlate with viral load and $CD4^+$ T-cell count and could be considered a biomarker of immunodeficiency and non-AIDS events.

METHODS

Patients

This was an observational, longitudinal, multicenter study. Ninety-three HIV-1–positive patients (Table I) were enrolled in 8 infectious diseases units located in northwestern Italy (Liguria and Piedmont) and divided into 4 groups with the following characteristics:

- *Naive patients with a new diagnosis of HIV infection (n = 63)*: plasma samples collected from patients at baseline (in 57/63 patients) and after 3, 6, and 12 months of therapy (in all 63 naive patients);
- *Elite controllers (EC [n = 19])*: patients with undetectable viral load for 2 or more years without ART;
- *Long-term nonprogressors (LTNPs [n = 7])*: patients with viral load ranging from 136 to 7100 HIV-RNA copies/mL and $CD4^+$ T-cell counts of 350/mm^3 or greater without ART; and
- *patients chronically HIV infected and affected by tumor (n = 4).*

All parameters were collected in a relational database connected through a Web-based interface.[24,25] The normalization of laboratory value ranges applied by the different units was achieved through the *z* score method, thus ensuring data harmonization.[26]

Fifty-three HIV-negative patients with cancer were given a diagnosis and enrolled at the Internal Medicine and Clinical Immunology Unit and at the Urologic Unit of the University of Genoa–IRCCS Azienda Ospedaliero Universitaria San Martino–IST-Istituto Nazionale per la Ricerca sul Cancro of Genoa. Patients were affected by renal (n = 21), bladder (n = 23), prostate (n = 2), skin (n = 1), acute myeloid leukemia (n = 1), thyroid (n = 1), breast (n = 1), myeloma (n = 1), ovary (n = 1), and bone (n = 1) cancers.

TABLE I. Clinical data of HIV-infected patients enrolled in the study

Patient no.	Sex	Age (y)	Group	Stage	Viral load (copies/mL)	$CD4^+$ T cells/μL	CD4/CD8 ratio	% $CD8^+$ Treg/$CD3^+$ T cells	$CD8^+$ Treg cells/μL	Comorbidity
1	M	31	Naive	C3	1,250	42	0.09	10.71	62	Kaposi sarcoma
2	F	49	Naive	C3	12,700	4	1.6	4.6	5	CMV, PjP, wasting syndrome
3	M	33	Naive	A2	61,600	453	0.3	1.5	26	
4	M	43	Naive	A1	26,200	790	0.6	4.02	92	
5	F	41	Naive	A2	1,230	386	1.1	2.78	24	
6	F	48	Naive	C3	NA	2	0	7.39	30	PjP
7	M	48	Naive	B3	1,060,000	10	0.1	1.2	15	
8	M	40	Naive	A3	103,000	18	0.3	7.5	116	
10	M	26	Naive	B3	858,000	32	0	4.34	28	
11	M	31	Naive	C3	285,000	121	0.1	5.3	44	
12	M	50	Naive	B2	77,800	403	0.5	2.3	29	
13	M	58	Naive	A3	390,702	15	0.02	6.5	46	
14	F	61	Naive	C3	270,964	12	0.08	18.06	30	CMV, PjP
15	M	31	Naive	A3	131,032	58	0.53	3.89	6	
16	M	60	Naive	C1	66,324	564	0.43	5.08	100	Kaposi sarcoma
17	F	40	Naive	A2	311,107	224	0.08	12.51	370	
18	M	26	Naive	A1	13,574	545	0.42	0.48	11	
19	M	53	Naive	A2	62	348	0.55	0.63	7	
20	M	74	Naive	A2	4,239	476	0.75	0.12	NA	
21	M	45	Naive	A1	286,466	612	0.65	5.8	82	
22	M	49	Naive	A3	680,326	49	0.05	7.79	88	
23	M	57	Naive	B3	491,308	137	0.26	0.38	3	
24	M	25	Naive	A1	41,714	770	0.64	3.15	68	
25	M	24	Naive	B1	29,958	510	0.27	4.90	149	
26	M	46	Naive	B3	54,982	90	0.51	0.86	3	
27	M	60	Naive	B3	243,348	140	0.25	0.18	1	
28	M	40	Naive	C3	174,190	40	0.02	3.07	68	PjP
29	M	43	Naive	C3	584,734	180	0.28	5.09	49	TB, wasting syndrome
30	M	46	Naive	B3	563,272	160	0.23	0.21	2	
31	M	46	Naive	B2	120,995	200	0.22	0.53	NA	
32	M	51	Naive	C3	72,889	10	0.02	4	16	PjP, CMV, cryptococcosis, wasting syndrome
33	M	64	Naive	C3	694,516	62	0.2	4.6	17	PjP, wasting syndrome
34	M	39	Naive	B1	673,214	550	0.48	5.54	96	
35	M	56	Naive	B3	11,744	130	0.25	10.08	73	
36	M	54	Naive	B2	38,537	300	0.5	0.14	1	
37	M	76	Naive	B2	262,500	369	0.3	6.14	112	
38	F	27	Naive	C3	62	82	0.1	0.84	14	PjP
39	M	54	Naive	A3	1,300	196	0.6	8.54	53	
40	F	52	Naive	A1	20	709	1	0.0918	1	
41	M	45	Naive	A1	20	525	0.8	0.059	1	
42	M	50	Naive	A2	12,400	377	0.4	4.45	72	
43	M	58	Naive	A2	8,100	284	0.3	8.8	122	
44	M	35	Naive	A2	4,900	279	0.4	1.05	10	
45	M	47	Naive	C3	41,000	80	0.1	3.964	22.5	CMV, cerebral toxoplasmosis
46	F	47	Naive	A3	1,100	76	0.2	3.345	24	
47	M	32	Naive	C3	311,200	8	0	2.43	33	PjP
49	M	42	Naive	C3	2,000	20	0	0.9744	6	Cerebral cryptococcosis, cerebral toxoplasmosis
50	M	41	Naive	A3	1,400,000	183	0.2	5.46	83	
51	M	26	Naive	B	13,700	NA	NA	12.76	NA	
53	F	62	Naive	A1	NA	624	0.6	3.8	68	
55	F	58	Naive	B2	4,400	276	0.2	1.84	31	
56	M	58	Naive	A1	45	567	0.3	0.6825	22	
57	F	67	Naive	A2	2,297	369	0.49	4.90	62	
58	M	53	Naive	A2	1,638,957	365	1.06	11.81	51	
59	M	52	Naive	A2	420,215	318	0.33	3.90	56	
60	M	39	Naive	A1	32,840	809	0.47	2.9	74	
61	F	52	Naive	A3	215,000	122	0.2	12.61	91	
63	M	64	Naive	B3	285,400	32	0.1	13.63	104	

(Continued)

TABLE I. *(Continued)*

Patient no.	Sex	Age (y)	Group	Stage	Viral load (copies/mL)	$CD4^+$ T cells/μL	CD4/CD8 ratio	% $CD8^+$ Treg/$CD3^+$ T cells	$CD8^+$ Treg cells/μL	Comorbidity
66	M	53	Naive	A1	3,389	737	1.64	0.53	8	
67	M	60	Naive	A1	44,719	725	0.54	3.99	83	
68	M	41	Naive	C3	13,600	9	0.01	1.71	12	Cerebral toxoplasmosis
70	M	41	Naive	A2	65,759	280	NA	1.09	NA	
72	M	50	Naive	C3	166,300	37	0.1	6.41	31	CMV, cerebral toxoplasmosis
73	M	56	EC	A2	16	482	0.8	0.016	0.2	
74	F	53	EC	A2	40	400	0.5	0.042	0.5	
75	F	55	EC	A1	40	1,170	0.7	0.19	3.1	
76	M	53	EC	A2	40	460	0.7	0.063	0.7	
77	M	57	EC	A1	40	710	NA	0	0	
78	M	56	EC	A1	40	500	NA	0.54	NA	
79	M	45	EC	B1	40	1,010	0.6	0.0688	NA	
80	M	66	EC	A1	0	1,163	1.3	0.8593	17	
81	F	60	EC	A1	10	662	1.4	0.2725	2.7	
82	F	60	EC	A1	0	652	1.4	0.2784	3	
83	M	50	EC	B1	40	674	0.2	0.6688	25	
84	M	53	EC	A2	40	454	0.8	0.88	8.5	
85	F	54	EC	A1	12	520	0.6	0.1193	1.6	
86	M	55	EC	A1	30	1,278	0.9	0.231	6.4	
87	F	55	EC	A1	40	1,120	1.0	0.8979	NA	
88	M	65	EC	A2	0	245	0.6	0.1217	0.8	
89	F	38	EC	A1	0	1,586	1.3	0.0578	1.8	
90	M	56	EC	A1	8	601	0.8	0.0515	0.6	
91	F	54	EC	A2	40	469	0.6	0.2062	2.7	
92	M	53	LTNP	A1	3,000	734	0.7	1.048	16	
93	F	66	LTNP	A1	416	713	1.2	3.9729	55	
94	M	43	LTNP	A2	3,200	368	0.3	2.964	53	
95	M	36	LTNP	A1	136	776	1.3	0.1903	2.7	
96	M	50	LTNP	A2	1,299	490	0.4	5.2038	NA	
97	F	47	LTNP	A1	7,000	511	NA	0.7031	NA	
98	M	61	LTNP	A2	7,220	430	1.1	18.368	NA	
99	M	58	Tumor in HIV	C3	7	38	0.1	7.7894	22	
100	M	59	Tumor in HIV	C3	20	94	0.3	7.9662	NA	
101	M	58	Tumor in HIV	C2	100	200	0.37	2.912	NA	
102	M	47	Tumor in HIV	C3	544,600	33	0.1	2.29	NA	

CMV, Cytomegalovirus infection; *F*, female; *M*, male; *NA*, not available; *PjP*, *Pneumocystis jirovecii* pneumonia; *TB*, tuberculosis.

Seventeen pretreatment hepatitis C virus (HCV)–monoinfected patients were enrolled at the Infectious Diseases Unit of the University of Genoa-San Martino IRCCS Hospital.

One hundred seventy-three healthy donors were also enrolled at the Internal Medicine and Clinical Immunology Unit of the University of Genoa–San Martino IRCCS Hospital and at the Istituto di Ricerca Genetica e Biomedica (IRGB), CNR, Monserrato, as control subjects.

