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Human immunodeficiency virus type I-specific CD8⁺ T cell subset abnormalities in chronic infection persist through effective antiretroviral therapy

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Abstract

Background: Effective highly active antiretroviral therapy (HAART) reduces human immunodeficiency virus (HIV) replication, restores CD4⁺ T lymphocyte counts and greatly reduces the incidence of opportunistic infections. While this demonstrates improved generalized immune function, rapid rebound to pre-treatment viral replication levels following treatment interruption indicates little improvement in immune control of HIV replication. The extent to which HAART can normalize HIV-specific CD8⁺ T cell function over time in individuals with chronic infection remains an important unresolved issue. In this study, we evaluated the magnitude, general specificity and character of HIV specific CD8⁺ T cell responses at four time points across 2-9 years in 2 groups of chronically infected individuals separated on the basis of either effective antiretroviral suppression or ongoing replication of HIV.

Methods: Peripheral blood mononuclear cells (PBMC) were stimulated with overlapping 15mer peptides spanning HIV Gag, Pol, Env and Nef proteins. Cells producing interferon- γ (IFN- γ) or interleukin-2 (IL-2) were enumerated by ELISPOT and phenotyped by flow cytometry.

Results and Conclusions: The magnitude of the HIV-specific CD8⁺ T cell response ranged from < .01 to approximately 1.0% of PBMC and was significantly greater in the group with detectable viral replication. Stronger responses reflected higher numbers of CD8⁺CD45RA⁻ effector memory cells producing IFN- γ , but not IL-2. Magnitude, general specificity and character of the HIV-specific CD8⁺ T cell response changed little over the study period. While antiretroviral suppression of HIV in chronic infection reduces HIV-specific CD8⁺ T cell response magnitude in the short term, it had no significant effect on response character over periods up to 9 years.

Background

Human immunodeficiency virus (HIV) infection progressively impairs immune function, including that of the HIV-specific CD8⁺ T cells that partially control HIV replication. This impairment manifests in phenotypic and functional changes affecting key CD8⁺ T cell properties such as proliferation, differentiation, cytokine production profile, signal transduction, and survival [1-8]. Compared to cytomegalovirus (CMV)-specific CD8⁺ T cells, HIV-specific CD8⁺ T cells are highly skewed towards effector

memory (Tem) status, suggesting reduced differentiation into CD45RA⁺ terminal effectors (Ttd) [9]. In addition, limited HIV-specific CD8⁺ T cell proliferation suggests defective generation and/or maintenance of central memory T cells (Tcm) [10]. Understanding the fate of these subset abnormalities in HIV-infected individuals on highly active antiretroviral therapy (HAART) is important for defining the need for complementary treatment and for gauging practical limits to immune recovery.

When effective, HAART increases CD4⁺ T lymphocyte counts, reduces opportunistic infections and according to early studies, normalizes immune responses [11,12]. However, more specific analyses have recently raised doubt as to whether HIV-specific CD4⁺ and CD8⁺ T cell

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responses recover characteristics normally associated with effective control of intracellular pathogens [13-18]. Early control of HIV replication may be required to preserve HIV-specific CD4⁺ T cells and fully functional HIV-specific CD8⁺ T cells appear peculiar to those long term non progressors (LTNP) who maintain low HIV replication levels without treatment [19]. Thus, a more pessimistic view is that the level to which HIV-specific T cell responses deteriorate prior to treatment represents an intractable deficit, insensitive to HAART-related immune recovery. This issue remains controversial as two recent studies reached diametrically opposite conclusions of clear HAART-related improvement versus no change [20,21]. These conflicting views could reflect different lenses, a broad range of pre-HAART deterioration in the groups studied and inherent human variation in thresholds beyond which the possibility or rate of recovery lessens.

In this study, we investigated evolution of the magnitude, specificity and memory subset distribution of HIV-specific CD8⁺ T cell responses over a 2 to 9 year period in two groups: one with continuous suppression of HIV replication on HAART; and another with continuously detectable HIV replication. We reasoned that contrasting trajectories of the 2 groups would highlight any impact HAART was having towards reversal or prevention of HIV-specific CD8⁺ T cell abnormalities and help resolve this critical issue.

Methods

Subjects

Study participants recruited through the St. John's General Hospital HIV Clinic, St. John's, Newfoundland, Canada were selected on the basis of ≥ 24 sequential months of either complete suppression of HIV replication below levels detectable by plasma virus load testing (< 2.6 or $< 1.7 \log_{10}$ copies HIV RNA/ml plasma by Roche [™]Amplicor standard or ultrasensitive testing respectively) or ≥ 24 sequential months of detectable plasma virus loads. Subjects were grouped accordingly and at least 4 sequential PBMC samples, over a period as stipulated above, were selected for analysis. Other relevant immunological and virological characteristics of the study subjects were obtained from patient charts and noted accordingly in results. All participants provided informed consent for whole blood donation, immunological studies and researcher access to medical laboratory records. The Memorial University Faculty of Medicine Human Investigation Committee gave ethical approval for this study.

Peripheral blood mononuclear cell (PBMC) isolation

Acid-citrate-dextrose treated whole blood obtained by forearm venipuncture at regular intervals concurrent with medically scheduled bloodwork was diluted 1:2 with

phosphate buffered saline (PBS) and PBMC isolated by Ficoll-Hypaque (GE Healthcare Bio-Science) density gradient centrifugation. Samples were cryopreserved in lymphocyte medium consisting of RPMI 1640 with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine, 10 mM HEPES buffer solution and 2×10^{-5} M 2-mercaptoethanol (all from Invitrogen) with FBS increased to 20% and supplemented with 10% dimethylsulfoxide (Sigma).

