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Higher levels of HIV DNA in *memory* and *naive* CD4$^+$ T cell subsets of viremic compared to non-viremic patients after 18 and 24 months of HAART

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Abstract

The degree of infection of *memory* and *naive* CD4$^+$ T cells in patients treated with HAART and with durable undetectable or detectable viral load in plasma was evaluated. The following two groups of patients were analyzed cross-sectionally: (i) patients with undetectable HIV RNA plasma levels during follow-up (responders); (ii) patients with no reduction or with rebound in HIV RNA levels during treatment (non-responders). Patients were examined following 6, 12, 18 and 24 months of HAART, respectively, by quantifying: (i) plasma HIV RNA load; (ii) CD4$^+$ T cells; (iii) *memory* and *naive* CD4$^+$ T cells; (iv) HIV DNA levels in *memory* and *naive* CD4$^+$ T cells. HIV RNA plasma levels were significantly higher in non-responders vs responders at each time point ($P < 0.02$), while CD4$^+$ T cell counts as well as *memory* and *naive* CD4$^+$ T cell levels were comparable in both viremic and non-viremic patients. However, higher HIV DNA values were observed in both *memory* and *naive* CD4$^+$ T cells of non-responders vs responders after 18 and 24 months of HAART ($P < 0.02$), suggesting an increased amount of HIV-infected *naive* CD4$^+$ T cells and a sustained high degree of infection of *memory* CD4$^+$ T cells. Immunological reconstitution

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following HAART might potentially be hampered in viremic patients despite the absolute increase in CD4$^+$ T cell counts.

Keywords: HIV DNA; memory CD4$^+$ T cells; naive CD4$^+$ T cells; HAART

1. Introduction

The introduction of combined antiretroviral therapies has proven to be effective in decreasing HIV replication for a prolonged period of time and to be associated with a dramatic increase in CD4$^+$ T cell counts. Indeed, the clinical benefit of HAART is documented by the nearly complete disappearance of AIDS-related opportunistic infections, suggesting an improvement of the immune system functions. However, both the extent of the immune system function restoration as well as the impact of HIV residual replication on the immune system reconstitution are not yet well understood. Particularly striking is the common observation of a dissociation between the progressive increase in CD4$^+$ T cell counts and consistently detectable HIV RNA levels in plasma (Kaufman et al., 1998; Levits, 1998). Provided that antiretroviral drugs do not appear to have a direct effect on stimulating CD4$^+$ T cell production or release, one can argue that residual HIV replication might have only a marginal influence on dynamics of CD4$^+$ T cells. In keeping with this hypothesis is the observation of similar dynamics of CD4$^+$ T cell subsets during HAART (redistribution of *memory* CD4$^+$ T cell within 3–6 months of treatment and proliferation of *naive* CD4$^+$ T cells within 6–9 months of therapy) in both non-viremic and viremic patients (Gray et al., 1998; Pakker et al., 1998; Mezzaroma et al., 1999; Connick et al., 2000).

However, some authors reported the beneficial effect of HAART only in expanding the CD4$^+$ T repertoire present before treatment (Connors et al., 1997), whereas others linked the extent of immune function restoration to the amplitude and duration of viral load reduction (Levits, 1998; Li et al., 1998; Pakker et al., 1998). These data have not been confirmed by other groups (Connick et al., 2000), highlighting the controversial aspects of this issue. From a virological standpoint, activated *memory* CD4$^+$ T cells are more susceptible to HIV infection (Sleasman et al., 1996; Spina et al., 1997). However, *naive* CD4$^+$ T cells may also be infected by HIV (Ostrowski et al., 1999). To date, only scant data on kinetics of HIV RNA in plasma in comparison with HIV DNA in peripheral blood mononuclear cells (PBMC) during HAART are available (Ibanez et al., 1999; Lillo et al., 1999; Fessel et al., 2000) and no comparison between the plasma virologic response to HAART and levels of HIV DNA in CD4$^+$ T cell subsets has been presented.

The aim of this study was to determine in both viremic and non-viremic patients during HAART the levels of: (i) *memory* and *naive* CD4$^+$ T cells; and (ii) HIV infection of these two cell subsets.

