For timing of HAART is less more? CD4+/CD8+ ratio and CD4+ percentage as surrogate markers for more complex immunological features

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SUMMARY

There is disagreement on the optimal timing of HAART initiation based on absolute CD4+ T-cell count (CD4+ count). We investigated if naïve patients with CD4+ T-cell percentage (%CD4+) <29% or CD4+/CD8+ ratio <1 display signs of immune deterioration notwithstanding CD4+ count ≥500 cells/µl. We found that these patients show B-cell aberrations and an impaired control of Torque Teno Virus replication. By contrast, patients with CD4+≥500/µl, %CD4+≥29% and CD4+/CD8+≥1 displayed features of healthy subjects. Results obtained suggest that a combination of these parameters could be an adequate surrogate marker of immunological competence. This will be helpful in deciding when to start HAART.

KEY WORDS: Asymptomatic HIV-infected patients, CD4+ count, CD4+ percentage, CD4+/CD8+ ratio, HAART initiation.

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The optimal timing of highly active antiretroviral therapy (HAART) initiation in HIV-infected persons is still unclear. The latest guidelines recommend making a decision for the use of HAART on the basis of CD4+ absolute T-cell count (CD4+ count) taking into account the asymptomatic or symptomatic status of the patients. For asymptomatic individuals therapy initiation is suggested when CD4+ count is lower than 500/µl, whereas in the symptomatic it is recommended even at higher CD4+ counts (Antinori *et al.*, 2012). Moreover, a moderate recommendation by experts in the field suggests initiating therapy when CD4+ counts >500/µl, independently of the presence of symptoms (Working Group of the Office of AIDS Research Advisory Council 2012; European AIDS Clinical Society, 2012). This suggestion is strengthened by recent studies showing that if CD4+ count is maintained equal to or greater than 500-750 cells/µl, an event that usually occurs if patients start HAART early, the expected disease-free survival is comparable in HIV-positive patients and in the general population (Lewden *et al.*, 2012; Mocroft *et al.*, 2013). Moreover, some evidence suggests that early HAART helps to preserve immune functions, for instance the longevity of B cell responses in vaccinated HIV-1-infected children (Pensieroso *et al.*, 2009). Therefore, to better estimate the "immunological threshold" for HAART initiation, other parameters have to be taken into account. In par-

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ticular, in clinical practice, there is increasing evaluation of $CD4^+$ T cell percentage (%$CD4^+$) as an additional parameter to help decide whether to initiate therapy. From 1993, the HIV classification system has allowed for the use of the %$CD4^+$ in alternative to $CD4^+$ count. Threshold value of %$CD4^+$ calculated to obtain optimal concordance with $CD4^+$ count ≥500/µl was of 29% (MMWR Recommendation Report, 1992). %$CD4^+$>29% may therefore reinforce the indication to delay HAART initiation in asymptomatic patients when $CD4^+$ count ≥500/µl. Several studies have shown that, besides $CD4^+$ count, %$CD4^+$ and $CD4^+/CD8^+$ T-cell ratio ($CD4^+/CD8^+$) predict the risk of both AIDS and non-AIDS-related morbidities (Guiguet *et al.,* 2009; Clifford *et al.*, 2009; Syrjälä *et al.,*1991; DAD Study Group *et al.*, 2007). Moreover, Torti *et al.* (2012) showed that in HAART-treated HIV-infected persons experiencing a prolonged period of virus suppression, $CD4^+$ count, %$CD4^+$ and $CD4^+/CD8^+$ at baseline are all independent predictors of immune recovery.

Phenotypic and functional markers help to form a picture of patient immune status. In particular, we recently demonstrated (Fogli *et al.*, 2012) that exhausted memory B cells, characterizing the late stages of HIV infection (Moir *et al.*, 2008), emerge early during the disease being already present in asymptomatic HIV-infected patients naive for HAART with $CD4^+$ count ≥350/µl. B cell phenotypic aberrations were associated with a high load of a harmless virus endemic in the healthy population, the Torque Teno Virus (TTV) (Bendinelli *et al.*, 2001), whose control of replication has been considered a surrogate marker of immune competence (Focosi *et al.*, 2010).