ELISPOT Assays

Individual PBMC samples from 6 non-infected controls and series of 4 PBMC samples from 30 HIV-infected individuals in the 2 groups were stimulated with pools of overlapping 15mer peptide sets spanning HIV clade B Gag, Pol, Env and Nef (National Institutes of Health AIDS Reference Reagent Repository). Individual peptides were dissolved at 10 mg/ml in DMSO and then pooled in sets of 49 (Nef), 123 (Gag), 125 (Pol1), 123 (Pol2), 128 (Env1), and 83 (Env2) in unsupplemented RPMI at a final concentration of 1.0 μ g/ml of each peptide. Samples were thawed and cultured overnight before counting to ensure accurate numbers of viable cells were used. For interferon- γ (IFN- γ) assays, microtitre plates (Multiscreen; Millipore) were coated overnight at 4°C with 100 μ l of 7.5 μ g/ml anti-IFN- γ monoclonal antibody (mAb) 1-D1K (Mabtech) and then washed 3 times with PBS. For interleukin-2 (IL-2) assays, plates were coated overnight at 4°C with 100 μ l of 15 μ g/ml anti-IL-2 mAb IL-2-/249 (Mabtech) and then washed 3 times with PBS. Cells were added at 4.0 or 1.0×10^5 /well in 100 μ l medium in single or dual replicates for IL-2 and IFN- γ detection respectively. Peptides (20 μ l/pool) were added to a final concentration of 0.1 μ g/ml with the volume in each well adjusted to 200 μ l with medium. After overnight (16 hr) incubation, the plates were washed 6 times as above and 100 μ l of 1 μ g/ml biotinylated 7-B6-1 or IL-2-II (Mabtech) added for 2 h. Plates were then washed 6 times and 100 μ l/well of a 1/1000 dilution of streptavidin-alkaline phosphatase (AP) (Mabtech) was added for 1 h. Plates were again washed 6 times and 100 μ l/well of chromogenic AP substrate (BioRad) was added. After 30 min, plates were washed with tap water to stop reactions and then air-dried. Spots were counted with an Immunoscan reader (Cellular Technology Limited) and results reported as IFN- γ spot forming cells (sfc)/ 10^6 PBMC (when ≥ 50) or IL-2 sfc/ 10^6 PBMC (when ≥ 0) after subtraction of background (sfc/ 10^6 PBMC cultured overnight in medium alone).

Flow cytometry

When sufficient cells were available, 1×10^6 PBMC were incubated overnight with each individual peptide pool or in medium alone as negative control under the same con-

ditions as ELISPOT except for addition of 10 µg/ml brefeldin A (Sigma) after 1 h of incubation. If ELISPOT results indicated ≥ 500 sfc/ 10^6 PBMC, the corresponding samples and controls were processed for flow cytometry. Cells were washed with PBS plus 1% FCS and 5 mM EDTA, then surface stained with anti-CD8-PerCP (clone RPA-T8, Biolegend) and anti-CD45RA-PE (clone JS-83, eBioScience). After washing, cells were fixed, permeabilized (Dako intrastain), and stained with anti-IFN- γ -APC (clone 4S-B3, eBioScience). At least 200,000 events were acquired on a FACS Calibur flow cytometer (Becton Dickinson) and CD8⁺ cells gated and analyzed for IFN- γ and CD45RA expression with Cellquest Pro software (Becton Dickinson).

Statistical analysis

Statistical analysis was carried out with GraphPad Prism 4.03 Software. Normality of data distribution was assessed by the Kolmogorov-Smirnov test. When data was normally distributed, means were presented for comparison. If data did not fit a normal distribution, medians were presented for comparison. Repeated measures ANOVA was used to compare the frequency of HIV-specific CD8⁺ T cells producing IFN- γ or IL-2 between and within groups at different time points and to compare between groups the percentage of CD45RA⁺ HIV-specific CD8⁺ T cells producing IFN- γ . Paired Student's *t* test was used to compare initial and final CD4⁺ T lymphocyte counts and initial and final percentages of CD45RA⁺ HIV-specific CD8⁺ T cells producing IFN- γ within groups. Probability values < 0.05 were considered significant.

Results

Subjects' characteristics

General characteristics of the two HIV-infected groups prior to and over the course of the study are reported in table 1 (undetectable viral replication) and table 2 (detectable viral replication). All subjects were chronically infected for a documented duration of from at least 5 to > 15 years. Most individuals in both groups had endured relatively high levels of HIV replication and had progressed to advanced HIV infection at some point as illustrated by zenith viral loads and CD4 nadirs < 200/ μ L peripheral blood. Those in the uncontrolled replication group had plasma virus loads throughout the study course that were substantially lower than their zenith, suggesting partial efficacy of their antiretroviral therapy as the recorded zenith was not associated with acute infection. Three individuals (187, 195 and 209) in the uncontrolled infection group had never received antiretroviral therapy up to and through the course of this study, while all others in both groups had received HAART in the form of triple therapy with drugs of at least 2 classes.

The documented duration of undetectable plasma virus load in the controlled infection group ranged from 3 to 84 months preceding this study.

Evolution of the magnitude and specificity of CD8⁺ anti-HIV T cell responses

ELISPOT testing of control PBMC (Figure 1) and 4 sequential PBMC samples from 15 individuals with persistently undetectable virus load (Figure 2) and 15 individuals with persistently detectable virus load (Figure 3) was carried out as shown. The cumulative number of IFN- γ sfc/ 10^6 PBMC stimulated by the peptide pools representing HIV Gag, Pol, Env and Nef was taken as overall magnitude of the CD8⁺ anti-HIV T cell response. Cells producing IFN- γ in response to the HIV peptides were almost exclusively CD8⁺ as no increase over control values in the percentage of non-CD8⁺ T cells producing IFN- γ was detectable by flow cytometry in any of the samples tested (data not shown). The fractional contribution of each individual HIV protein to overall magnitude indicated general specificity. The six uninfected controls had cumulative responses from 0-61 (median = 23) IFN- γ and 0-38 (median = 4) IL-2 sfc/ 10^6 PBMC in response to the HIV peptide pools, demonstrating the specificity and low background of the peptide ELISPOT assay. In the undetectable virus load group, overall magnitude of the CD8⁺ anti-HIV T cell response ranged from 67 sfc/ 10^6 PBMC to 5166 sfc/ 10^6 PBMC (Figure 4). Median overall CD8⁺ anti-HIV T cell response magnitude in this group remained consistent over the course of study, beginning at 874 and finishing at 895 sfc/ 10^6 PBMC, indicating no long-term decay in the magnitude of the CD8⁺ anti-HIV T cell response, despite apparently effective antiretroviral therapy. Mean CD4⁺ T cell count rose from 511 to 729/ μ L peripheral blood over the same time period (*P* = .002). If > 50% of the total IFN- γ sfc were specific for 1 of the 4 major HIV proteins, we considered the response against this protein immunodominant. At the first study point Gag was the dominant CD8⁺ T cell antigen for 6, Pol for 1, Nef for 2 and Env for 0 individuals in the undetectable virus group. This pattern persisted through the fourth study point with Gag immunodominant for 6, Pol for 2, Nef for 2 and Env for 0 individuals (Figure 5). In the absence of detectable HIV replication, several dramatic shifts in general antigenic dominance still occurred, from Gag to Pol (subject 50) and Pol to Gag (subject 128) (Figure 5). These cases of shifting immunodominance or epitope spreading together with persistently high HIV-specific CD8⁺ T cell responses suggest immune stimulation by ongoing viral replication at levels undetectable by clinical virus load testing.