The study was carried out in compliance with the Helsinki Declaration and approved by the Ethical Committee of San Martino Hospital in Genoa (P.R.251REG2014). All enrolled patients provided written informed consent.

Measurement of plasma viral load in HIV-infected patients

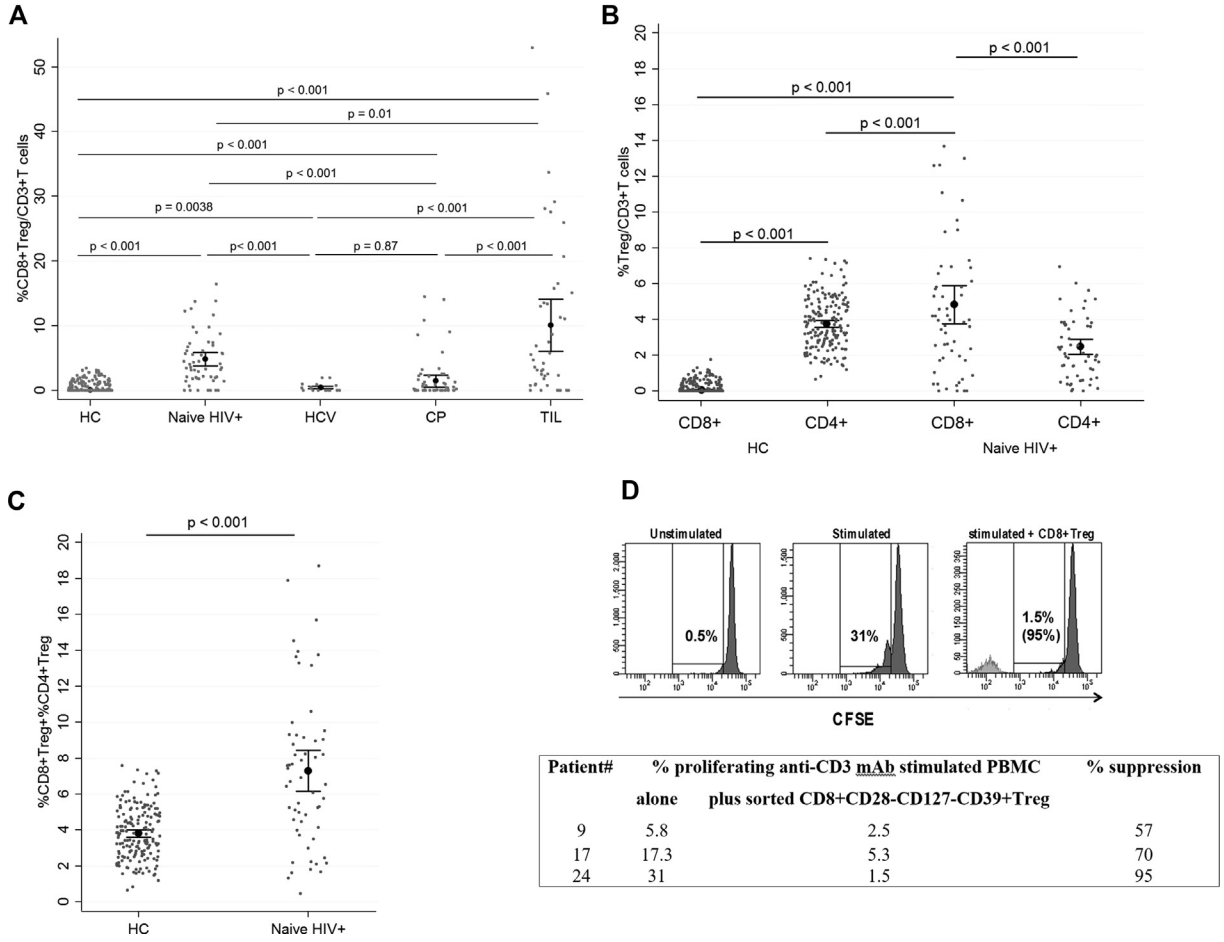
Plasma viral load, measured in HIV RNA copies with a cutoff set at 40 copies/mL, was quantified by using the K-PCR-HIV1 (Siemens Health Care, Erlangen, Germany) and the Nucleosens HIV (bioMerieux, Marcy-l'Étoile, France) kits.

Purification of PBMCs and tumor lymphocyte infiltrates

PBMCs were purified from heparinized blood samples by means of centrifugation on Ficoll-Hypaque gradient (Biochrom AG, Berlin, Germany) for 30 minutes at 1800 rpm. Lymphocytes from surgical specimens were purified by filtering minced tissues with a sterile cell strainer (Falcon; BD Biosciences, San Jose, Calif) and running the collected cells on Ficoll gradient.

mAbs and immunofluorescence analyses

Immunofluorescence analyses were performed on 100 μL of fresh blood samples, incubating the cells with specific fluorochrome-conjugated mAbs at 4°C for 30 minutes in the dark. Red cells were lysed with 2 mL of FACS Lysing Solution (BD Biosciences) and resuspended in 200 μL of the same solution. The following mAbs were used to analyze Treg cell subsets: phycoerythrin (PE)–conjugated anti-CD127, clone HIL-7R-M21 (BD Biosciences); fluorescein isothiocyanate (FITC)–conjugated anti-CD45RA, clone HI100 (BD Biosciences); peridinin chlorophyll protein complex–cyanin 5.5 (PerCP-Cy5.5)–conjugated anti-CD28, clone CD28.2 (BioLegend, San Diego, Calif); PE-cyanin 7–conjugated anti-CD25, clone M-A251 (BD Biosciences); allophycocyanin (APC)–conjugated anti-CD39, clone TU66 (BD Biosciences); APC-H7–conjugated anti-CD4, clone RPA-T4 (BD Biosciences); Brilliant Violet (BV) 421–conjugated anti-CD8, clone RPA-T8 (BD Biosciences); and BV510-conjugated anti-CD3, clone UCHT1 (BD Biosciences). The following mAbs were used to analyze activation status of T-cell subsets: FITC-conjugated anti-CD8, clone RPA-T8 (BD Biosciences); PE-conjugated anti-CD38, clone HB7 (BD Biosciences);



Patient#	% proliferating anti-CD3 mAb stimulated PBMC		% suppression
	alone	plus sorted CD8+CD28-CD127-CD39+Treg	
9	5.8	2.5	57
17	17.3	5.3	70
24	31	1.5	95

FIG 1. Frequency of Treg cell subsets in patients affected by HIV or HCV infection, patients with cancer, and healthy control subjects. $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells and $CD4^+CD25^{hi}CD127^{lo}$ Treg cells were named $CD8^+$ and $CD4^+$ Treg cells in the figure, respectively. **A,** Comparison among the frequencies of $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells in the circulation of healthy control subjects *(HC)*, naive HIV-infected patients *(Naïve HIV+)*, untreated HCV-monoinfected patients *(HCV)*, patients with cancer *(CP)*, and patients with tumor-infiltrating lymphocytes *(TIL)*. **B,** Comparison between $CD8^+CD28^-CD127^{lo}CD39^+$ and $CD4^+CD25^{hi}CD127^{lo}$ Treg cell frequencies in the circulation of healthy control subjects *(HC)* and naive HIV-infected patients *(Naïve HIV+)*: $CD8^+$ and $CD4^+$ indicate $CD8^+CD28^-CD127^{lo}CD39^+$ and $CD4^+CD25^{hi}CD127^{lo}$ Treg cells, respectively. **C,** Comparison of the sums of circulating $CD8^+CD28^-CD127^{lo}CD39^+$ and $CD4^+CD25^{hi}CD127^{lo}$ Treg cell frequencies between healthy control subjects *(HC)* and naive HIV-infected patients *(Naïve HIV+)*. **D,** Representative proliferation suppression assay performed with circulating $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells purified from patient 24. The percentages of T-cell proliferation under unstimulated *(left panel)* or anti-CD3 UCHT1 mAb–stimulated (*central* and *right panels*) conditions are shown; in the *right panel* the percentage of proliferation inhibition by $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells is shown in parentheses. The *box* shows the percentages of suppressive activity on T-cell proliferation achieved with sorted $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells from patients 9, 17, and 24. All experiments were performed with autologous PBMCs as responder cells. *CFSE*, Carboxyfluorescein succinimidyl ester.

PerCP-Cy5.5–conjugated anti–HLA-DR, clone L243; and APC-conjugated anti-CD3, clone UCHT1 (BD Biosciences). Samples were analyzed by using a BD FACSCanto II flow cytometer (BD Biosciences) with BD FACSDiva software (version 6.0; BD Biosciences). Expression of the Ki-67 marker was analyzed, incubating 1×10^6 PBMCs with Aqua Dead (Molecular Probes, Thermo Fisher, Waltham, Mass) for 15 minutes at room temperature. Cells were washed with PBS–BSA 0.01% and incubated with the following mAbs for surface staining: BV510-conjugated anti-CD3, clone UCHT1 (BD Biosciences); APC-H7–conjugated anti-CD8, clone RPA-T8 (BD Biosciences); BV-605–conjugated anti-CD28, clone CD28.2 (BD Biosciences); PerCP-Cy5.5–conjugated anti-CD127, clone HIL-7R-M21 (BD Biosciences); and APC-conjugated anti-CD39, clone TU66 (BD Biosciences). After staining, the cells were fixed and permeabilized by using the Transcription Buffer Set (BD PharMingen) before intranuclear staining with FITC mouse anti–Ki-67 (BD Biosciences) for 30 minutes in the dark. The cells were washed with 1 mL of PBS–BSA 0.01% and resuspended in 300 μL of PBS. Samples were analyzed by using a BD Fortessa X20 flow cytometer (BD Biosciences) with the BD FACSDiva software (version 8.0; BD Biosciences).

Evaluation of HLA-A2–positive patients

Fifty microliters of fresh blood was incubated with 10 μL of unconjugated anti–HLA-A2 mAb, clone BB7.2, for 20 minutes at room temperature.[27] The cells were then washed and incubated with the FITC-conjugated goat anti-mouse IgG_1 secondary antibody (SouthernBiotech, Birmingham, Ala)

for 30 minutes at room temperature. Red cells were lysed with 2 mL of FACS Lysing Solution (BD Biosciences) and resuspended in 200 μL of the same solution. Tubes were analyzed by using a BD FACSCanto II flow cytometer (BD Biosciences) with BD FACSDiva software (version 6.0; BD Biosciences).

