Immunological and virological responses in HIV-1-infected adults at early stage of established infection treated with highly active antiretroviral therapy

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Objective: To evaluate the immunological and virological responses to highly active antiretroviral therapy (HAART) in blood and lymphoid compartments of HIV-1-infected patients at an early stage of infection.

Design: An open-label, observational, non-randomized, prospective trial of outpatients attending the Centre of Clinical Investigation in Infectious Diseases, Centre Hospitalier Universitaire Vaudois, University of Lausanne, Switzerland.

Subjects: Forty-one antiretroviral-naive HIV-1-infected adults with 400 CD4 T cells/μl or greater and 5000 plasma HIV-1-RNA copies/ml or greater were enrolled, and 32 finished the study. Forty-nine HIV-negative individuals were included as controls. All subjects gave written informed consent.

Interventions: All patients received abacavir 300 mg by mouth every 12 h and amprenavir 1200 mg by mouth every 12 h for 72 weeks.

Mainoutcome measures: The extent of immune reconstitution in blood and lymph nodes after 72 weeks of HAART was evaluated, and compared with immunological measures of 49 HIV-negative subjects.

Results: Virus replication was effectively suppressed (-3.5 $\log_{10}$ at week 72). Substantial increments of CD4 T cell count in blood and percentage in lymph nodes were observed over time, and these measures were comparable to HIV-negative subjects by week 24 in blood and by week 48 in lymph nodes. The increase was equally distributed between naive and memory CD4 T cells. Recovery of HIV-specific CD4 responses occurred in 40% of patients.

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Conclusion: The initiation of HAART at an early stage of established HIV infection induces systemic quantitative normalization of CD4 T cells, a partial recovery of HIV-specific CD4 cell responses, and effective and durable suppression of virus replication.

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Keywords: Early established infection, HAART, HIV, HIV-specific CD4 cell responses, immune reconstitution, lymph node, plasma viraemia

Introduction

The introduction of HIV-1 protease inhibitor-containing regimens [1–4] as the first choice antiretroviral therapy has substantially changed the natural course of HIV-1 infection. The beneficial clinical effects, such as reduced morbidity and mortality associated with therapeutic combinations including protease inhibitors, have been clearly demonstrated [5]. However, the experience of the past 3 years in the use of protease inhibitors has revealed a number of limitations, such as the complexity of the therapeutic regimens, the long-term toxicity [6–8], the substantial rate of virological failure [9,10], particularly in advanced stages of HIV-1 infection, and the failure to induce HIV-1 eradication [11–15] within 2–3 years as previously estimated [16]. On the basis of these observations, widely accepted principles regarding the time of initiation of antiretroviral therapy, i.e. as early as possible to prevent disease progression, and the choice of the initial therapy, i.e. the most potent available regimen in order to induce effective and durable suppression of virus replication, have been challenged [17].

The evaluation of the immune reconstitution achievable after combination therapy including protease inhibitors has been mostly limited to the analysis of the blood compartment [18] and to individuals at an advanced stage of HIV-1 infection [19–22]. Variable degrees of immune reconstitution have been reported [19–22], and the findings that the large increments of CD4 cell count were mostly caused by memory rather than by naive CD4 cells has led to the hypothesis that HIV-1 infection severely impairs the regenerative capacity of naive CD4 cells [21], similar to patients treated with chemotherapy and receiving bone-marrow transplantation [23].

In the present study, the use of a new potent therapeutic combination, including a reverse transcriptase inhibitor (abacavir) [24–29] and a protease inhibitor (amprenavir) [30], allowed to perform an extensive assessment of the immune reconstitution achievable in both blood and lymph node compartments in patients at an early stage of established HIV-1 infection.

Materials and methods

Study design and patients

The CNAB2006 study was an open-label, observational, non-randomized study at a single site (Lausanne) in Switzerland. Eligible patients have been treated with abacavir (300 mg twice a day) and amprenavir (1200 mg twice a day) in combination, and followed for 72 weeks. Of note is the fact that amprenavir was combined with only one inhibitor of reverse transcriptase, because historical data indicated that the antiviral effect of monotherapy with abacavir was at least as effective as zidovudine and lamivudine in combination. This study was planned to enrol 50 subjects. After 6 months it became apparent that there was a very low withdrawal rate and the majority of lymph nodes were successfully biopsied, and therefore the recruitment number was subsequently amended (protocol amendment dated 19 March 1997 and 23 December 1997).

A total of 41 antiretroviral-naive adults were recruited. Subjects included in the study had to be at least 18 years of age, had to have documented HIV-1 infection, one screening CD4 cell count of 400/mm^3 or greater measured within 14 ± 7 days before study drug administration, one screening plasma HIV-1-RNA level of 5000 copies/ml or greater within 14 ± 7 days before study drug administration, no previous antiretroviral therapy, and women of childbearing potential had to have a negative pregnancy test. All subjects had to provide written informed consent. All subjects have been followed for 72 weeks.

Clinical and laboratory monitoring

The CD4 cell count and plasma HIV-1-RNA level were determined twice at baseline 1 (minus 5 days from the initiation of therapy) and at baseline 2 (day 0, i.e. day of initiation of therapy). Excisional lymph node biopsies were performed at baseline 2. Subjects were monitored at weeks 2 and 4, and every 4 weeks until week 24, and after week 24, every 12 weeks until week 72. Ultrasound-guided lymph node aspirations were performed at weeks 24, 36 and 48.