In the detectable virus load group, overall CD8⁺ anti-HIV T cell response magnitude ranged from 608 to 9856

Table 1: General characteristics and changes for subjects with undetectable HIV replication

ID	Duration of infection	aVL zenith	bCD4 nadir	cDuration of suppression	dCD4i	CD4f	eMonths study course	fCD8+ IFN γ + CD45RA+i	CD8+ IFN γ + CD45RA+f
27	>15 yrs	5.13	308	21 months	702	1364	90	13%	26%
30	>15 yrs	4.53	314	84 months	630	1089	28	ND	ND
50	>15 yrs	5.45	18	7 months	220	627	108	52%	50%
62	>15 yrs	4.61	154	32 months	738	756	28	10%	15%
90	>15 yrs	6.22	73	24 months	280	352	96	79%	84%
109	>15 yrs	3.66	145	72 months	740	665	24	22%	16%
128	>12 yrs	4.53	364	3 months	570	912	66	44%	52%
134	>12 yrs	5.01	96	72 months	850	1020	24	ND	ND
143	>10 yrs	4.77	400	24 months	400	682	60	ND	22%
149	>9 yrs	5.67	9	36 months	338	264	24	ND	ND
155	>9 yrs	5.10	210	3 months	368	408	54	10%	10%
174	>7 yrs	5.53	380	12 months	494	768	30	ND	ND
176	>7 yrs	<2.6	245	12 months	361	750	36	ND	ND
193	>6 yrs	<2.6	40	12 months	405	324	24	23%	13%
200	>5 yrs	>5.88	192	3 months	567	957	24	ND	ND

^aThe highest recorded virus load (VL) for each subject in log₁₀ copies HIV RNA/mL plasma.

^bThe lowest recorded peripheral blood CD4⁺ T lymphocyte count for each subject.

^cThe length of time before analysis of HIV-specific CD8⁺ T cell responses for which each subject experienced suppression of viremia below detectable levels.

^dThe number of CD4⁺ T lymphocytes/ μ L peripheral blood for each subject at beginning (CD4_i) and end (CD4_f) of period over which HIV-specific CD8⁺ T cell responses were assessed.

^eTotal time period over which four assessments of HIV-specific CD8⁺ T cell responses occurred.

^fPercentage of HIV-specific CD8⁺ T cells producing INF- γ that expressed CD45RA at beginning (i) and end (f) of period over which HIV-specific CD8⁺ T cell responses were assessed.

sfc/10⁶ PBMC and increased (non-significant) from a median of 1587 to a median of 1889 sfc/10⁶ PBMC over the course of the study (Figure 4). Median CD8⁺ anti-HIV T cell response magnitude was significantly higher in the detectable virus load group than in controlled virus replication group ($P < .0001$). The mean CD4⁺ T cell count was unchanged at 553 versus 524/ μ L peripheral blood at initial and final time points. Although these subjects had detectable virus replication throughout the study, all but three (187, 195 and 209) received partially suppressive antiretroviral therapy, either persistently or intermittently. At the first study point, Gag was the dominant CD8⁺ T cell antigen for 2, Pol for 1, Nef for 1 and Env for 1 individual in the detectable virus load group. By the fourth study point, Gag was immunodominant for 3, Pol for 3, Nef for 0 and Env for 0 individuals (Figure 6).

Evolution of memory status of CD8⁺ anti-HIV T cell responses

Central memory (Tcm) HIV-specific CD8⁺ T cells are rare in progressive HIV infection and difficult to accurately enumerate by flow cytometry [10]. Therefore, we

measured IL-2 producing cells by ELISPOT to estimate HIV-specific CD8⁺ Tcm numbers (Figures 1, 2, 3). As expected, these were much less frequent than IFN- γ producing HIV-specific CD8⁺ T cells. Although at lower absolute levels, response distribution across the 4 HIV antigens tested and overall expansion/contraction of the HIV-specific CD8⁺ T cell IL-2 response generally mirrored that of the HIV-specific CD8⁺ T cell IFN- γ response. For 12 individuals with the most prominent IL-2 sfc responses, individual peptides stimulating IL-2 were identified using peptide matrices from the appropriate peptide set. Optimal 9mers previously identified within the 15mers http://www.hiv.lanl.gov/content/immunology/ctl_search were then shown to stimulate IL-2 production, confirming that CD8⁺ class I-restricted T cells were responsible for the IL-2 production. In the undetectable virus load group, the magnitude of the CD8⁺ anti-HIV T cell IL-2 response ranged from 0 to 508 sfc/10⁶ PBMC (Figure 7). Over the course of the study, the median overall CD8⁺ anti-HIV T cell IL-2 response magnitude remained similar at 56 and 50 sfc/10⁶ PBMC at

Table 2: General characteristics and changes for subjects with detectable HIV replication

ID	Duration of infection	^a VL zenith	^b CD4 nadir	^c VL study course	^d CD4i	CD4 ^f	^e Months study course	^f CD8+ IFN γ + CD45RA+i	CD8+ IFN γ + CD45RA+f
11	>15 yrs	>5.88	63	4.58	308	335	60	15%	16%
17	>15 yrs	4.48	296	4.12	518	490	30	ND	ND
35	>15 yrs	4.77	176	3.37	660	736	48	35%	12%
39	>15 yrs	5.64	24	2.70	754	961	30	6%	14%
51	>15 yrs	5.61	40	3.29	390	297	36	33%	25%
57	>15 yrs	5.48	176	4.25	420	506	36	14%	8%
77	>15 yrs	5.00	207	3.80	692	288	24	ND	ND
78	>15 yrs	>5.88	32	4.53	110	184	36	24%	23%
85	>14 yrs	4.60	307	2.65	684	663	66	11%	11%
98	>13 yrs	5.73	69	3.79	484	435	48	ND	ND
116	>12 yrs	5.56	44	3.22	319	378	48	23%	23%
125	>12 yrs	5.06	59	4.52	496	209	42	31%	ND
187	>7 yrs	3.93	1064	2.89	1127	1419	36	37%	ND
195	>5 yrs	4.93	462	4.54	518	462	24	27%	25%
209	>5 yrs	4.97	500	4.72	812	500	24	ND	ND

^aThe highest recorded virus load (VL) for each subject in log₁₀ copies HIV RNA/mL plasma.

^bThe lowest recorded peripheral blood CD4⁺ T lymphocyte count for each subject.

^cVirus load (log₁₀ copies HIV RNA/mL plasma) averaged over the 4 time points for each subject.

^dThe number of CD4⁺ T lymphocytes/ μ L peripheral blood for each subject at beginning (CD4_i) and end (CD4_f) of period over which HIV-specific CD8⁺ T cell responses were assessed.

^eTotal time period over which four assessments of HIV-specific CD8⁺ T cell responses occurred.