Frequency of gag-specific CD8$^+$ T lymphocytes

PBMCs (2×10^6) were incubated with A*02:01 HIV gag PE-conjugated 1-10 FLGKIWPSYK pentamers (TC Metrix, Epalinges, Switzerland) or PE-conjugated hTert ILAKFLHWL pentamers (ProImmune, Oxford, United Kingdom) as a negative control for 15 minutes at 4°C. Cells were washed with PBS–BSA 0.01% and incubated with Live Dead/Aqua Dead (Molecular Probes, Thermo Fisher) for 15 minutes at room temperature. Cells were washed with PBS–BSA 0.01% and incubated with the following mAbs for surface staining: FITC-conjugated anti-CD8, clone RPA-T8 (BD Biosciences); PerCP-Cy5.5–conjugated anti-CD28, clone CD28.2 (BioLegend); APC-conjugated anti-CD39, clone TU66 (BD Biosciences); BV421-conjugated anti-CD127, clone HIL-7R-M21 (BD Biosciences); and APC-H7–conjugated anti-CD3, clone UCHT1 (BD Biosciences). After staining, the tubes were washed with 1 mL of PBS–BSA 0.01% and resuspended in 300 μL of BD FACS Lysing solution (BD Biosciences). The samples were analyzed by using a BD FACSCanto II flow cytometer (BD Biosciences) with BD FACSDiva software (version 6.0; BD Biosciences).

Sorting of CD8$^+$CD28$^-$CD127^{lo}CD39$^+$ Treg cells

CD8$^+$ T lymphocytes, purified from PBMCs by means of immunomagnetic bead separation with microbeads conjugated with mAb specific for the CD8 antigen (Dynal CD8 positive isolation kit; Invitrogen, Life Technologies, Paisley, United Kingdom), were labeled with FITC–conjugated anti-CD127, clone HIL-7R-M21 (BD Biosciences), PerCP-Cy5.5–conjugated anti-CD28, clone CD28.2 (BioLegend); PE-conjugated anti-CD39, clone TU66 (BD Biosciences); and BV421-conjugated anti-CD8, clone RPA-T8 (BD Biosciences). Cells were sorted by using the BD FACSAria (BD Biosciences) with BD FACSDiva software (version 6.0; BD Biosciences).

Proliferation suppression assay

The suppression activity of sorted CD8$^+$CD28$^-$CD127^{lo}CD39$^+$ Treg cells was evaluated by monitoring the inhibition of dye dilution in PBMCs from healthy donors stained before the test with carboxyfluorescein succinimidyl ester (5 μmol/L; Molecular Probes). After staining, cells were pulsed with the anti-CD3 UCTH-1 mAb (5 μg/mL; BD Biosciences) and cultured for 5 days in a 96-well U-bottom plate (1×10^5 cells/well) in the presence (or not) of sorted CD8$^+$CD28$^-$CD127^{lo}CD39$^+$ Treg cells (1×10^5 cells/well). Then the samples were washed in PBS and analyzed by using a BD FACSCanto II flow cytometer (BD Biosciences) with BD FACSDiva software (version 6.0; BD Biosciences). Dead cells were excluded from analysis by adding 7-aminoactinomycin D (BD Biosciences) before acquisition. Suppression activity was expressed as the percentage reduction of proliferation in the presence of CD8$^+$CD28$^-$CD127^{lo}CD39$^+$ Treg lymphocytes compared with levels of proliferation observed in control cultures of PBMCs performed in the absence of Treg cells. A suppression activity of 25% or greater was considered significant.

Statistical analyses

Sample size calculation was based on the difference of CD8$^+$ Treg cell frequencies among the 7 groups (healthy control subjects, ECs, naive HIV$^+$ patients, patients with HCV, patients with cancer, LTNPs, and patients with tumor-infiltrating lymphocytes). A total of 231 subjects were needed to detect a medium effect size from ANOVA (effect size, f = 0.25) with a statistical power of 80% and a level of significance (α) of 5%. With these α values and power, a sample size in the naive group of greater than 60 patients permits detection of a correlation coefficient of greater than 0.35 as significant.

ANOVA was used to statistically compare CD8$^+$CD28$^-$CD127^{lo}CD39$^+$ Treg cell frequency in the different categories of subjects (healthy control subjects and groups of patients). A cube-root transformation to this parameter was previously adopted to make distribution less skewed. Single *P* values from each comparison were adjusted for multiple comparisons by using the false discovery rate approach. Similarly, independent-samples *t* tests (for analyses performed on data collected from different patients) or paired-samples *t* tests (for analyses performed on data collected from the same patient) were used to compare CD8$^+$CD28$^-$CD127^{lo}CD39$^+$ Treg cell frequency, CD4$^+$CD25^{hi}CD127^{lo} Treg cell frequency, and their sum in healthy control subjects and naive HIV-infected patients.

The Pearson correlation coefficient was calculated between CD8$^+$CD28$^-$CD127^{lo}CD39$^+$ Treg cells and viremia, CD4$^+$ T-cell counts, and CD4$^+$ T-cell percentages in naive HIV-infected patients. Correlation was assessed by using both only baseline assessments and all longitudinal time points values (including baseline values). Correlation using all longitudinal time point values was also calculated between CD8$^+$CD28$^-$CD127^{lo}CD39$^+$ Treg cell frequency and frequency of either CD8$^+$CD38$^+$ or CD8$^+$CD38$^+$HLA-DR$^+$ T lymphocytes.

A linear mixed model with random intercept was performed to assess the longitudinal change of CD8$^+$ Treg cell counts, viremia, and CD4$^+$ T-cell counts and percentages. Univariable and multivariable models with CD8$^+$ Treg cell counts as a dependent variable and viremia, CD4$^+$ T-cell counts, or CD4$^+$ T-cell percentages as independent variables were run to confirm the association between CD8$^+$ Treg cell counts and the other 3 clinical parameters.

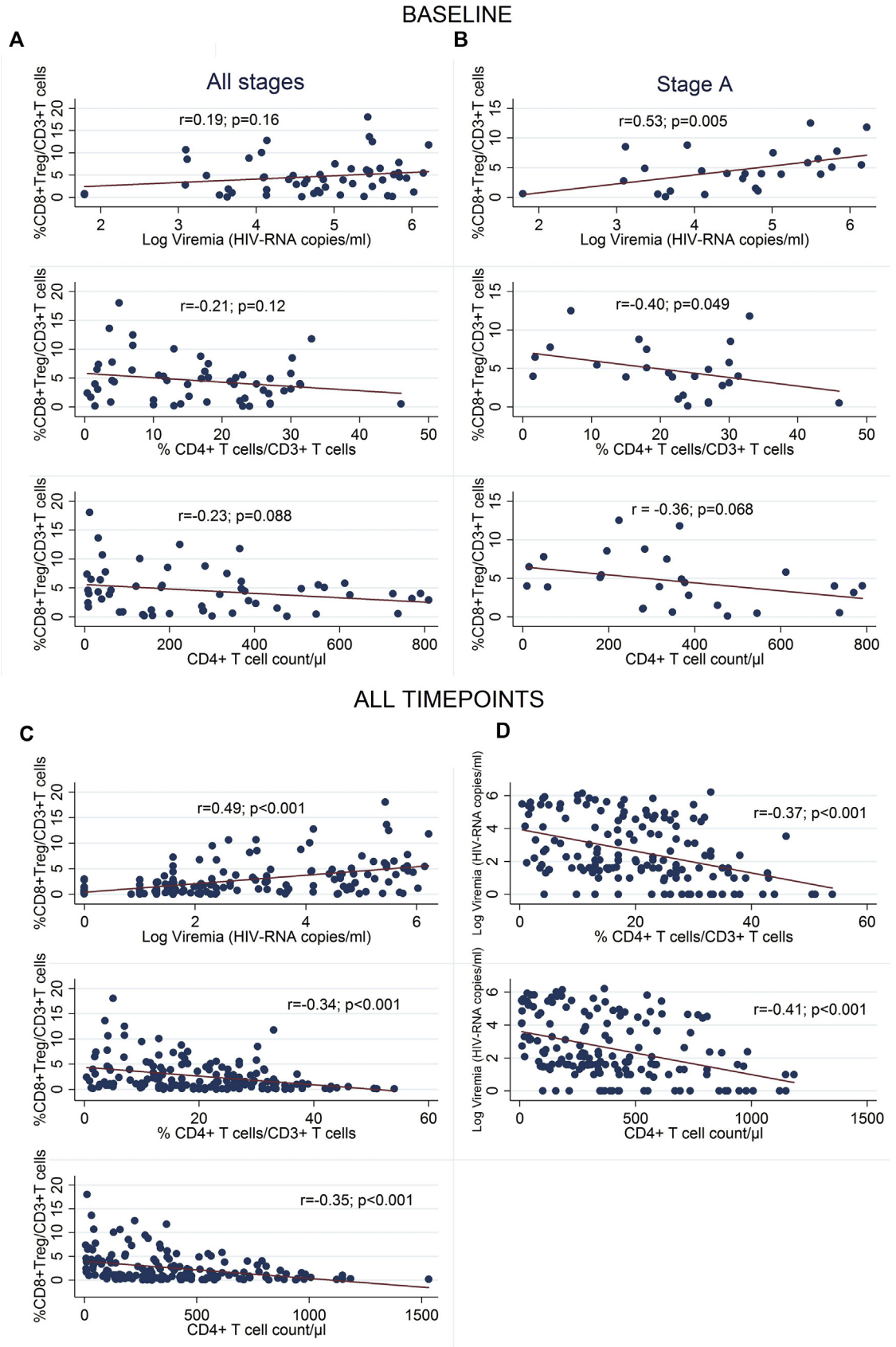
In linear mixed models all possible functions of time (linear, quadratic, cubic, log, and square root) were considered to better predict the longitudinal shape of CD8$^+$ Treg cells, viremia, and CD4$^+$ T-cell counts and percentages. Viremia was transformed and represented on a $\log_{10}$ scale. Bayesian information criterion values were used to identify the functions of time with the better fit to observed data.

SEs were calculated from the linear mixed model and used to define the CIs of predictions at all time points. A *P* value of less than .05 was considered statistically significant. Stata (v.13; StataCorp, College Station, Tex) and R (p.adjust function; R Foundation for Statistical Computing) software were used for computation.

