Long-term highly active antiretroviral therapy in chronic HIV-1 infection: evidence for reconstitution of antiviral immunity

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In this study we investigated the long-term effect of highly active antiretroviral therapy (HAART) on HIV-specific CD4⁺ T-cell responses in comparison with virus-specific CD4⁺ T-cell responses against the persistent herpes viruses cytomegalovirus (CMV) and Epstein–Barr virus (EBV). To this end, HIV- and herpes virus-specific cellular immune responses were measured longitudinally in 10 seroconverters with long-term follow-up including 55 months of successful suppression of viral load by HAART. HIV- and CMV-specific CD4⁺ T cells producing interferon-γ (IFNγ) or interleukin-2 (IL-2) were analysed as well as proliferative capacity. EBV-specific CD4⁺ T cells were determined using a 12-day *ex vivo* assay. Initiation of HAART resulted in a transient increase of HIV-specific IL-2⁺IFNγ⁺CD4⁺ T cells and, to a lesser extent, IL-2⁺CD4⁺ T cells. Long-term HAART resulted in an increase in HIV-, CMV- and EBV-specific CD4⁺ T-cell proliferative capacity. The increase in HIV- and herpes-virus-specific CD4⁺ T-cell proliferative capacity after 55 months of HAART suggests that the improved proliferative response is not specific for HIV, but reflects a more general improvement of antiviral immune responses, which is induced by HAART.

Introduction

Highly active antiretroviral therapy (HAART) has changed the course of HIV-1 infection, with clinical benefits for the patients [1–3]. Treatment with HAART results in a decrease in viral load often to undetectable levels which is paralleled by an increase in total numbers of CD4⁺ T cells [4–7]. Furthermore, successful treatment with HAART is characterized by a decrease in the expression of the activation markers HLA-DR and CD38 on both CD4⁺ and CD8⁺ T cells [8]. In addition to a strong decline in CD4⁺ T-cell numbers, activation of CD4⁺ and CD8⁺ T cells has been demonstrated to be predictive for progression to AIDS [9–11], a decrease in the expression of these markers suggests a better prognosis of HIV-1-infected individuals after successful treatment with HAART.

The effect of HAART on HIV-specific CD8⁺ cytotoxic T cells has been studied extensively [12–17], demonstrating that the HAART-induced decrease in HIV load is paralleled by a decrease in HIV-specific CD8⁺ T cells. However, results are inconclusive with respect to HIV-specific CD4⁺ T cells. Some studies reported detectable, but not enhanced, HIV-specific CD4⁺ T-cell responses in individuals receiving antiretroviral therapy in chronic HIV-1 infection [18–20], whereas responses to recall antigens or mitogens were generally found to be strongly restored by HAART [8,21,22]. Other studies reported a restoration of HIV-specific CD4⁺ T-cell proliferative capacity [23–28] as well as increased HIV-specific production of interleukin-2 (IL-2), but not interferon-γ (IFNγ) [29,30].

Also, the effect of HAART on the phenotype of HIV-specific CD4⁺ T cells is not yet fully understood. It has been reported that HAART treatment reverses the observed skewing of HIV-specific CD4⁺ T cell to the non-proliferating effector memory phenotype [29,30]. Other studies, however, failed to confirm these results [31].

Until now, most studies investigating the effect of HAART during chronic HIV-1 infection on HIV-specific cellular immune responses analysed T-cell functions after 1 year of HAART, although one study reported the effect of 4 years of HAART on total CD4⁺ T-cell responses [32]. However, as HAART was initiated in 1996, many individuals have been treated for

more than 7 years now. To our knowledge this is the first study in which the effect of an extended period of HAART (55 months) on HIV-specific CD4+ T-cell function is analysed.

Further novelty lies in the analysis of cellular immune responses against Epstein–Barr virus (EBV) and cytomegalovirus (CMV) after treatment with HAART to determine the effect of HAART on T-cell responses against widespread persistent herpes viruses.

Materials and methods

Study participants

Ten HIV-1 seropositive men of the Amsterdam Cohort on HIV-1 infection and AIDS with a known seroconversion date were selected based on human leukocyte antigen (HLA) type. All study participants were treatment naive at entry and did not develop AIDS-defining illnesses. Patients started therapy based on physician's advise or personal choice. None of the individuals were coinfected with hepatitis C. When therapy was initiated, the median CD4+ T-cell count was 320 cells/μl (range 150–500), the median CD8+ T-cell count was 1,100 cells/μl (range 800–2,190) and the median viral load was 28,500 copies/ml (range 1,000–310,000). Individuals were analysed early in infection (median 19.5 months after seroconversion; 72 months before the start of HAART; T1), just before HAART (median 82 months after seroconversion; 11 months before HAART, T2), soon after the start of HAART (median 100 months after seroconversion; 7.5 months after HAART; T3) and late in infection (median 154 months after seroconversion; 55.5 months after HAART; T4). Follow-up of each individual is described in more detail in Table 1. From all time points, cryopreserved peripheral blood mononuclear cells (PBMCs) were available for the analyses of cellular immune responses. All samples from individual study participants were assayed in parallel to avoid day-to-day assay variations. Viability and recovery were similar between early and late time points. HAART was defined as a regimen containing at least two nucleotide or nucleoside reverse transcriptase inhibitors (NRTIs) and one protease inhibitor (PI), or one NRTI and two PIs. Most of the patients did not switch their regimen dramatically. In some instances a reverse transcriptase inhibitor of PI was switched for a different reverse transcriptase inhibitor of PI due to toxicity. This did not effect their response to therapy, as viral load remained suppressed in all individuals. HLA type, Centre of Disease Control stages, follow-up and the HAART regimen of all study participants are summarized in Table 1. From all participants of the Amsterdam Cohort studies on HIV-infection and AIDS informed written consent was obtained and this study has been approved by the medical ethical committee.