We therefore sought to investigate if HAART-naïve patients with %$CD4^+$ <29% and $CD4^+/CD8^+$ <1 display signs of immune deterioration (either as immune-phenotype patterns and lack of control of TTV replication) despite having an absolute $CD4^+$ count ≥500/µl and, vice versa, if preservation of these parameters may be considered a surrogate of immune competence. To this purpose, surface phenotypic analysis of peripheral blood B lymphocytes was performed by 4-color flow cytometry on whole blood samples depleted of erythrocytes by lysis (FACS Lysing Solution, BD Biosciences). Directly fluorochrome-conjugated monoclonal antibodies specific to CD19, CD21, CD27 and CD10 (BD Pharmingen) were then used to evaluate the percentage of circulating exhausted tissue-like memory B cells ($CD21^{low}CD27^-$ cells, gated on $CD19^+CD10^-$lymphocytes), and mature activated B cells or plasma blasts ($CD21^{low}CD27^+$ cells, gated on $CD19^+$ $CD10^-$ lymphocytes). To determine TTV load, viral DNA was extracted from 200 µl of plasma samples and amplified using a single step universal TaqMan real-time PCR assay that targets a highly conserved UTR region of the viral genome. Amplification of this genome fragment enables to detect all the known TTV genotypes. The lower limit of sensitivity was 10^3 TTV DNA copies/ml of plasma (Maggi *et al.*, 2003). All samples evaluated in this study were assayed simultaneously in triplicate. Statistical analysis was performed using Mann-Whitney U-test. P≤0.05 was used as conventional level of significance.

Forty-two HIV-infected patients naïve to antiretroviral drugs, asymptomatic and without any history of opportunistic infections or malignancies were enrolled at the Section of Infectious and Tropical Diseases of the University of Brescia, where a written informed consent was obtained. The study was approved by the Ethical Committee of the University Hospital of Brescia according to the declaration of Helsinki. Thirty-four healthy controls (HC) were selected among the laboratory workers. Patient characteristics are described in Table 1. In all the subjects enrolled $CD4^+$ count never dropped below 350/µl. Within the HIV-infected patient cohort, 21 patients had $CD4^+$ count <500/µl (median: 469, range: 353-496) and 21 patients had $CD4^+$ count ≥500/µl (median: 621, range: 500-2049). Among the $CD4^+$ count ≥500/µl patients 9 were characterized by %$CD4^+$ ≥29% and $CD4^+/CD8^+$ ≥1, 9 had %$CD4^+$ <29% and $CD4^+/CD8^+$ <1, and 3 had just one of the 2 parameters over the threshold.

We first investigated if signs of B cell exhaustion were present in asymptomatic HAART-naïve HIV-infected patients with preserved $CD4^+$ count (≥500/µl). As expected, when patients with $CD4^+$ count <500/µl were compared to the HC cohort, we observed a significantly higher percentage of $CD21^{low}$ B cells, either exhausted tissue-like memory B cells ($CD10^-$

TABLE 1 - *Characteristics of asymptomatic HAART-naïve HIV-infected patient cohorts.*

Demographics	
Age (years)	42 (21-55)[1]
Male gender	29 (69%)[2]
Caucasian	42 (100%)[2]
Mode of transmission	
Heterosexual	21 (50%)[2]
Homosex/bisexual	13 (31%)[2]
Intravenous drug use	6 (14%)[2]
Other/unknown	2 (5%)[2]
HCV Abs	
Positive	11 (36%)[2]
Negative	31 (74%)[2]
HIV viremia (RNA copies/ml)	6584 (102-160116)[1]
Absolute CD4⁺ count (cell/μl)	
CD4 350/500 (n=21)	469 (353-496)[1]
CD4 ≥500 (n=21)	621 (500-2049)[1]
CD4+ percentage	
CD4 ≥29% (n=19)	33.3 (29-49.8)[1]
CD4<29% (n=23)	24.1 (11.9-28.3)[1]
CD4+/CD8+ ratio	
CD4/CD8 ≥1 (n=10)	1.1 (1.1-1.8)[1]
CD4/CD8 <1 (n=32)	0.6 (0.3-1)[1]
Group A patients (n=9)	
CD4 count	545 (510-2049)[1]
CD4 percentage	25.7 (11.9-28.5)[1]
CD4/CD8 ratio	0.5 (0.3-0.7)[1]
HIV viremia	6390 (105-62078)[1]
Group B patients (n=9)	
CD4 count	684 (500-857)[1]
CD4 percentage	36.8 (33.3-42.9)[1]
CD4/CD8 ratio	1.1 (1.1-1.8)[1]
HIV viremia	487 (74-58322)[1]