Safety

For the assessment of safety, measures of evaluation included adverse events and clinical laboratory values,

and were evaluated according to the AIDS Clinical Trials Group toxicity scale. Eight subjects (19%) in the treatment cohort experienced at least one serious adverse event (serious adverse events n = 10). However, six out of 10 serious adverse events reported were not attributable to study medication (inguinal abscess, injury to right leg, bronchitis, bilateral inguinal hernia, cholecystectomy, depression). An additional serious adverse event (grade 4 hypercholesterolaemia and hypertriglyceridaemia) was thought not to be caused by antiviral drugs because it was clearly related to alcohol abuse; this event resolved once the patient discontinued the use of alcohol, while antiviral therapy was not interrupted. One patient (Centers for Disease Control and Prevention grade B at enrolment) developed a lymph node Kaposi's sarcoma 8 weeks after the initiation of therapy; in this case, it was not clear whether the condition was undetected although present before the initiation of the trial or had developed after the initiation of antiviral therapy. Finally, a grade 4 increase in liver function tests after 20 and 36 weeks of therapy (aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT)) occurred in two HIV/hepatitis C virus co-infected patients. Although it was not clear what contribution to this event was made by antiviral drugs and the presence of active hepatitis C infection, the discontinuation of amprenavir and its replacement with combivir twice daily (zidovudine 300 mg plus lamivudine 150 mg) was accompanied by a significant decrease of liver function tests in both patients. Results from these two patients were kept in the analysis.

Seven subjects (17%) had at least one treatment emergent grade 3 (five subjects) and grade 4 (two subjects) clinical chemistry toxicity. Only three subjects (7%) withdrew from the study as a result of an adverse event (two rash and one biological pancreatitis). Two out of three subjects withdrew from the study experienced an abacavir hypersensitivity reaction, which required discontinuation of the study drug after being rechallenged with either amprenavir only or abacavir only, respectively. Nausea was the most commonly reported (63%) drug-related adverse event during the first 2 weeks of treatment, and other common adverse events included diarrhoea (37%), headache (24%), paraesthesia oral/perioral (22%), fatigue (17%), and vomiting (15%). However, none of these resulted in treatment limiting and discontinuation, and the adverse events either disappeared or their frequency significantly dropped over time (nausea was at 7% by week 24). No significant changes over baseline were observed in the plasma levels (non-fasted) of triglycerides (normal range 0.84–1.94 mmol/l; mean $\pm$ SD 2.11 ± 2.95 at baseline; 1.99 ± 1.33 at 24 weeks; and 1.96 ± 1.24 at 48 weeks; 1.78 ± 1.28 at 72 weeks) throughout the duration of the follow-up, whereas cholesterol levels (normal range 3.1–5.1 mmol/l) were significantly increased at weeks 24 (mean $\pm$ SD 5.43 ± 1.24 mmol/l), 48 (mean $\pm$ SD 5.59 ± 1.29 mmol/l) and 72 (5.48 ± 1.4 mmol/l) over baseline (4.89 ± 1.09, $P < 0.001$ for all comparisons). The data reported are updated to 1 September 1999, and no cases of lipodystrophy were reported up to this data cut-off point.

HIV-1-negative subjects

CD4 cell counts were determined in a cohort of 49 HIV-negative individuals aged between 18 and 77 years (median 36.3 years). HIV-negative subjects were matched for age and sex with the treatment cohort. Lymph nodes (inguinal nodes) were collected from 15 HIV-negative subjects (11 men and four women), who underwent vascular (varicose vein stripping) and general (uncomplicated bilateral inguinal herniorraphy and osteosynthesis for ankle fracture) surgery, and CD4 and CD8 cell percentages were assessed on mononuclear cells isolated from lymph nodes.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated on Becton Dickinson's Vacutainer CPT tubes containing sodium citrate, Ficoll Hypaque density fluid, and a polyester gel barrier.

Lymph node biopsy, isolation of lymph node mononuclear cells and ultrasound-guided lymph node aspiration

Mononuclear cells from lymph nodes were isolated as previously described [31]. Tissue specimens were minced with a scalpel and the cells were teased out, counted and prepared for the determination of virological and immunological measures. Ultrasound-guided lymph node aspirations were performed as previously described [32].

Cytofluorometric analysis

Cytofluorometric analysis was performed as previously described [33,34]. Anti-CD3, anti-CD4, anti-CD8, anti-CD38, anti-CD45RA and anti-CD45RO monoclonal antibodies conjugated either with fluorescein isothiocyanate, or phycoerythrin, or perdinin chlorophyll were used (Becton Dickinson, San Jose, CA, USA). The determination of CD4 and CD8 cell percentages was performed on freshly isolated blood and lymph node mononuclear cells, whereas the determination of CD4CD45RA and CD45RO positive cells was analysed retrospectively on appropriately stored mononuclear cell samples.

Determination of plasma HIV-1-RNA concentration

The determination of plasma HIV-1-RNA concentration was performed with a branched (b) chain DNA assay [35] with a limit of detection of 500 RNA copies/ml in all the plasma samples collected during the study follow-up. The plasma samples with HIV-1-RNA concentrations below 500 copies were also

analysed retrospectively with an ultrasensitive assay with a limit of detection of 5 copies/ml. The ultrasensitive HIV-1-RNA assay used has been adapted [36] from the modified Amplicor HIV Monitor Assay (Roche, Basel, Switzerland) with an initial centrifugation step before RNA extraction that allows an increase in the input of viral RNA. Briefly, 1 ml plasma were centrifuged at 50 000 ***g*** (Heraeus Biofuge 28 RS, rotor 3740; Heraeus AG, Osterode, Germany) at 4°C, for 80 min. Supernatants were then discarded, and the subsequent steps were performed according to the manufacturers' instructions, implementing slight modifications such as a reduction (7.3 times) in the input of internal quantitative standard, a change in the volume of specimen diluent (55 versus 400 μl), and a prolongation of 15 min of the substrate incubation step. In preliminary experiments, in order to validate the assay, replicates (22 for each dilution) of known numbers, i.e. 20, 10, 5, and 2.5 HIV-1-RNA copies were quantified. In three separate experiments, quantification was possible in 100% of the replicates containing 20, 10, and 5 copies, whereas it was possible in 65% of the replicates containing 2.5 copies. On the basis of these results, the limit of detection of the assay was set at 5 HIV-1-RNA copies/ml. The mean coefficient of variation for samples containing known numbers of 20, 10 and 5 HIV-RNA copies/ml was 40% (range 23–52%). The range of quantification was between 5 and 2000 HIV-1-RNA copies.