Tetramer staining and flow cytometry

MHC class I tetramers complexed with virus specific-peptides were produced as previously described [33,34]. Immunodominant peptides from HIV [35–37], EBV [38,39] and CMV [40] were selected and synthesized by the Netherlands Cancer Institute (Amsterdam, the Netherlands). Next, the peptides were complexed with the corresponding HLA-A201, B801 and B5701 proteins. Refolded HLA peptide complexes were biotinylated and subsequently tetramerized by the addition of streptavidin-allophycocyanin (APC) or streptavidin-phycoerythrin (PE). Four-colour fluorescence analysis was performed. Briefly, PBMCs were thawed and $1–1.5\times10^6$ cells were stained in phosphate buffered saline supplemented with 0.5% (v/v) bovine serum albumin (PBA) with αCD8–PerCP (Beckton Dickinson, San José, Calif., US), αCD27–FITC (Sanquin Reagents, Amsterdam, the Netherlands) and two different HLA–peptide complexes, conjugated to

Table 1. Characteristics of the HIV-1 infected homosexual men who participated in this study

Patient	CDC stage	HLA type	Follow-up (months)	Time on HAART (months)	HAART regimen
36	B*	A1, A32, B8, B44	120	47	3TC, Ind, Saqu
156	B	A11, A28, B8, B38	203	58	3TC, Ind, Saqu
164	B	A1, A24, B8, B44	145	50	AZT, 3TC, ddC, Ind, Rit
188	B	A2, B7	140	48	3TC, ddC, Ind, Rit
523	C†	A1, A33, B8, B44	104	42	3TC, ddC, Ind, Saqu
545	B	A1, A24, B8	152	53	AZT, 3TC, ddC, Ind, Saqu
1194	C	A11, A24, B8, B62	164	80	AZT, 3TC, Saqu
1113	B	A1, A11, B8, B57	156	71	3TC, ind, Saqu
1140	B	A2, A11, B40, B52	182	60	AZT, 3TC, ddC, Ind, Saqu
1186	B	A1, A24, B7	188	76	AZT, 3TC, ddC, Ind, Saqu

*Asymptomatic HIV infection. †Hairy leukoplakia. 3TC, lamivudine; AZT zidovudine; CDC, Centre of Disease Control; ddC, zalcitabine; HAART, highly active antiretroviral therapy; HLA, human leukocyte antigen; Ind, indinavir; Rit, ritonavir; Saqu, saquinavir.

either PE or APC. To determine the level of immune activation, 3×10^5 PBMCs were stained with αCD4–APC, αCD8–PerCP, αHLA–DR–FITC (Beckton Dickinson) and αCD38–PE (Sanquin Reagents). Cells were then fixed in Cellfix (Beckton Dickinson) and at least 200,000 events were acquired using a FACSCalibur flow cytometer (Beckton Dickinson). Lymphocytes were gated by forward and sideward scatter. Data were analysed using the software program CELLQuest (Beckton Dickinson).

Intracellular cytokine staining after antigenic stimulation

Cryopreserved PBMCs were thawed using RPMI-1640 medium supplemented with penicillin, streptomycin and 20% inactivated FCS and subsequently washed in 10% FCS medium. Cells were aliquoted at 2×10^6 cells per ml in round bottom tubes (polystyrene, Falcon, Beckton Dickinson). Cells were stimulated with a gag-peptide pool (15mers with 11 overlap, HXB2, NIH AIDS Research and Reagent program, Bethesda, Md., US) or a 1/100 dilution of CMV lysate (Microbix Biosystems Inc, Toronto, ON, Canada) in the presence of co-stimulation: 2 μg/ml αCD28 (Sanquin Reagents) and 2 μg/ml αCD49d (Pharmingen, San José, Calif., US). The concentration of the individual peptides within the pool was 2 μg/ml. As a positive control, phorbol 12-myristate 13-acetate (PMA)/ Ionomycin was used. After 1 h, Brefeldin A (Beckton Dickinson) was added and cells were incubated for another 5 h at 37°C, 5% CO_2. Next, cells were fixed and permeabilized (permeabilization kit, Beckton Dickinson) and stained with αCD3–PerCP, αCD4–APC, αIL-2–PE and αIFNγ–FITC (Beckton Dickinson) for 20 min at 4°C. At least 300,000 events were acquired by flowcytometry as described before. Frequencies of IFNγ and IL-2-producing cells were reported after subtraction of the frequencies in medium controls.

Antigen-specific proliferation

In vitro T-cell proliferation to HIV peptide pools was measured using CFSE (5,6-carboxyfluorescein diacetate succinimidyl ester; Molecular Probes, Leiden, the Netherlands) according to the manufacturer's protocol. Briefly, PBMCs were thawed and cells were labelled using CFSE for 8 min, labelling was stopped using human pool serum (HPS). Cells were washed and 2×10^6 cells in 0.5 ml RPMI-1640 medium were aliquoted in round bottom tubes. Cells were then stimulated with a gag peptide pool or 1/100 dilution of CMV lysate. As a positive control, 0.02 μg αCD3 and 2 μg αCD28 (Sanquin Reagents) was used. After 6 days of incubation at 37°C, 5% CO_2, cells were stained using αCD4–APC and αCD3–PerCP (Beckton Dickinson) as described before. Stimulation indices (SIs) were calculated by dividing the % proliferation $CD3^+CD4^+$ T cells after stimulation by the % proliferation of unstimulated cells.

Detection of EBV-specific $CD4^+$ T cells

As the frequency of EBV-specific $CD4^+$ T cells *ex vivo* was too low to detect, EBV-specific T cells were cultured for 12 days, in the presence of EBNA1 or BZLF peptide pools and IL-2 as previously described [41]. Next, cells were re-stimulated with the EBNA1 peptide pool (concentration of each peptide 2 μg/ml; 15 mers with 11 overlap, carboxy-terminal region of EBNA1, Jerini AG, Berlin, Germany) to enumerate the $IFN\gamma^+CD4^+$ T cells using the same protocol as used for the stimulation of HIV- and CMV-specific $CD4^+$ T cells. Specific central memory $CD4^+$ T cells capable of both proliferation and IFNγ production in response to antigen are measured by this method, and were shown to correlate with protection against hepatitis C virus and malaria [42,43].

Plasma HIV-1 RNA determination

Plasma HIV-1 RNA was measured using the NucliSens HIV-1 QT assay (bioMérieux, Boxtel, the Netherlands) with a detection limit of 1,000 copies/ml and the Amplicor HIV monitor (Roche Diagnostic Systems Inc, Branchburg, NJ, US) with a detection limit of 400 copies/ml. From August 1999 onward, plasma HIV-1 RNA was determined by the more sensitive Quantiplex bDNA 3.0 assay (Bayer Corporation, Tarrytown, NY, US) resulting in a detection limit of 50 copies/ml.