^fPercentage of HIV-specific CD8⁺ T cells producing INF- γ that expressed CD45RA at beginning (i) and end (f) of period over which HIV-specific CD8⁺ T cell responses were assessed.

time points 1 and 4 respectively. In the detectable virus load group, the magnitude of the CD8⁺ anti-HIV T cell IL-2 response ranged from 0 to 940 sfc/10⁶ PBMC and remained stable at a median of 43 and 55 sfc/10⁶ PBMC at time points 1 and 4 respectively (Figure 7). There was no significant difference in the magnitude of CD8⁺ HIV-specific IL-2 sfc/10⁶ PBMC between groups and no significant changes in the magnitude of the response in either group over the course of the study. Viral replication reduced the fraction of HIV-specific CD8⁺ Tcm with no impact on absolute number. Long term suppression of HIV replication had little impact on either the fraction or absolute number of CD8⁺ HIV-specific IL-2 sfc.

Since the contribution of CD45RA⁺ naïve T cells to in vitro HIV-specific T cell responses is minimal, terminally differentiated HIV-specific CD8⁺ effector cells can be distinguished by co-expression of intracellular IFN- γ and surface CD45RA following stimulation with HIV peptides. When cells were available and ELISPOT results indicated enough IFN- γ producing cells for phenotypic analysis of HIV-specific CD8⁺ T cells by flow cytometry, we estimated the fraction of Ttd through CD45RA

expression on HIV-specific CD8⁺ T cells producing IFN- γ (Figure 8). In the undetectable virus load group, the CD45RA⁺ percentage of the CD8⁺ anti-HIV T cell response ranged from 13% to 84% (Table 1), but did not increase from a mean of 32% over the course of the study. In the detectable virus load group, the CD45RA⁺ percentage of the CD8⁺ anti-HIV T cell response ranged from 6% to 37% and fell from a mean of 23% to 17% over the course of the study. This decrease was not statistically significant. We observed a generally low percentage of CD8⁺ anti-HIV T cells expressing CD45RA⁺ throughout both groups of subjects, corroborating previous reports on impaired terminal differentiation of HIV-specific CD8⁺ T cells [9,14].

Discussion and Conclusions

The unusually low fractions of HIV-specific CD8⁺ Ttd and Tcm detected in progressive infection suggest impaired differentiation may be a key factor in HIV-specific CD8⁺ T cell dysfunction and progression to acquired immune deficiency syndrome (AIDS) [9,10,14]. Since CD8⁺ T cells themselves are rarely HIV-infected, their impairment relates indirectly to HIV replication. Thus,

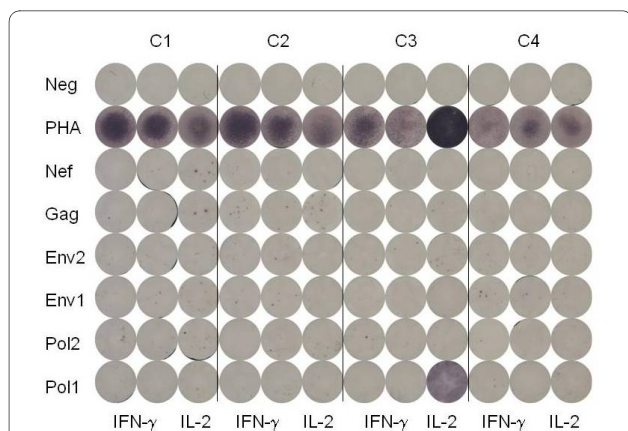


Figure 1 Detection of HIV-specific IFN- γ and IL-2 spot forming cells (sfc) in uninfected controls. Individual PBMC samples from four (C1, C2, C3 and C4) of six uninfected individuals tested by ELISPOT with medium alone (Neg), PHA and overlapping peptide pools from HIV Gag, Pol, Env and Nef for IFN- γ (duplicate) and IL-2 (single replicate) sfc are shown.

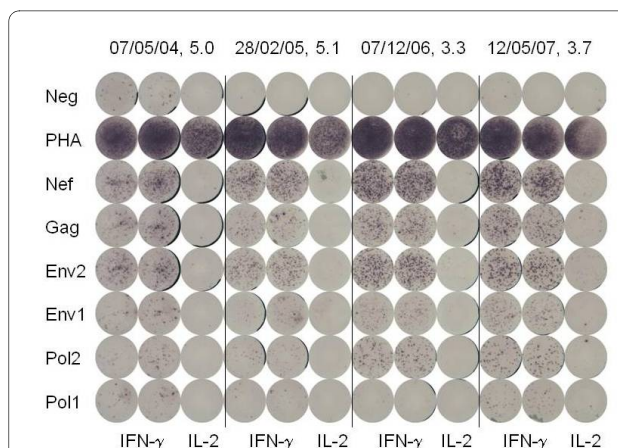


Figure 3 Detection of HIV-specific IFN- γ and IL-2 spot forming cells in HIV-infected individuals with continuously detectable HIV replication. Four PBMC samples taken from a representative HIV-infected individual with continuously detectable HIV replication on dates shown on top of the figure together with concurrent log₁₀ copies HIV RNA/ml plasma were tested as described in figure 1.

whether HAART normalizes HIV-specific CD8⁺ T cell differentiation in chronic infection remains unknown. In this study, we used overlapping peptides encompassing all of HIV Gag, Pol, Env and Nef to broadly examine HIV-specific CD8⁺ T cell responses and address the impact successful antiretroviral suppression of HIV replication had on these responses. We compared evolution of the magnitude, specificity and character of HIV-specific CD8⁺ T cell responses in two groups of individuals, both chronically infected with HIV, but distinguished by continuously detectable versus undetectable HIV replication over study periods from 2-9 years.

We found that the HIV-specific CD8⁺CD45RA⁺ population, which includes Tdt, were relatively scarce throughout the detectable virus replication group and most of the undetectable virus replication group. While such paucity was previously described in studies focused on individual epitopes, the use of genome wide peptide pools herein clarifies its broader relevance to the HIV-specific CD8⁺ T cell response [9,14]. The percentage of CD45RA⁺ effectors, together with the absolute number of HIV-specific CD8⁺ T cells remained relatively constant in both groups, consistent with establishment of stable ratios under

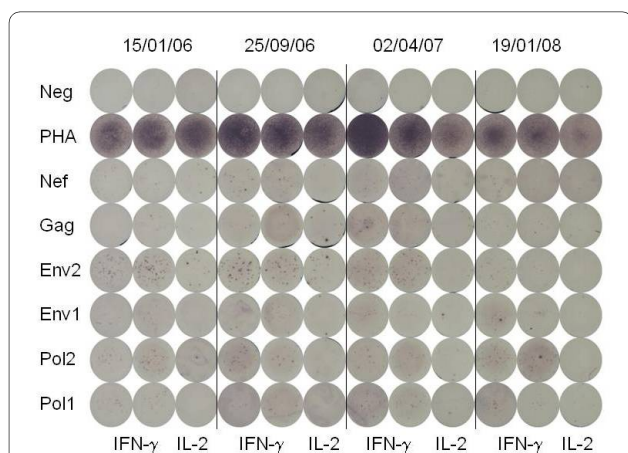


Figure 2 Detection of HIV-specific IFN- γ and IL-2 spot forming cells in HIV-infected individuals with continuous suppression of viral replication. Four PBMC samples taken on dates shown on top of the figure from a representative HIV-infected individual with continuous suppression of viral replication were tested as described in figure 1.

