TNF-α levels in HIV-infected patients after long-term suppressive cART persist as high as in elderly, HIV-uninfected subjects

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Background: Chronic and systemic inflammatory alterations occur in HIV-infected patients and elderly uninfected subjects and in both scenarios these alterations are associated with the development of chronic morbidities and mortality. However, whether the levels of inflammatory alterations in untreated HIV-infected patients and elderly individuals are similar is unknown. Moreover, whether long-term antiretroviral therapy normalizes inflammatory alterations compared with HIV-uninfected persons of different age is not known.

Methods: We analysed soluble inflammatory levels [high-sensitivity C-reactive protein, interferon (IFN)-γ, tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-8 and IL-17] in a cohort of viraemic HIV-infected patients compared with (i) age-matched, (ii) elderly and (iii) non-survivor elderly, uninfected healthy controls. We longitudinally analysed the effect of long-term 48 and 96 week suppressive combined antiretroviral therapy (cART) on the soluble inflammatory levels compared with those found in control subjects.

Results: Baseline IL-6 and IL-8 levels were at similar or lower concentrations in untreated patients compared with healthy elderly individuals. However, TNF-α and IFN-γ levels broadly exceeded those found in survivors and non-survivor elderly individuals. Long-term suppressive cART normalized most of the inflammatory markers, with the exception of TNF-α levels, which persisted as high as those in elderly non-survivor controls.

Conclusions: Chronic inflammatory alterations associated with HIV infection are maintained at a different level from those of ageing. The persistent alteration of TNF-α levels in HIV-infected patients might cause tissue damage and have implications for developing non-AIDS-defining illnesses, even when HIV replication is long-term controlled by cART.

Keywords: HIV infection, inflammation, ageing

Introduction

Ageing and HIV infection are associated with profound changes in the immune system, with marked similarities. Both induce several defects that are particularly associated with T cell function, such as thymic involution, which reduces the numbers of circulating naive T cells.1–3 Although less described, persistent inflammation and a hypercoagulable altered state have been reported for HIV infection and ageing.6 Untreated HIV infection is associated with persistently high plasma interleukin (IL)-6 and tumour necrosis factor (TNF)-α concentrations.5 Additionally, chronic elevation of plasma inflammatory markers has been described in HIV-uninfected elderly subjects.6 Furthermore, these alterations are strongly associated with the development of morbidity and mortality in the elderly and in those with HIV infection.7–9 However, no previous studies have compared the inflammatory alterations of elderly individuals with those of naive, HIV-infected patients to address whether these alterations are similar and/or occur at the same magnitude for antiretroviral treatment.

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Once patients are treated with combined antiretroviral therapy (cART), the suppression of HIV replication results in a substantial decrease in AIDS-related morbidity and mortality,\textsuperscript{10} resulting in an increased life expectancy\textsuperscript{11} and a more aged HIV-infected population. However, cART mediates an immune restoration that, unfortunately, is incomplete even in long-term virologically suppressed patients.\textsuperscript{12} Thus, effectively treated patients show an immune dysfunction that includes inflammatory alterations,\textsuperscript{13} which translates to higher risk for developing chronic diseases (termed non-AIDS morbidities) when compared with similarly aged, HIV-uninfected persons.\textsuperscript{14} Cross-sectional and longitudinal studies have shown that short-term cART reduces but does not normalize systemic inflammatory alterations.\textsuperscript{5} However, whether long-term antiretroviral therapy normalizes or reduces these alterations compared with HIV-uninfected persons of different age is not known.

Thus, the aim of this study was to compare the levels of inflammatory markers in HIV-infected persons immediately before and after 48 and 96 weeks of long-term suppressive treatment, and also compare them with the inflammatory states of age-matched, elderly HIV-uninfected controls.

### Methods

#### Study participants

Naive-for-cART HIV-infected patients were retrospectively selected from the Infectious Disease Unit at Virgen del Rocío University Hospital in Seville (Spain). Thirty-nine asymptomatic and consecutive Caucasian patients were included according to sample availability from the HIV BioBank of the Spanish AIDS Research Network. Individuals were analysed at baseline ($n=39$, HIV<sub>0</sub>) and after 48 ($n=39$, HIV<sub>48</sub>) and 96 weeks ($n=19$, HIV<sub>96</sub>) of suppressive CART (defined as persistent undetectable viral load).