Lymphocyte proliferative assay

The response to varying non-HIV-specific and HIV-specific antigens was tested in a subgroup of 24 patients over the 72 weeks of antiviral therapy. Antigens tested include: tetanus toxoid (TT Ag) (0.34 IU/ml; NIBSC, Herts, UK); purified protein derived of *Mycobacterium tuberculosis* (PPDT Ag) (1 μg/ml; NIBSC); cytomegalovirus, CF Antigen (CMV Ag) (1 : 2000; BioWhittaker, Walkersville, MD, USA); Herpes virus hominis, CF antigen (HSV Ag) (1 : 400; BioWhittaker); *Candida albicans*, cytoplasmatic proteins (4 μg/ml; NIBSC); HIV-1 p24 (p24 Ag) (1 μg/ml; Protein Sciences Corporation, Meriden, CT, USA); and inactivated total HIV-1 antigens (1 μg/ml; Immune Response Corporation, Carlsbad, CA, USA) [37]. The lymphocyte proliferative assays were performed on frozen PBMC obtained at varying timepoints, i.e. baseline and weeks 12, 20, 36, and 72. Proliferation assays were performed as previously described [38] with few modifications. Briefly, PBMC were resuspended in RPMI 1640 medium containing 10% human AB serum (Sigma, Saint-Louis, MO, USA) and L-glutamine. Approximately 10^5 cells per well were cultured in a 96-well U-bottom plate in the presence of the different antigens. As a positive control, cells were stimulated in RPMI medium containing 1 μg/ml phytohaemagglutinin and 10% IL-2. As a negative control, cells were incubated without antigens. Each condition was tested in quadruplicate. Cells were incubated at 37°C for 5 days; then 1 μCi per well of ^{3}H-thymidine (NEN, Boston, MA, USA) was added and incubated for 20 h. At day 6, plated cells were recovered on a FilterMate Harvester for UniFilter-96 microplates (GF/C type membrane) (Canberra Packard SA, Zurich, Switzerland). After recovery, 50 μl of Micro-Scint-O (Canberra Packard SA) was added to each well. Plates were then sealed and counted on a TopCount (Canberra Packard SA). The stimulation index (SI) was defined as the ratio between the mean counts-per-minute (cpm) of stimulated cells and the mean cpm of the negative control. An SI greater or equal to 3 was considered positive.

Statistical analysis

The primary measures of antiretroviral drug activity were changes in plasma HIV-1 RNA and CD4 cell count in blood and percentage in lymph nodes over time. Changes in CD4 cell count in blood and percentage in lymph nodes in the treatment cohort were compared with those in a cohort of 49 HIV-1-negative healthy subjects. The HIV-1-RNA values underwent $\log_{10}$ transformation before analysis. The proportion of individuals who had HIV-1-RNA levels of either less than 500 or 5 copies/ml was calculated over time, considering as denominator either the number of on-treatment persons (as-treated analysis) or the number of enrolled subjects (intent-to-treat analysis). In the intent-to-treat analysis, subjects who withdrew from the study were considered as treatment failures. Within-subject value changes were compared by paired *t*-tests. Results were analysed by the chi-square test in the case of dichotomous data and by Student's *t*-test in the case of continuous variables. All *P* values were two-tailed, and a *P* value of less than 0.05 was considered to indicate statistical significance.

Results

Patients

Forty-one HIV-1-infected adults were enrolled in the study and followed for 72 weeks. Baseline mean ± SD CD4 and CD8 cell counts were 756 ± 225 and 1221 ± 609 cells/mm^3, respectively, and baseline mean plasma viraemia was 4.42 ± 0.45 $\log_{10}$ HIV-1-RNA copies/ml. Seventeen per cent (seven out of 41) subjects withdrew from the study. Four subjects discontinued the study treatment prematurely because of protocol-defined adverse events: two within 2 weeks after the initiation of treatment, one after 8 weeks, and one after 24 weeks. Three subjects discontinued the study treatment voluntarily at weeks 20 and 36 (two subjects) after the initiation of treatment. Finally, two additional subjects were not available for the week 72 visit; however, they were again monitored at week 84.

Changes in plasma HIV-1-RNA concentrations

By using an assay with a limit of detection of 500 HIV-1-RNA copies/ml, i.e. 2.7 $\log_{10}$ copies/ml, a rapid and persistent decrease in the plasma HIV-1-RNA concentrations was observed throughout the duration of follow-up in the study treatment cohort; 1.59 $\log_{10}$ copies/ml reduction from baseline values at week 2 (n = 41), 1.64 $\log_{10}$ reduction at week 24 (n = 36), 1.73 $\log_{10}$ reduction at week 48 (n = 34) and 1.73 $\log_{10}$ reduction at week 72 (n = 32) (Fig. 1). The use of a modified RNA assay with a limit of detection of 5 HIV-1-RNA copies/ml, i.e. 0.70 $\log_{10}$ copies/ml, allowed a better appreciation of the responses of the plasma HIV-1-RNA concentrations to treatment. Because the range of quantification of this assay was between 5 and 2000 RNA copies, only plasma samples with less than 500 RNA copies were quantified with the ultrasensitive assay. By using the ultrasensitive assay, the decrease in the plasma HIV-1-RNA concentrations was 1.59 $\log_{10}$ copies/ml at week 2 (n = 24), 2.72 $\log_{10}$ copies/ml at week 12 (n = 37), 3.15 $\log_{10}$ copies/ml at week 24 (n = 36), 3.55 $\log_{10}$ copies/ml at week 48 (n = 34), and 3.51 $\log_{10}$ copies/ml at week 72 (n = 32) (Fig. 1). The changes in plasma HIV-1-RNA concentrations observed between baseline and any timepoint after the initiation of treatment were highly significant ($P < <0.0001$, paired *t*-test).