RESULTS

Increased CD8$^+$CD28$^-$CD127^{lo}CD39$^+$ Treg cell frequencies in the circulation of chronically HIV-infected patients with viremia controlled by therapy

CD8$^+$CD28$^-$CD127^{lo}CD39$^+$ Treg cell frequencies in peripheral blood of HIV-infected patients were analyzed in 63 ART-naive patients in comparison with those of 173 healthy subjects. The majority (125/173) of healthy subjects had no detectable CD8$^+$CD28$^-$CD127^{lo}CD39$^+$ Treg cells, or if they did, their frequency did not exceed 0.2% of total CD3$^+$ T lymphocytes (Fig 1, *A*). In contrast, 63 (100%) of 63 naive HIV-infected patients showed CD8$^+$CD28$^-$CD127^{lo}CD39$^+$ Treg cells in their circulation: in these patients the frequency of CD8$^+$CD28$^-$CD127^{lo}CD39$^+$ Treg cells was increased (mean, 4.8%) and was significantly ($P < .001$) greater than that observed in healthy control subjects (Fig 1, *A*). The CD8$^+$CD28$^-$CD127^{lo}CD39$^+$ Treg cell frequency of HIV-infected patients was also compared with that of circulating or tumor-infiltrating CD8$^+$CD28$^-$CD127^{lo}CD39$^+$ Treg cells in patients with cancer.[23] Surprisingly, the frequency found in the circulation of HIV-infected patients was significantly ($P < .001$) higher than that present in the peripheral blood of 46 patients with cancer (mean, 1.7%) and was only lower than that observed within tumor infiltrates (in our hands the highest frequency thus far measured; Fig 1, *A*).[23] These results suggest that a massive


BASELINE
A
All stages
r=0.19; p=0.16
%CD8+Treg/CD3+T cells
Log Viremia (HIV-RNA copies/ml)
r=-0.21; p=0.12
% CD4+ T cells/CD3+ T cells
r=-0.23; p=0.088
CD4+ T cell count/µl
B
Stage A
r=0.53; p=0.005
r=-0.40; p=0.049
r = -0.36; p=0.068
ALL TIMEPOINTS
C
r=0.49; p<0.001
r=-0.34; p<0.001
r=-0.35; p<0.001
D
Log Viremia (HIV-RNA copies/ml)
r=-0.37; p<0.001
r=-0.41; p<0.001

expansion/recirculation of these cells occurs in HIV-infected patients, as further supported by absolute counts of circulating $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells (Table I) and their expression of the CCR4 chemokine receptor (specific for CCL22, which is a known attractant for Treg cells; see Fig E1 in this article's Online Repository at www.jacionline.org).[28]

The frequency of circulating $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells in 17 patients before treatment affected by HCV infection (another chronic viral infectious disease) was also analyzed to verify the specificity of this phenomenon for HIV infection. The results show that in HCV-infected patients the frequency of circulating $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells is significantly ($P < .001$) lower than in HIV-infected patients (Fig 1, *A*), strengthening the possibility that the preferential expansion of these cells in HIV-infected patients is specifically related to HIV itself and its peculiar interactions with the immune system. Moreover, in naive HIV-infected patients the frequency of $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells was significantly greater than that of $CD4^+CD25^{hi}CD127^{lo}$ Treg cells in matched measurements performed in individual patients. Interestingly, the frequency of $CD4^+CD25^{hi}CD127^{lo}$ Treg cells was greater than that of $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells in the circulation of healthy control subjects (Fig 1, *B*). Moreover, when the $CD4^+CD25^{hi}CD127^{lo}$ and $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cell subsets were considered together, they accounted for an increase in the circulation that was consistent in naive HIV-infected patients compared with healthy subjects (Fig 1, *C*). Hence HIV infection always associates with expansion of the Treg cell compartment.

$CD4^+CD25^{hi}CD127^{lo}$ Treg cells have been proposed to be functionally deficient in HIV-infected patients: this phenomenon could also apply to $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells.[15] Hence we tested the functional activity of $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells sorted from the peripheral blood of HIV-infected patients. Fig 1, *D*, shows that these cells efficiently inhibited T-cell proliferation induced by stimulation with an anti-CD3 mAb. Interestingly, $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells from representative HIV patient 21 showed more effective suppression activity than $CD4^+CD25^{hi}CD127^{lo}$ Treg cells from a healthy donor, confirming their functional and activated status (see Fig E2 in this article's Online Repository at www.jacionline.org). Altogether, these results suggest that HIV infection selectively expands/recruits fully functional $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells in the circulation, likely contributing to the maintenance of immunodeficiency.

Induction of $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells is HIV related

A direct relationship existing between the increased $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cell frequency and HIV activity was interrogated next. To this end, the statistical correlations between $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cell frequencies and the 3 traditional biomarkers of disease, HIV viral load, absolute $CD4^+$ T-cell count, and percentage of $CD4^+$ T cells, were analyzed in matched measurements performed in individual HIV-infected patients. Analyses performed on naive, untreated HIV-infected patients (baseline assessment) showed a weak and nonsignificant level of correlation between $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cell frequency and the 3 traditional parameters (Fig 2, *A*). However, stratifying the patients in relation to the current Centers for Disease Control and Prevention classification (stage A vs stages B and C), clear correlations were detected between $CD8^+$ Treg cell frequencies and either HIV viral load, absolute $CD4^+$ T-cell count, or percentage of $CD4^+$ T cells in stage A patients (Fig 2, *B*) but not in stage B or C patients (not shown). Importantly, when the analyses included values collected longitudinally at baseline and after 3, 6, 9, and 12 months from initiation of ART, highly significant correlations were observed in the whole cohort of patients (Fig 2, *C*, and see Fig E3 in this article's Online Repository at www.jacionline.org). In particular, $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cell frequency and viremia showed a tighter statistical association ($r = .45$) than those between either absolute T-cell counts ($r = -0.32$) or $CD4^+$ T-cell percentages ($r = -0.30$) with viremia (Fig 2, *D*). Altogether, these results suggest that the enrichment of $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells in the circulation might be a virus-dependent phenomenon possibly related as a cofactor of $CD4^+$ T-cell loss to the pathogenesis of immunodeficiency. Inverse associations between the frequency of $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells and the $CD4^+$ T-cell count or percentage support this hypothesis.

In HIV-infected patients $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells are actively proliferating cells because they express the Ki-67 marker (Fig 3, *A*). It is possible that the expanded pool of actively proliferating $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells in HIV-infected patients could represent a population of chronically stimulated $CD8^+$ T cells reacting against HIV itself. To test this, circulating HIV-specific $CD8^+$ T lymphocytes were identified by staining with HLA-A2 pentamers loaded with the HIV $gag_{1\text{-}10}$ peptide: phenotypic analysis revealed that a percentage of gag-reactive T lymphocytes ranging from 2% to 34% in 11 naive $HLA\text{-}A2^+$ HIV-infected patients had the $CD8^+CD28^-CD127^{lo}CD39^+$ phenotype (Fig 3, *B*, and Table II). Interestingly, in all tested patients the percentage of gag-reactive $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells decreased after initiation of ART (Table II). Hence this finding suggests that the virus can drive the commitment of T lymphocytes to regulatory functions with the acquisition of a $CD8^+CD28^-CD127^{lo}CD39^+$ phenotype, which might contribute to the

FIG 2. Correlation of $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cell frequency with HIV viremia, $CD4^+$ T-cell percentages, and absolute $CD4^+$ T-cell counts in naive HIV-infected patients. $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells and $CD4^+CD25^{hi}CD127^{lo}$ Treg cells were named $CD8^+$ and $CD4^+$ Treg cells in this figure, respectively. Correlations were performed either considering only data collected before ART initiation (*BASELINE*; **A** and **B**) or data achieved at all the examined time points (*ALL TIMEPOINTS*, including baseline and 3, 6, 9, and 12 months after initiation of ART; **C** and **D**). Viremia is expressed as HIV RNA copies/mL on cube root transformation *(c.r.)*. Fig 2, *A*, Correlations performed with data collected from all patients at baseline. Fig 2, *B*, Correlations performed with data collected only from stage A patients at baseline. Fig 2, *C* and *D*, Correlations performed with data collected from all patients at all time points (n = 158; baseline and 3, 6, 9, and 12 months after initiation of ART). Correlations were calculated by using the Pearson correlation coefficient. Viremia is expressed as HIV RNA copies/mL and represented on a $\log_{10}$ scale.