HIV-infected patients were compared with age-matched, HIV-uninfected healthy subjects (‘Young’ (Y) group, $n=26$) and with elderly subjects belonging to the previously described CARRERITAS cohort.\textsuperscript{15} Briefly, free-living volunteers and nursing home residents from Seville (Spain) were asked to participate in this cohort. Inclusion criteria included subjects aged $\geq 65$ years and self-sufficient health status. Exclusion criteria included diagnosis of dementia or any of the following situations during the last 6 months: (i) clinical data of active infections; (ii) hospital admission; (iii) antitumour therapy; or (iv) any treatment that could influence their immune status (mainly corticosteroids). Demographic data and blood samples were obtained at baseline (2008) and at the end of follow-up (2010), when all participants were contacted again to assess survival rates. For this study, consecutive baseline (at the cohort inclusion) samples from the CARRERITAS cohort were selected for two elderly control groups according to sample availability: individuals $\geq 65$ years who died during the 2 year follow-up of this cohort were selected as the ‘Elderly Survivor’ group ($n=26$). Individuals $>65$ years old who died during the follow-up despite being healthy at baseline were selected as the ‘Elderly Non-survivor’ group ($n=26$).

All necessary institutional or ethical review board approvals were obtained and written informed consent was obtained from all study participants.

#### Laboratory methods

Absolute CD4 T cell counts (cells/mm$^3$) were determined using an Epics XL-MCL flow cytometer (Beckman-Coulter, Brea, CA, USA). The plasma HIV-1 RNA concentration (HIV-RNA copies/mL) was measured using quantitative PCR (COBAS Ampliprep/COBAS Taqman HIV-1 test, Roche Molecular Systems, Basel, Switzerland) according to the manufacturer’s protocol. The detection limit for this assay was 20 HIV-RNA copies/mL. Plasma samples were tested for a hepatitis B virus (HBV)-related marker [hepatitis B surface antigen (HBsAg)] using an HBV ELISA (Siemens Healthcare Diagnosis, Malvern, PA, USA). Hepatitis C virus (HCV) RNA was detected using a commercially available PCR procedure (COBAS Amplicor, Roche Diagnosis, Barcelona, Spain) with a detection limit of 15 IU/mL. HCV exposure (anti-HCV) was detected using an HCV ELISA (Siemens Healthcare Diagnosis).

#### Measurement of inflammatory markers

Soluble plasma concentrations of the inflammatory markers interferon (IFN)-γ, IL-1β, IL-6, IL-8, IL-17 and TNF-α were determined in duplicate and included variability interplate controls (<20% variability was considered) using a two-site sandwich ELISA technique (R&D, San Diego, CA, USA). Plates were read using the ImageQuant LAS 4000 Mini imaging system (GE Healthcare Bio-Sciences, Uppsala, Sweden). The results were analysed using Q-view software (Quansys Bioscience, Logan, UT, USA) by automatically choosing the best curve-fitting regression models. High-sensitivity C-reactive protein (hsCRP) levels were determined through an immunoturbidimetric assay of sera using COBAS 701\textsuperscript{8} (Roche Diagnostics, Mannheim, Germany).

#### Statistical analysis

All continuous variables are expressed as median (IQR). Categorical variables are expressed as $n$ (%). Differences between groups were analysed using the $\chi^2$ test for categorical variables and the Mann–Whitney U-test, Friedman test and Wilcoxon test were used for unmatched and matched continuous variables. $P$ values $<0.05$ were considered significant. Statistical analysis was performed using SPSS version 20 (SPSS, Chicago, IL, USA) and Prism version 5.0 (GraphPad Software) was used to generate graphs.

### Results

#### Characteristics of the study subjects

The demographic and immunovirological characteristics of all study participants are shown in Table 1. CMV serostatus was highly prevalent in all study groups and was 92%, 81%, 92% and 96% in HIV-infected patients, young, elderly and elderly non-survivor groups, respectively. There were no statistical differences in CMV status among the different study groups. The median CD4 T cell count was 350 cells/mm$^3$ for HIV-infected patients at baseline and 9 and 39 (23%) had CD4 T cell counts $<200$ cells/mm$^3$, despite a median time of diagnosis of only 3.2 months. After 48 weeks of cART, CD4 T cell counts reached a median of 583 cells/mm$^3$ and 3 of 39 (8%) had active HCV infection. All study participants were negative for HBsAg. The median log$_{10}$ HIV RNA copies/mL was 4.5 at baseline and HIV suppression was achieved for all patients at a maximum of 48 weeks of therapy.
Naive HIV-infected patients showed higher TNF-α and IFN-γ levels than elderly subjects

