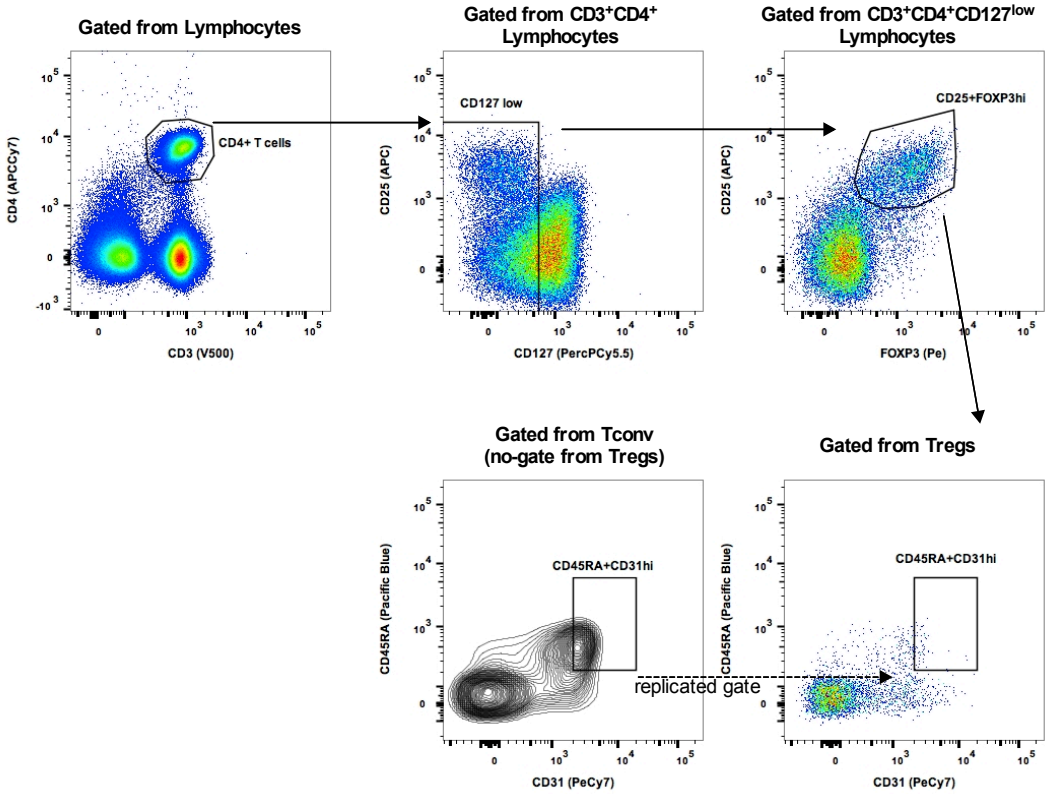
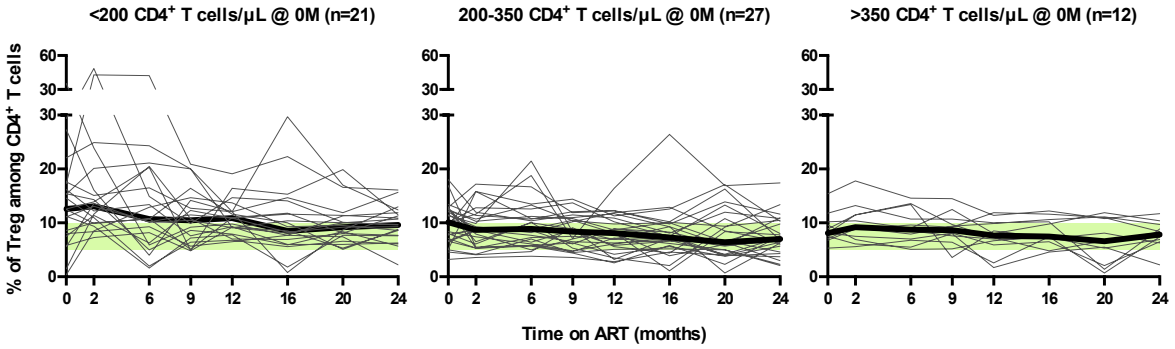
SUPPLEMENTARY FIGURES AND TABLES

Figure S1.