Determination of $CD4^+$ and $CD8^+$ T-cell numbers

PBMCs were isolated from heparinized venous blood by density gradient centrifugation on Ficoll–Paque (Amersham Pharmacia, Uppsala, Sweden). Lymphocyte immunophenotyping was accomplished by flow cytometry.

Statistical analyses

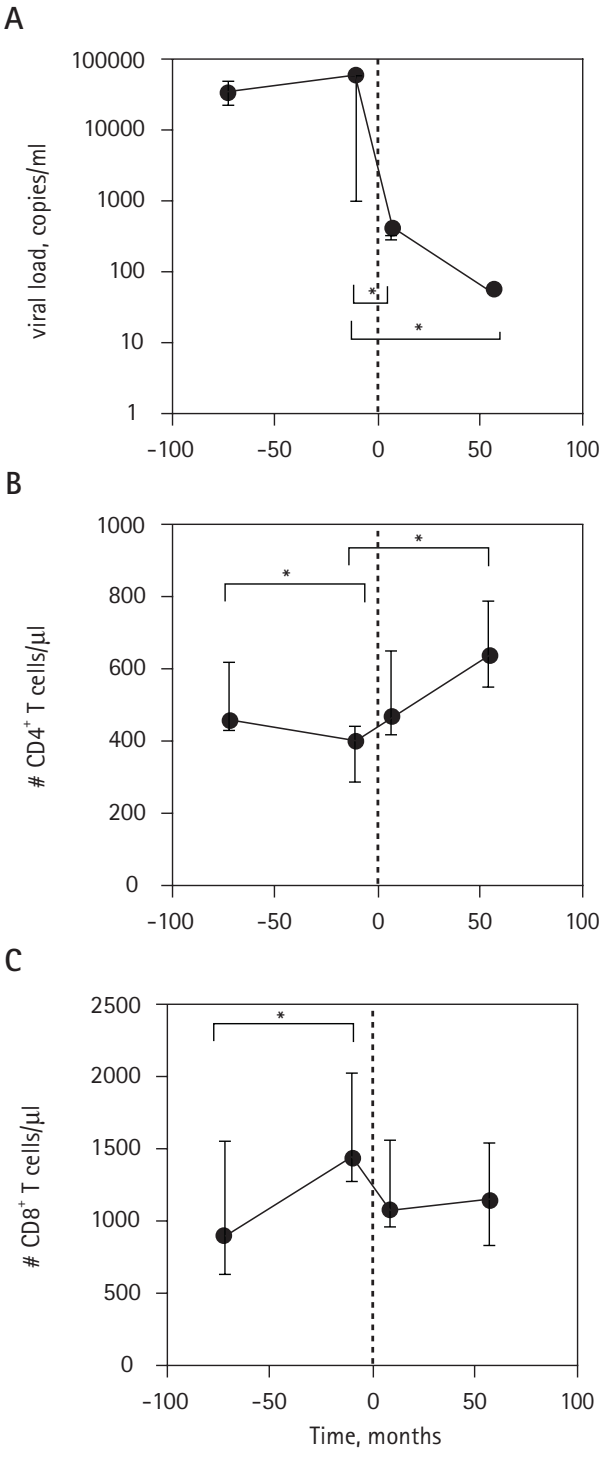
Non-parametric tests were performed to avoid the assumption of normally distributed data sets. Wilcoxon tests were performed to determine differences over time. Correlations were tested using the Spearman's correlation test. All statistical analyses were performed using the software program SPSS 10.0 (SPSS Inc, Chicago, Ill, US).

Results

Effect of long-term HAART on viral load, $CD4^+$ T-cell numbers and activation markers

Treatment with HAART in this selected group of virological responders resulted in a decrease in viral load from a median of 57,000 at 10.5 months before to 400

Figure 1. Effect of long-term HAART on viral load, CD4⁺ and CD8⁺ T-cell numbers



Viral load **(A)**, total numbers of CD4⁺**(B)** and CD8⁺**(C)** T cells in 10 HIV-1 infected individuals at two time points before and two time points after the start of highly active antiretroviral therapy (HAART) are shown. For each time point, the median is indicated, together with the 25th and 75th percentiles. Initiation of HAART is indicated by a dotted line. Significant changes in CD4⁺ T-cell responses and viral load were calculated using the Wilcoxon signed ranks test and are indicated by an asterix.

copies/ml at 7.5 months after HAART (P=0.009, Wilcoxon) and 50 copies/ml at 55.5 months after HAART (P=0.04; T2 vs T4, Figure 1A). Total CD4⁺ T-cell numbers tended to increase from a median of 400 cells/µl just before HAART to 460 cells/µl at 7.5 months after HAART (P=0.059) and 550 cells/µl at 55.5 months after HAART (P=0.107; T2 vs T4, Figure 1B). No effect of HAART on total CD8⁺ T-cell numbers was observed (Figure 1C).

Furthermore, HAART treatment resulted in a decrease in the fraction of HLA-DR⁺CD38⁺ CD4⁺ T cells from 11.2% 10.5 months before HAART to 3.8% 7.5 months after HAART (P=0.037) and 3.9% at 55.5 months after HAART (P=0.037; T2 vs T4, Table 2). Also the expression of CD38 and HLA-DR on CD8⁺ T cells decreased by HAART from 19.5% 10.5 months before, to 7.5% 10 months after HAART (P=0.013) and 2.7% at 55.5 months after HAART (P=0.009; T2 vs T4, Table 2). Thus, long-term HAART resulted in a strong and persistent decrease in viral load, an increase in CD4⁺ T-cell numbers and a decrease in the expression of activation markers on CD4⁺ and CD8⁺ T cells, related to a decrease of systemic immune activation.