IL-1β and IL-17 levels were below the detection limit of the assay. Figure 1 represents the results for the remaining inflammatory markers. These levels were first analysed in naive HIV-infected subjects (HIV0) and compared (solid lines) with those of age-matched controls (Y). All markers with the exception of hsCRP and IL-6 (Figure 1a and b) showed significantly altered levels in naive HIV-infected patients. IL-6 levels (Figure 1b) showed an increasing trend ($P = 0.137$) and when the analysis was restricted to patients with a time of diagnosis $1$ year (represented with open circles, $n = 14$), the differences in IL-6 concentrations reached statistical significance ($P = 0.003$). IL-8 plasma levels were also increased in naive HIV-infected patients when compared with age-matched controls (Figure 1c). In addition, the TNF-α and IFN-γ levels (Figure 1d and e) were strongly increased in the naive HIV-infected group when compared with the age-matched controls.

We explored whether the inflammatory alterations found in viraemic HIV-infected patients were similar to or different from those during age-related chronic inflammation and found different patterns. The hsCRP and IL-6 levels were not increased in HIV-infected patients when compared with elderly uninfected controls (Figure 1a and b). In addition, HIV-infected patients showed significantly lower hsCRP and IL-6 plasma levels than the uninfected, elderly non-survivor group. On the other hand, IL-8 levels, which were increased in HIV-infected patients when compared with young controls, were also significantly lower than those observed in elderly individuals (Figure 1c).

Remarkably, TNF-α and IFN-γ (Figure 1d and e) showed significantly higher levels in HIV-infected patients than in survivors and non-survivor elderly individuals.

HIV-infected patients showed TNF-α levels as high as elderly non-survivor controls, despite long-term suppressive cART

Long-term suppressive cART normalized most (IL-8, IL-6 and IFN-γ) of the inflammatory markers (Figure 1, dashed lines). hsCRP levels were not modified despite treatment. IL-8 concentrations were normalized to the levels of the healthy, age-matched controls after 48 weeks of cART; however, 96 weeks of treatment were needed to normalize IL-6 and IFN-γ concentrations (Figure 1a, c, b and e, respectively).

In contrast, long-term suppressive cART failed to normalize TNF-α concentrations (Figure 1d). When compared with the young controls, HIV-infected patients showed significantly high TNF-α levels ($P < 0.001$) after 48 or 96 weeks of suppressive treatment (Figure 1d). TNF-α levels progressively decreased in response to cART, but, after 96 weeks, they remained as high as the levels found in the elderly non-survivor group (Figure 1d).

Despite the small number of patients, we did not find differences in inflammatory alterations between HIV-infected patients coinfected or not with HCV at baseline or after 48 or 96 weeks of cART (data not shown). Likewise, no differences were found between patients receiving nucleoside reverse transcriptase

<table>
<thead>
<tr>
<th>Table 1. Baseline characteristics of HIV-infected patients and healthy subjects</th>
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<tr>
<td><strong>HIV-infected patients (n = 39)</strong></td>
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<tr>
<td>Age (years), median (IQR)</td>
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<tr>
<td>Male, n (%)</td>
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<tr>
<td>Positive for CMV IgG antibodies, n (%)</td>
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<tr>
<td>Transmission route, n (%)</td>
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<tr>
<td>sexual</td>
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<td>injection drug use</td>
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<td>CD4 T cell count (cells/mm³), median (IQR)</td>
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<td>CD4 T cell count &lt;200 cells/mm³, n (%)</td>
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<td>Nadir CD4 T cell count (cells/mm³), median (IQR)</td>
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<td>CDC-C event, n (%)</td>
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<td>HIV RNA (log₁₀ copies/mL), median (IQR)</td>
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<tr>
<td>Time since HIV diagnosis (months), median (IQR)</td>
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<tr>
<td>HCV RNA detected, n (%)</td>
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<td>cART type, n (%)</td>
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<td>NRTI containing</td>
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NA, not applicable; Y, young; ES, elderly survivor; ENS, elderly non-survivor.

Sexual includes male–male sex and heterosexual sex.
inhibitor (NRTI)-containing cART versus an NRTI-sparing regimen \[ n = 23 \ (59\%) \text{ versus } n = 16 \ (41\%), \text{ respectively} \] (data not shown).

**Discussion**

The results presented herein showed a heterogeneous and different level of chronic inflammatory alteration between HIV infection and ageing.