2. Materials and methods

2.1. Patients

Two groups of HIV-1-infected individuals were enrolled in the study at the Clinics of Infectious Diseases of the Università di Pavia, Pavia; Ospedale di Circolo, Busto Arsizio, Varese; Istituti Ospedalieri Cremonesi, Cremona; and Ospedali Riuniti di Bergamo, Bergamo, Italy, in the period 1998–2000: (i) 58 patients receiving HAART for at least 6 months who reached undetectable HIV RNA values in plasma (<50 copies/ml) within 4 months of treatment and remained consistently non-viremic afterwards (responders); (ii) 44 patients receiving HAART for at least 6 months and showing either no reduction ($n=35$, 79.5%) or rebound ($n=9$, 20.4%) in HIV RNA plasma levels during treatment (non-responders). Rebound was defined as an increase in HIV RNA plasma levels from undetectable (<50 copies/ml) to ≥ 50 copies/ml, lasting for at least 3–6 months prior to analysis.

Responders and non-responders were diagnosed as HIV-infected since a median time of 83.5 (range 49–156) and 87.0 (range 51–122) months, respectively. At the time of analysis, according to CDC classification, 23/58 (39%), 31/58 (53%) and 4/58 (7%) responders as well as 13/44 (29%), 22/44 (50%) and 9/44 (20%) non-responders were classified as B1, B2 and B3, respectively.

Responders and non-responders received HAART consisting of two reverse transcriptase (RT) nucleoside analog inhibitors (NRTI) + 1 or two protease inhibitors (PI), or two NRTI + 1 RT non-nucleoside analog inhibitor (NNRTI). Patients were stratified on the basis of treatment duration into four groups: patients having received HAART for 6 ($n = 17$ responders, $n = 10$ non-responders), 12 ($n = 20$ responders, $n = 10$ non-responders), 18 ($n = 11$ responders, $n = 15$ non-responders) and 24 months ($n = 10$ responders, $n = 9$ non-responders), respectively.

Twelve out of 58 (20.6%) responders and 8/44 (18.1%) non-responders received HAART as a first antiretroviral regimen, while 46/58 (79.2%) responders and 36/44 (81.7%) non-responders were submitted to either monotherapy with zidovudine followed by dual therapy with zidovudine + didanosine or stavudine + didanosine or zidovudine + lamivudine [5/58 (8.6%) responders and 4/44 (9.0%) non-responders, respectively] or were directly administered dual-therapy [41/58 (70.6%) responders and 32/44 (72.7%) non-responders, respectively] for a median time of 36 (range 9–72) months.

Inclusion criteria required patients to have $\geq$ 50/μl $CD4^+$ T cells at time of blood sampling in order to allow $CD4^+$ T cell subset purification (see below). On this basis, in the indicated period only three patients with $CD4^+$ T cell counts < 50/μl were excluded from the study.

2.2. Flow cytometry

Absolute $CD3^+CD4^+$ and $CD3^+CD8^+$ T cell counts were determined in EDTA-treated peripheral blood samples by using an Ortho Cytoron Absolute flow cytometer (Ortho Diagnostic Systems Inc., Raritan, NJ) and a combination of *anti*-CD3-CyP, *anti*-CD4-FITC and *anti*-CD8-PE monoclonal antibodies (Ortho Diagnostic System Inc.), according to the Manufacturer's instructions.

Three-color flow cytometry was also performed for supplemental $CD4^+$T lymphocyte subsets identification. A panel of monoclonal antibodies (Mabs) to the following cell surface markers were used: CD4-PerCP, $CD45RA^+$-FITC, $CD45R0^+$-FITC and $CD62L^+$-PE (Pharmingen, San Diego, CA). $CD4^+$T cell subset expressing $CD45RA^+$ and $CD62L^+$ were regarded as truly *naive* $CD4^+$ T cells, whereas $CD4^+$T cell expressing $CD45R0^+$, as well as T cells expressing $CD45RA^+$ but lacking $CD62L^+$, were regarded as *memory* lymphocytes. After staining of whole blood with monoclonal antibodies, samples were lysed with FACS Lysing Solution (Becton Dickinson, San Jose, CA). Ten-thousand lymphocytes were analyzed on a FACSCalibur (Becton Dickinson) equipment. The absolute counts of the above mentioned $CD4^+$T cell subsets were obtained using the TRUCount system (Becton Dickinson).