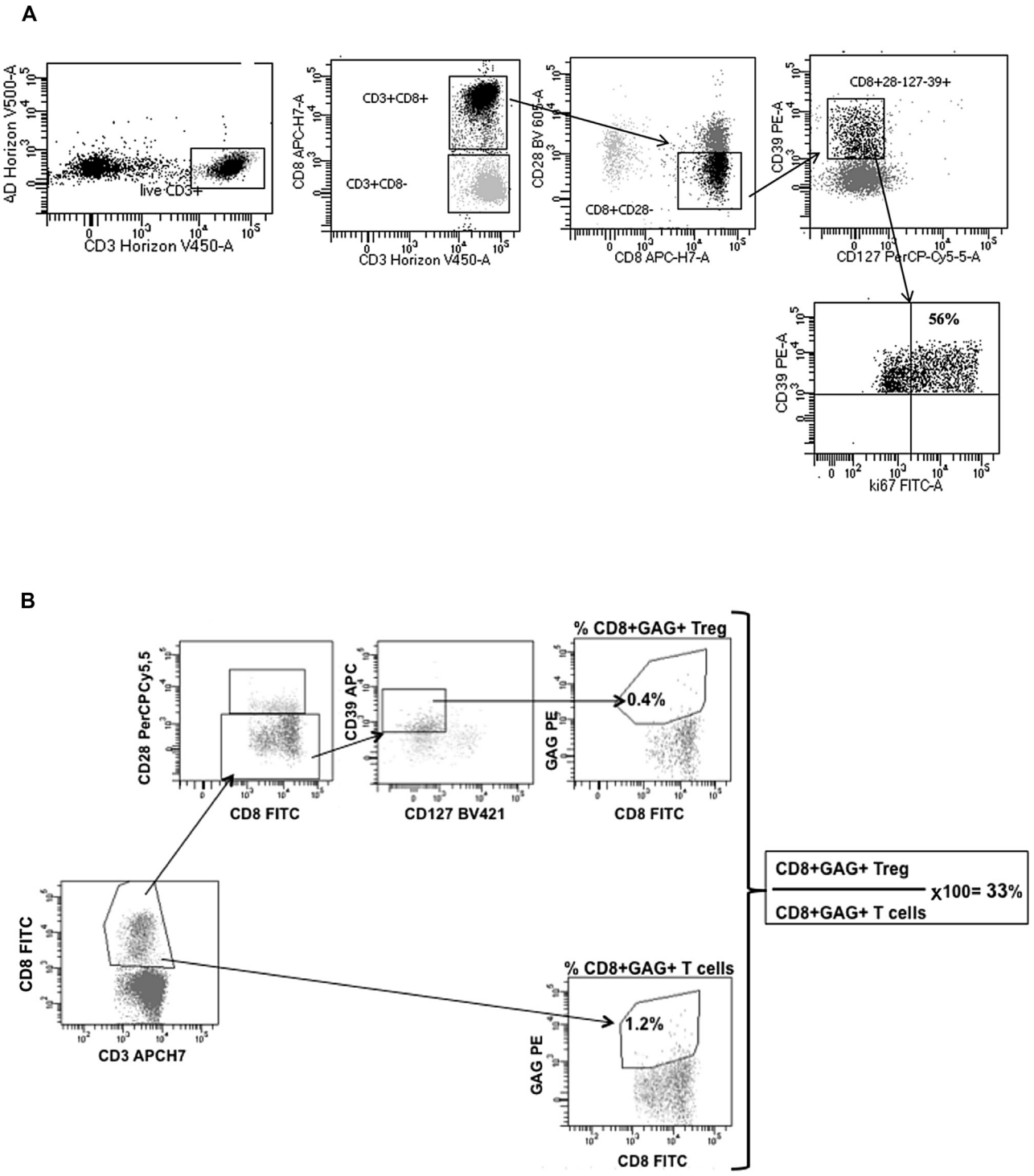


FIG 3. Analyses of Ki-67 expression on $CD8^+CD28^-CD127^{lo}CD39^+$ cells and $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cell binding to HLA-A2 $gag_{1\text{-}10}$ pentamers. **A,** Gating strategy to measure the fraction of Ki-67^+ cells among $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells from representative HIV-infected patient 72. Comparable findings were achieved in all tested HIV-infected patients. **B,** analysis of $CD8^+$ T-cell and $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cell binding to HLA-A2 $gag_{1\text{-}10}$ pentamers performed with cells from representative HIV-infected patient 56. *Upper panels* show HLA-A2 $gag_{1\text{-}10}$ pentamer–positive $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells within the total $CD8^+$ T-cell subpopulation; *lower panels* show HLA-A2 $gag_{1\text{-}10}$ pentamer–positive cells within the total $CD8^+$ T-cell subpopulation. Percentages of positivity are indicated. The *box* shows the calculation for achieving the percentage of $CD8^+CD28^-CD127^{lo}CD39^+GAG^+$ Treg cells within the total $CD8^+GAG^+$ T-cell subpopulation.

TABLE II. HLA-A2-GAG pentamer–positive $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells in naive HIV-infected patients

	Before ART		After ART instauration	
Patient no.	$CD8^+GAG^+$ T cells/$CD8^+$ T cells (%)	$CD8^+CD28^-CD127^{lo}CD39^+$ GAG^+ T cells/$CD8^+GAG^+$ T cells (%)	$CD8^+GAG^+$ T cells/$CD8^+$ T cells (%)	$CD8^+CD28^-CD127^{lo}CD39^+$ GAG^+ T cells/$CD8^+GAG^+$ T cells (%)
1	1.3	21	0.3	0
3	0.4	23	0.2	0
10	0.5	13	ND	ND
11	0.2	2	0.2	0
15	0.4	7	ND	ND
28	0.9	18	ND	ND
51	0.5	15	ND	ND
53	0.4	11	0.4	1
58	1.2	33	0.2	8
63	0.3	2.3	0.2	0
64	0.4	10	ND	ND

ND, Not done.

immunosuppression of HIV-infected subjects. The precise molecular and cellular interactions leading to such a commitment, as well as the anatomic site at which it occurs, remain to be elucidated.

$CD8^+CD28^-CD127^{lo}CD39^+$ Treg cell frequency is a possible biomarker of HIV infection

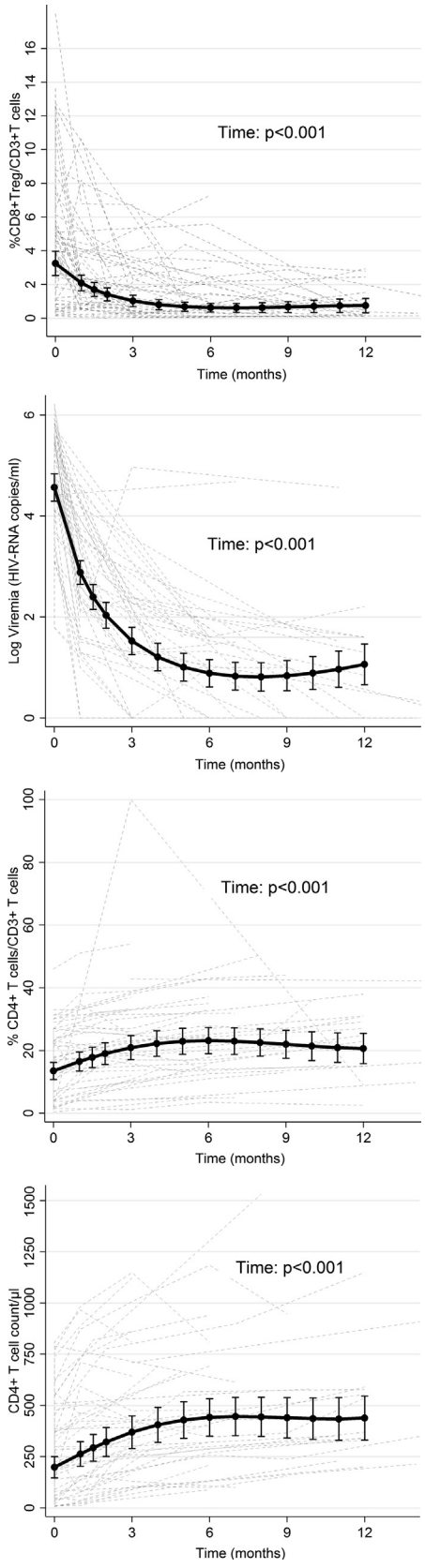
The fact that the correlations between the frequency of $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells and viral load, as well as $CD4^+$ T-cell counts or percentages, were only seen longitudinally in ART-treated patients but not at baseline of patients earmarked to commence ART leads to the hypothesis that $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cell frequency itself could represent a new dynamic biomarker to monitor the course of the disease. To explore this possibility, we undertook a longitudinal analysis of the frequency of $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells in 63 naive HIV-infected patients before and after the administration of an ART regimen. A progressive significant reduction of circulating $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cell frequency was observed after the beginning of treatment (Fig 4), a behavior reminiscent of programmed death 1 expression on T lymphocytes in HIV-infected patients.[29,30] In parallel, a progressive significant reduction of viremia and an increase in both $CD4^+$ T-cell counts and percentages were observed (Fig 4) The reduction of circulating $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cell frequencies remained significant ($P = .03$), even after adjustment for viremia and $CD4^+$ T-cell counts in the multivariable model. These findings suggest that during HIV infection, viremia gauges the expansion of T-cell subsets linked to regulatory function.

Arguably, $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cell frequency can be used as a new biomarker for HIV infection and disease progression. To corroborate this idea, we determined the frequency of $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells in a cohort of untreated chronically HIV-infected patients divided into ECs (n = 19; all with undetectable viral load, with a lower limit of detection <40 copies/mL) and LTNPs (n = 7; viral load range, 136-7100 HIV RNA copies/mL). Interestingly, 14 (73.7%) of 19 ECs did not have $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells in their circulation. Moreover, the frequency of $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells in the EC group, but not in LTNP group, was significantly lower than that in naive HIV-infected patients (Fig 5).

Interestingly, 6 (9.5%) of 63 naive HIV-infected patients did not show a decrease in $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cell frequency after initiation of ART (see Table E1 in this article's Online Repository at www.jacionline.org). We focused on the clinical status of these patients. In particular, patients 20 and 27, who did not respond to ART therapy, had an increase in viral load after 6 months of ART; patients 2, 29, 32, and 33 had wasting syndrome (patient 33 also had an unresolved opportunistic infection, cryptococcosis) despite the decrease of viremia and the increase in absolute $CD4^+$ T-cell count. This suggests that an increase in $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cell frequency might be a marker of virologic or clinical worsening independent of viremia and $CD4^+$ T-cell count. Accordingly, high circulating $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cell frequencies (7.7%, 7.9%, and 2.2%, respectively) were found in 3 chronically HIV-infected patients at the time of tumor diagnosis (patients 69 and 72: skin and visceral disseminated Kaposi; patient 70: non-Hodgkin lymphoma) despite HIV RNA values of less than 100 copies/mL. Another patient (patient 71) affected by lung cancer in remission after chemotherapy showed a rapid increase in $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cell frequency (from 0% to 2.9%) at the time of diagnosis of brain metastasis. Altogether, these findings suggest that the $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cell frequency might represent a new biomarker to assess ART lack of effect and/or identify those patients in whom the virologic response is not associated with a full clinical response because of concomitant opportunistic infections or tumors.

Correlation between $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cell frequency and immune activation

HIV-related comorbidities in patients receiving ART with viremia control remain a clinical concern because they reduce life expectancy of HIV-infected patients compared with the general population.[31,32] Comorbidities, such as central nervous system vasculopathy and atherosclerosis, cardiovascular disease, loss of bone mineral density, cognitive impairment, and HIV-related malignant diseases, are likely related to chronic activation of the immune system and to a condition of chronic inflammation that has been reported in HIV-infected patients.[33-39] Here the frequency of circulating $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells, but not that of $CD4^+CD25^{hi}CD127^{lo}$ Treg cells



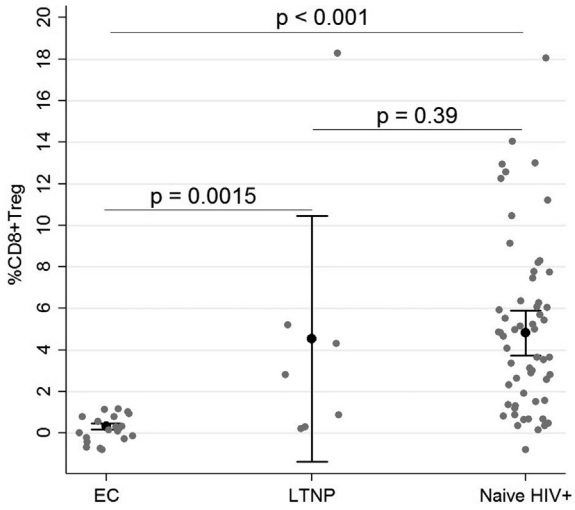


FIG 5. $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cell frequency in ECs, LTNPs, and naive HIV-infected patients. $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells were named $CD8^+$ Treg cells in the figure. Comparison among the $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cell frequencies in the circulation of ECs, LTNPs, and naive HIV-infected *(Naïve HIV+)* patients at baseline are shown.