Effect of HAART on HIV-specific cellular immune responses

To investigate the long-term effect of HAART on cellular immune responses, numbers of HIV-specific CD8⁺ T cells were analysed as well as HIV-specific CD4⁺ T-cell function before and after initiation of HAART. One year of HAART resulted in a (non-significant) decrease in HIV-specific CD8⁺ T-cell numbers from 12,890/ml at 10.5 months before HAART to 6,319/ml at 7.5 months after HAART (P=0.066, Table 2). After 5 years of HAART, HIV-specific CD8⁺ T-cell numbers were significantly lower than pre-HAART levels (7,533/ml at 55.5 months after HAART, P=0.017). Expression of CD27 on HIV-specific CD8⁺ T cells was not influenced by HAART (Table 2). Treatment with HAART had variable effects on the Gag-specific cytokine production. In Figure 2A, a representative fluorescence activated cell sorter (FACS) plot of HIV-specific cytokine production before and during treatment is shown. An overview of percentages of HIV-, CMV- and PMA/Ionomycin-induced production of IFNγ, IL-2 and IL-2&IFNγ is shown in Table 3. Next, indexed values of cytokine producing CD4⁺ T cells were calculated, with the percentage cytokine production determined early in infection (19.5 months after seroconversion) set at 100% (Figure 3). HIV-specific IFNγ⁺CD4⁺ T cells tended to decrease from 28.6% 10.5 months before HAART to 14.3% 7.5 months after HAART (P=0.249) and 14.3% 55.5 months

Table 2. Effect of long term HAART on activation markers, numbers and differentiation status of HIV-specific CD8+ T cells

	Time*	%CD4+38+DR+†	%CD8+38+DR+‡	# tetramers§	%CD8+CD27-¶
72 months before HAART (T1)	19.5	4.4	15.5	11,160	58.2
Range	(8–75)	(1.9–9.2)	(9.5–23.6)	(0–42,903)	(19.9–80.3)
10.5 months before HAART (T2)	82.4	11.2$	19.5	12,890	53.2
Range	(48–144)	(1.2–17.4)	(2.7–40.2)	(0–187,488)	(33.4–76.0)
7.5 months after HAART (T3)	99.7	3.8♠	7.5♠	6,319	51.3
Range	(67–158)	(1.7–14.5)	(3.0–29.1)	(0–53,192)	(12.9–78.2)
55.5 months after HAART (T4)	153.7	3.9♠	2.7♠	7,553♠	56.4
Range	(104–203)	(7.8–52.5)	(1.3–14.5)	(0–31,458)	(32.8–78.5)

*Median months after seroconversion. †Median expression of activation markers on CD4+ T cells. ‡Median expression of activation markers on CD8+ T cells. §Median numbers of HIV-specific CD8+ T cells per µl (sum of the responses for the different tetramers used in each individual). ¶Median percentage of HIV-specific CD27 negative CD8+ T cells. $Significantly different from first time point. ♠Significantly different from pre highly active antiretroviral therapy (HAART; $P<0.05$).

Table 3. Cytokine production by CD4+ T-helper cells before and during HAART

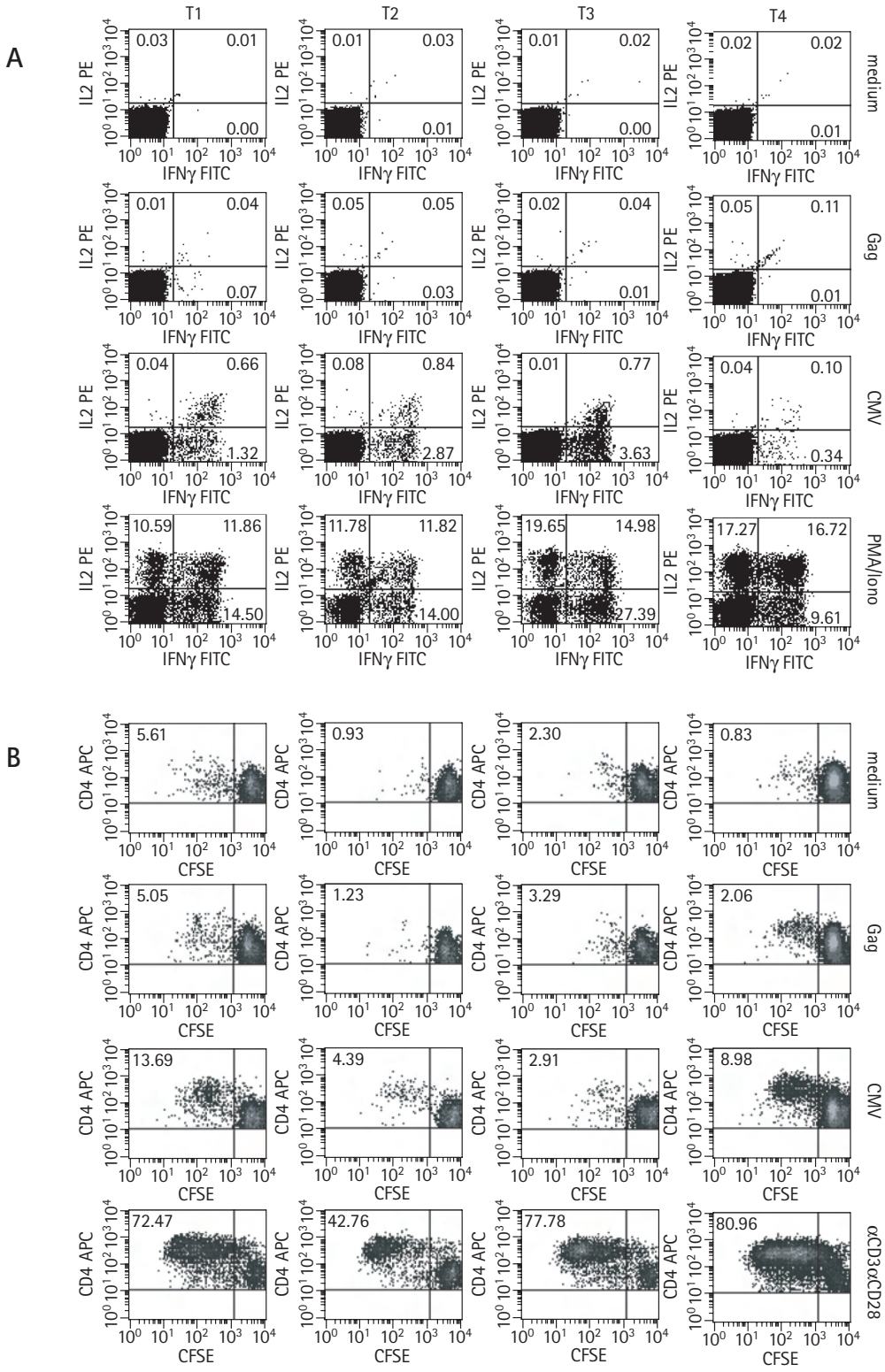
	72 months before HAART (T1)		10.5 months before HAART (T2)		7.5 months after HAART (T3)		55.5 months after HAART (T4)	
	Median	Range	Median	Range	Median	Range	Median	Range
Gag								
%IFNγ	0.05*	(0–0.24)	0.03	(0–0.09)	0.01	(0–0.08)	0.01	(0–0.04)
%IL-2	0.03	(0–0.15)	0	(0–0.06)	0.02	(0–0.17)	0.02	(0–0.10)
%IL-2&IFNγ	0.10	(0–0.40)	0.05	(0.02–0.17)	0.09	0.02–0.20	0.06	(0–0.22)
Proliferation	1.0†	(0.6–14.0)	1.1	(0.6–77.8)	1.4	(0.5–22.2)	2.3	(1–45)
CMV								
%IFNγ	0.90	(0.13-7.72)	1.53	(0.07–21.37)	0.95	(0.02–10.76)	0.28	(0–14.12)
%IL-2	0.03	(0-0.43)	0.02	(0–1.27)	0.01	(0–0.61)	0.02	(0–0.12)
%IL-2&IFNγ	0.24	(0-0.94)	0.23	(0–1.59)	0.39	(0–0.87)	0.08	(0–2.98)
Proliferation	3.56	(1.2–41.9)	3.8	(1.0–10.0)	3.1	(0.8–15.2)	12.3	(2.6–34.9)
PMA/Iono								
%IFNγ	32.43	(5.62–45.55)	34.44	13.36–66.64	26.92	(8.44–66.67)	28.51	(12.17–64.56)
%IL-2	13.95	(6.03–30.03)	11.38	7.56–23.28	11.64	(7.85–37.58)	19.43	(3.87–36.51)
%IL-2&IFNγ	7.89	(3.24–11.25)	5.70	3.09–12.28	5.73	(2.95–13.50)	11.25	(3.56–20.93)
Proliferation	10.9	(3.9–70.3)	12.2	(1.0–77.8)	13.2	(0.8–48.0)	19.6	(10.0–39.0)