2.3. Memory and naive $CD4^+$ T cell purification

In detail, 50 ml of EDTA-treated blood were obtained from each patient. After PBMC separation on Ficoll-hypaque gradient (Arnika Diagnostic Linee, Novamed, Jerusalem, Israel), $CD4^+$ T cell population was indirectly enriched by depletion of $CD8^+$, $CD14^+$, $CD16^+$, $CD19^+$ and $CD45R0^+$ or $CD45RA^+$ by saturating cell markers with specific mouse MAbs and then capturing them with magnetic beads for immunomagnetic separation coated with *anti*-mouse MAbs, according to the Manufacturer's instructions (Dynal, Oslo, Norway). $CD45R0^+$ T cells were obtained from $CD45RA^+$-depleted $CD4^+$ T cell population, and immediately stored at -80°C. Similarly, $CD45RA^+$ T cells were obtained from $CD45R0^+$-depleted $CD4^+$ T population. These cells were further fractionated by positive selection of $CD62L^+$ subpopulation by labeling with *anti*-$CD62L^+$ and subsequent binding to magnetic beads. $CD45RA^+CD62L^+$ T cells were immediately stored at -80°C.

The purity ($> 95\%$) of the resulting subpopulations was confirmed by flow cytometry.

2.4. Virologic assays

Plasma HIV RNA levels were routinely determined every 3 months by using the Quantiplex (Chiron Corporation, Emeryville, CA, USA) assay according to the Manufacturer's instructions. Detection limit of Quantiplex assay was 50 HIV RNA copies/ml plasma.

HIV DNA levels in *memory* and *naive* $CD4^+$ T cell subsets were determined on 1×10^5 cell aliquots by quantitative PCR (Bagnarelli et al., 1994) after purification of $CD45R0^+$ and $CD45RA^+CD62L^+$ expressing cells.

2.5. Statistical analysis

Median time of HIV infection prior to HAART was compared between the two groups of patients using the *t*-test. In addition, median time of exposure to antiretroviral treatments prior to HAART between responders and non-responders was analyzed using the *t*-test. Finally, the distribution of responders and non-responders patients according to CDC classification was analyzed using the Pearson χ^2-test.

$CD4^+$ T cell counts as well as *memory* and *naive* $CD4^+$ T cell levels in responder and non-responder patients were expressed as median values, while HIV RNA plasma levels as well as HIV DNA copy numbers in the two lymphocyte subsets were expressed as geometric mean values $\pm$ 95% confidence intervals. HIV RNA plasma levels and $CD4^+$ T cell counts were compared between responder and non-responder patients prior to initiation of HAART using the Mann Withney *U*-test for non-parametric data. Differences in levels of HIV load, $CD4^+$ T cell counts, number of *memory* and *naive* $CD4^+$ T cells as well as HIV DNA in *memory* and *naive* $CD4^+$ T cells in responder patients with respect to non-responders after 6, 12, 18 and 24 months of HAART were also analyzed using the Mann Withney *U*-test. Patients with undetectable HIV RNA load were considered as having 25 HIV RNA copies/ml.

3. Results

3.1. Patients characteristics

Prior to HAART, responder and non-responders patients were comparable in terms of HIV RNA plasma levels and $CD4^+$ T cell counts (Table 1). In addition, no difference in the duration of HIV infection and previous exposure to antiretroviral drugs was documented between responders and non-responders (*t*-test, $P \geq 0.05$). Finally, at the time of analysis no difference in the distribution of patients according to CDC classification was observed between the two groups (Pearson χ^2-test, $P = 0.1$).

Table 1
Comparison of HIV RNA plasma levels and $CD4^+$ T cell counts of responders and non-responders prior to HAART

Patients	Geometric mean of HIV RNA copy no./ml plasma (±95% confidence intervals)		Median $CD4^+$ T cells /μl blood (range)	
N= 58 responders:	6,908 (±31,059)	p> 0.05[a]	271 (12-999)	p> 0.05
N=44 non-responders:	7,985 (±86,460)		176 (6-1,058)	

[a] Mann Withney U test for non-parametric data.