In accordance with other authors, our results demonstrate the profound disruption that HIV causes in the inflammatory state of untreated patients when compared with similar-aged controls. In addition, this is the first study to show the different magnitudes of systemic inflammatory alterations between ageing and HIV infection. However, these results must be put into the context of the present cohort (reduced sample size, 23% of the patients had CD4 T cell counts <200 cells/mm\(^3\), the median nadir CD4 T cell was 293 cells/mm\(^3\), 8% of patients had a CDC-C event and short time since diagnosis of HIV); therefore, caution should be used when generalizing the results and making comparisons with previously published reports.

Chronic immune activation and systemic inflammation are likely to be the major causes of systemic ageing of physiological functions and the driving mechanisms for developing chronic diseases, termed age-related diseases, in HIV-uninfected elderly subjects. These observations have also been described for HIV and have led to growing concern that HIV-infected persons suffer from accelerated or premature immunosenescence and ageing of the inflammatory system. Persistent viral infections such as CMV or HIV that stimulate and exhaust the immune system may therefore play a major role in driving the ageing of the immune system. Our cohort presents high CMV seroprevalence in agreement with other Spanish cohorts, estimated to be 93.0% in adulthood. Thus, the results presented herein suggest that although untreated HIV-infected patients and elderly uninfected subjects presented an altered chronic inflammatory state, HIV infection and not CMV coinfection might be the major cause favouring the inflammatory differences that we described for TNF-\(\alpha\) and IFN-\(\gamma\), which broadly exceed those of ageing. In this sense, we cannot exclude the possibility that the high hsCRP and IL-6 levels seen in elderly groups could be attributable to CMV reactivations and new
reinfections that occur more frequently in older people than in the young.20

Interestingly, we noticed that these alterations occurred shortly after infection (the median time since HIV diagnosis in the cohort was 3.2 months). Remarkably, we noticed that these alterations seemed to be time dependent. In this regard, only naive patients with >1 year since diagnosis showed significantly higher IL-6 levels compared with age-matched controls. Accordingly, higher IL-6 levels have been reported in naive patients compared with controls in data from a Strategies for Management of Antiretroviral Therapy study,21 but this result is in contrast to other studies.22 Therefore, our results suggest that these discordant results could be attributable to differing times since HIV diagnosis in patients from different cohorts. Consequently, these results showed the precocity of the inflammatory alterations and support the onset of cART as soon as possible.

Antiretroviral treatment tended to reduce systemic inflammation. However, only IL-8 levels were normalized after the first year of therapy. In contrast, 2 years of suppressive cART (>1 year of virological suppression) were needed to normalize IFN-γ and IL-6 levels. However, the TNF-α level remained elevated, despite 96 weeks of suppressive cART. Although we cannot exclude the possibility that the TNF-α level could normalize after a longer period of treatment, one possible explanation for this heterogeneous profile could be that TNF-α is secreted by different cell types but mainly by monocytes upon TLR4 lipopolysaccharide (LPS)-mediated activation. Furthermore, it is known that low LPS concentrations cause the activation and release of high amounts of TNF-α by these cells in vitro23 and we recently reported that long-term cART did not normalize levels of sCD14, which is another monocyte activation marker.24 Thus, these activated monocytes might be the major source of TNF-α. Persistent inflammatory and hypercoagulable states that remain altered, even when HIV replication has been well controlled by cART for a long time, can damage several tissues, which leads to cumulative harm that might end in chronic diseases or non-AIDS illnesses in HIV-infected patients. In this sense, the TNF-α concentration predicts cardiovascular risk and mortality in non-HIV-infected elderly subjects.8,24,25 Hence, these results support approaches involving strategies to reduce systemic TNF-α levels and may be important for reducing cardiovascular disease rates. Our results extend knowledge of inflammatory alterations after 96 weeks of suppressive treatment and provide further evidence of incomplete immune restoration mediated by cART even in long-term virologically suppressed patients.12,25 The potential mechanisms driving persistent inflammation could be: (i) microbial translocation caused by barrier defects in gut-associated lymphoid tissue related to HIV pathogenesis; (ii) residual HIV proteins in virologically suppressed patients that could directly activate the innate immune system; (iii) reactivation of endogenous pathogens such as Mycobacterium tuberculosis and herpesviruses; or (iv) dys-functional immunoregulatory factors.

In summary, our results demonstrate that the inflammatory alterations associated with chronic HIV infection are maintained at a different level from those during ageing. The persistent alteration of inflammatory markers, even after long-term suppressive cART, make it necessary to focus further investigations on the mechanisms that are the sources of this inflammation and potential therapeutic targets to reduce non-AIDS-defining illnesses and restore health to effectively treated patients.

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Transparency declarations

None to declare.

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