The proportion of subjects with plasma HIV-1-RNA concentrations below 500 copies/ml was similar in both the as-treated and the intent-to-treat analyses (Fig. 2a). Plasma HIV-1-RNA concentrations were above 5 copies/ml in all subjects receiving the study treatment during the first 12 weeks of treatment. At week 16 after the initiation of treatment, a small proportion of subjects (5% in both the as-treated and the intent-to-treat analyses) had plasma HIV-1-RNA concentrations below 5 copies/ml (Fig. 2b). However, the proportion of subjects with plasma HIV-1-RNA concentrations below 5 copies/ml progressively increased at week 24 (36% in the as-treated versus 32% in the intent-to-treat analysis), at week 48 (47 versus 39%) and at week 72 (50 versus 39%) (Fig. 2b). Finally, the proportion of subjects with a plasma viraemia

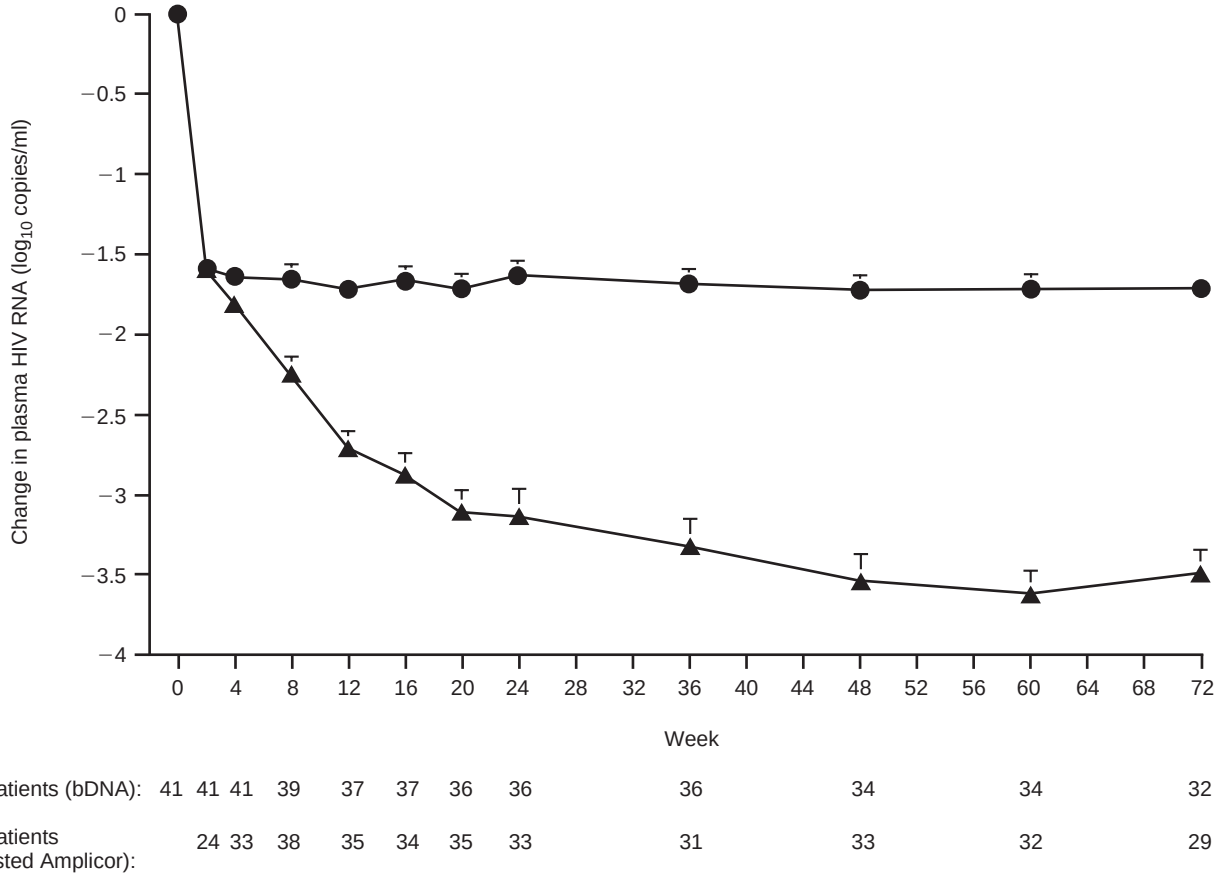

Fig. 1. Mean change from baseline in plasma HIV-1-RNA concentration. The HIV-1-RNA concentration was determined in all the plasma samples with an assay with a limit of detection of 500 HIV-1-RNA copies/ml. Only the plasma samples with less than 500 HIV-1 RNA copies were analysed with an ultrasensitive assay with a limit of detection of 5 HIV-1-RNA copies. The number of subjects who could be evaluated at each timepoint is shown. Bars indicate mean ± SE. ——●—— Limit: 500 copies/ml; ——▲—— limit: 5 copies/ml.

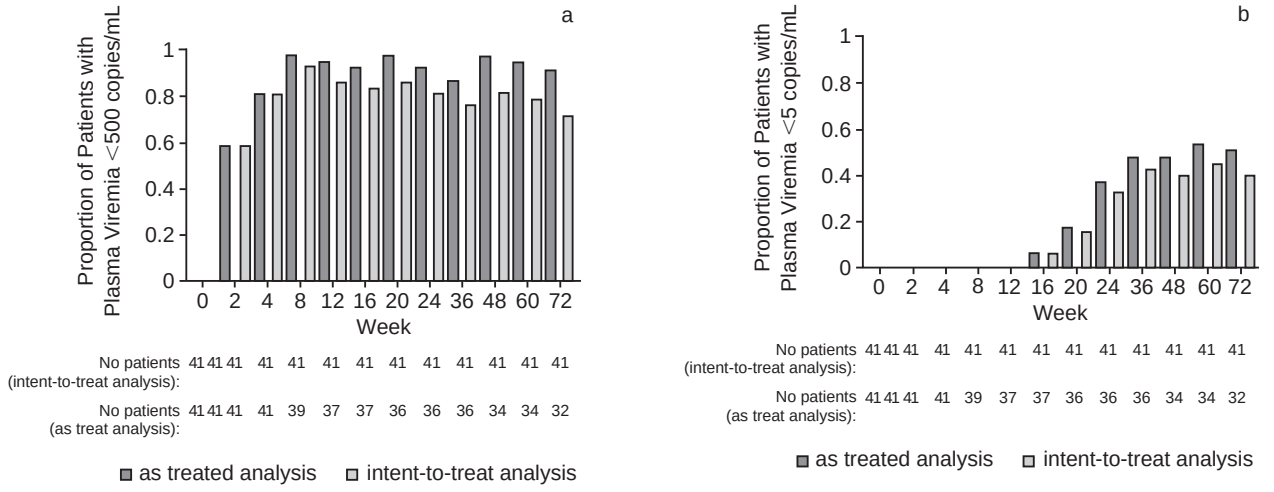

Fig. 2. (a) Proportion of subjects with plasma HIV-1 RNA concentration of less than 500 copies/ml. (b) Proportion of subjects with plasma HIV-1 RNA concentration of less than 5 copies/ml. The number of subjects who could be evaluated at each timepoint is shown. ■ As-treated analysis; □ intent-to-treat analysis.