(data not shown), was significantly correlated with the frequency of $CD8^+CD38^+$ and $CD8^+CD38^+HLA\text{-}DR^+$ T lymphocytes (Fig 6), suggesting that HIV infection sustains a chronic immune cell activation that is not strictly related to viremia levels but that is paralleled by progressive expansion of circulating $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells.

DISCUSSION

The results of this study show that (1) functional $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cell counts are markedly increased in the circulation of HIV-infected patients, (2) monitoring their frequency in the peripheral blood of HIV-infected patients might represent a clinical biomarker, and (3) these $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells at least partially originate from an HIV-specific T-cell pool.

Thus far, the most studied Treg cell subtypes are those belonging to the $CD4^+$ T-cell compartment.[40] However, regulatory lymphocytes are also present within the $CD8^+$ T-lymphocyte subpopulation. Indeed, several $CD8^+$ Treg cell subsets have been described both in animals and human subjects.[20,41] Similar to what occurs to $CD4^+$ Treg cells, some of these subsets generate within the thymus (natural $CD8^+$ Treg cells), whereas others develop at the periphery.[20,41-43] Interestingly, high expression of CD28 antigen seems not to be compatible with commitment of

FIG 4. Longitudinal assessment of $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cell frequencies, viremia, and $CD4^+$ T-cell percentages and absolute counts. $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells were named $CD8^+$ Treg cells in the figure. Trends of the following biomarkers of disease in naive HIV-infected patients during 12 months of follow-up after ART initiation are shown, respectively (top to bottom side): $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cell frequency, viremia, $CD4^+$ T-cell percentage, and $CD4^+$ T-cell count. *P* values refer to the differences from baseline values during the entire follow-up. Predictions with CIs were estimated from a linear mixed model.

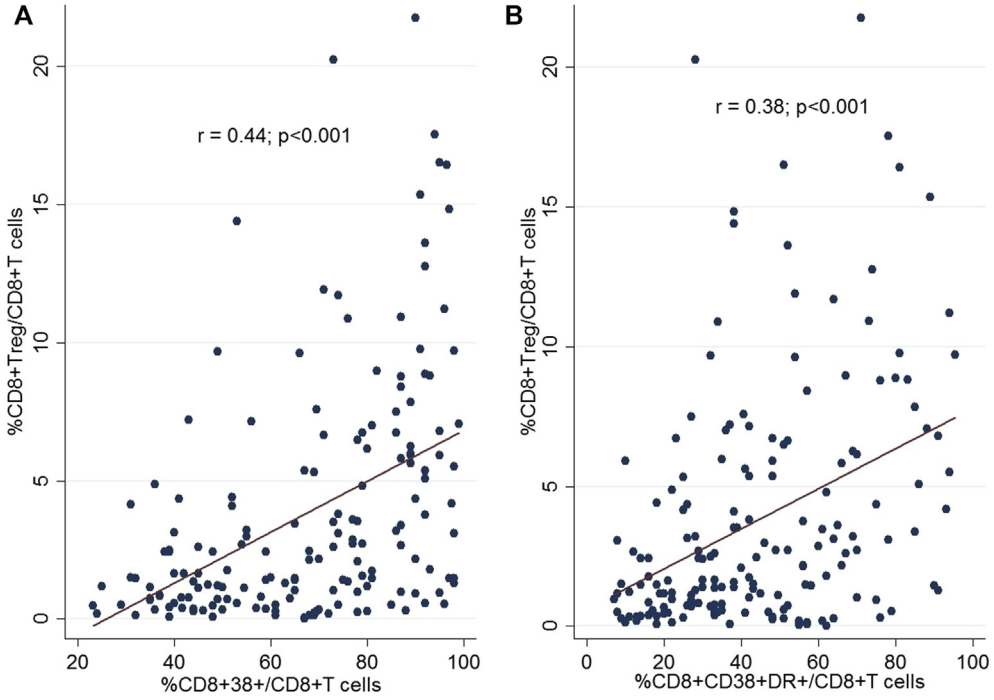


FIG 6. Correlation between circulating frequencies of $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells and activated $CD8^+$ T lymphocytes. $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells were named $CD8^+$ Treg cells in the figure. **A,** Correlation between $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cell and $CD8^+CD38^+$ T-lymphocyte frequencies in the circulation of naive HIV-infected patients. Data were collected before and after initiation of ART at different time points. **B,** Correlation between $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cell and $CD8^+CD38^+DR^+$ T-lymphocyte frequencies in the circulation of naive HIV-infected patients. Data were collected before and after instauration of ART at different time points.

$CD8^+$ T cells to regulatory functions because all $CD8^+$ Treg cell subsets thus far known are characterized by partial or complete downmodulation of this antigen.[42] Phenotypically, no specific markers have been identified thus far uniquely identifying $CD8^+$ Treg cells. Recently, we found that CD39 can be highly involved in the function of $CD8^+$ Treg cells: this allowed us to characterize a restricted phenotypic panel, $CD8^+CD28^-CD127^{lo}CD39^+$, which for the first time identifies invariably a specific $CD8^+$ Treg cell subpopulation.[23] These cells do not express forkhead box protein 3, CD25, and cytotoxic T lymphocyte–associated protein 4, distinguishing them from natural $CD8^+$ Treg cells.[23,44] Moreover, they are negative for NKG2a, CD103, and CD122; are HLA class I restricted; and exert their suppressive activity through soluble factors, indicating a different nature from Qa1/HLA-E–restricted $CD8^+$ Treg cells.[22,23,45-48] Interestingly, they express CD45RA but not CCR7 and hence can be considered terminally differentiated T cells.[22,49] Analyzing different series of cancer specimens, we found these cells to be highly concentrated within the tumor microenvironment, where they can exert remarkable immunosuppressive activity because of their capacity to target both T-cell proliferation and cytotoxicity.[21-23,50]

Here, being aware of the pathogenic involvement of the CD39/adenosine pathway in HIV infection, as well as the function of $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells, we investigated the presence of these cells in the circulation of HIV-infected patients and possible correlations between their frequency and established markers of disease activity.[51] Indeed, high levels of association were observed between $CD8^+CD28^+CD127^{lo}CD39^+$ Treg cell frequency and validated markers of disease, such as HIV viral load, absolute $CD4^+$ T-cell counts, and percentages of $CD4^+$ T cells, suggesting that $CD8^+CD28^+CD127^{lo}CD39^+$ Treg cell frequency could also represent a biomarker of disease. Interestingly, these associations were seen in patients already undergoing ART but not at baseline in naive patients, with the exception of stage A patients. Importantly, $CD8^+CD28^+CD127^{lo}CD39^+$ Treg cell frequency was shown to be related not only to the markers of disease but also to the occurrence of comorbidity (for which they were early predictors). Hence it is conceivable that the statistical association with other markers of disease is interfered with by perturbing events (namely comorbidities), so that it can be detectable when the values of these markers are reset and harmonized by ART, a condition likely comparable with that of stage A asymptomatic patients.

The potential pathogenic and clinical relevance of $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells in patients with HIV infection is also suggested by the following considerations: (1) their frequency increases when ART effects on viremia and clinical response are not met and (2) their frequency correlates with signs of chronic immune cell activation. Concerning the first consideration, although $CD8^+CD28^+CD127^{lo}CD39^+$ Treg cell frequency correlated generally with viral load, it was increased in patients with tumors, despite their suppressed viral load. This observation suggests that monitoring $CD8^+CD28^+CD127^{lo}CD39^+$ Treg cell frequency might have a 2-fold use: (1) it might provide indications on the efficacy of

ART, discriminating patients who will respond to ART from those who will not when the effects of ART on viremia are not immediately clear, and (2) it might behave like an "alarm bell" when perturbations occur because of superimposed diseases (ie, opportunistic infections and tumors), thus allowing early detection of pathological manifestations in patients with ART-suppressed viremia.

Concerning the second consideration, relative to the correlation between $CD8^+CD28^+CD127^{lo}CD39^+$ Treg cell frequency and markers of chronic immune cell activation, certainly this relationship will deserve further investigation to pinpoint the underlying mechanisms. Perhaps 2 speculations can be advanced as possible explanations: either $CD8^+CD28^+CD127^{lo}CD39^+$ Treg cells and markers of chronic immune cell activation increase at the same time because of factors directly dependent on HIV (ie, because of an incomplete control of virus replication), or $CD8^+CD28^+CD127^{lo}CD39^+$ Treg cell expansion is the expression of a regulatory attempt of the immune system against T-cell activation and inflammation.

The finding of an inverted ratio between circulating $CD4^+$ T-cell and $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cell suggests that both events might be pathogenically related in determining the immunodeficiency typical of HIV infection. In this regard an issue thus far unsolved was whether Treg cells are expanded or decreased in HIV-infected patients.[10-19] Our study provides the answer to this issue because we found that the associated frequency of circulating $CD4^+CD25^{hi}CD127^{lo}$ and $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cell subsets is remarkably and consistently higher in HIV-infected patients than in healthy subjects. Hence these data indicate that expansion of the circulating Treg cell compartment represents a constitutive feature of HIV infection, corroborating the hypothesis that HIV-related immunodeficiency originates not only from the quantitative reduction in $CD4^+$ T-cell counts but also from a qualitative alteration of immune homeostasis because of an abnormal effector T/Treg cell ratio.