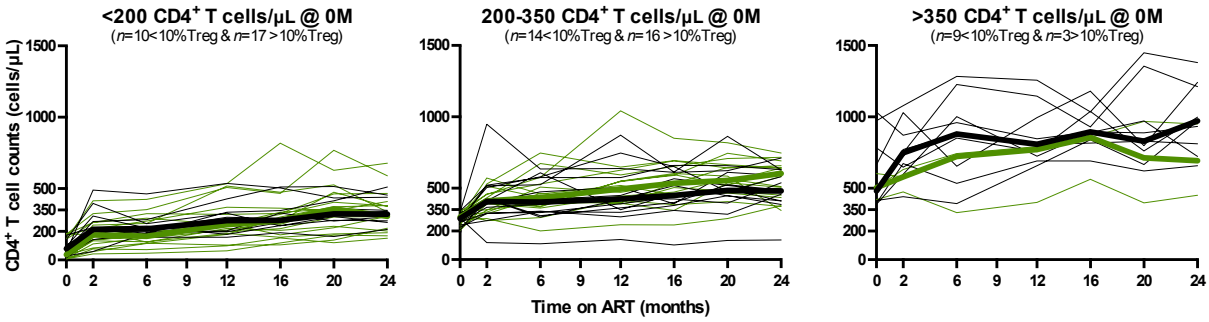
Flow cytometry gating strategy for the identification of Tregs ($CD3^+CD4^+CD127^{low}CD25^{high}Foxp3^+$) of and recent thymic emigrants ($CD45RA^+$ $CD31^{hi}$). $CD4^+$ T cells ($CD3^+CD4^+$; top left panel) were selected from lymphocytes (based on the FSC/SSC profile; not shown). From these, the $CD127^{low}$ population was gated (top middle panel) and Tregs identified as $CD25^{hi}Foxp3^+$ (top right panel). Recent thymic emigrants were selected as $CD45RA^+CD31^{hi}$ gated on Tconv (bottom left panel); the same gate was used to define the RTE among the Tregs (bottom right panel).

Figure S2.



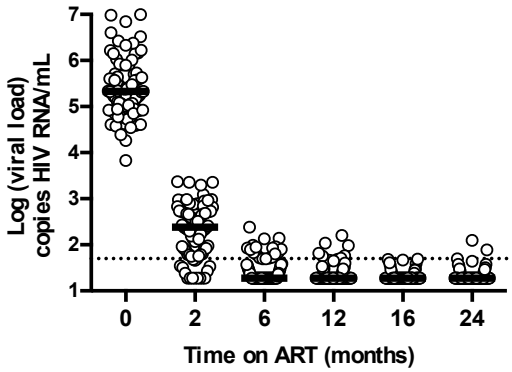
Longitudinal progression of Treg percentage throughout the first 24 months of ART excluding from the analysis all the individuals with at least one missing data-point. Stratification of HIV-infected individuals according to $CD4^+$ T cell counts at baseline. Each thin black line represents the Treg percentage evolution of a single individual, the bold black line represents the median of the individuals for each time-point and the shaded green horizontal bar represents the range of Treg percentage among $CD4^+$ T cells described for healthy individuals using similar markers (Simonetta *et al.*, J Infect Dis. 2012. 205:1510-1519). Friedman's test followed by Dunn's multiple comparison tests were applied, using as reference group the ART-naïve individuals: <200: $X^2_F(7)=19.11$, $p=0.0078$, $n=21$, $W_K=0.119$; 200-350: $X^2_F(7)=16.68$, $p=0.0196$, $n=27$, $W_K=0.129$; >350: $X^2_F(7)=11.36$, $p=0.1236$, $n=12$, $W_K=0.080$].

Figure S3.