below 50 copies/ml was 85 and 70% in the as-treated and the intent-to-treat analysis, respectively, at both weeks 48 and 72.

Changes in CD4 and CD8 cell counts in blood

A progressive and continuous increase in the mean CD4 cell count above baseline values was observed over time. After a slight non-significant decrease in CD4 cells during the initial 4 weeks of treatment, the mean CD4 cell count increased by 35 at week 8 (n = 39), by 90 at week 12 (n = 37; $P = 0.03$), by 134 at week 24 (n = 36; $P = 0.03$), by 160 at week 48 (n = 34; $P < 0.0001$), and by 240 cells/mm^3 at week 72 (n = 32; $P < 0.0001$, paired *t*-test) (Fig.

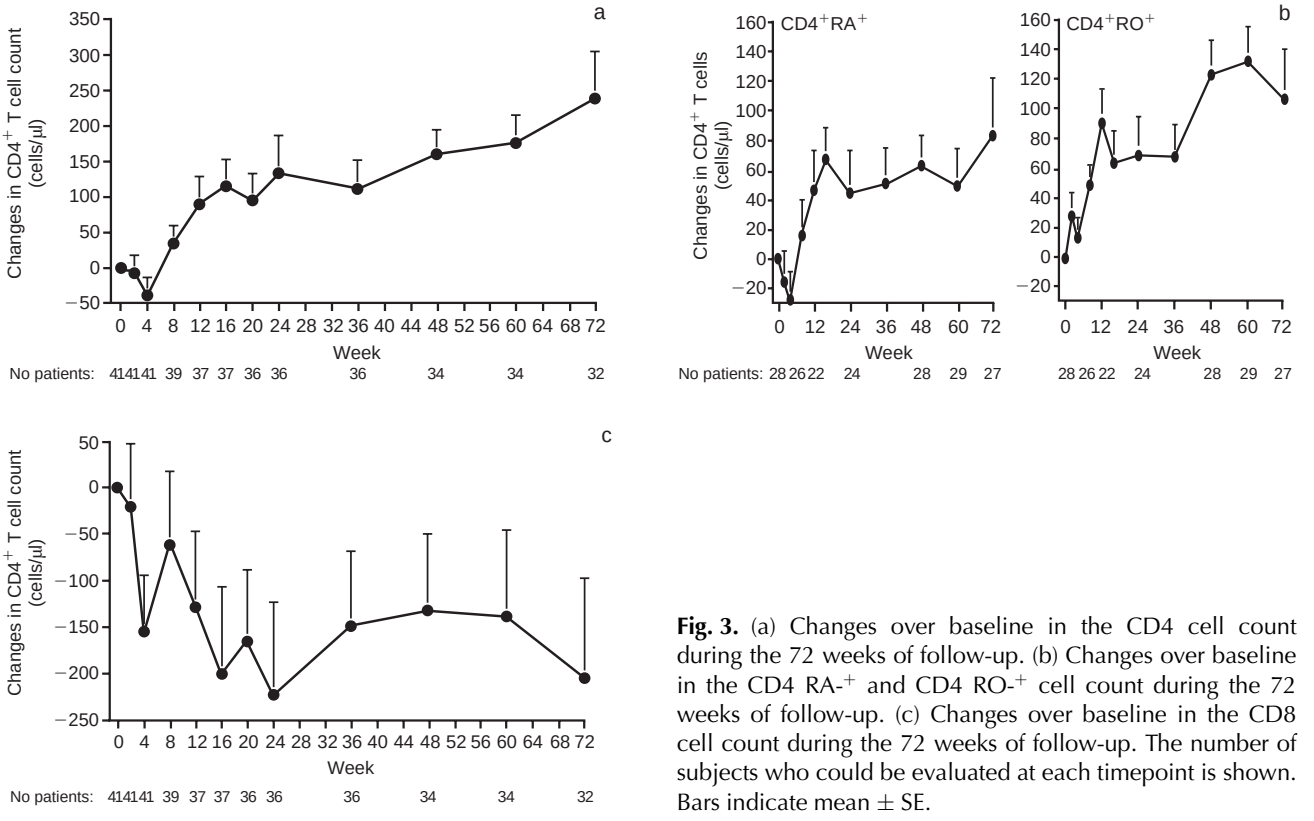

Fig. 3. (a) Changes over baseline in the CD4 cell count during the 72 weeks of follow-up. (b) Changes over baseline in the CD4 RA-$^+$ and CD4 RO-$^+$ cell count during the 72 weeks of follow-up. (c) Changes over baseline in the CD8 cell count during the 72 weeks of follow-up. The number of subjects who could be evaluated at each timepoint is shown. Bars indicate mean ± SE.

3a). Furthermore, the CD4 cell count in the treatment cohort (mean 756 ± 225, $n = 41$) was significantly lower than that measured in the HIV-negative cohort (967 ± 355, $n = 49$; $P = 0.0001$, *t*-test) at baseline. However, CD4 cell counts in treated patients over time were comparable to those of HIV-negative subjects by week 24 and onwards (mean CD4 cell count 878/mm^3 in the treatment cohort; mean CD4 cell count 878 at week 24, $P = 0.28$; 898 at week 48, $P = 0.3$; and 970 at week 72, $P = 0.9$, *t*-test).