[1]Median, range; [2]number, percentage.

$CD21^{low}CD27^{-}$) and mature activated or plasma blast B cells ($CD10^{-}CD21^{low}CD27^{+}$) (median: 8.1% vs. 2.1%, p<0.0001 and 8.05% vs. 2.4%, p<0.0001, respectively). Of note, signs of B cell exhaustion were also present in subjects with $CD4^{+}$ count ≥500/μl. In fact, the patient cohort with $CD4^{+}$ count ≥500/μl displayed expansions of aberrant B cell subpopulations (p<0.0001 when compared to HC) that were superimposable to the one observed in the cohort of subjects with $CD4^{+}$ <500/μl (median of $CD10^{-}CD21^{low}CD27^{-}$ B cells: 8.1% vs. 12.4%, Not Significant, NS; median of $CD21^{low}CD27^{+}$ B cells: 8.1% vs. 10%, NS) (Figure 1A).

Due to the strong positive correlation between the abnormal expansion of exhausted tissue-like memory B cells and the lack of viral replication control of TTV we previously observed (Fogli *et al.*, 2012), TTV viremia was also evaluated. TTV was detected in both patient cohorts with $CD4^{+}$ ≥500/μl and $CD4^{+}$ <500/μl (median: 5.7 $\log_{10}$ vs. 5.6 $\log_{10,}$ NS) and these numbers of TTV genome equivalents were significantly higher than those observed in HC (median: 4.1 $\log_{10}$, p≤0.005) (Figure 1B).

This evidence indicates that, independently from the absolute $CD4^{+}$ count, asymptomatic HAART-naïve HIV-infected patients have B cell aberrations as well as an impaired capability to control the replication of a commensal virus. Therefore, a high absolute $CD4^{+}$ count is not "per se" associated with a complete preservation of the immune system.

Ranking HIV-infected patients with $CD4^{+}$ count ≥500/μl in two groups, those with only $CD4^{+}$ count ≥500/μl (*group A*), and patients with $CD4^{+}$ count ≥500/μl *plus* %$CD4^{+}$ ≥29% *and* $CD4^{+}/CD8^{+}$ ≥1 (*group B*) highlights that $CD21^{low}$ B cell subsets ($CD10^{-}CD21^{low}CD27^{-}$and $CD21^{low}CD27^{+}$) were significantly higher in *group A* than in *group B* (median: 7.9% vs. 3.6%, p≤0.05, and 8.1% vs. 4.0, p≤0.05, respectively), despite patients in *group A* and in *group B* having no significant differences in absolute $CD4^{+}$ count (median 545 vs. 684 cells/μl, NS). Percentages of $CD21^{low}CD27^{-}$ and $CD21^{low}CD27^{+}$ B cells in *group B* were comparable to those observed in the HC cohort (median: 3.6% vs. 2.1%, NS, and 4.0% vs. 2.4%, NS, respectively), whereas the percentages of both $CD21^{low}$ B cell subsets in *group A* were significantly higher than HC (median: 7.9 vs. 2.1, p≤0.001, and median: 8.1 vs. 2.4, p≤0.001, respectively) (Figure 1C). In line with a more preserved immune cell status, in patients belonging to *group B* TTV load did not significantly differ from that detected in HC (median: 5.3 vs. 4.1, NS) whereas TTV plasma viremia was observed to be higher in *group A* compared to HC (median: 5.8 vs. 4.1, p≤0.005) (Figure 1D). HIV median viremia, as well as the number of patients showing a low-level viremia (<1000 HIV-RNA copies/ml) did not significantly differ between group A and group B [median viremia: 6390 copies/ml in group A (p25:103/ p75: 3565) vs 487 copies/ml in group B (p25:

362/p75: 32750), NS; 3 patients in group A vs 5 patients in group B, NS] (Table 1).