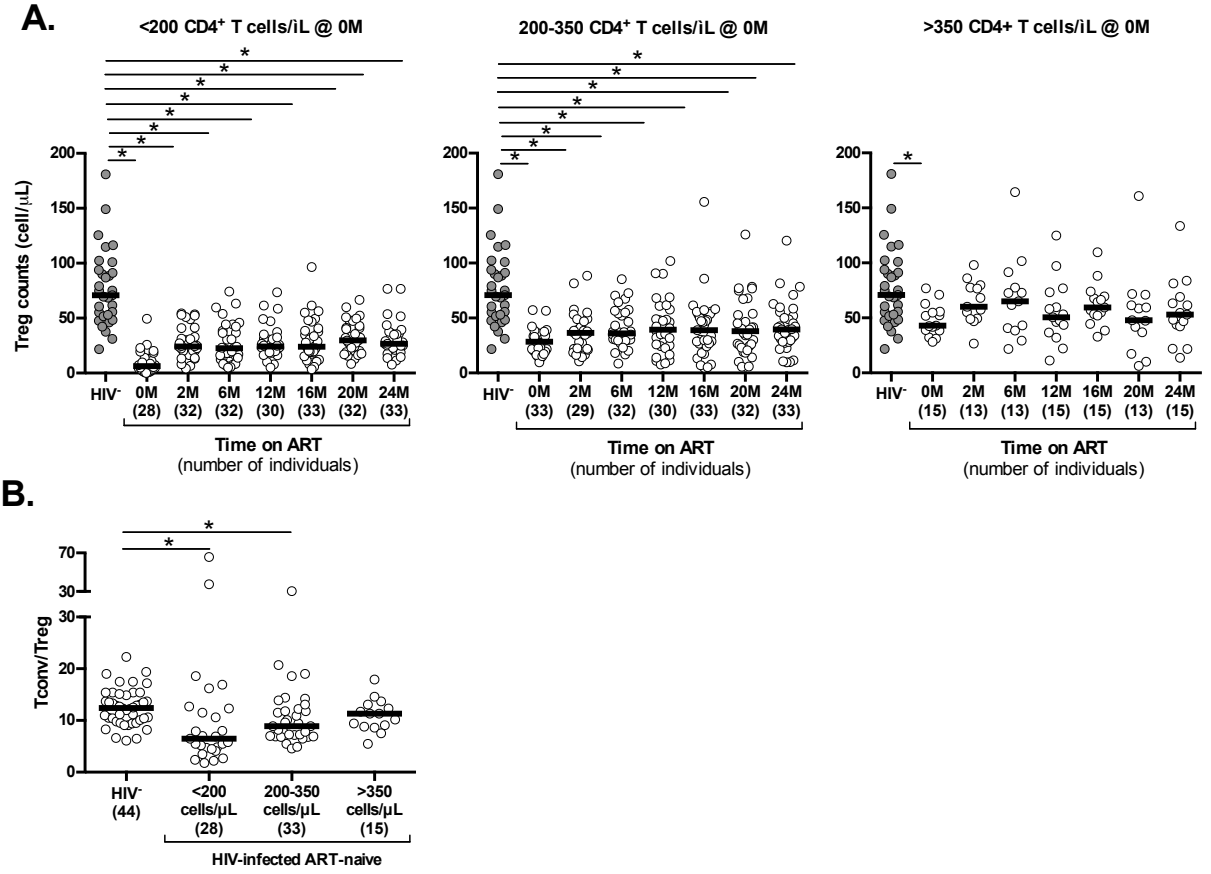
Longitudinal progression of CD4$^+$ T cell counts, based on Treg percentage at baseline, throughout the first 24 months of ART, excluding from the analysis all the individuals with at least one missing data-point. Stratification of HIV-infected individuals based on their CD4$^+$ T cell counts at baseline (<200, 200-350 and >350 cells/µL) and on their Treg percentage at baseline (<10%, grey lines; ≥10%, green lines) and evaluation of the CD4$^+$ T cell reconstitution throughout ART. Each thin line represents a single individual and bold lines represent the median of all individuals from each group at each specific time-point. A two-way ANOVA repeated measurements followed by Sidak's multiple comparison tests was performed to compare the CD4$^+$ T cell evolution of the two groups (<10% and ≥10% Treg at baseline) throughout ART and no significant differences were observed: <200: $F(1,25)=0.214$, $p=0.6476$, $\eta^2_P=0.021$; 200-350: $F(1,28)=0.80$, $p=0.3788$, $\eta^2_P=0.052$; $F(1,10)=2.88$, $p=0.1208$) , $\eta^2_P=0.350$.

Figure S4.



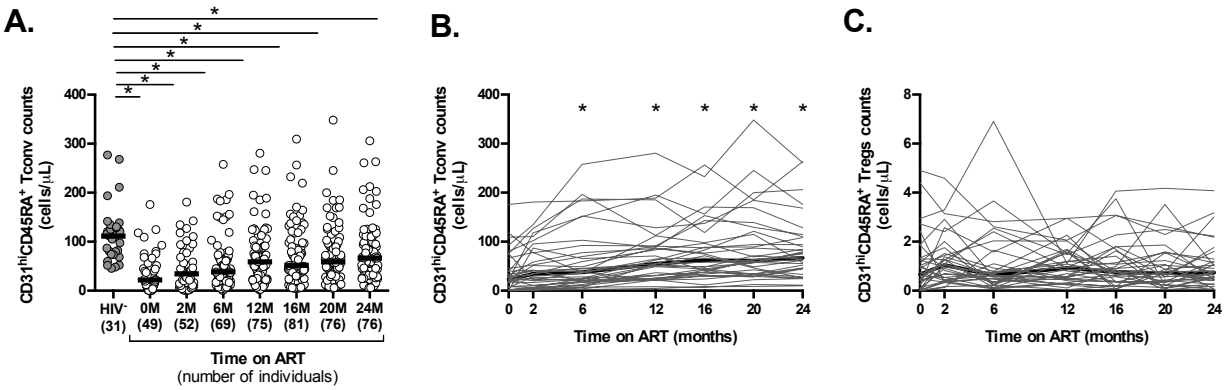
ART leads to a decrease in the plasma viral load of all treated HIV-infected individuals. Each dot represents a single individual, the horizontal black lines the median and the horizontal dashed line represents 50 copies of plasma HIV RNA/mL.