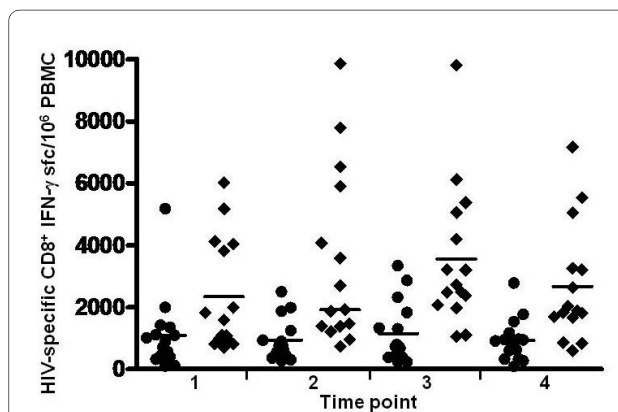


Figure 4 Frequency of HIV-specific CD8⁺ T cells producing IFN- γ in chronically infected individuals with ongoing HIV replication either detectable (◆) or suppressed below detectable levels (●). The total number of HIV-specific CD8⁺ T cells producing IFN- γ /10⁶ PBMC in response to Gag, Pol, Env and Nef peptide pools is shown for each individual at 4 serial time points with horizontal line through the group representing the median frequency.

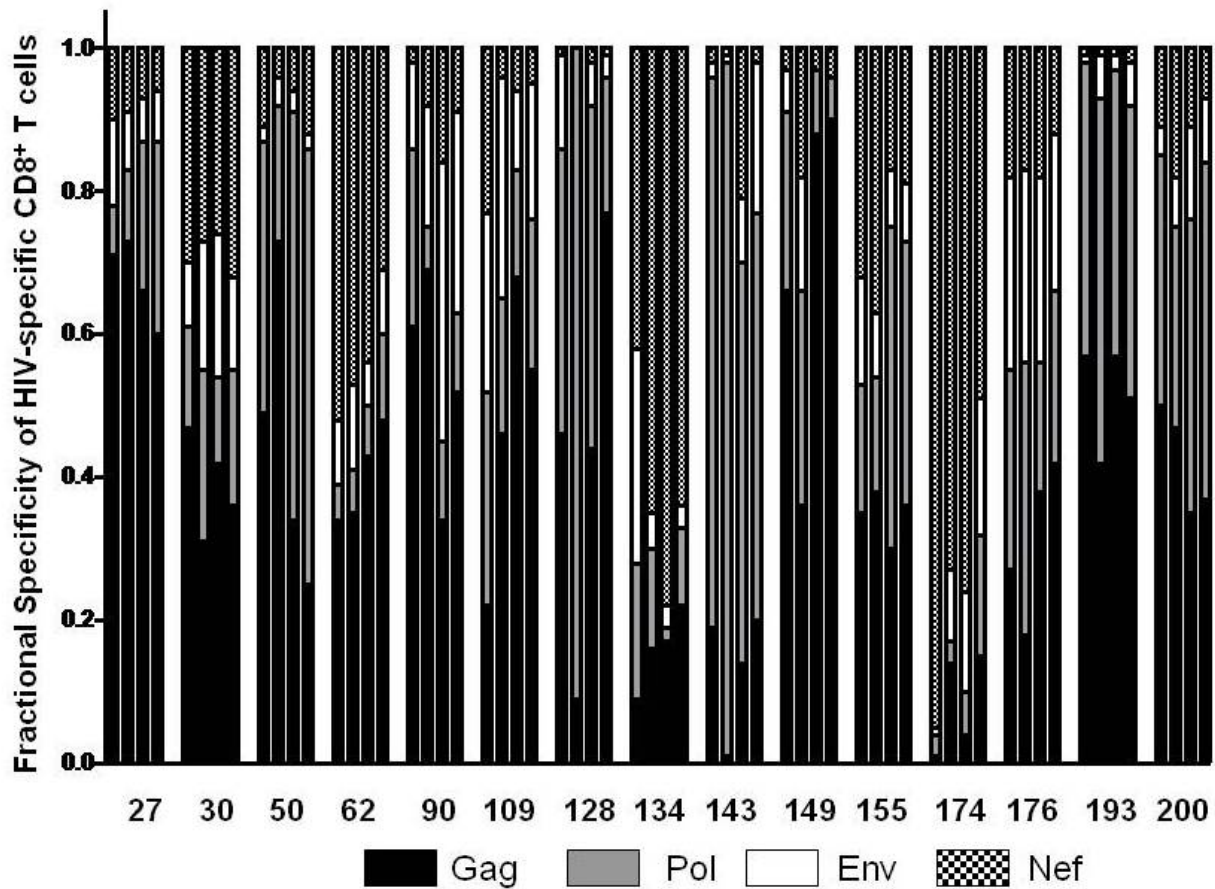


Figure 5 Fractional specificity of the overall HIV-specific CD8⁺ T cell response. The fraction of the overall HIV specific CD8⁺ T cell response attributed to each of the 4 major HIV proteins is shown by differential bar graph shading for individuals in the undetectable viral replication groups at each of 4 sequential time points tested.

chronic conditions of either antiretroviral suppression or HIV replication. While the overall similar character of HIV-specific CD8⁺ T cell responses in both groups also supports the notion of intransigence to antiretroviral therapy, we did not compare the same individuals before and after introduction of successful antiretroviral therapy. Therefore, improvements in the character of HIV-specific CD8⁺ T cell responses occurring early after onset of antiretroviral therapy would not have been detected in this study.

Although these responses would likely not be detectable by flow cytometry with individual peptides, we detected CD8⁺ HIV-specific IL-2 responses in nearly half of the chronically infected individuals we tested by ELISPOT with peptide sets covering the entire HIV genome. The absolute and relative frequencies of HIV-specific CD8⁺ T cells producing IL-2 were, nonetheless, very low in both groups, confirming that few, or in some cases, none of the HIV-specific CD8⁺CD45RA⁻ T cells were Tcm. The low numbers of HIV-specific IL-2 sfc

indicate that only a very small fraction of the HIV-specific CD8⁺CD45RA⁻ IFN- γ producing T cells are Tcm. Therefore, prolonged or repeated expansion of HIV-specific effector cells skews the HIV-specific CD8⁺ T cell population towards a predominant effector memory (Tem) phenotype and this skewing is not reversed by long-term suppression of HIV replication.