Changes in the naive and memory CD4 cell subsets were analysed by the expression of CD45RA and CD45RO surface markers, respectively. The memory CD4 cell count was above baseline values at all the timepoints analysed (Fig. 3b). The increase in memory CD4 cells was not significant at weeks 2 and 4, and it became statistically significant ($P = 0.002$, paired *t*-test) at week 8 (increase of 50 cells/mm^3; Fig. 3b). The memory CD4 cell count continued to increase at week 12 (93 cells/mm^3, $P = 0.001$, paired *t*-test) and remained stable at the later timepoints (increased by 70 at week 24, by 125 at week 48, and by 108 at week 72). The naive CD4 cell count slightly decreased during the first 4 weeks of treatment (Fig. 3b). However, from week 8 and onwards, the naive CD4 cell count increased progressively over time. The naive CD4 cell count increased by 16 cells at week 8, by 46 at week 12, by 44 at week 24, by 62 at week 48 ($P = 0.007$, paired *t*-test), and by 82 cells/mm^3 at week 72 (Fig. 3b).

The increments observed in the CD4 T cell subsets were paralleled by a constant decrease in the CD8 T cell count, inducing a decline of the mean CD8 T cell count from 1221 at baseline to 985 cells/mm^3 at week 72. During therapy, the mean CD8 T cell count decreased by 62 at week 8 ($n = 39$), by 128 at week 12 ($n = 37$), by 224 at week 24 ($n = 36$; $P = 0.03$), and by 206 cells/mm^3 at week 72 ($n = 32$; $P = 0.06$, paired *t*-test) (Fig. 3c). The mean CD8 T cell count measured in the HIV-negative cohort (582 ± 215, $n = 49$) was significantly lower than those measured in the treatment cohort both at baseline and at week 72 ($P < 0.0001$, *t*-test). However, in the treatment cohort, a normalization of the CD4/CD8 cell ratio occurred after therapy (1.2 at week 72 versus 0.7 at baseline, $P < 0.0001$, paired *t*-test). Finally, in a subgroup of 16 of the 41 treated patients, the proportion of CD8 T cells expressing the CD38 molecule significantly decreased after antiretroviral therapy. Compared with the baseline value (mean percentage $\pm$ SE, 41.1 ± 2.9), the decrease in CD8 CD38 T cell percentage observed at weeks 24 (29.9 ± 2.8), 48 (27.9 ± 2.9), and 72 (18.9 ± 2.4) was highly statistically significant ($P < 0.003$, paired *t*-test).

Changes in CD4 and CD8 cell percentages in lymph nodes

Lymph node cells were obtained from excisional lymph node biopsies on the day of the initiation of treatment and at week 72, and from ultrasound-guided lymph node aspirations at weeks 24, 36, and 48 in the study treatment cohort. The percentage of CD4 cells progressively increased over time (Fig. 4a). Compared with baseline values (37%), the increases in CD4 cell percentage observed at weeks 24 (48%), 36 (51%), 48 (53%) and 72 (54%) were highly significant ($P < 0.0001$, paired *t*-test) (Fig. 4a).

In order to evaluate the responses of the CD4 cell percentage to treatment in lymph nodes more accurately, the CD4 cell percentage in the treatment cohort was compared with that measured in 15 HIV-1-negative subjects who underwent an excisional lymph node biopsy during vascular and general surgery. At baseline, the mean $\pm$ SE CD4 cell percentage (59.1 ± 2.2) in the lymph nodes of HIV-1-negative subjects was significantly higher compared with that of the treatment cohort ($P < 0.0001$, *t*-test) (Fig. 4). However, the CD4 cell percentage progressively increased over time in the lymph nodes of the treatment cohort, and the differences in CD4 cell percentage between the treatment cohort and the HIV-1-negative subjects were no more significant at weeks 48 and 72 after the initiation of treatment ($P = 0.12$, *t*-test) (Fig. 4a).

Similar to what was observed for the CD8 T cell count in the peripheral blood, the mean lymph node CD8 T cell percentage progressively decreased after therapy (Fig. 4b), and these declines were highly statistically significant ($P < 0.0001$ for all comparisons, paired *t*-test). At baseline, the mean $\pm$ SE CD8 cell percentage (12.3 ± 1.1) in the lymph nodes of HIV-1-negative subjects was significantly lower than those measured in the treatment cohort both at baseline ($P < 0.0001$, *t*-test) and after 72 weeks of therapy ($P = 0.001$) (Fig. 4b).

Changes in lymphocyte proliferative responses

In a subset of 24 out of 41 patients enrolled in the trial, lymphocyte proliferative responses against both non-HIV-specific (CMV, HSV, and *C. albicans*) and HIV-specific antigens (native p24 Ag and inactivated HIV antigen) were investigated. Approximately 90% of patients had significant proliferative responses against CMV, HSV and *C. albicans* before the initiation of antiviral therapy, whereas approximately 50% of patients had significant responses against TT and PPDT. Mean $\pm$ SE stimulation indexes to CMV, HSV, *C. albicans*, TT and PPDT were 30.3 ± 6.1, 24.9 ± 3.9, 30.5 ± 5.3, 7.6 ± 2.6, and 16.8 ± 7.8, respectively. After the initiation of abacavir/amprenavir combination therapy an overall increase of the response against CMV, HSV, *C. albicans*, and PPDT antigens was

