HAART in HIV-infected patients: restoration of antigen-specific CD4 T-cell responses *in vitro* is correlated with CD4 memory T-cell reconstitution, whereas improvement in delayed type hypersensitivity is related to a decrease in viraemia

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Objective: To analyse prospectively the effect of highly active antiretroviral treatment (HAART) on CD4 T-cell responses *in vitro* and *in vivo* in HIV-infected patients.

Design: Prospective study with 49 protease inhibitor-naive adult patients. Data were collected at baseline and after 3 and 6 months of HAART.

Methods: *In vitro* CD4 T-cell reactivity was analysed by stimulation of peripheral blood mononuclear cells with several antigens. *In vivo* CD4 T-cell reactivity (delayed type hypersensitivity) was assessed by Multitest Merieux. Both measurements were correlated to CD4 (memory) T-cell count and HIV-1 viraemia.

Results: Restoration of specific CD4 T-cell proliferation was observed in most patients. The *in vitro* T-cell response was restored more frequently against antigens to which the immune system is constantly exposed (*Candida albicans*, *Mycobacterium tuberculosis*, *M. avium*) as compared with a low-exposure antigen (tetanus toxoid). Overall, delayed type hypersensitivity detection rate increased under HAART. Multivariate analysis showed improvement of antigen-specific T-cell proliferation to be significantly associated with an increase in memory CD4 T-cells, whereas improvement of the delayed type hypersensitivity response was associated with a decrease in plasma HIV-1 RNA.

Conclusions: HAART for 6 months restored antigen-specific CD4 T-cell response to several antigens. *In vitro* immune reconstitution was closely correlated with an increase in memory CD4 cells. Restoration of delayed type hypersensitivity was associated with suppression of viraemia. It appears that in addition to expansion of memory CD4 cells, suppression of viraemia following HAART may allow an improved inflammatory reaction, thus providing even stronger immune reconstitution.

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Introduction

Infection with HIV is characterized by a high rate of HIV replication in CD4 T cells and progressive loss and functional defects of CD4 T cells [1]. CD4 T cells of HIV-infected patients fail to respond to HIV proteins shortly after infection, and loose their antigen-specific responsiveness to opportunistic pathogens and to recall antigens; this is shown by a loss of delayed type hypersensitivity (DTH) response and of antigen-specific T-cell proliferation [2,3]. The reduced functional CD4 T cells and the consecutive breakdown of cellular immunity contribute to the emergence of opportunistic infections and may facilitate HIV-replication [4]. Highly active antiretroviral therapy (HAART) results in strong and sustained inhibition of HIV replication [5] and is associated with a rapid rise in CD4 T cells in patients with advanced disease [6]. However, little is known about the functional integrity of the reconstituted T-cells. Some data suggest that in patients with advanced immunodeficiency, reconstituted CD4 T cells respond to opportunistic pathogens [7]. However, it is not clear which antigen-specific CD4 T-cell responses improve or recover in these patients *in vitro* and *in vivo*. We conducted a prospective study to analyse CD4 T-cell responses by lymphocyte proliferation assays *in vitro* and by DTH reactivity *in vivo*.

Material and methods

Patients and study design

HIV-1-positive, protease inhibitor-naive patients from two participating centres (AIDS Unit, University Hospital Bern, and AIDS Unit, Kantonspital St. Gallen, Switzerland) were asked to participate in the study. Patients were stratified into two groups according to their baseline CD4 cell count (below or above 250×10^6/l). Treatment was started with a protease inhibitor-containing HAART regimen. CD4 cell counts, HIV RNA concentration and CD4 T-cell responses were measured prospectively at baseline, and after 3 and 6 months of HAART. Ten HIV-negative, untreated volunteers were used as control group. The study was approved by the local ethical review boards and all subjects gave written informed consent.

Flow cytometry

Phenotyping of CD4 and CD8 lymphocytes was performed on fresh EDTA-blood samples by 2 colour flow cytometry as described previously [8]. CD4 memory T-cells were identified by anti-CD45RO monoclonal antibody (Becton-Dickinson, Basel, Switzerland). Absolute CD4 and CD8 cell counts were calculated on the basis of absolute lymphocyte counts (Coulter Counter FTKS, Coulter, Hialeah, Florida, USA).