Figure S5



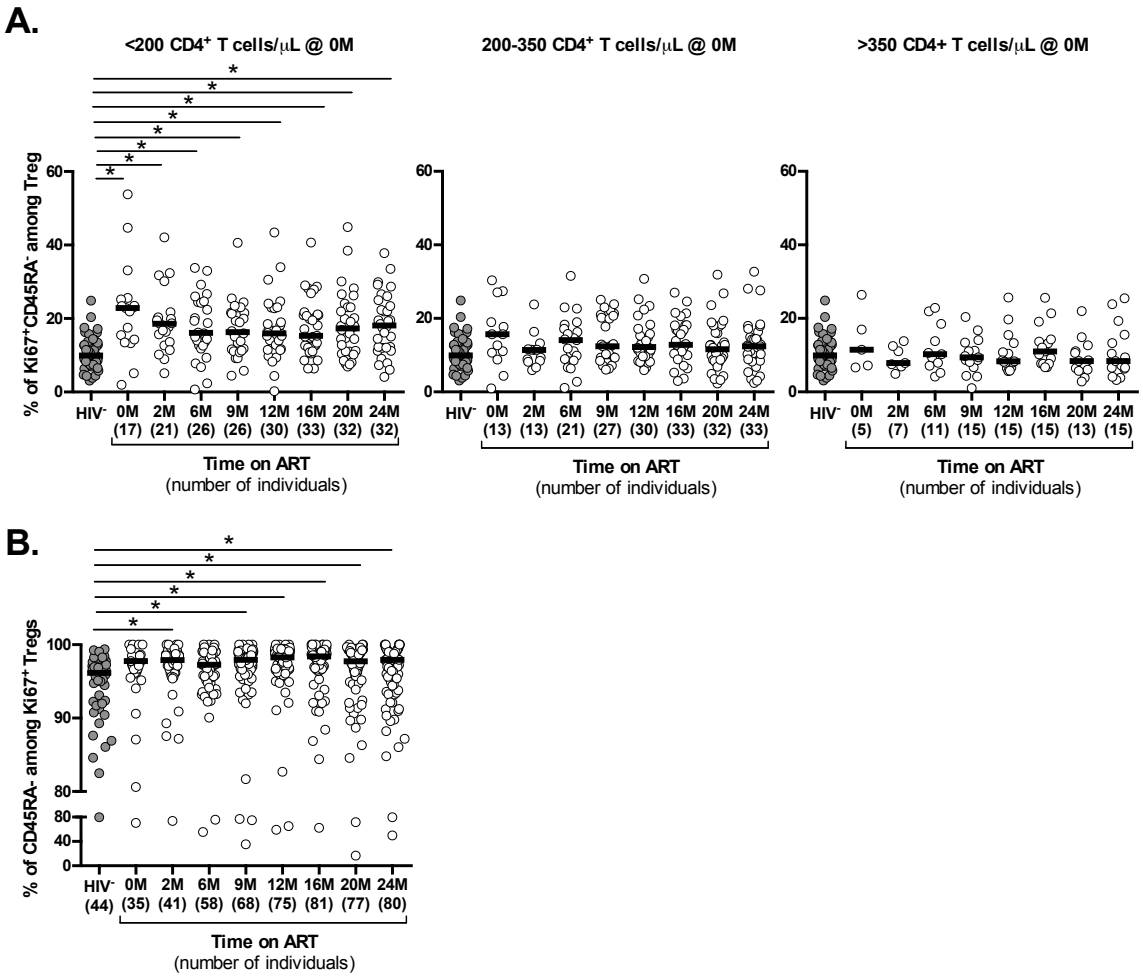
The loss of Tregs is less pronounced than the one of Tconv in HIV-infected individuals with severe lymphopenia. A. Stratification based on baseline $CD4^+$ T cell counts (<200, 200-350 and >350 cells/μL). **B.** Ratio of Tconv/Treg and comparison between uninfected controls and ART-naïve HIV-infected individuals. Each dot represents a single individual and the horizontal lines the median; on the x-axis legend, values in brackets refer to the number of individuals analysed at each time-point. Comparisons were performed using an one-way ANOVA or a Kruskal-Wallis test followed by Bonferroni's or Dunn's multiple comparison tests, respectively, using as reference group the uninfected individuals: **A.** <200: $X^2_{KW}(7,243)=96.90$, $p<0.0001$, $\eta^2_{KW}=0.387$; 200-350: $X^2_{KW}(7,245)=49.15$, $p<0.0001$, $\eta^2_{KW}=0.195$; >350: $F(7,122)=2.03$, $p=0.0569$, $\eta^2=0.104$; **B.** $X^2_{KW}(3,115)=17.30$, $p=0.0006$, $\eta^2_{KW}=0.147$.

Figure S6.



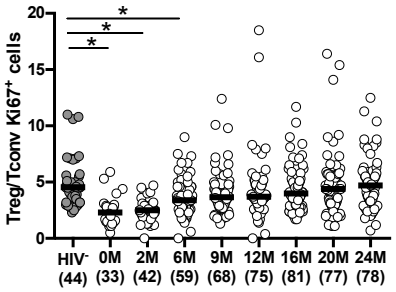
The number of Tconv recent thymic emigrants increases from baseline throughout ART, though no major alterations are observed for the Treg recent thymic emigrants. A. Comparison of Tconv recent thymic emigrants ($CD45RA^{+}CD31^{hi}$) counts between uninfected controls and HIV-infected individuals naïve for ART or on different time-points upon ART initiation. Each dot represents a single individual and the horizontal lines the median; on the x-axis legend, values in brackets refer to the number of individuals analysed at each time-point. Analysis performed using an one-way ANOVA test followed by Bonferroni's multiple comparison tests; $F(7,501)=8.013$ $p<0.0001$, $\eta^2=0.101$. **B.** Evolution of Tconv recent thymic emigrants ($CD45RA^{+}CD31^{hi}$) counts of HIV-infected individuals from baseline and throughout ART; **C.** Evolution of Treg recent thymic emigrants ($CD45RA^{+}CD31^{hi}$) counts of HIV-infected individuals from baseline and throughout ART. **B and C**. Each thin black line represents a single individual; the bold black line represents the median of the individuals for each time-point. The evolution of $CD45RA^{+}CD31^{hi}$ cells with ART was evaluated using Friedman's test followed by Dunn's multiple comparison tests using as reference group the ART-naïve individuals. **B.** $X^2_F(7)=99.30$, $p<0.0001$, $N=34$, $W_K=0.430$. **C.** $X^2_F(7)=5.49$, $p=0.4822$, $N=33$, $W_K=0.025$.