Identifying HIV-specific CD8⁺ Tcm other than by expression of surface markers such as CCR7 could conceivably underestimate Tcm numbers, but proliferative function as indicated by IL-2 production may be a more meaningful discriminator of HIV-specific CD8⁺ Tcm. The lower fraction of HIV-specific CD8⁺ Tcm thus identified in the group with detectable HIV replication actually reflected higher absolute Tem numbers in this group, not fewer Tcm. Absolute Tcm numbers changed little over time in either group, implying a rough equilibrium of HIV-specific T cell subset distribution under relatively stable conditions of antiretroviral suppression or HIV replication. Thus, it appears that in stable settings of

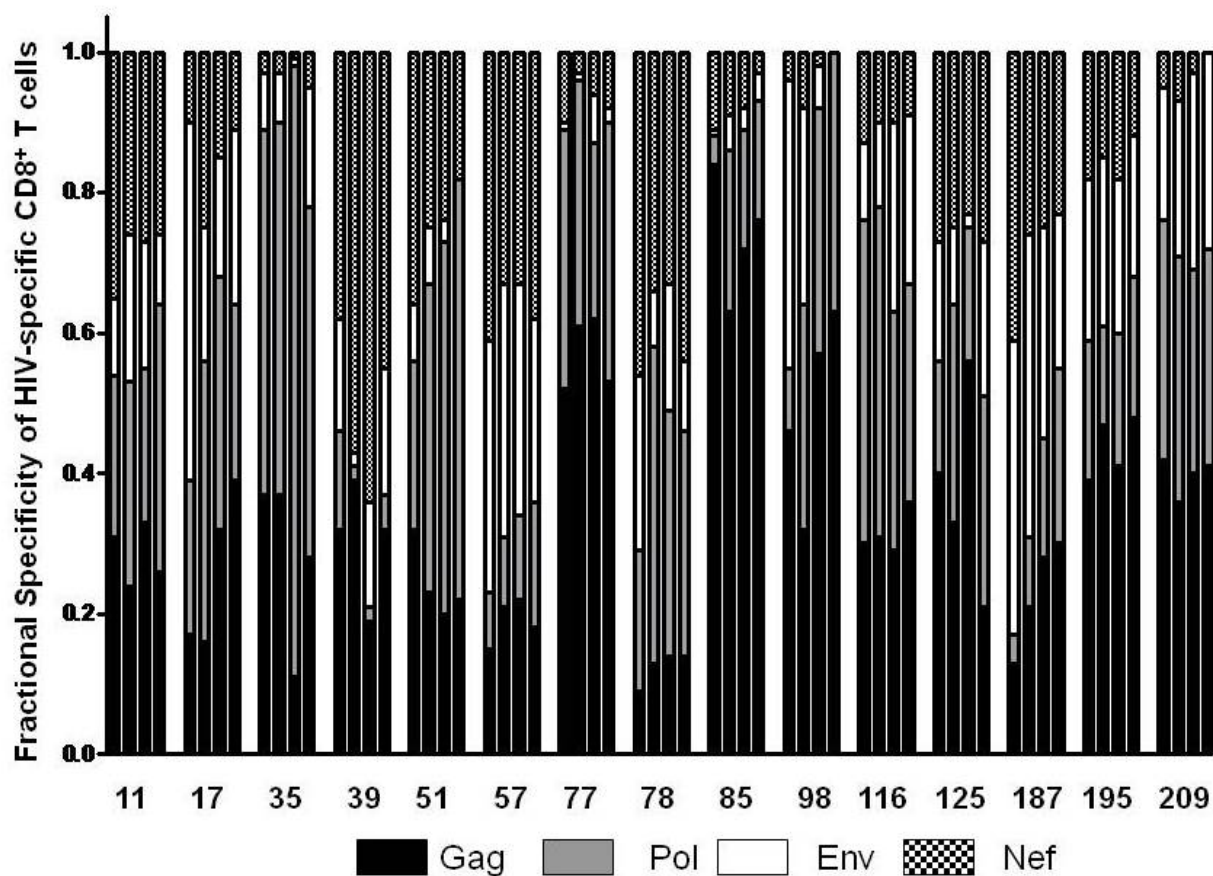
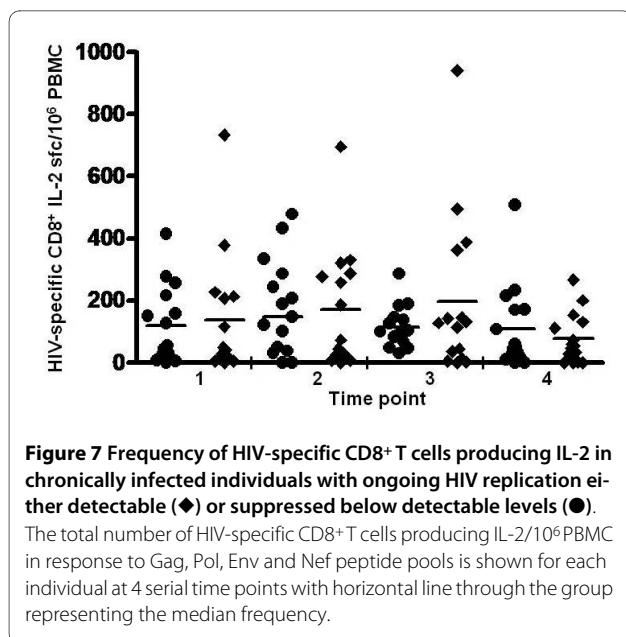


Figure 6 Fractional specificity of the overall HIV-specific CD8⁺ T cell response. The fraction of the overall HIV specific CD8⁺ T cell response attributed to each of the 4 major HIV proteins is shown by differential bar graph shading for individuals in the detectable viral replication groups at each of 4 sequential time points tested.