Antigens

Mycobacterium tuberculosis purified protein derivative was obtained from the Statens Serum Institute (Copenhagen, Denmark). *M. avium* purified protein derivate was a gift of J. Stenderup (Statens Serum Institute). *Candida albicans* antigen was provided by the Institute of Medical Microbiology, University of Bern, Switzerland. *Toxoplasma gondii* antigen (strain RH) was provided by B. Gottstein, Institute of Parasitology, University of Bern. Tetanus toxoid was supplied by J. Cryz, Serum und Impfinstitut Bern, Switzerland. All antigens were tested negative for endotoxin by limulus lysate assay. Optimal antigen concentrations were determined in proliferation assays with peripheral-blood mononuclear cells (PBMC) of healthy HIV-negative individuals.

Lymphocyte proliferation assay

PBMC were isolated from 20 ml heparinized blood samples by density gradient centrifugation using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). PBMC were frozen in foetal calf serum supplemented with 10% dimethylsulfoxide and stored in liquid nitrogen until use. For lymphocyte cultures, samples collected at all time-points were thawed simultaneously. PBMC were adjusted to 1×10^6/ml in RPMI 1640 supplemented with 10% human AB-serum. PBMC (1×10^6/well) were cultured in triplicate in a total volume of 200 µl in 96-well round-bottom plates for 5 days in a standard [^{3}H]thymidine proliferation assay. Stimulation indices were calculated as c.p.m. in culture with antigen divided by c.p.m. in culture without antigen. Specific proliferation was defined as a stimulation index > 3 and [^{3}H]thymidine incorporation > 1000 c.p.m.

DTH assay

Testing of DTH was performed with Multitest-Mérieux (Pasteur Mérieux, Paris, France) according to the manufacturer's instructions before and after 6 months of HAART. Assessment of reaction was always performed by one responsible investigator in the two participating centres. A DTH response was considered positive when an induration at the site of antigen application, but not at the control site, was palpable. Multitest-Meriéux contains seven antigens: *C. albicans*, tuberculin, tetanus toxoid, *Proteus mirabilis*, diphtheria, *Trichophyton* and *Streptococcus*. As only tetanus toxoid, *Proteus*, *Candida* and tuberculin produced positive and reproducible responses in HIV-negative individuals, these DTH responses were included in the analysis. The same batch was used throughout the whole study period.

Measurement of HIV-1 RNA

HIV-1 RNA concentration in blood was quantified in EDTA plasma by HIV reverse transcription (RT)–PCR (Amplicor HIV Monitor; Hoffmann-LaRoche, Basel Switzerland), with a limit of detection of approximately 200 copies/ml.

Table 1. Patient characteristics.

	CD4 T-cell count ($\times 10^6$/l)	
	< 250	> 250
Total	24	25
Male	15	16
Female	9	9
Age		
[mean (range)]	42 (31–63)	35 (29–50)
Risk group		
Men who have sex with men	6	4
Heterosexual	10	13
Injecting drug users	4	3
Other	4	4
Centers for Disease Control stage		
A	8	13
B	10	10
C	6	2
Pre-treatment status		
Naive	15	18
Pre-treated	9	7

Statistics

Statistical analysis on changes of HIV-RNA and CD4 T cell counts were performed by Wilcoxon rank test (comparison of patients at identical time points). Multivariate analysis was performed with stata software (version 5.0, Stata corporation, College station, Texas, USA). Changes in DTH or lymphocyte proliferative responses to the different antigens were tested with the two-sided McNemar test. Differences were considered significant at $P \leq 0.05$.

Results

Patients

Patient characteristics are shown in Table 1. Sixty-five patients were included in the study, but only 49 patients concluded the 6 month treatment period and were included in the final analysis. Thirty-three of these 49 patients (67%) were treatment-naive, and 16 out of 49 were treatment-experienced but protease inhibitor-naive. Twenty-four (49%) had a CD4 cell count $< 250 \times 10^6$/l.

Changes in viral load and CD4 T-cell counts

Table 2 shows changes in viral load and CD4 T-cell counts. Reduction of viral load was not different between patients. The median increase in total CD4 and in CD4 memory T-cell counts was higher in the low than in the high CD4 stratum. This difference was not statistically significant due to the high variability of the individual responses.