Figure S7.



Tregs undergoing proliferation are mostly memory Tregs. **A..** Stratification by $CD4^+$ T cell counts at baseline (<200, 200-350 and >350 cells/μL); **B.** Percentage of $CD45RA^-$ among $Ki67^+$Tregs. Each dot represents a single individual and the horizontal lines the median; on the x-axis legend, values in brackets refer to the number of individuals analysed at each time-point. Comparisons were performed using an one-way ANOVA or a Kruskal-Wallis test followed by Bonferroni's or Dunn's multiple comparison tests, respectively, using as reference group the uninfected individuals: **A.** <200: $X^2_{KW}(8,253)=38.60$, $p<0.0001$, $\eta^2_{KW}=0.148$; 200-350: $F(8,237)=1.49$, $p=0.1600$, $\eta^2=0.048$; >350: $F(8,131)=0.67$, $p=0.7177$, $\eta^2_{KW}=0.039$; **B.** $X^2_{KW}(8,542)=32.17$, $p<0.0001$, $\eta^2_{KW}=0.058$.

Figure S8.



Tregs have higher proliferation rates than Tconv. The ratio of Treg/Tconv Ki67^{+} cells was evaluated and compared between uninfected individuals and HIV-infected individuals ART-naïve or at different time-points of ART. Each dot represents a single individual and the horizontal lines the median; on the x-axis legend, values in brackets refer to the number of individuals analysed at each time-point. Comparisons were performed using a Kruskal-Wallis test followed by Dunn's multiple comparison tests: $X^2_{KW}(8,545)=101.6$ $p<0.0001$, $\eta^2_{KW}=0.183$.

Table S1. Summary of characteristics and results of different studies regarding Tregs features/dynamics in HIV-infected patients on ART.

Markers used to identify Tregs among $CD4^+$ T cells	Cross-sectional (previous time on ART) or longitudinal study (follow-up period/time-points on ART)	Number of HIV-infected individuals on ART	Baseline $CD4^+$ T cell counts (cells/μL) median (range)	Baseline viral load (Log; copies/mL) median (range)	Treg's percentage among $CD4^+$ T cells			Year of publication	Ref.
					Uninfected *vs.* ART-naïve HIV-infected patients	Uninfected *vs.* HIV-infected at different times on ART	Among HIV-infected throughout ART (only for longitudinal studies)		
$CD25^{high}$	Longitudinal (0, 4, 12 and 24 weeks)	26	208 (23-368)	5.0 (2.7-6.0[b])	Higher on HIV-infected	Higher on HIV-infected at 24 weeks	No significant changes	2008	[20]
$CD25^+CD127^{low}$	Longitudinal [a] (from 10 to 53 weeks)	12	<100 (n.d.)	n. a.	Higher on HIV-infected	Higher on HIV-infected up to 40 weeks on ART	A slight decrease was observed on Tregs percentage throughout the 40 weeks of ART, but it remained higher then uninfected controls.	2007	[24]
	Longitudinal (1 and 5 years)	15	136 (5-289)	5.0 (3.9-5.2[b])	n.d.	Higher on HIV-infected	Treg percentage were higher in HIV-infected individuals then uninfected controls, at 1 and 5 years on ART. No significant alterations were observed between 1 and 5 years of ART.	2009	[21]
	Longitudinal (2 years)	58	293 (n.d.)	4.4 (n.d.)	n.a.	n.a.	The initial increased proportion of Tregs became atenuated.	2015	[26]
$CD25^+FOXP3^+$	Cross-sectional (>1 year)	20	n.d.	n.d.	Higher on HIV-infected	No significant differences between ART-naïve and individuals on ART	n.a.	2012	[17]
	Longitudinal (0, 2, 4, 8, 12, 24, 36 and 48 weeks)	18	75 (17-263)	5.1 (3.7-5.7)	Higher on HIV-infected	Higher on HIV-infected until 8 weeks on ART	Initially increased Tregs proportion normalized along ART	2011	[19]
	Longitudinal (0, 4, 12, 24, 48 and 60 weeks)	17	215 (24-341)	4.4 (3.2-5.6)	Higher on HIV-infected	Comparison only with week 60 - no significant difference	Lower than baseline (week 0) after 12 weeks on ART	2012	[27]
$CD25^{+/high}CD127^-FOXP3^+$	Cross-sectional (aviremic >1 year)	31	n.d.	n.d.	Higher on HIV-infected	Higher on HIV-infected	n.a.	2011	[18]
	Longitudinal (0, 2, 4, 8, 24 and >46 weeks)	11	288 (20-615)	4.7 (3.6-5.9[b])	Higher on HIV-infected	n.d.	Lower than baseline (week 0) after 24 weeks of ART	2011	[22]
	Cross-sectional (>1 year)	53	n.d.	n.d.	n.a.	No differences in the median percentage of Tregs but a wider distribution of Tregs percentage among HIV-infecetd compared to healthy controls	n.a.	2013	[25]