*Percentage of cytokine+ CD3+ CD4+ T cells. †Simulation index as described in Materials and methods. CMV, cytomegalovirus; HAART, highly active antiretroviral therapy; IFNγ, interferon-γ, IL-2, interleukin-2 PMA/Iono, phorbol 12-myristate 13-acetate/Ionomycin.

after HAART (P=0.866, T2 vs T4). For HIV-specific CD4+ T cells producing single IL-2 or IL-2&IFNγ, a different pattern was observed. Treatment with HAART initially resulted in a slight increase in the indexed fraction of HIV-specific IL-2+ CD4+ T cells from 45% just before to 75% at 7.5 months after HAART (P=0.12; T2 vs T3). Also IL-2+&IFNγ+ CD4+ T cells increased after start of HAART (from 39.7% to 51.3%, P=0.046, T2 vs T3). However, this HAART-induced increase in IL-2+ and IL-2+&IFNγ+ CD4+ T cells was only transient, as 55.5 months after the initiation of HAART slightly lower amounts of HIV-specific IL-2 producing CD4+ T cells (66.7%, P=0.106; T2 vs T4) and significantly lower numbers of IL-2+&IFNγ+ CD4+ T cells (20%, P=0.008; T2 vs T4) were found compared with pre-HAART levels. Overall frequencies of HIV-specific CD4+ T cells show similar kinetics as the single-cytokine producing T cells after HAART (data not shown).

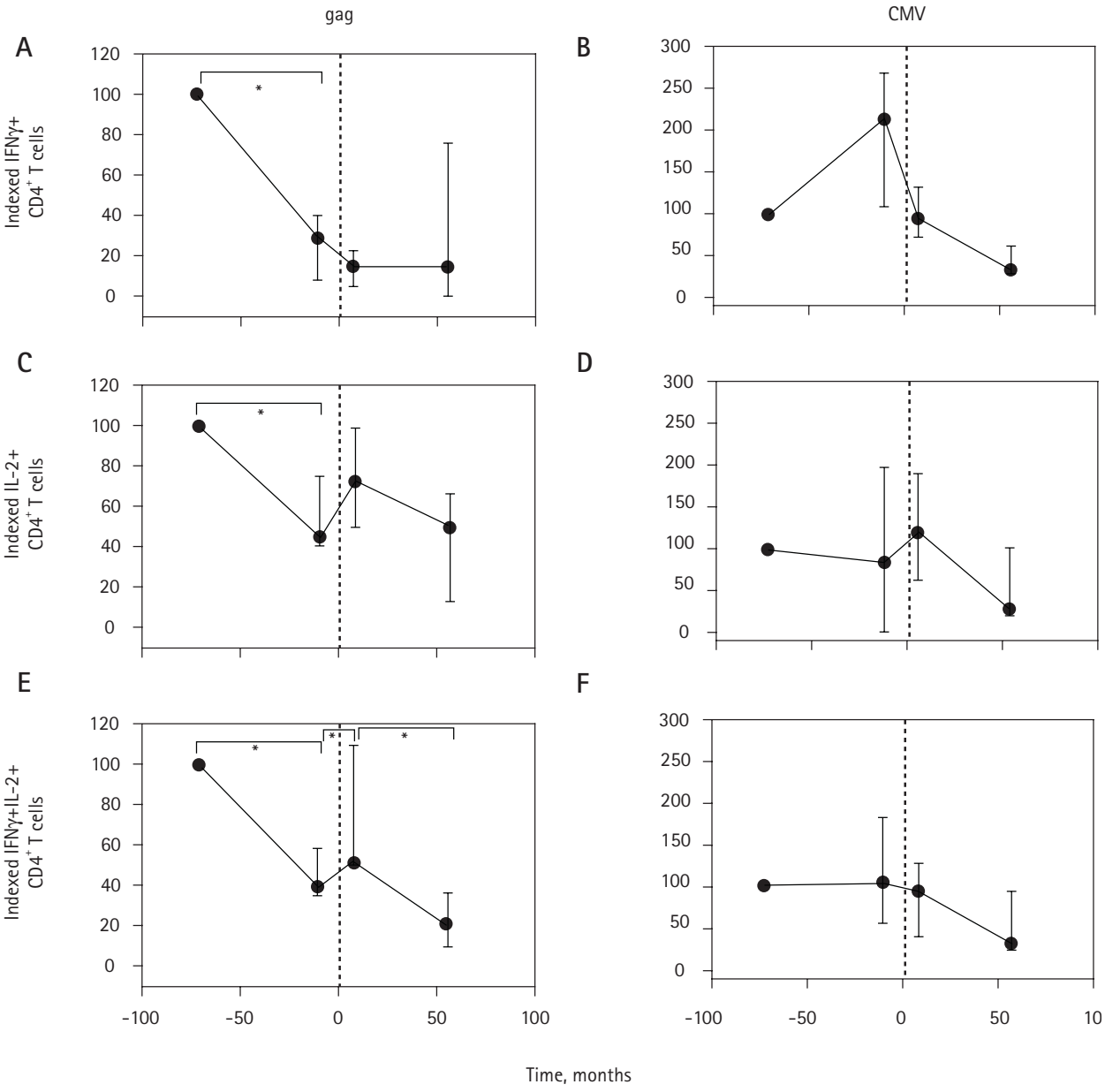
Thus, HAART resulted in a decrease in HIV-specific CD8+ T-cell numbers and HIV-specific IFNγ+ CD4+ T cells, which was sustained by 5 years of HAART. Interestingly, HIV-specific IL-2+&IFNγ+ CD4+ T cells, and to a lesser extent IL-2+CD4+ T cells showed a transient increase after the start of HAART. However, after 5 years these responses had decreased again, even below pre-HAART levels.