CD4 T-cell proliferation

Patients in the low CD4 stratum

Antigen-specific proliferation was detectable in 12 out of 24 (50%) patients in the low CD4 stratum before HAART (Table 3). Recovery of antigen-specific CD4 T-cell proliferation was observed in most patients after 3 months of HAART (Fig. 1a). This response was antigen-dependent and most pronounced with *C. albicans* (0 versus 3 months, $P = 0.003$; 0 versus 6 months, $P = 0.001$), *M. tuberculosis* (0 versus 3 months, $P = 0{,}022$; 0 versus 6 months, $P = 0.012$) and *M. avium* (0 versus 3 months, $P = 0{,}07$; 0 versus 6 months, $P = 0.039$). A comparison of the improvement between ubiquitous antigens and tetanus toxoid revealed a significantly better response for *Candida* only (3 months, $P = 0.001$; 6 months, $P = 0.039$). The number of antigens to which PBMC of individual patients responded increased from a median of 12.5% before HAART to a median of 66% after 6 months of therapy ($P = 0.0001$).

Patients in the high CD4 stratum

CD4 T-cell proliferation in response to opportunistic antigens was constantly present in most patients before initiation and during 6 months of HAART at a rate comparable to that in HIV-negative controls (Fig. 1a). Thus, a high proportion of patients with CD4 counts

Table 2. Changes in viral load and CD4 T-cells.

	CD4 T-cell count ($\times 10^6$/l)		P < 250 versus > 250
	< 250	> 250	
Patients	n = 24	n = 25	
Naive/pre-treated	15/9	18/7	
HIV-1 RNA [median (range)] ($\log_{10}$ copies/ml plasma)			
Before HAART	5.12 (2.00 to 5.93)	4.38 (2 to 5.22)	NS
Δ 3 months	–2.32 (1.49 to –3.88)	–2.16 (0 to –3.19)	NS
Δ 6 months	–2.69 (1.49 to –3.88)	–2.16 (0.52 to –3.19)	NS
CD4 T cell count [median (range)] ($\times 10^6$ cells/l)			
Before HAART	101 (10 to 249)	401 (255 to 800)	
Δ 3 months	51 (–43 to 361)	20 (–143 to 295)	NS
Δ 6 months	125 (–46 to 433)	49 (–145 to 515)	NS
CD4 memory T cell count[a] [median (range)] ($\times 10^6$ cells/l)			
Before HAART	77 (8 to 172)	261 (92 to 464)	
Δ 3 months	41 (–34 to 169)	11 (–137 to 208)	NS
Δ 6 months	73 (–41 to 135)	–3 (–153 to 435)	NS

[a]CD45RO T cells. NS, Not significant.

Table 3. Improvement of CD4 T-cell reactivity *in vitro* and *in vivo*.

Patients	Duration of HAART (months) 0	3	6
Antigen-specific T cell proliferation			
HIV-positive			
CD4 cells $< 250 \times 10^6$/l	50	83	96
HIV-positive			
CD4 cells $> 250 \times 10^6$/l	96	96	92
HIV-negative	100	100	100
DTH responses			
HIV-positive			
CD4 cells $< 250 \times 10^6$/l (n=17)	41		64
HIV-positive			
CD4 cells $> 250 \times 10^6$/l (n=9)	66		77
HIV-negative	100		100

The percentages of patients with specific T-cell proliferation to any antigen, and the percentage of patients with delayed type hypersensitivity (DTH) responses to Tetanus toxoid, purified protein derivative of *Mycobacterium tuberculosis*, *Candida albicans* or *Proteus mirabilis* are shown.

$> 250 \times 10^6$/l had functional CD4 T cells specific for important pathogens despite ongoing viral replication.

DTH-response

DTH-testing was performed in 26 HIV-infected patients before and after 6 months of HAART and in parallel in eight HIV-negative individuals. The response rate increased after 6 months of HAART but was still inferior to the *in vitro* response rate and to the DTH response in HIV-negative volunteers (Table 3). Analysis of DTH responses to specific antigens (Fig. 1b) revealed an increase with antigens in patients in the high CD4 stratum. In contrast, in patients with low CD4 counts, recovery of DTH reactivity was limited to a frequently exposed antigen (*Proteus*). There was an increased response to purified protein derivative of (*M. tuberculosis*) in healthy individuals after 6 months, probably due to a booster effect of the previous skin testing in this BCG vaccinated population.

Predictors of improvement of antigen-specific T-cell proliferation and DTH response

The factors associated with an improvement of proliferation and DTH response were analysed in a logistic model, which included the increase of total CD4 counts as well as memory CD4 cell count as binary response variable, and the decrease in $\log_{10}$ plasma HIV-1 RNA as continuous variable between time 0 and after 6 months of treatment. Only patients with all of the variables available were included in the analysis. Fig. 2 shows the median changes in CD4, CD4 memory T-cell numbers and of HIV-1 RNA levels of patients with and without improvement of proliferation or DTH. Table 4 shows the multivariate analysis of these data: improvement in antigen-specific T-cell proliferation was associated significantly with an increase in memory CD4 T-cell number but not with increase in CD4 T-cell number or decrease in HIV-1 RNA. In contrast, improvement in DTH response was associated significantly only with a decrease in plasma HIV-1 RNA.