n.d. stands for information "not depicted" in the manuscript

n.a. stands for information "not aplicable"

[a] The authors also performed a cross-sectional study comparing a group of 29 untreated HIV-infected patients with 20 healthy controls, but few data is provided.

[b] Maximum HIV viral load correspond to the upper detection limit.

Table S2. Demographic characterization of the cohorts.

Variable	HIV-infected (n=81)	Control (n=44)
Mean Age, years (range) [1]	41 (22-67) [2]	40 (22-56)
Men (n)	77.8% (63) [3]	68.0% (30)
HIV transmission mode		
Intravenous drug users (n) [4]	23.4% (19)	NA
Men who have sex with men (n)	28.4% (23)	NA
Heterosexuals (n)	45.7% (37)	NA
Unknown (n)	2.5% (2)	NA
Mean $CD4^+$ T cell count, cells/μL (range) [1]	273 (8-1033)	NA
<200 cells/μL, mean (range; n)	84 (8-193; 33)	NA
200-350 cells/μL, mean (range; n)	287 (205-341; 33)	NA
≥350 cells/μL, mean (range; n)	567 (388-1033; 15)	NA
Mean $\log_{10}$ viral load, copies/mL (range) [1]	5.33 (3.8-7.0)	NA
Individuals on criteria for AIDS n) [5]	44.4% (36)	NA
Hepatitis C virus-positive individuals (n) [6]	22.2% (18)	NA
Hepatitis B virus-positive individuals (n)	1.2% (1)	NA
HIV subtype [1,7]		
A (n)	3.7% (3)	NA
B (n)	46.9% (38)	NA
C (n)	11.1% (9)	NA
G (n)	25.9% (21)	NA
Others (n) [8]	4.9% (4)	NA
Unknown (n)	7.5% (6)	NA
Viral mutations of resistance (n) [7]	18.5% (15)	NA
ART regimen components		
2NRTIs		
TDF+FTC (n)	61	NA
ABC+3TC (n)	19	NA
ZDV+3TC (n)	1	NA
3rd Drug (NNRTI or PI/r)		
NNTRI: EFV/NVP (n)	68/1	NA
PI/r: DRVr/LPVr (n)	10/2	NA

[1]At baseline;

[2]No significant difference between the mean ages of the two groups (t=0.6250, p=0.5336, unpaired T-test with Welch correction);

[3]No significant difference between the percentages of males on the two groups (Fisher exact probability test; p=0.285);

[4]Five of these patients presented also heterosexual risk for HIV transmission;

[5]Accordingly to the classification defined by the Center for Disease Control and Prevention [30];

[6]Positive for hepatitis C virus antibodies and RNA;

[7]Not assessed for 6 HIV-infected individuals;

[8]Includes two individuals with the A/D subtype, one individual with the F and other with the CRF02_AG subtypes.

Abbreviations: ABC, abacavir; DRVr, ritonavir boosted darunavir; EFV, efavirenz; FTC, emtricitabine; LPVr, ritonavir boosted lopinavir; NA, not applicable; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside (or nucleotide) analogue reverse transcriptase inhibitors; NVP, nevirapine; PI/r, ritonavir boosted protease inhibitor; TDF, tenofovir disoproxil fumarate; ZDF, zidovudine; 3TC, lamivudine.

Table S3. Tregs percentage among CD4$^+$ T cells and CD4$^+$ T cell counts for each HIV-infected individual enrolled in the study. Estimated values were calculated as the mean of the neighbour values and are underlined. Four individuals have no Treg percentage at baseline for technical issues.