Figure 2. Representative FACS plots showing cytokine production and proliferation of CD4+ T cells before and during HAART



(A) A representative staining of IFNγ+, IL-2+ and IL-2+&IFNγ+ CD4+ T cells is shown for the unstimulated control, after stimulation with a gag-peptide pool, cytomegalovirus (CMV) lysate and a combination of PMA (phorbol 12-myristate 13-acetate)/Iono (Ionomycin). Results are gated on the CD3+CD4+ cells. Percentages of cytokine positive CD4+ T cells are indicated. **(B)** A representative staining of labelled cells after a 6 day culture in the presence of medium (unstimulated control), a gag-peptide pool, CMV lysate and a polyclonal stimulation (aCD3&aCD28). Gating was performed on the CD3+CD4+ T cells. Percentages of cells that have divided at least once, are indicated in the upper left corner. APC, allophycocyanin; CFSE, 5,6-carboxyfluorescein diacetate succinimidyl ester; FACS, fluorescence-activated cell sorter; HAART, highly active antiretroviral therapy; T1, 72 months before HAART; T2, 10.5 months before HAART; T3 7.5 months after HAART; T4 55.5 months after HAART.

Figure 3. Effect of long-term HAART on HIV- and CMV-specific cytokine producing CD4+ T cells



CD4+ T-cell responses were determined in 10 HIV-1 infected individuals at two time points before and two time points after the start of highly active antiretroviral therapy (HAART). Responses were related to responses determined at the first time point. For each time point, the median indexed response is shown, together with the 25th and 75th percentiles. Gag-specific production of interferon γ [IFNγ; **(A)**, interleukin (IL) 2 **(C)** and IL2+IFNγ **(E)**] is shown as well as the cytomegalovirus (CMV)-specific production of IFNγ **(B)**, IL-2 **(D)** and IL-2&tIFNγ **(F)**. Initiation of HAART is indicated by a dotted line. Significant changes in CD4+ T-cell responses were calculated using the Wilcoxon signed ranks test and are indicated by an asterix.

Long-term effect of HAART on CMV-specific cellular immune responses

To determine the effect of HAART on immune responses against persistent herpes viruses, numbers of EBV- and CMV-specific CD8+ T cells were measured, as well as CMV-specific CD4+ T-cell function. No differences in EBV-specific CD8+ T-cell numbers after HAART were observed [14,300/ml 10.5 months before HAART; 15,300/ml at 7.5 months after HAART; 14,000/ml at 55.5 months after HAART, P=0.26 and P=0.77 respectively; data not shown]. Because of the HLA type of the individuals, we were only able to measure CMV-specific CD8+ T cells in 3 out of 10 individuals. In this small group of individuals we did not

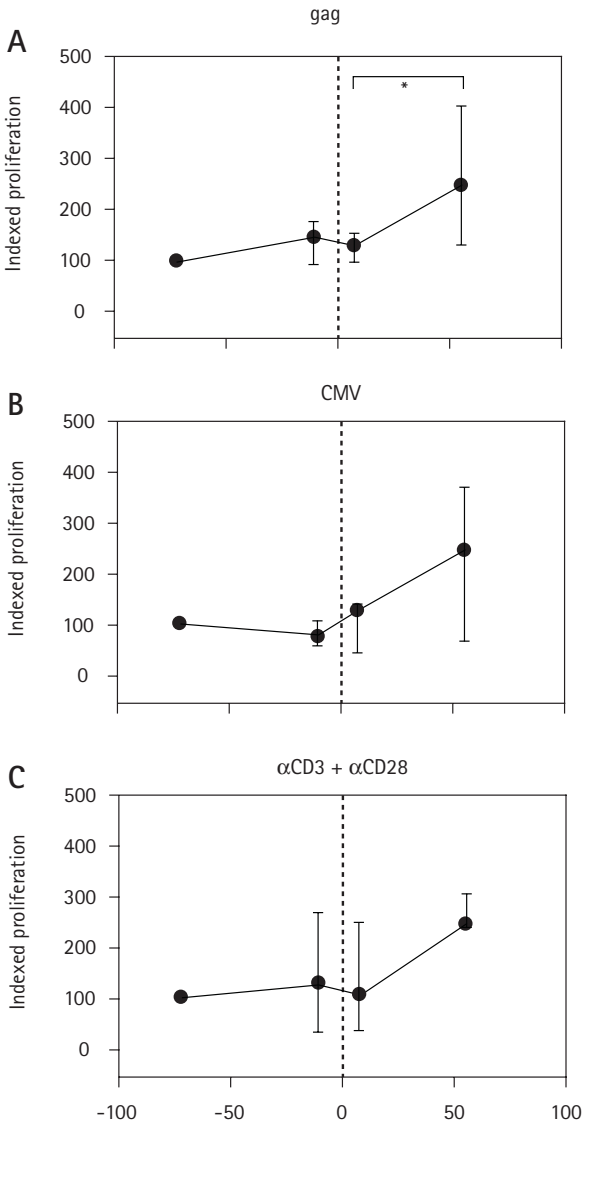
observe evidence for an effect of HAART on CMV-specific CD8$^+$ T-cell numbers (data not shown). However, we were able to determine CMV-specific cytokine-producing CD4$^+$ T cells in all 10 individuals (Figure 2B; Table 3). Again indexed values were calculated, with the percentage cytokine production determined early in infection (19.5 months after seroconversion) set at 100% (Figure 3). CMV-specific IFNγ^+CD4$^+$ T-cell percentages strongly increased before the initiation of HAART, to 214.8% 10.5 months before HAART (P=0.037; T1 vs T2). Following treatment with HAART, these cells tended to decrease to 94.1% (P=0.09; T2 vs T3) at 7.5 months after HAART and to 33.2% at 55.5 months after HAART (P=0.007; T2 vs T4). CMV-specific IL-2$^+$ and IL-2$^+$&IFNγ^+CD4$^+$ T cells did not change upon the initiation of treatment. Interestingly, during HAART both IL-2$^+$ and IL-2$^+$&IFNγ^+ CD4$^+$ T cells decreased from 121 to 27.9% and 93 to 30.2% respectively), although this was not significant (P=0.39 and P=0.57 respectively).