It could be speculated that since it is yet uncertain whether antiretroviral therapy should be started at $CD4^+$ count ≥500/μl, the consideration of additional parameters like $\%CD4^+$ and $CD4^+/CD8^+$, that are readily available in clinical practice, may help to pinpoint the timing of therapy initiation by selecting patients whose immune status may be more compromised. The present results appear to reinforce previous clinical findings showing that patients with higher $\%CD4^+$ and $CD4^+/CD8^+$ may have a better prognosis independently from the absolute $CD4^+$ count (Burcham *et al.*, 1991; Gebo *et al.*, 2004; Taylor *et al.*, 1989). However, we are aware that since an estimated date of HIV infection for the patient cohorts analyzed in this study was not available, differences observed might be also influenced by a different duration of untreated HIV infection.

Albeit most studies focused on $CD4^+$ count increase as the main prognostic sign of immunological recovery after initiation of antiretroviral

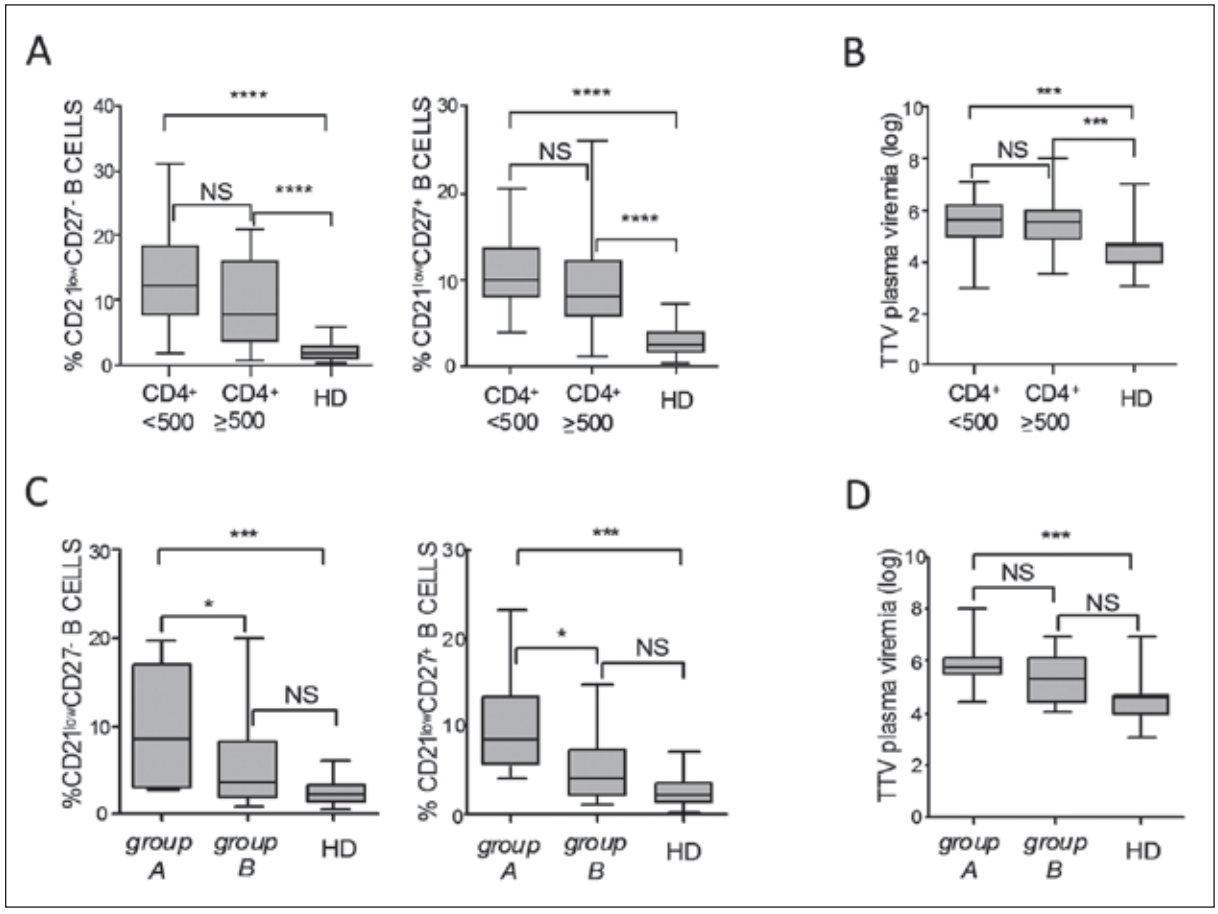

FIGURE 1 - *(A) HAART-naïve HIV-infected patients with either $CD4^+$ count <500/μl and $CD4^+$ count ≥500/μl show increased percentage of $CD21^{low}CD27^-$ (left panel) and $CD21^{low}CD27^+$ (right panel) B cell subsets compared to healthy controls (HC). (B) HAART-naïve HIV-infected patients with either $CD4^+$ count <500/μl and $CD4^+$ count ≥500/μl show higher TTV levels compared to HC. (C) HAART-naïve HIV-infected patients with $CD4^+$ count ≥500/μl, $\%CD4^+$ <29% and $CD4^+/CD8^+$ <1 (group A) show a higher percentage of $CD21^{low}CD27^-$ (left panel) and $CD21^{low}CD27^+$ (right panel) B cell subsets compared to HC whereas patients with $CD4^+$ count ≥500/μl, $\%CD4^+$ ≥29% and $CD4^+/CD8^+$ ≥1 (group B) have B cell subsets superimposable to HC. (D) Patients belonging to group A show higher TTV levels compared to HC whereas TTV viremia did not show significant differences between patients belonging to group B and HC. Graphs represent box-plot analysis with indication of median, 25^{th}-75^{th} and 10^{th}-90^{th} percentiles, and p value (****: p≤0.0001; ***: p≤0.001; *: p≤0.05; NS: Not Significant).*