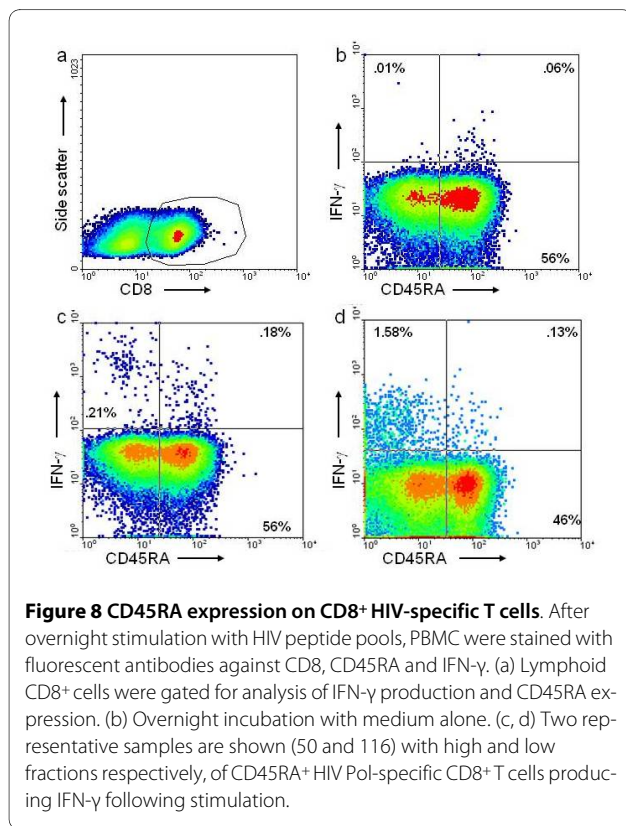
either viral replication or antiretroviral suppression, individuals reach a steady-state HIV-specific CD8⁺ T cell response, analogous and related to virus load set-point [22]. This equilibrium would shift with acute expansion of Tem upon de novo viral replication or contraction of Tem with introduction of effective HAART. In our study, where initial samples were drawn at least 3 months after establishment of viral suppression or viral replication, the absolute magnitude, general specificity and subset distribution of the CD8⁺ anti-HIV T cell response remained remarkably constant. This suggests an enormous reserve of CD8⁺ HIV-specific effector cells, although previous or partially effective treatment probably also played a role in the durability of responses in our study group. In progressive infection, HIV-specific CD8⁺ T cell activation, subset distribution and elimination maintains a set point that slowly decays towards true numerical deficits only in advanced infection. Set point maintenance in treated non-progressing subjects with undetectable viral loads indicates occult viral replication sufficient for continuous

recruitment of relatively short-lived CD8⁺ HIV-specific Tem or some residual conditioning of the immune system that perpetuates aberrant differentiation and subset distribution of HIV-specific CD8⁺ T cells. Such low level replication, as was recently confirmed in both elite controllers and treated individuals with clinically undetectable virus loads, may be an important component of immune deficits that persist in chronic, treated HIV infection [19,23].

In humans, there are clear associations between initial suppression of viral replication and emergence of HIV-specific CD8⁺ T cells as well as between advanced disease progression and decline of HIV-specific CD8⁺ T cells [24-26]. In macaques, depletion of CD8⁺ T cells in macaque models of HIV infection directly demonstrated the role that CD8⁺ T cells play in lowering virus load in acute infection and delaying disease progression in chronic infection [27]. However, the coexistence of strong anti-HIV CD8⁺ T cell responses with substantial levels of HIV replication that we and others have observed raises ques-



tions as to the long term contribution of these natural responses [28]. Some data indicate that clonal restriction of the HIV-specific CD8⁺ T cell response is the earliest predictor of rapid disease progression, suggesting that the initial dynamics of CD8⁺ T cell-mediated suppression of viral replication versus viral dissemination, diversification



and immune destruction play a major role in determining the steady-state parameters of chronic infection [17,29]. Our data indicate that antiretroviral treatment in chronic HIV infection shifts the steady state towards slower decay and lower HIV-specific CD8⁺ Tem frequencies without affecting the overall character of the HIV-specific CD8⁺ T cell response. Without supplementary immunological conditioning to enhance the efficacy of HIV-specific immunity, the system will return to its original trajectory upon treatment failure or withdrawal.

In summary, we found no evidence that effective antiretroviral therapy influences any feature other than magnitude of HIV-specific CD8⁺ T cell responses. Low frequencies of CD8⁺ HIV-specific Tcm and low fractions of CD45RA⁺ Tdt persisted over prolonged periods of effective HAART, suggesting that abnormal characteristics of the HIV-specific CD8⁺ T cell response imprinted following acute infection endure immune restoration associated with HAART. However, none of the subjects in our study received antiretroviral therapy during acute infection, which may be more likely to influence HIV-specific CD8⁺ T cell differentiation and subset distribution than treatment during chronic infection. Novel strategies addressing HIV-specific CD8⁺ T cell response character will be required to achieve true immune restoration in chronic HIV infection.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JP performed most of the ELISPOT and flow cytometry assays, coordinated sample selection and collated data. KZ and NAH performed additional ELISPOT assays. MEG processed and archived blood samples and collated clinical laboratory data. MDG designed the study, directed its conduct, interpreted data and prepared the manuscript. All authors read and approved the final manuscript.

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References

1. Kuerten S, Nowacki TM, Kleen TO, Asaad RJ, Lehmann PV, Tary-Lehmann M: **Dissociated production of perforin, granzyme B, and IFN-gamma by HIV-specific CD8(+) cells in HIV infection.** *AIDS Res Hum Retroviruses* 2008, **24**(1):62-71.
2. Kuerten S, Asaad RJ, Schoenberger SP, Angelov DN, Lehmann PV, Tary-Lehmann M: **The TRAIL of helpless CD8+ T cells in HIV infection.** *AIDS Res Hum Retroviruses* 2008, **24**(9):1175-1183.