Patient ID	CD4$^+$ T cell counts							%Tregs among CD4$^+$ T cells							
	0	2	6	12	16	20	24	0	2	6	9	12	16	20	24
AH001	11	59	116	180	301	373	382	31,5	6,9	12,0	6,6	7,7	8,8	7,9	6,4
AH003	22	135	128	186	159	182	191	19,5	6,0	12,0	7,5	7,5	7,6	7,0	6,0
AH004	292	571	460	519	451	467	435	11,5	9,3	8,2	10,2	11,9	12,9	8,5	6,5
AH005	257	413	370	549	604	585	344	12,9	5,3	7,3	7,5	7,0	9,3	5,8	6,6
AH006	43	177	274	252	304	367	363		14,0	13,4	15,7	10,2	13,4	11,7	14,8
AH007	147	414	425	534	819	629	679	12,5	9,9	10,8	5,1	11,0	11,8	8,1	11,3
AH009	310	427	544	555	451	625	600	10,9	8,3	5,7	8,7	12,4	1,2	7,4	13,6
AH010	316	285	325	365	379	675	473	9,0	7,4	7,4	6,6	6,8	7,0	7,8	9,6
AH011	182	176	208	198	220	301	304	12,6	13,9	20,3	4,9	16,4	15,3	19,9	11,5
AH012	230	347	508	472	532	646	641	12,2	10,7	13,3	10,4	9,8	10,0	6,5	9,8
AH013	30	60	190	204	329	273	303	2,6	21,7	23,3	19,3	15,2	11,4	10,2	7,8
AH014	334	948	635	639	612	624	711	4,6	4,1	4,7	9,1	5,6	4,4	10,8	6,0
AH015	291	120	111	141	102	134	138	6,7	8,7	7,7	6,5	6,3	6,8	4,2	7,3
AH016	974	1078	1284	1257	1035	1449	1380	7,9	9,1	7,9	9,4	9,9	10,6	11,1	9,7
AH017	408	448	486	524	468	443	374	15,4	17,8	14,6	14,5	11,4	12,2	10,8	11,7
AH018	338	456	576	1042	850	818	747	12,5	10,3	11,2	10,8	8,7	10,1	9,4	10,5
AH021	239	529	746	649	693	665	644	12,6	7,9	5,0	7,0	8,9	7,7	4,0	6,4
AH022	268	321	435	395	344	538	506	13,4	11,4	10,7	12,1	12,3	9,8	13,9	11,6
AH023	314	459	530	626	691	641	658	18,0	12,0	10,4	11,5	10,9	8,1	12,0	10,8
AH025	278	287	201	245	243	288	378	12,7	6,5	10,0	8,9	3,2	7,2	6,4	3,0
AH026	665	1030	655	996	1180	799	1242	6,4	6,6	6,6	7,7	3,7	5,0	3,1	1,1
AH027	415	441	393	658	840	667	972	7,5	6,1	5,5	8,0	1,7	4,6	5,5	2,2
AH030	330	390	419	449	567	519	477	5,0	4,2	7,0	4,5	5,1	5,2	5,0	2,3
AH031	228	323	339	300	345	318	458	6,5	8,5	8,9	10,5	8,1	3,9	6,0	7,4
AH032	303	333	464	400	504	480	452	12,1	14,7	12,4	13,7	13,0	12,2	11,5	10,8
AH033	68	270	282	428	512	515	454	7,3	11,6	14,5	17,4	8,7	11,0	6,2	6,7
AH034	306	400	475	507	392	612	588	3,2	3,6	3,8	3,6	2,8	4,6	4,0	3,7
AH035	25	229	178	285	250	360	299	11,1	13,1	10,2	11,5	10,9	11,6	11,4	10,9
AH036	263	327	330	392	439	494	411	7,8	8,7	9,2	8,4	8,2	1,1	8,4	7,8
AH037	92	163	169	133	322	368	322		12,5	21,6	17,4	12,2	3,6	8,8	6,6
AH039	9	77	72	100	155	121	152	17,3	42,9	42,2	20,9	19,1	22,3	16,6	16,1
AH040	422	475	329	402	563	398	452	10,2	10,4	9,0	12,5	8,0	9,8	11,9	10,4
AH041	397	674	535	692	692	622	659	8,1	11,5	13,6	9,7	7,3	4,8	6,7	8,3
AH042	262	366	386	426	425	397	538	9,6	15,8	13,5	14,4	11,3	12,6	17,1	10,3
AH044	56	489	463	537	505	434	439	0,4	8,5	12,9	14,7	13,7	9,3	9,5	8,6
AH045	481	771	880	809	817	830	811	8,1	7,5	7,0	8,1	5,9	6,5	2,1	6,5
AH046	388	598	1002	725	890	972	724	9,9	9,5	9,2	8,5	6,4	5,0	7,3	3,0
AH047	321	428	308	363	399	524	486	9,4	9,6	9,8	7,5	7,2	6,1	8,6	7,0
AH048	63	205	126	221	207	236	202	22,1	24,9	24,3	20,0	12,1	29,7	17,0	12,4