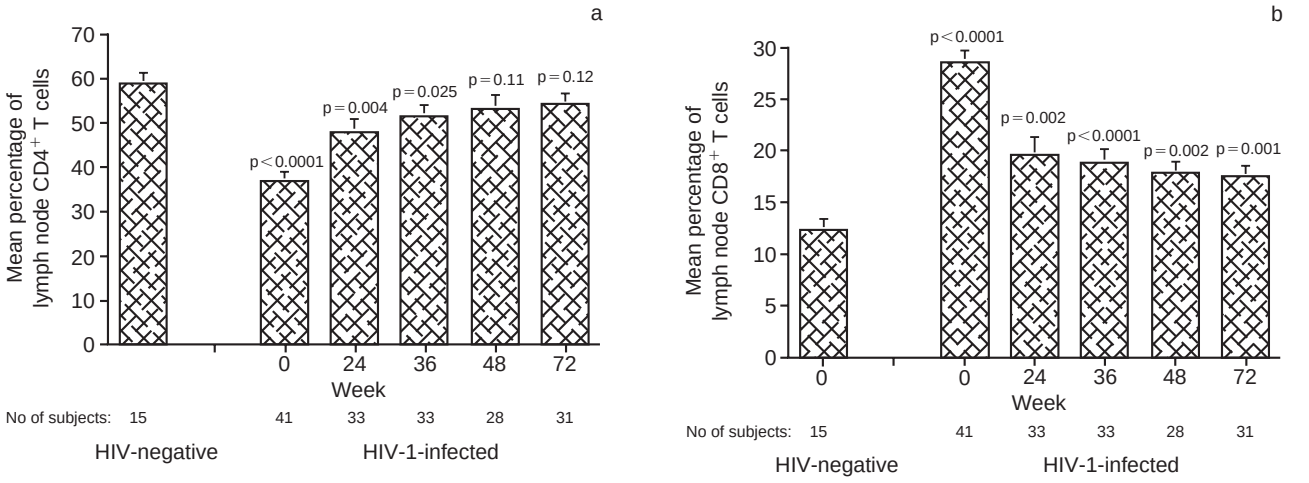

Fig. 4. (a) CD4 cell percentage in lymph nodes of 15 HIV-negative subjects and the CD4 cell percentage in lymph nodes of the treatment cohort at baseline and at different timepoints during antiviral therapy. (b) CD8 cell percentage in lymph nodes of 15 HIV-negative subjects and the CD8 cell percentage in lymph nodes of the treatment cohort at baseline and at different timepoints during antiviral therapy. The number of subjects who could be evaluated at each timepoint is shown. Bars indicate mean ± SE. T-test *P*-values are shown.

observed during the first 6 months of therapy. Afterwards, between weeks 24 and 72, these responses tended to decrease progressively, with mean ± SE stimulation indexes to CMV, HSV, *C. albicans* and PPDT of 19.2 ± 4.3, 15.3 ± 2.7, 17.7 ± 2.8, and 10.5 ± 3.9 respectively, after 72 weeks of antiviral therapy (Fig. 5a). No significant changes have been observed in the response to TT Ag over time (Fig. 5a).

Significant HIV-specific responses (i.e. a stimulation index greater than or equal to 3), against both native p24 Ag and inactivated HIV Ag, were present in approximately 10% of patients before the initiation of therapy. During antiviral therapy, a positive response to p24 Ag and inactivated HIV Ag was detected in 30 and 45% of patients, respectively (Fig. 5b).

Discussion

This study has extensively investigated: (i) the efficacy of antiretroviral therapy at an early stage of established HIV-1 infection (i.e. HIV-1-infected subjects with

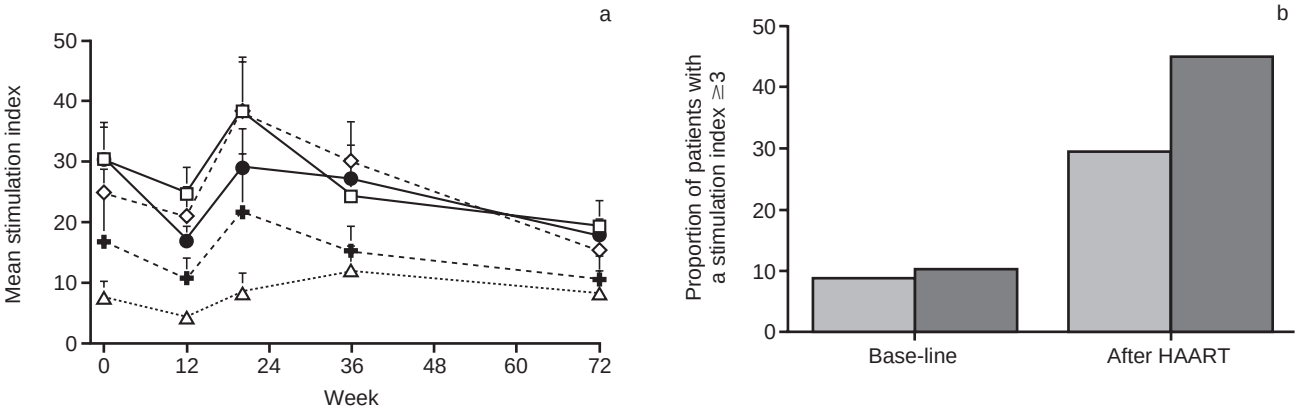

Fig. 5. (a) Changes in the mean stimulation index to cytomegalovirus ——□——, herpes simplex virus ——◇——, *Candida albicans* ——●——, tetanus toxoid ——△——, and purified protein derived of *Mycobacterium tuberculosis* ——+—— antigens over time in 24 patients treated with abacavir and amprenavir in combination. Bars indicate mean ± SE. (b) Proportion of subjects with a positive response (simulation index ≥ 3) to native p24 (■) and inactivated total HIV (■) antigens at baseline and after antiviral therapy in 24 patients treated with abacavir and amprenavir in combination.

CD4 cell counts $\geqslant 400$ cells/mm^3); (ii) the use of an assay with a limit of detection of 5 HIV-1-RNA copies/ml of plasma for the evaluation of the suppression of virus replication after antiretroviral therapy; (iii) the monitoring of immunological measures in peripheral blood and lymphoid tissue; and (iv) the extent of reconstitution of CD4 cells in blood and lymph nodes by comparison with a cohort of HIV-negative individuals.