Interestingly, the pool of $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells in HIV-infected patients has been shown to contain cells reactive against HIV antigens. This finding, which is consistent with similar observations on $CD4^+$ Treg cells, leads to speculation that HIV-specific T cells are functionally committed to regulatory functions when they encounter the respective antigens.[52] This event might progressively spread tolerance to non-HIV antigens contributing to the onset of immunodeficiency, a phenomenon reminiscent of the process termed "infectious tolerance."[53] The elucidation of HIV-driven mechanisms "deviating" HIV-reactive T cells toward a regulatory function might allow identification of the involved molecular pathways that could represent useful targets for new therapeutic agents. Indeed, the discovery of a stably expanded Treg cell compartment in patients with HIV infection associated with the previous knowledge relative to the increased T-lymphocyte expression of regulatory molecules, such as programmed death 1, might point to new therapeutic approaches based on Treg cell–targeting agents as checkpoint inhibitors.[29,30]

We thank Professors M. Zanetti and S. Kinloch for their helpful revision of data and precious suggestions. We thank Professor F. Cucca who supervised the Istituto di Ricerca Genetica e Biomedica cohort phenotyping.

Key messages

- **HIV sustains immunodeficiency through expansion of $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells.**
- **Monitoring the frequency of these cells in the circulation is a new marker of response to ART when effects on viremia and clinical response are not met.**

REFERENCES

1. Phetsouphanh C, Xu Y, Zaunders J. CD4 T cells mediate both positive and negative regulation of the immune response to HIV Infection: complex role of T follicular helper cells and regulatory T cells in pathogenesis. Front Immunol 2015;5:681.
2. Haase AT. Population biology of HIV-1 infection: viral and CD4+ T cell demographics and dynamics in lymphatic tissues. Annu Rev Immunol 1999;17:625-56.
3. INSIGHT START Study Group, Lundgren JD, Babiker AG, Gordin F, Emery S, Grund B, Sharma S, et al. Initiation of Antiretroviral Therapy in Early Asymptomatic HIV Infection. N Engl J Med 2015;373:795-807.
4. Mandalia S, Westrop SJ, Beck EJ, Nelson M, Gazzard BG, Imami N. Are long-term non-progressors very slow progressors? Insights from the Chelsea and Westminster HIV cohort, 1988-2010. PLoS One 2012;7:e29844.
5. Rey D, de Mautort E, Saussine C, Hansmann Y, Waller J, Herbrecht R, et al. Isolated renal Aspergillus abscess in an AIDS patient with a normal CD4+ cell count on highly active antiretroviral therapy. Eur J Clin Microbiol Infect Dis 1999;18:137-41.
6. Mori S, Polatino S, Estrada-Y-Martin RM. Pneumocystis-associated organizing pneumonia as a manifestation of immune reconstitution inflammatory syndrome in an HIV-infected individual with a normal CD4+ T-cell count following antiretroviral therapy. Int J STD AIDS 2009;20:662-5.
7. Sakaguchi S. Regulatory T cells: key controllers of immunologic self-tolerance. Cell 2000;101:455-8.
8. Sakaguchi S. Regulatory T cells: history and perspective. Methods Mol Biol 2011;707:3-17.
9. Chevalier MF, Weiss L. The split personality of regulatory T cells in HIV infection. Blood 2013;121:29-37.
10. Veiga-Parga T, Sehrawat S, Rouse BT. Role of regulatory T cells during virus infection. Immunol Rev 2013;255:182-96.
11. Weiss L, Donkova-Petrini V, Caccavelli L, Balbo M, Carbonneil C, Levy Y. Human immunodeficiency virus-driven expansion of CD4+CD25+ regulatory T cells, which suppress HIV-specific CD4 T-cell responses in HIV-infected patients. Blood 2004;104:3249-56.
12. Kinter AL, Horak R, Sion M, Riggin L, McNally J, Lin Y, et al. CD25+ regulatory T cells isolated from HIV-infected individuals suppress the cytolytic and nonlytic antiviral activity of HIV-specific CD8+ T cells in vitro. AIDS Res Hum Retroviruses 2007;23:438-50.
13. Jiao Y, Fu J, Xing S, Fu B, Zhang Z, Shi M, et al. The decrease of regulatory T cells correlates with excessive activation and apoptosis of CD8+ T cells in HIV-1-infected typical progressors, but not in long-term non-progressors. Immunology 2009;128(suppl 1):e366-75.
14. Eggena MP, Barugahare B, Jones N, Okello M, Mutalya S, Kityo C, et al. Depletion of regulatory T cells in HIV infection is associated with immune activation. J Immunol 2005;174:4407-14.
15. Oswald-Richter K, Grill SM, Shariat N, Leelawong M, Sundrud MS, Haas DW, et al. HIV infection of naturally occurring and genetically reprogrammed human regulatory T-cells. PLoS Biol 2004;2:E198.
16. Angin M, Sharma S, King M, Murooka TT, Ghebremichael M, Mempel TR, et al. HIV-1 infection impairs regulatory T-cell suppressive capacity on a per-cell basis. J Infect Dis 2014;210:899-903.
17. Schulze Zur Wiesch J, Thomssen A, Hartjen P, Tóth I, Lehmann C, Meyer-Olson D, et al. Comprehensive analysis of frequency and phenotype of T regulatory cells in HIV infection: CD39 expression of FoxP3+ T regulatory cells correlates with progressive disease. J Virol 2011;85:1287-97.
18. Ndhlovu LC, Loo CP, Spotts G, Nixon DF, Hecht FM. FOXP3 expressing CD127lo CD4+ T cells inversely correlate with CD38+ CD8+ T cell activation levels in primary HIV-1 infection. J Leukoc Biol 2008;83:254-62.
19. Whiteside TL. Clinical Impact of Regulatory T cells (Treg) in Cancer and HIV. Cancer Microenviron 2015;8:201-7.

20. Filaci G, Fenoglio D, Indiveri F. CD8(+) T regulatory/suppressor cells and their relationships with autoreactivity and autoimmunity. Autoimmunity 2011; 44:51-7.
21. Filaci G, Fenoglio D, Fravega M, Ansaldo G, Borgonovo G, Traverso P, et al. CD8+ CD28- T regulatory lymphocytes inhibiting T cell proliferative and cytotoxic functions infiltrate human cancers. J Immunol 2007;179:4323-34.
22. Filaci G, Fravega M, Negrini S, Procopio F, Fenoglio D, Rizzi M, et al. Nonantigen specific CD8+ T suppressor lymphocytes originate from CD8+CD28- T cells and inhibit both T-cell proliferation and CTL function. Hum Immunol 2004;65:142-56.
23. Parodi A, Battaglia F, Kalli F, Ferrera F, Conteduca G, Tardito S, et al. CD39 is highly involved in mediating the suppression activity of tumor-infiltrating CD8+ T regulatory lymphocytes. Cancer Immunol Immunother 2013;62:851-62.
24. Fraccaro P, Dentone C, Fenoglio D, Giacomini M. Multicentre clinical trials' data management: a hybrid solution to exploit the strengths of electronic data capture and electronic health records systems. Inform Health Soc Care 2013; 38:313-29.
25. Fraccaro P, Pupella V, Gazzarata R, Dentone C, Cenderello G, De Leo P, et al. The Ligurian Human Immunodeficiency Virus Clinical Network: a web tool to manage patients with human immunodeficiency virus in primary care and multicenter clinical trials. Med 2 0 2013;2:e5.
26. Chuang-Stein C. Summarizing laboratory data with different reference ranges in multi-center clinical trials. Drug Inform J 1992;26:77-84.
27. Parham P, Brodsky FM. Partial purification and some properties of BB7.2. A cytotoxic monoclonal antibody with specificity for HLA-A2 and a variant of HLA-A28. Hum Immunol 1981;3:277-99.
28. Tanaka A, Sakaguchi S. Regulatory T cells in cancer immunotherapy. Cell Res 2017;27:109-18.
29. Yamamoto T, Price DA, Casazza JP, Ferrari G, Nason M, Chattopadhyay PK, et al. Surface expression patterns of negative regulatory molecules identify determinants of virus-specific CD8+ T-cell exhaustion in HIV infection. Blood 2011; 117:4805-15.
30. Cockerham LR, Jain V, Sinclair E, Glidden DV, Hartogenesis W, Hunt PW, et al. Programmed death-1 expression on $CD4^+$ and $CD8^+$ T cells in treated and untreated HIV disease. AIDS 2014;28:1749-58.
31. Freguja R, Gianesin K, Mosconi I, Zanchetta M, Carmona F, Rampon O, et al. Regulatory T cells and chronic immune activation in human immunodeficiency virus 1 (HIV-1)-infected children. Clin Exp Immunol 2011;164:373-80.
32. Losina E, Schackman BR, Sadownik SN, Gebo KA, Walensky RP, Chiosi JJ, et al. Racial and sex disparities in life expectancy losses among HIV-infected persons in the united states: impact of risk behavior, late initiation, and early discontinuation of antiretroviral therapy. Clin Infect Dis 2009;49:1570-8.
33. Benjamin LA, Bryer A, Emsley HC, Khoo S, Solomon T, Connor MD. HIV infection and stroke: current perspectives and future directions. Lancet Neurol 2012; 11:878-90.
34. Calza L. HIV Infection and Myocardial Infarction. Curr HIV Res 2016;14: 456-65.
35. Gazzola L, Bellistri GM, Tincati C, Ierardi V, Savoldi A, Del Sole A, et al. Association between peripheral T-Lymphocyte activation and impaired bone mineral density in HIV-infected patients. J Transl Med 2013;11:51.
36. Kaplan RC, Sinclair E, Landay AL, Lurain N, Sharrett AR, Gange SJ, et al. T cell activation and senescence predict subclinical carotid artery disease in HIV-infected women. J Infect Dis 2010;203:452-63.
37. Deeks SG. HIV infection, inflammation, immunosenescence, and aging. Annu Rev Med 2011;62:141-55.
38. Dentone C, Di Biagio A, Parodi A, Bozzano F, Fraccaro P, Signori A, et al. Innate immunity cell activation in virologically suppressed HIV-infected maraviroc-treated patients. AIDS 2014;28:1071-4.
39. Guihot A, Dentone C, Assoumou L, Parizot C, Calin R, Seang S, et al. Residual immune activation in combined antiretroviral therapy-treated patients with maximally suppressed viremia. AIDS 2016;30:327-30.
40. Josefowicz SZ, Rudensky A. Control of regulatory T cell lineage commitment and maintenance. Immunity 2009;30:616-25.
41. Cosmi L, Liotta F, Lazzeri E, Francalanci M, Angeli R, Mazzinghi B, et al. Human CD8+CD25+ thymocytes share phenotypic and functional features with CD4+CD25+ regulatory thymocytes. Blood 2003;102:4107-14.
42. Vuddamalay Y, van Meerwijk JP. CD28- and CD28lowCD8+ regulatory T cells: of mice and men. Front Immunol 2017;8:31.
43. Maggi E, Cosmi L, Liotta F, Romagnani P, Romagnani S, Annunziato F. Thymic regulatory T cells. Autoimmun Rev 2005;4:579-86.
44. Fenoglio D, Ferrera F, Fravega M, Balestra P, Battaglia F, Proietti M, et al. Advancements on phenotypic and functional characterization of non-antigen-specific CD8+CD28- regulatory T cells. Hum Immunol 2008;69:745-50.
45. Filaci G, Bacilieri S, Fravega M, Monetti M, Contini P, Ghio M, et al. Impairment of CD8+ T suppressor cell function in patients with active systemic lupus erythematosus. J Immunol 2001;166:6452-7.
46. Lu L, Kim HJ, Werneck MB, Cantor H. Regulation of CD8+ regulatory T cells: interruption of the NKG2A-Qa-1 interaction allows robust suppressive activity and resolution of autoimmune disease. Proc Natl Acad Sci U S A 2008;105: 19420-5.
47. Kim HJ, Verbinnen B, Tang X, Lu L, Cantor H. Inhibition of follicular T-helper cells by CD8(+) regulatory T cells is essential for self tolerance. Nature 2010; 467:328-32.
48. Kim HJ, Barnitz RA, Kreslavsky T, Brown FD, Moffett H, Lemieux ME, et al. Stable inhibitory activity of regulatory T cells requires the transcription factor Helios. Science 2015;350:334-9.
49. Sallusto F, Lenig D, Förster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature 1999;401:708-12.
50. Parodi A, Traverso P, Kalli F, Conteduca G, Tardito S, Curto M, et al. Residual tumor micro-foci and overwhelming regulatory T lymphocyte infiltration are the causes of bladder cancer recurrence. Oncotarget 2016;7:6424-35.
51. Nikolova M, Carriere M, Jenabian MA, Limou S, Younas M, Kök A, et al. CD39/adenosine pathway is involved in AIDS progression. PLoS Pathog 2011;7: e1002110.
52. Angin M, King M, Altfeld M, Walker BD, Wucherpfennig KW, Addo MM. Identification of HIV-1-specific regulatory T-cells using HLA class II tetramers. AIDS 2012;26:2112-5.
53. Kleijwegt FS, Laban S, Duinkerken G, Joosten AM, Koeleman BPC, Nikolic T, et al. Transfer of regulatory properties from tolerogenic to proinflammatory dendritic cells via induced autoreactive regulatory T cells. J Immunol 2011;187:6357-64.