In conclusion, HAART did not influence EBV-specific CD8$^+$ T-cell numbers. CMV-specific IFNγ^+ CD4$^+$ T cells tended to decrease at 7.5 months after the initiation of HAART. In the long term, frequencies of all CMV-specific cytokine-producing CD4$^+$ T cells were diminished compared with frequencies early in HIV infection.

Restoration of HIV- and herpes-virus-specific CD4$^+$ T-cell proliferative capacity after 5 years of HAART

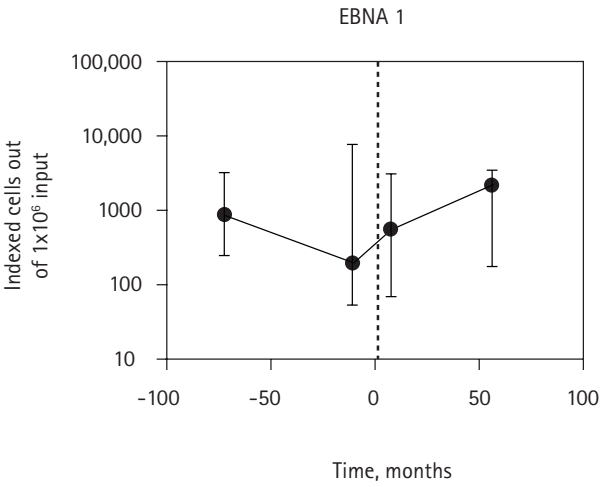
With respect to the effect of HAART on the proliferative capacity of HIV- and herpes virus-specific CD4$^+$ T cells, discrepant results have been reported. In this study, proliferative responses of CD4$^+$ T cells after stimulation with Gag, CMV and EBV, and polyclonal stimulation by a combination of αCD3 and αCD28 were analysed early in HIV-1 infection, approximately 1 year before HAART and 7.5 months and 55.5 months after the initiation of HAART (Figure 1B, Table 3). Indexed values were calculated, with the proliferative responses determined early in infection set as 100%. HIV-specific proliferation was not changed after 7.5 months of HAART (P=0.508). However, after 5 years of HAART an increase in the *in vitro* proliferative capacity was observed from 146.5% at 10.5 months before HAART to 249% at 55.5 months of HAART (P=0.028; Figure 4A). Similar results were observed for CMV-specific CD4$^+$ T-cell proliferative responses which increased from 79.6% 10.5 months before to 126% 7.5 months after HAART (P=0.57), and 247% at 55.5 months after HAART (P=0.04) (Figure 4B). Also the responses to polyclonal stimuli tended to increase during 5 years on HAART from 63.5 (7.5 months after) to 253% (55.5 months after, P=0.09; Figure 4C). Furthermore, when the expansion of EBV- (EBNA1) specific CD4$^+$ T cells was measured using a 12-day *in vitro* expansion assay, similar results were found; after 55.5 months of therapy the CD4$^+$ T-cell responses increased from

Figure 4. Effect of long term HAART on HIV- and non-HIV-specific CD4$^+$ T cell proliferative capacity



Proliferation of gag-specific CD4$^+$ T cells **(A)**, cytomegalovirus (CMV)-specific CD4$^+$ T cells **(B)** and polyclonal stimulated CD4$^+$ T cells **(C)** was determined using 5,6-carboxyfluorescein diacetate succinimidyl ester in 10 HIV-1 infected individuals. Stimulation indices were calculated as described in Materials and methods. Responses were related to responses determined at the first time point. For each time point, the median indexed proliferation is shown, together with the 25th and 75th percentiles. Initiation of highly active antiretroviral therapy (HAART) is indicated by a dotted line. Significant changes in CD4$^+$ T-cell responses were calculated using the Wilcoxon signed ranks test and are indicated by an asterix.

Figure 5. Effect of long term HAART on EBV-specific CD4+ T cells



CD4+ T cells from 10 HIV-1 infected individuals were expanded after 12 days of culture with an Epstein-Barr virus (EBV) EBNA1 peptide pool. On the Y axis is plotted the number of specific CD4+ T cells determined after 12 days expansion *in vitro* per $1x10^6$ peripheral blood mononuclear cells input. Responses were related to responses determined at the first time point. For each time point, the median indexed number of specific CD4+ T cells after 12 day expansion is shown, together with the 25th and 75th percentiles. Initiation of highly active antiretroviral therapy (HAART) is indicated by a dotted line. Significant changes in CD4+ T-cell responses were calculated using the Wilcoxon signed ranks test and are indicated by an asterix.

21.6% (10.5 months before) to 241.7% (P=0.02; Figure 5).

Thus, long-term HAART resulted in an increased proliferation of HIV-specific CD4+ T cells. In parallel, proliferation of CMV, EBV as well as polyclonally stimulated CD4+ T cells increased. This may indicate a general immune reconstitution after more than 5 years of treatment with HAART.