The antiviral efficacy of the abacavir/amprenavir therapeutic combination was at least comparable to that observed in previous studies using two nucleoside analogues plus a protease inhibitor [1,2]. In the intent-to-treat analysis, 70% of subjects had levels of viraemia below 50 copies/ml at 24 weeks, a proportion similar to that reported in individuals with more advanced disease treated with triple combination therapy [1,2,36]. After 48 and 72 weeks of abacavir/amprenavir combination therapy, the proportion of patients with a plasma viraemia below 50 copies/ml was still 70% (intent-to-treat analysis). However, this suppression of virus replication did not correspond to unquantifiable viraemia because approximately 50% of subjects had levels of viraemia between 50 and 5 HIV-1-RNA copies/ml at weeks 24, 48 and 72. The time to decrease of plasma HIV-1-RNA concentrations below 5 copies ranged between 16 and 48 weeks in the subjects who never discontinued treatment. Therefore, in agreement with recent observations [11,12,39], plasma HIV-1-RNA concentrations below 50 copies do not necessarily correspond to complete viral latency, thus indicating the presence of persistent residual virus replication [40,41].

The extent of immune reconstitution after therapeutic regimens containing protease inhibitors, has been mostly investigated in patients at advanced stages of disease. Normalization of the CD4 cell count was rarely observed, and naive CD4 cells slowly increased only after prolonged (12 months) treatment [21,22]. In the present study, we demonstrated that the initiation of treatment at an early stage of established infection results in a substantial increase in the CD4 cell count [1,2] and, most importantly, CD4 cell counts in the treatment cohort were no longer significantly different from those in the HIV-negative cohort by week 24. The rapid normalization of the CD4 cell count thus occurs after the initiation of effective antiretroviral therapy at an early stage of established infection.

The normalization of CD4 cells was further confirmed by the monitoring of the CD4 cell percentage in the lymph nodes, which, in the treatment cohort, was comparable to that of the HIV-negative cohort by week 48. The increase in the CD4 cell percentage truly reflected new production and reconstitution of CD4 cell numbers in the lymph nodes. In a subset of subjects enrolled in the present study, it was demonstrated that the proportion of proliferating CD4 cells in the lymph nodes significantly increased (approximately sixfold compared to baseline) after 24–36 weeks of treatment [42].

A substantial increase in naive CD4 cells in the blood was observed over time, and the kinetic of this increase is similar to that observed in memory CD4 cells. The increase of naive CD4 cells accounted for at least 50% of the total increase in the CD4 cell count and the naive : memory CD4 cell ratio was normalized by week 24. These results indicated that the dynamics of regeneration of naive CD4 cells are different between the early and advanced stage of HIV infection, and that the regenerative machinery of naive CD4 cells is not impaired in HIV-1-infected individuals at an early stage of established infection. Furthermore, the rapid increase in naive CD4 cells observed in subjects at an early stage of established chronic infection after highly active antiretroviral therapy (HAART), was consistent with a recent study [43], which demonstrated the production of CD4 cells from the thymus in HIV-1-infected patients treated with HAART.

Normalization of the CD4 T cell subsets was accompanied by significant declines in the CD8 cell populations, both in the peripheral blood and in lymphoid tissue. Although both peripheral blood and lymph node CD8 T cells were still significantly higher in the treatment cohort compared with HIV-negative controls, the mean CD4 : CD8 cell ratio value (1.2) was within the normal range after 72 weeks of therapy. Of note was the fact that, in a subgroup of 16 patients, the proportion of CD8 T cells expressing the CD38 molecule significantly decreased after antiretroviral therapy, consistent with a reduction in immune activation after the effective suppression of virus replication.

The majority of HIV-1-infected patients enrolled in this study had significant lymphocyte proliferative responses against CMV, HSV, *C. albicans*, TT and PPDT. During the first 6 months of antiviral therapy, these responses increased overall. Of note is the fact that this increase parallels, at least temporally, the maximal increase in the proportion of proliferating CD4 T cells after therapy, observed in a subgroup of patients enrolled in this trial [42]. Subsequently, these responses tend to decrease over time, consistent with previous findings [44]. The HIV-specific lymphocyte proliferative responses recovered in a proportion of patients after therapy. As a matter of fact, the response to p24 Ag and to inactivated HIV Ag, which was present in approximately 10% of patients before the initiation of therapy, became positive in 30 and 45% of patients, respectively, during the 72 weeks of therapy. Of note is the fact that the ability to recover HIV-1-specific CD4 cell proliferative responses did not corre-

late with any immunological (CD4 cell count and percentage, memory and naive CD4 cell percentages) and virological (plasma viraemia) measures in blood and the lymph nodes at baseline. These results suggest that the institution of antiviral therapy at an early stage of infection may lead to the recovery of HIV-specific responses, although in less than 50% of patients.

Major abnormalities in lipid metabolism and signs of lypodistrophy were observed in cohorts of HIV-1-infected subjects at an advanced stage of infection receiving a protease inhibitor-containing regimen [4,6–8]. After 72 weeks of abacavir/amprenavir combination therapy, no case of self-reported or observed physical changes associated with lipodystrophy was noted. Considering very stringent cut-offs to define the levels of cholesterol (> 5.0 mmol/l) and triglycerides (> 1.6 mmol/l) associated with cardiovascular morbidity, approximately 25 and 50% of patients had levels of triglycerides or cholesterol above these cut-offs. These figures are significantly lower than those reported by Carr *et al.* [8]. This difference might be due to several factors, including the fact that patients in this cohort began antiviral therapy at an early stage of HIV-1 infection, that patients were all naive to antiviral drugs, and finally that amprenavir might have a less significant impact on the occurrence of lipodystrophy and lipid values, based on studies carried out in mice [45]. However, it is possible that these abnormalities may emerge over a longer duration of treatment.

The efficacy of protease inhibitor-sparing regimens, i.e. two nucleoside plus one non-nucleoside inhibitor of reverse transcriptase, as a first-line treatment in HIV-1-infected patients at an early stage of chronic infection, is currently being investigated. The results shown in the present study provide the basis for evaluating the virological and immunological efficacy of therapeutic regimens without protease inhibitors in individuals with a high (> 400 cells) CD4 cell count.

Conclusion

The present study demonstrates that the initiation of HAART at an early stage of established HIV-1 infection is associated not only with a durable suppression of virus replication but, more importantly, with a rapid quantitative systemic normalization of the CD4 cells and a partial recovery of HIV-specific CD4 cell responses.

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