AH049	415	420	424	488	599	533	503	6,8	8,9	9,6	9,2	11,9	11,6	11,0	8,4
AH050	303	514	435	389	452	481	581	12,9	17,2	16,7	11,9	12,2	10,2	16,3	6,8
AH051	784	649	852	757	1039	761	998	7,1	9,3	8,5	7,6	7,0	7,1	8,1	6,3
AH052	107	165	213	300	290	360	348	8,0	8,4	9,3	5,7	6,5	7,7	7,4	2,2
AH053	32	244	256	518	475	526	337	15,3	20,1	21,1	20,0	9,1	10,4	10,0	10,7
AH054	286	505	579	747	652	477	581	7,6	8,5	9,3	6,5	8,2	7,2	6,3	6,7
AH058	326	272	295	414	385	406	373	10,1	12,8	12,6	13,7	10,2	7,5	13,3	8,0
AH059	1033	872	960	847	895	888	933	5,3	5,6	6,8	6,4	2,6	7,4	0,7	7,4
AH060	341	516	524	872	614	864	629	5,1	15,8	10,8	11,0	11,7	9,3	0,7	6,3
AH061	339	410	398	398	537	546	642	16,9	5,5	5,8	6,3	4,2	5,6	3,8	5,7
AH062	205	391	361	444	473	565	512	15,5	10,0	10,4	10,8	10,0	9,3	2,5	8,3
AH063	146	262	293	248	335	358	300	13,4	8,7	7,9	6,8	7,6	8,3	4,9	7,6
AH064	603	580	724	772	865	713	694	11,8	13,2	10,7	10,7	10,0	10,2	1,4	8,9
AH065	319	433	673	593	654	708	716	12,1	8,7	6,4	8,7	6,9	7,2	5,7	7,0
AH066	12	112	155	209	274	475	375	29,4	48,6	14,9	9,9	14,7	14,1	10,1	9,6
AH067	504	631	744	791	857	968	950	10,4	8,8	5,2	7,6	5,5	6,1	5,6	5,1
AH068	540	680	887	1093	866	911	1097	9,6	6,9	5,1	5,2	6,7	7,5	6,5	7,4
AH069	334	350	397	546	595	521	624	11,3	9,9	21,5	8,4	5,8	6,6	6,7	4,6
AH070	269	521	574	575	660	659	630	8,4	7,4	6,1	4,8	6,1	7,3	3,9	4,3
AH073	37	85	118	104	106	140	222	35,4	23,9	5,9	9,2	9,8	7,7	11,3	11,9
AH074	61	201	341	354	331	337	380	18,1	20,0	21,8	17,9	13,9	9,3	14,1	13,6
AH075	319	412	608	350	462	468	468	7,1	5,3	5,8	4,7	7,2	6,2	5,0	2,1
AH076	296	352	407	550	604	623	544	7,8	8,9	8,6	6,1	8,9	8,3	5,9	11,0
AH077	471	748	1227	1145	930	1355	1213	8,9	11,5	13,4	3,6	8,5	7,3	5,3	6,9
AH078	208	426	297	422	518	447	412	6,6	5,3	5,7	4,4	2,6	5,8	2,3	7,6
AH079	47	142	166	253	176	226	307	15,4	14,3	5,0	8,6	7,9	5,9	8,4	5,7
AH080	14	167	197	197	263	327	302	7,5	11,4	6,3	12,3	10,9	8,3	9,2	9,3
AH081	181	262	324	320	327	463	467	11,2	9,4	1,6	7,2	7,8	1,8	7,7	7,6
AH082	176	218	272	370	363	347	409	14,6	11,0	2,0	6,4	6,8	5,6	6,6	6,1
AH083	181	325	353	508	467	768	589	27,3	16,1	4,0	14,2	12,1	0,8	8,7	13,0
AH084	243	273	336	332	379	448	387	8,0	6,1	10,1	5,3	3,5	2,1	9,2	13,4
AH085	8	43	47	65	119	174	170	17,6	15,1	16,5	12,7	12,1	14,7	11,9	15,7
AH086	92	202	159	159	191	165	214	8,6	10,0	10,7	16,4	11,6	10,0	5,1	8,8
AH087	42	142	188	335	347	414	511	1,5	11,6	20,5	10,8	7,0	6,9	8,0	7,7
AH088	174	227	238	180	241	277	274	5,6	13,6	10,1	10,5	9,1	8,1	10,0	9,7
AH089	143	396	254	328	240	304	263	5,1	13,4	3,3	8,3	10,7	8,3	8,9	9,6
AH090	20	143	165	249	320	404	380	16,2	12,2	13,4	7,6	9,3	6,0	6,0	6,3
AH091	97	157	167	192	213	247	218		8,5	9,7	5,4	2,7	7,2	6,7	6,3
AH092	164	461	310	628	401	514	428		11,6	14,3	8,7	3,2	12,0	9,7	10,2
AH093	193	404	602	774	707	766	726		6,7	10,5	0,8	2,4	7,8	3,9	2,0
AH095	94	308	222	253	266	319	341	5,8	7,3	8,2	4,8	9,2	8,5	5,3	8,6
AH096	216	424	375	550	590	745	692	10,1	11,6	18,8	7,1	16,4	26,4	16,9	17,4

Table S4. Univariate analysis of the $CD4^+$ T cell counts at 24 months of ART onset. Variables with a p<0.200 on the univariate analysis were included in the hierarchical linear models (Table 2).

	Univariate analysis (*p*)
Age	0.197
Gender	0.330
Log (HIV viral load) @ 0M	0.059
Log (HIV viral load) @ 24M	0.165
Chronic co-morbility	0.039
HLA-DR^- among $CD4^+$ T cells @ 24M	0.003
%Tregs cells @ 0M	0.057
$CD4^+$ T cell count @ 0M	0.000