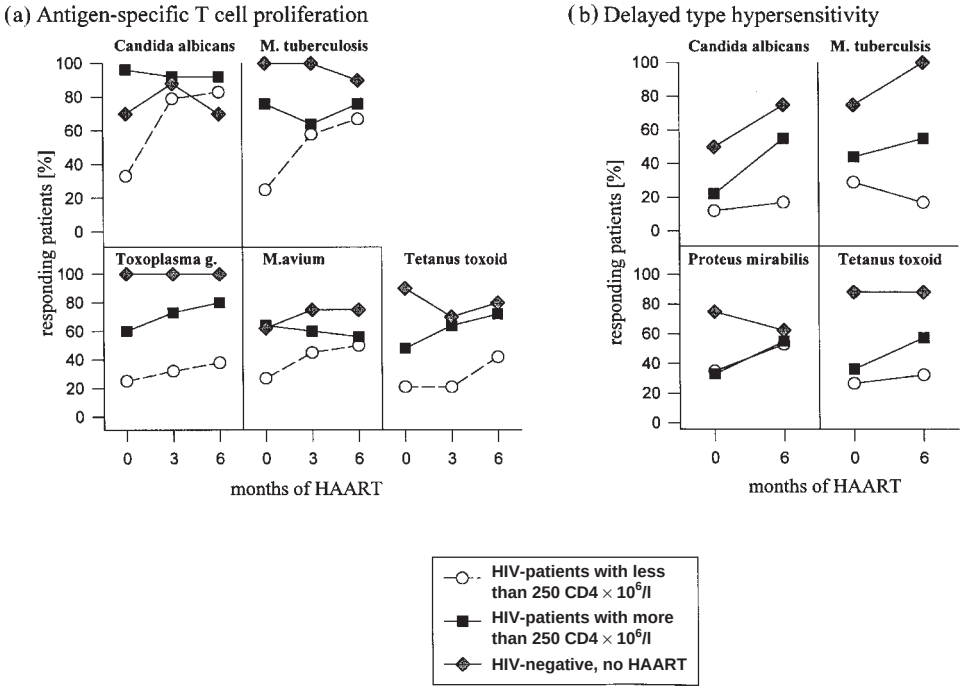

Fig. 1. CD4 T-cell response *in vitro* and *in vivo*. (a) Recovery of specific CD4 T-cell proliferation. (b) Recovery of DTH-responses. The ordinate shows the percentage of HIV-infected patients and HIV-negative individuals with responses to the antigens indicated.

Table 4. Improvement of proliferation and delayed type hypersensitivity response before and after 6 months of HAART as predicted in a logistic model including increase of absolute CD4 cell count and absolute memory CD4 cell count as binary response variable and decrease in $\log_{10}$ plasma HIV RNA/ml as continuous variable. Results of univariate and multivariate analysis including all listed variables are shown.

	Improvement in proliferation n = 46				Improvement in delayed type hypersensitivity response n = 23			
	Univariate		Multivariate		Univariate		Multivariate	
Variable	Odds ratio	*P*	Odds ratio	*P*	Odds ratio	*P*	Odds ratio	*P*
Increase in CD4 T cell count [median (range)]	1.5 (0.3–6.8)	0.6	0.3 (< 0.1–2.8)	0.3	1.0 (0.1–18)	1.0	0.5 (< 0.1–956)	0.9
Increase in CD4 T cell memory count [median (range)]	3.9 (1.1–14.0)	0.04	7.0 (1.8–41.5)	0.03	1.1 (0.1–9.6)	0.9	0.3 (< 0.1–21.5)	0.6
Decrease in HIV-1 RNA (per 1 $\log_{10}$ decrease) [median (range)]	1.1 (0.7–1.7)	0.6	0.6 (0.7–1.6)	0.9	2.8 (1.1–7.3)	0.03	3.6 (1.2–10.2)	0.02