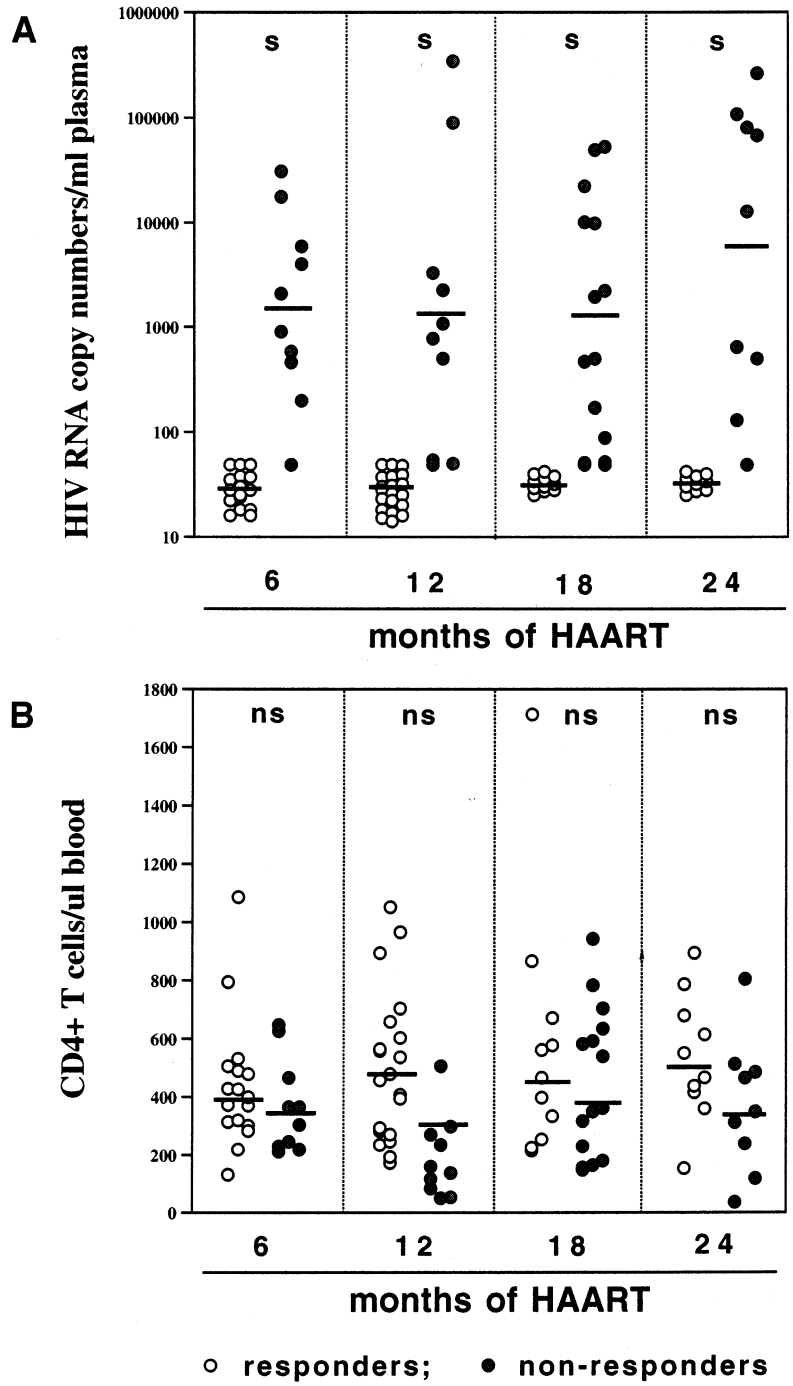

Fig. 1. HIV RNA plasma levels (A) and CD4$^+$ T cell counts (B) in responder and non-responder patients at 6, 12, 18 and 24 months of HAART. The geometric mean of HIV RNA levels (A) as well as the median values of CD4$^+$ T cell counts (B) are indicated by a dash in the relevant plots. On the top of each panel section, the level of statistical significance (s, significant; ns, not significant) between responders and non-responders is shown.

3.2. HIV RNA plasma levels and CD4$^+$ T cell counts in responders and non-responders during HAART

As expected, a significant difference was observed between responder and non-responder HIV RNA plasma values at each time point ($P \leq 0.02$, Fig. 1A). On the other hand, no significant difference in median CD4$^+$ T cell counts was observed between responders and non-responders at each time point ($P \geq 0.14$, Fig. 1B).

3.3. Memory and naive CD4$^+$ T cell counts

As shown in Fig. 2A and B, although responders had mostly higher median *memory* (CD45R0$^+$) and *naive* (CD45RA$^+$CD62L$^+$) CD4$^+$ T cell counts than non-responders, the difference between the two groups at each time point was not significant ($P \geq 0.05$).