Discussion

In this study we investigated the effect of long-term HAART on HIV-specific T-cell responses and compared these results with responses against CMV and EBV. We selected for patients who were successfully treated with HAART, as was reflected by a decrease in viral load, and an increase in CD4+ T-cell numbers. The expression of the activation markers HLA-DR and CD38 on these CD4+ and CD8+ T cells was decreased after 5 years of HAART. Furthermore, 5 years of treatment with HAART resulted in a decrease in HIV-specific CD8+ T-cell numbers. Previous studies showed decreases in HIV-specific CD8+ T-cell responses after 1 year of HAART [12–17]. Also HIV-specific IFNγ+CD4+ T cells decreased, and this effect was sustained after 5 years of HAART treatment. HIV-specific IL2+ and IL-2+&IFNγ+CD4+ T-cell responses showed a transient increase after the start of HAART, which was only temporary as these responses declined after 5 years of HAART, even below pre-HAART levels. The increase in IL-2+CD4+ T cells by 1 year of HAART had been reported previously in cross-sectional studies, suggesting a restoration of HIV-specific CD4+ T-cell responses by HAART [29,30]. However, this longitudinal analysis demonstrates that HAART does not induce sustained increased HIV-specific CD4+ T-helper activity.

The different effect of HAART on the various populations of HIV-specific cytokine producing cells may partly explain contradictions in the previously reported results of the effect of HAART on HIV-specific CD4+ T-cell responses. Pitcher *et al.* demonstrated a decrease of HIV-specific CD4+ T cells after 1 year of HAART [44], whereas an increase in HIV-specific CD4+ T cells has been described by others [29,30]. However, Pitcher *et al.* used the production of IFNγ as a read out of HIV-specific CD4+ T-cell function, whereas the latter studies also measured IL-2 production. Our results clearly show a discrepancy between HIV-specific IFNγ and IL-2 producing CD4+ T cells after HAART, which indicates that the observed effect of HAART on HIV-specific CD4+ T cells clearly depends on the population of cytokine-producing CD4+ T cells that is analysed.

Our data fit the differentiation model of CD4+ T cells described by Younes *et al.* [45], which is based on the linear differentiation models proposed by Lanzavecchia *et al.* [46]. Younes *et al.* assume that antigen (HIV) drives the differentiation from naive to central memory T cells (TCMs), which produce IL-2 and have self-renewal capacity, to effector memory T cells (TEMs), which lack proliferative potential upon re-stimulation by antigen. In individuals with a high viral load, TCMs are preferentially induced to differentiate into the IFNγ-only-producing TEM cells. Upon initiation of HAART, viral load strongly decreases and cells are no longer driven to this end-stage IFNγ-only-producing population, but remain of the IL-2-producing TCM phenotype. Indeed, in our study, after 7.5 months of HAART a decrease in HIV-specific IFNγ+CD4+ T cells was observed (reflecting the diminished differentiation to end-stage IFNγ-only cells) while the HIV-specific IL-2+ and IFNγ+IL-2+ CD4+ T cells increased (reflecting the preservation of the TCM population). However, after 5 years of HAART both IFNγ+, IL-2+ and IL-2+&IFNγ+CD4+ T cells decrease. This probably reflects a situation with very low antigen exposure where naive T cells are no longer primed to differentiate.

Interestingly, before HAART an increase in the CMV-specific IFNγ+CD4+ T cells is observed, which most likely reflects a higher rate of CMV reactivation

(in parallel to increased immune activation markers) resulting in increasing antigenic load. Ten months after the initiation of HAART, a decrease in IFNγ-producing CMV-specific CD4+ T cells was observed. As described before, CMV-specific IL-2+ and IL-2+&IFNγ+ T cells were not influenced by 7.5 months of HAART [29]. Eventually, after 5 years of HAART, CMV-specific cytokine-producing CD4+ T cells declined to levels below baseline (19.5 months after seroconversion). As HAART results in a loss of CMV viraemia [47,48], this decrease in functional CMV-specific CD4+ T cells may simply reflect the low level of antigen after 5 years of HAART. Low levels of CMV-reactivation after HAART may also explain the observed decrease in CMV-specific CD4+ T cells after a short period on HAART, as the lower level of CMV reactivation may fail to drive the CMV-specific CD4+ T cells to the IFNγ-producing TEM subtype, while the IL-2-producing TCM population is still replenished.

Long-term treatment with HAART resulted in increased proliferative capacity of HIV-specific CD4+ T cells. Increased proliferation of HIV-specific CD4+ T cells was previously observed in some studies [23–28], whereas other studies could not confirm these results [21,22]. The discrepancy may be explained by the drug regimen and viral load levels attained by HAART. Also different techniques to measure proliferation *ex vivo* and the nature of the stimuli may play a role. Furthermore, duration of therapy may influence the results, as the increase in proliferation in the present study was only observed after 5 years of therapy, and not after 1 year. Most previous studies analysed HIV-specific CD4+ T-cell responses up to approximately 1 year of HAART.

Interestingly, CMV- and EBV-specific as well as polyclonal CD4+ T-cell proliferative responses were increased after 5 years of HAART. This may indicate a general reconstitution of antiviral immune responses after long-term HAART, instead of an increase in only HIV-specific CD4+ T-cell proliferative responses. To determine from which time point exactly proliferative responses are restored, future studies should investigate the 1–5 year time span in more detail.

In conclusion, initiation of HAART resulted in a transient increase in HIV-specific IL-2+&IFNγ+CD4+ T cells and, to a lesser extent, IL-2+CD4+ T cells. Moreover, after 5 years of successful HAART HIV-specific proliferative responses were increased. CMV- and EBV-specific CD4+ T-cell responses were restored as well. This suggests that the improved proliferative response is not specific for HIV, but reflects a more general HAART-induced improvement of antiviral immune responses.

Acknowledgements

This study is part of the Amsterdam Cohort Studies on HIV infection and AIDS; a collaboration of the Municipal Health Service, The Academic Medical Centre and Sanquin Research at CLB. The authors thank M.Th.L. Roos, L. Dekker and co-workers for the processing of patient samples. Furthermore, we thank G. Van Schijndel for the synthesis of all tetramers used in this study and J.A. Borghans for critically reading the manuscript. The Gag-derived overlapping peptide pool (15-mers with 11 overlap, HXB2) was obtained through the NIH AIDS Research and Reference Reagent program, Division of AIDS, NIAID, NIH.

This study was financially supported by AIDS Funds Netherlands, grant 5005 and grant CLBD2000-2164 of the Dutch Cancer Society.

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Received 29 March 2005, accepted 28 November 2005