3. Wang C, Wen T, Routy JP, Bernard NF, Sekaly RP, Watts TH: **4-1BBL induces TNF receptor-associated factor 1-dependent Bim modulation in human T cells and is a critical component in the costimulation-dependent rescue of functionally impaired HIV-specific CD8 T cells.** *J Immunol* 2007, **179**(12):8252-8263.
4. Chen G, Shankar P, Lange C, Valdez H, Skolnik PR, Wu L, Manjunath N, Lieberman J: **CD8 T cells specific for human immunodeficiency virus, Epstein-Barr virus, and cytomegalovirus lack molecules for homing to lymphoid sites of infection.** *Blood* 2001, **98**(1):156-164.
5. Lieberman J, Shankar P, Manjunath N, Andersson J: **Dressed to kill? A review of why antiviral CD8 T lymphocytes fail to prevent progressive immunodeficiency in HIV-1 infection.** *Blood* 2001, **98**(6):1667-1677.
6. Wherry EJ, Day CL, Draenert R, Miller JD, Kiepiela P, Woodberry T, Brander C, Addo M, Klenerman P, Ahmed R, *et al.*: **HIV-specific CD8 T cells express low levels of IL-7Ralpha: implications for HIV-specific T cell memory.** *Virology* 2006, **353**(2):366-373.
7. Shin H, Wherry EJ: **CD8 T cell dysfunction during chronic viral infection.** *Curr Opin Immunol* 2007, **19**(4):408-415.
8. Jagannathan P, Osborne CM, Royce C, Manion MM, Tilton JC, Li L, Fischer S, Hallahan CW, Metcalf JA, McLaughlin M, *et al.*: **Comparisons of CD8+ T cells specific for human immunodeficiency virus, hepatitis C virus, and cytomegalovirus reveal differences in frequency, immunodominance, phenotype, and interleukin-2 responsiveness.** *J Virol* 2009, **83**(6):2728-2742.
9. Champagne P, Ogg GS, King AS, Knabenhans C, Ellefsen K, Nobile M, Appay V, Rizzardi GP, Fleury S, Lipp M, *et al.*: **Skewed maturation of memory HIV-specific CD8 T lymphocytes.** *Nature* 2001, **410**(6824):106-111.
10. Migueles SA, Laborico AC, Shupert WL, Sabbaghian MS, Rabin R, Hallahan CW, Van Baarle D, Kostense S, Miedema F, McLaughlin M, *et al.*: **HIV-specific CD8+ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors.** *Nat Immunol* 2002, **3**(11):1061-1068.
11. Autran B, Carcelain J, Li TS, Blanc C, Mathez D, Tubiana R, Katlama C, Debre P, Leibowitch J: **Positive effects of combined antiretroviral therapy on CD4+ T cell homeostasis and function in advanced HIV disease.** *Science* 1997, **277**(5322):112-116.
12. Angel JB, Parato KG, Kumar A, Kravcik S, Badley AD, Fex C, Ashby D, Sun E, Cameron DW: **Progressive human immunodeficiency virus-specific immune recovery with prolonged viral suppression.** *J Infect Dis* 2001, **183**(4):546-554.
13. Rosenberg ES, Billingsley JM, Caliendo AM, Boswell SL, Sax PE, Kalams SA, Walker BD: **Vigorous HIV-1-specific CD4+ T cell responses associated with control of viremia.** *Science* 1997, **278**(5342):1447-1450.
14. Hess C, Altfeld M, Thomas SY, Addo MM, Rosenberg ES, Allen TM, Draenert R, Eldridge RL, van Lunzen J, Stellbrink HJ, *et al.*: **HIV-1 specific CD8+ T cells with an effector phenotype and control of viral replication.** *Lancet* 2004, **363**(9412):863-866.
15. Emu B, Sinclair E, Favre D, Moretto WJ, Hsue P, Hoh R, Martin JN, Nixon DF, McCune JM, Deeks SG: **Phenotypic, functional, and kinetic parameters associated with apparent T-cell control of human immunodeficiency virus replication in individuals with and without antiretroviral treatment.** *J Virol* 2005, **79**(22):14169-14178.
16. Potter SJ, Lacabaratz C, Lambotte O, Perez-Patrigeon S, Vingert B, Sinet M, Colle JH, Urrutia A, Scott-Algara D, Boufassa F, *et al.*: **Preserved central memory and activated effector memory CD4+ T-cell subsets in human immunodeficiency virus controllers: an ANRS EP36 study.** *J Virol* 2007, **81**(24):13904-13915.
17. Streeck H, Jolin JS, Qi Y, Yassine-Diab B, Johnson RC, Kwon DS, Addo MM, Brumme C, Routy JP, Little S, *et al.*: **Human immunodeficiency virus type 1-specific CD8+ T-cell responses during primary infection are major determinants of the viral set point and loss of CD4+ T cells.** *J Virol* 2009, **83**(15):7641-7648.
18. Northfield JW, Loo CP, Barbour JD, Spotts G, Hecht FM, Klenerman P, Nixon DF, Michaelsson J: **Human immunodeficiency virus type 1 (HIV-1)-specific CD8+ T(EMRA) cells in early infection are linked to control of HIV-1 viremia and predict the subsequent viral load set point.** *J Virol* 2007, **81**(11):5759-5765.
19. Pereyra F, Palmer S, Miura T, Block BL, Wiegand A, Rothchild AC, Baker B, Rosenberg R, Cutrell E, Seaman MS, *et al.*: **Persistent Low-Level Viremia in HIV-1 Elite Controllers and Relationship to Immunologic Parameters.** *J Infect Dis* 2009, **200**(6):984-990.
20. Rehr M, Cahenzli J, Haas A, Price DA, Gostick E, Huber M, Karrer U, Oxenius A: **Emergence of polyfunctional CD8+ T cells after prolonged suppression of human immunodeficiency virus replication by antiretroviral therapy.** *J Virol* 2008, **82**(7):3391-3404.
21. Lopez M, Soriano V, Rallon N, Cascajero A, Gonzalez-Lahoz J, Benito JM: **Suppression of viral replication with highly active antiretroviral therapy has no impact on the functional profile of HIV-specific CD8(+) T cells.** *Eur J Immunol* 2008, **38**(6):1548-1558.
22. Mellors JW, Rinaldo CR Jr, Gupta P, White RM, Todd JA, Kingsley LA: **Prognosis in HIV-1 infection predicted by the quantity of virus in plasma.** *Science* 1996, **272**(5265):1167-1170.
23. Shiu C, Cunningham CK, Greenough T, Muresan P, Sanchez-Merino V, Carey V, Jackson JB, Ziemniak C, Fox L, Belzer M, *et al.*: **Identification of ongoing human immunodeficiency virus type 1 (HIV-1) replication in residual viremia during recombinant HIV-1 poxvirus immunizations in patients with clinically undetectable viral loads on durable suppressive highly active antiretroviral therapy.** *J Virol* 2009, **83**(19):9731-9742.
24. Borrow P, Lewicki H, Hahn BH, Shaw GM, Oldstone MB: **Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection.** *J Virol* 1994, **68**(9):6103-6110.
25. Carmichael A, Jin X, Sissons P, Borysiewicz L: **Quantitative analysis of the human immunodeficiency virus type 1 (HIV-1)-specific cytotoxic T lymphocyte (CTL) response at different stages of HIV-1 infection: differential CTL responses to HIV-1 and Epstein-Barr virus in late disease.** *J Exp Med* 1993, **177**(2):249-256.
26. Koup RA, Safrit JT, Cao Y, Andrews CA, McLeod G, Borkowsky W, Farthing C, Ho DD: **Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome.** *J Virol* 1994, **68**(7):4650-4655.
27. Jin X, Bauer DE, Tuttleton SE, Lewin S, Gettie A, Blanchard J, Irwin CE, Safrit JT, Mittler J, Weinberger L, *et al.*: **Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques.** *J Exp Med* 1999, **189**(6):991-998.
28. Draenert R, Verrill CL, Tang Y, Allen TM, Wurcel AG, Boczanowski M, Lechner A, Kim AY, Suscovich T, Brown NV, *et al.*: **Persistent recognition of autologous virus by high-avidity CD8 T cells in chronic, progressive human immunodeficiency virus type 1 infection.** *J Virol* 2004, **78**(2):630-641.
29. Pantaleo G, Demarest JF, Schacker T, Vaccarezza M, Cohen OJ, Daucher M, Graziosi C, Schnittman SS, Quinn TC, Shaw GM, *et al.*: **The qualitative nature of the primary immune response to HIV infection is a prognosticator of disease progression independent of the initial level of plasma viremia.** *Proc Natl Acad Sci USA* 1997, **94**(1):254-258.

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