3.4. Quantification of HIV DNA in memory and naive CD4$^+$ T cell subsets

As shown in Fig. 3A, geometric mean HIV DNA levels in *memory* CD4$^+$ T cells were consistently lower in responders compared to non-responders. However, the difference between the two groups was significant only at 18 and 24 months of HAART ($P \leq 0.02$).

At each time point, geometric mean HIV DNA values in *naive* CD4$^+$ T cells were lower in responders than in non-responders (Fig. 3B). However, the difference between values detected in responders vs non-responder patients was significant only at 18 and 24 months of HAART ($P \leq 0.02$).

Finally, when analyzing the distribution of HIV DNA amounts in *memory* and *naive* CD4$^+$ T cell subsets by dividing the total HIV DNA copy number by the cell number, it was found that the median number of *memory* and *naive* CD4$^+$ T cells required to detect at least one copy of HIV DNA in non-responders vs responders was significantly lower after 18 and 24 months of HAART between the two groups. In fact, the required number of *memory* CD4$^+$ T cells was 1309 (range, 170–100 000) vs 8680 (range, 585–50 000) at 18 months, and 598 (range, 282–2564) vs 2128 (range, 490–20 000) at 24 months, whereas the required number of *naive* CD4$^+$ T cells was 2593 (range, 111–50 000) vs 30 555 (range, 521–100 000) at 18 months, and 735 (range, 187–50 000) vs 14 285 (range, 203–100 000) at 24 months, respectively ($P \leq 0.04$).

4. Discussion

In HIV-infected individuals treated with HAART a sharp increase in CD4$^+$ T cell counts is documented in nearly all patients after a few months of treatment, including those with persistently detectable HIV RNA in plasma (Kaufman et al., 1998; Levits, 1998). However, the CD4$^+$ T cell increase appears somewhat related to the magnitude of the virologic suppression (Levits, 1998; Li et al., 1998; Pakker et al., 1998), while the possible interference of residual viral replication with respect to the degree of immune function restoration following HAART is still debated (Levits, 1998; Li et al., 1998; Pakker et al., 1998; Connick et al., 2000).

We evaluated levels of total CD4$^+$ T cells as well as *memory* and *naive* CD4$^+$ T cells subsets in patients receiving HAART for 6, 12, 18 and 24 months and with consistently undetectable HIV RNA levels in plasma in comparison with viremic patients. In addition, we compared the level of HIV infection in *memory* and *naive* CD4$^+$ T cell subpopulations in non-viremic and viremic patients receiving HAART.

Results of this cross-sectional study demonstrated that, at each timepoint, CD4$^+$ T cell counts were not statistically different between responders and non-responders, thus confirming previous findings showing an apparent dissociation between virological response to treatment and CD4$^+$ T cell restoration (Kaufman et al., 1998). Similar results were obtained when analyzing the CD4$^+$ T cell subsets. In fact, no significant difference in *memory* and *naive* CD4$^+$ T cell counts in non-responders vs responders was found.