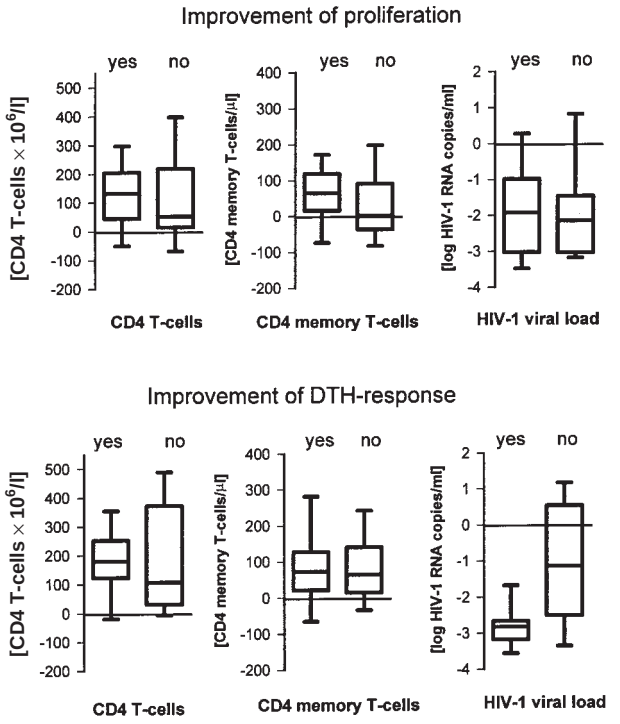

Fig. 2. Predictors of improvement of proliferation and delayed type hypersensitivity (DTH) response. Box plot representation of the median changes (± 25 to 75 percentiles; error bars indicate 10th and 90th percentiles) of CD4, CD4 memory and HIV-1 RNA levels of patients after 6 months of HAART with and without improvement of proliferation or DTH response.

Discussion

In this study HAART over a period of 6 months improves specific CD4 T-cell responses *in vitro* and *in vivo* in adult HIV-infected patients. Improvement of antigen-specific CD4 T-cell proliferation was observed for all opportunistic pathogens tested. Together with previous observations [6,7], our data indicate that HAART-induced improvement of CD4 T-cell reactivity is directed against multiple, clinically relevant pathogens. Restoration of antigen-specific T-cell proliferation was more frequently observed than restoration of DTH reactivity. Although intrinsic differences among both tests for measuring cellular immune function may account for this discrepancy, the multivariate analysis performed points to distinct biological mechanisms occurring under HAART. In fact, improvement of proliferation is easily explained by a higher number of memory cells available which is not strictly associated with suppression of viraemia. On the other hand, suppression of viraemia appears to be a prerequisite for improvement of DTH. Several explanations are possible for the latter observation: High HIV-1 levels are associated with elevated tumour necrosis factor-α levels, which may disturb cell recruitment [9]. HIV itself may induce long-lasting changes in antigen-presenting cell functions in the skin, thus altering DTH. Furthermore, even if the CD4 cell reactivity is reconstituted, high levels of HIV may still have a suppressing effect on the rather complex cascade of events following T-cell triggering which leads to a DTH reaction in the skin. A possible mechanism for this observation could be an interference by HIV virions or soluble gp160 proteins with chemokine receptors required for the recruitment of inflammatory cells [10]. However, our data need confirmation in a larger sample of HIV-infected subjects under HAART.

CD4 T-cell responses *in vitro* were observed more frequently after exposure to antigens of opportunistic pathogens (in particular *C. albicans*) than with tetanus toxoid before and after HAART. These findings suggest that reactivity of circulating CD4 T-cells is skewed in favour of highly exposed antigens in chronic HIV-infection, in agreement with earlier observations [11–13].

The differences seen in both patient strata regarding CD4 T-cell proliferation to opportunistic pathogens before initiation of HAART are in good correlation with clinical data. For example, only 35% of patients with CD4 cell counts $< 250 \times 10^6$/l (a group at high risk of severe *Candida* infections) but 96% of subjects

with higher CD4 cell counts showed proliferation against *C. albicans*. The rapid recovery of the *Candida*-specific CD4 T-cell proliferation is in accordance with epidemiological data showing a rapid decrease in the incidence of candidal oesophagitis after a few months of HAART treatment in patients with advanced immune dysfunction [14]. Furthermore, the beneficial effect of HAART extends to other antigens as well, as shown most recently after stopping primary and secondary prophylaxis against *Pneumocystis carinii*, and, possibly, *T. gondii* and cytomegalovirus [15–17].

In conclusion, HAART results in a significant restoration of multi-specific CD4 T-cell responses, but the degree of this effect is different when *in vitro* or *in vivo* methods are used. Functional studies of CD4 T cells might help to guide decisions regarding discontinuation of prophylactic medication against opportunistic infections in patients with undetectable viral load under HAART.

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