therapy, some studies have described the potential usefulness of %CD4$^+$ and CD4$^+$/CD8$^+$ in estimating immune recovery. Hulgan *et al.* (2005), showed, for instance, that %CD4$^+$ <17% before initiation of the first HAART regimen was associated with subsequent clinical disease progression even in persons with absolute CD4$^+$ count ≥350/µl at baseline (Burcham *et al.*, 1991).
Moreover, Castagna *et al.* (2010), in a retrospective observational study performed on 24 week-antiretroviral-treated patients, observed that patients who started therapy with a higher %CD4$^+$ had a greater CD4$^+$ count recovery than other patient cohorts.
Lastly, Torti *et al.* (2012) observed that lack of increase in any of the immune indicators %CD4$^+$ and CD4$^+$/CD8$^+$ or CD4$^+$ count appear to be deleterious for the achievement of excellent immune recovery in long-term HAART-treated patients.
Overall, the present study, limited in size and observational in design, appears to reinforce the hypothesis that patients who have CD4$^+$ ≥500/µl, % CD4$^+$ ≥29% and CD4$^+$/CD8$^+$ ≥1 do not suffer from HIV-related immune deterioration. Albeit clinical recommendations of HAART initiation should be based on differences in clinical outcomes, our results suggest that evaluation of CD4$^+$ T cell number combined with % CD4$^+$ and CD4$^+$/CD8$^+$ may contribute to a better definition of HAART initiation timing.
Our data are in line with recent findings (Serrano-Villar *et al.*, 2013a; Serrano-Villar *et al.*, 2013b) regarding the impact of CD4/CD8 T cell ratio on immune activation and aging. However, to confirm these conclusions larger studies including clinical end-points are still needed. Finally, results obtained in this study suggest that restoration of not only CD4$^+$ count, but also of %CD4$^+$ and the CD4$^+$/CD8$^+$ ratio should be considered in evaluating the immune-reconstitution effects of HAART.

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