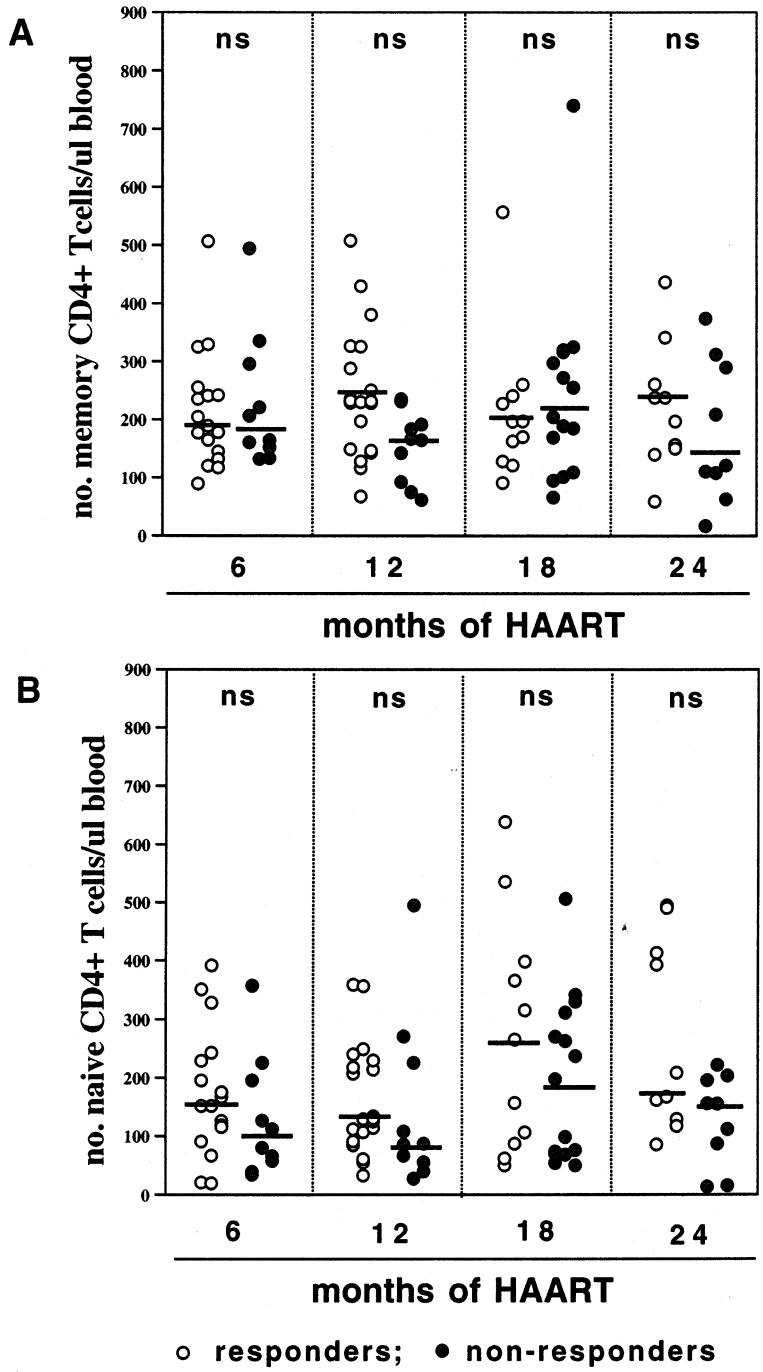

Fig. 2. Number of *memory* (A) and *naive* (B) $CD4^+$ T cell counts in responder and non-responder patients at 6, 12, 18 and 24 months of HAART. The median values of *memory* and *naive* $CD4^+$ T cell counts (A, B) are indicated by a dash in the relevant plots. On the top of each panel section, the level of statistical significance (s, significant; ns, not significant) between responders and non-responders is shown.