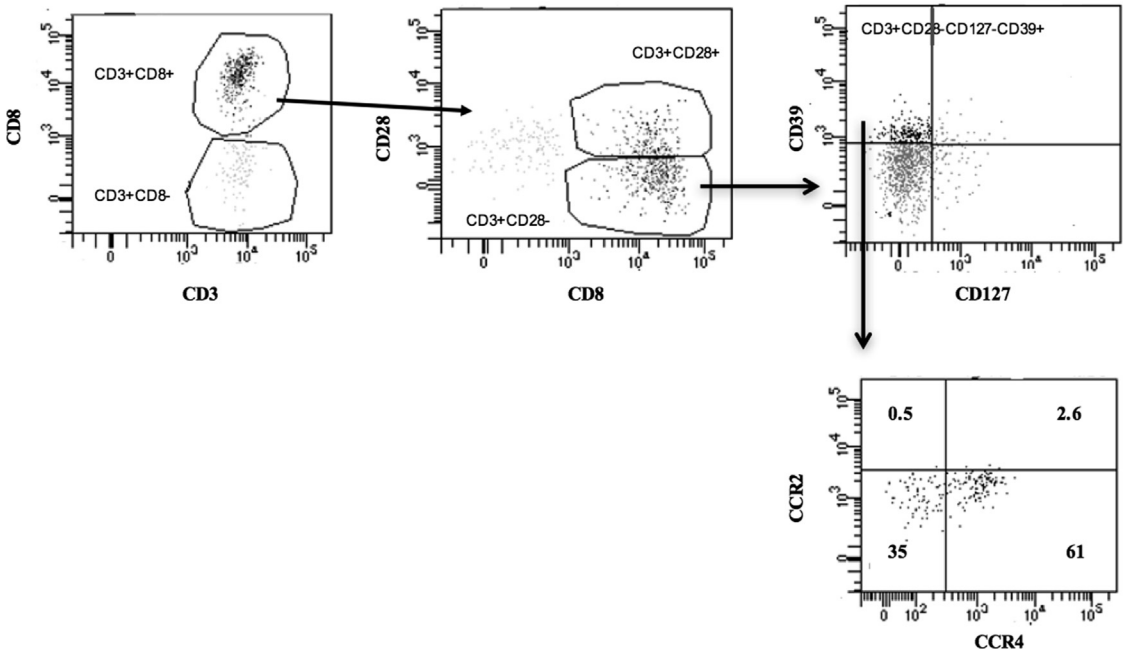


FIG E1. Expression of CCR2 and CCR4 chemokine receptors by $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells from representative patient 14. Comparable findings were achieved in all tested HIV-infected patients. Percentages of $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells positive for the 2 chemokine receptors are shown in the respective boxes of plotting.

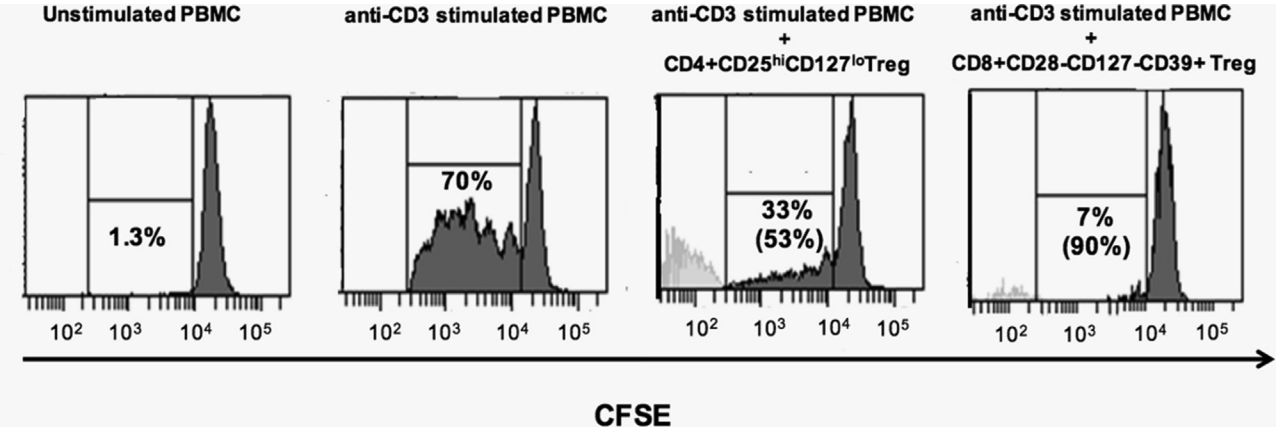


FIG E2. Comparison between suppression activity of $CD4^+CD25^{hi}CD127^{lo}$ Treg cells from a healthy donor and that of $CD8^+CD28^-CD127^{lo}CD39^+$ Treg cells from representative HIV-infected patient 21. *CFSE*, Carboxyfluorescein succinimidyl ester.

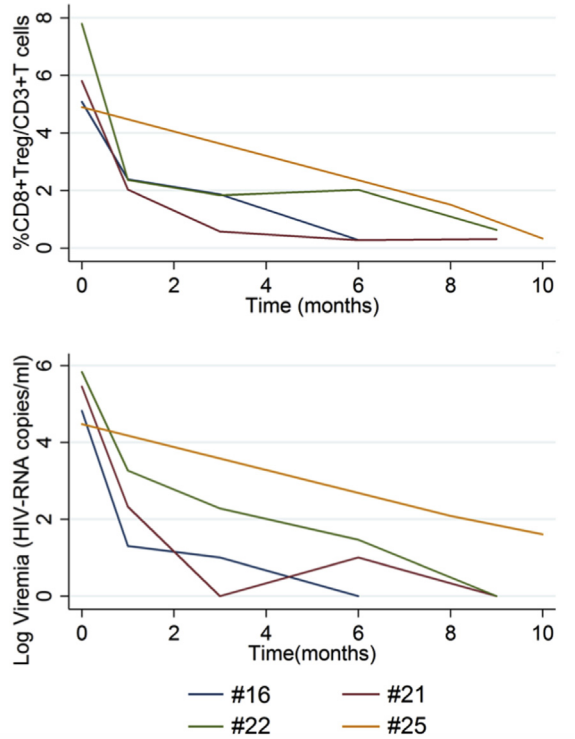


FIG E3. Longitudinal analyses of $CD8^+CD28^+CD127^{lo}CD39^+$ Treg cell frequencies and viremia in representative patients 16, 21, 22, and 25.

TABLE E1. Follow-up in 6 patients did not show a remarkable decrease of $CD8^+CD28^+CD127^{lo}CD39^+$ Treg cell frequency during ART

No. of patients	Timing (mo)	VL (copies/mL)	$CD4^+$ T cells/μL	% $CD8^+$ Treg cells/ $CD3^+$ T cells	Clinical data
2	0	12,700	8	4.6	CMV, PjP, wasting syndrome
	6	325	47	4.4	
20	0	4,239	476	0.1	Failure to ART
	6	92,783	429	2.3	
27	0	243,348	140	0.2	Failure to ART
	6	160	270	1.5	
29	0	584,734	180	5.1	Pulmonary tuberculosis, wasting syndrome
	6	40	410	5.6	
32	0	72,889	10	4	PjP, cryptococcosis, CMV, wasting syndrome
	6	40	210	7.3	
33	0	694,516	62	4.6	PjP, wasting syndrome
	6	40	310	4.4	

CMV, Cytomegalovirus; *PjP*, *Pneumocystis jirovecii* pneumonia; *VL*, viral load.