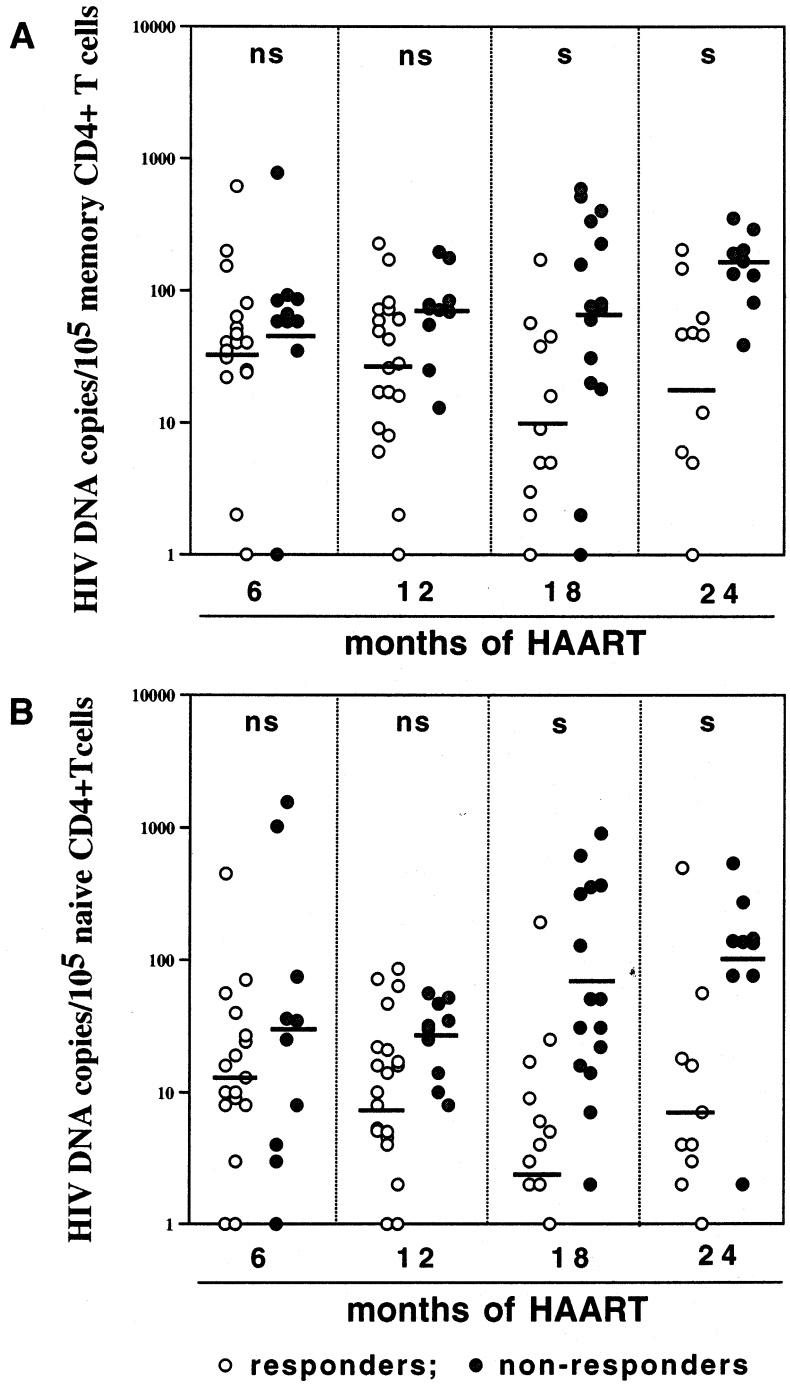

Fig. 3. HIV DNA copy number in *memory* (A) and *naive* (B) $CD4^+$ T cell counts in responder and non-responder patients at 6, 12, 18 and 24 months of HAART. The geometric mean of HIV DNA levels (A, B) are indicated by a dash in the relevant plots. On the top of each panel section, the level of statistical significance (s, significant; ns, not significant) between responders and non-responders is shown.

In contrast, despite similar $CD4^+$ T cell counts, non-responders showed significantly higher geometric mean HIV DNA levels in the $CD4^+$ T cell subpopulations than responders after 18 and 24 months of HAART. These findings suggest a different kinetics of proviral DNA in *memory* and *naive* $CD4^+$ T cells in responders vs non-responders during HAART and may point to an increased proportion of cells infected by HIV in viremic patients. This higher infection rate might ultimately have a direct impact on the increased turnover of $CD4^+$ T cell in viremic patients (Lyles et al., 1999). In fact, at the same timepoints the number of *memory* $CD4^+$ T cells required for detection of at least one copy of HIV DNA was 6.63- and 3.55-fold lower in non-responders than responders, respectively. Similarly, the number of *naive* $CD4^+$ T cells carrying at least one copy of HIV DNA was 11.78- and 19.43-fold lower in non-responders vs responders. In this respect, a viral burden-driven global immune system activation, leading to increased proliferation of both $CD4^+$ and $CD8^+$ T cells and subsequent enhancement of cell death has been recently described (Lempicki et al., 2000). In addition, Fessel et al. (2000) reported that higher levels of HIV DNA as well as infectious virus could be observed in 1×10^6 PBMC aliquots of HAART-treated viremic patients with decreased $CD4^+$ T cell counts as compared to non-viremic patients with an increase in $CD4^+$ T cell counts. HIV DNA levels were not determined in the $CD4^+$ T cell subsets. However, these findings appear to be in keeping with our observation of increased cell turnover associated with higher degree of HIV infection.

Although obtained in a cross-sectional study, our results show a different dynamics of infection in *memory* and, to a greater extent, *naive* $CD4^+$ T cells between viremic and non-viremic HIV-infected individuals. We believe that our results might be helpful in the understanding of virologic factors which may hamper long-term immune reconstitution during HAART. In fact, our data show an increase in the rate of HIV infection in $CD4^+$ T cell subsets after prolonged suboptimal inhibition of HIV replication. The relevance of this finding in terms of long-term disease progression and possible reappearance of opportunistic infections remains to be determined in larger prospective studies.

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