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Effect of highly active antiretroviral therapy on biomarkers of B-lymphocyte activation and inflammation

Deborah L. Regidor^{a,b,d}, Roger Detels^{a,d}, Elizabeth C. Breen^c, Daniel P. Widney^{b,d}, Lisa P. Jacobson^e, Frank Palella^f, Charles R. Rinaldo^g, Jay H. Bream^h, and Otoniel Martínez-Maza^{a,b,d}

^aDepartment of Epidemiology, UCLA School of Public Health

^bDepartments of Obstetrics & Gynecology and Microbiology, Immunology & Molecular Genetics, David Geffen School of Medicine at UCLA

^cDepartment of Psychiatry & Biobehavioral Sciences, Semel Institute for Neuroscience and Human Behavior, David Geffen School of Medicine at UCLA

^dUCLA AIDS Institute, University of California at Los Angeles, Los Angeles, California

^eDepartment of Epidemiology, Bloomberg School of Public Health, Johns Hopkins University

^fDivision of Infectious Diseases, Feinberg School of Medicine, Northwestern University

^gGraduate School of Public Health, University of Pittsburgh

^hDepartment of Molecular Microbiology and Immunology, Bloomberg School of Public Health, Johns Hopkins University, USA

Abstract

Objective—Chronic inflammation and B-cell hyperactivation are seen in HIV infection, contributing to an increased risk for the accrual of genetic errors that may result in B-cell lymphoma. The primary objective of this study was to determine the effect of highly active antiretroviral therapy (HAART) on serum levels of molecules that are associated with immune activation and/or inflammation, including several that are associated with B-cell activation, specifically IL-6, sCD30, sCD27, IgG, IgA, CXCL13 (B lymphocyte chemoattractant, BLC), a B-lymphocyte chemokine involved in B-cell trafficking, as well as C-reactive protein, an acute-phase protein.

Design—We used a retrospective cohort study design, measuring serum levels of these markers at each of four 1-year intervals, 2 years before and 2 years after HAART initiation, in a subgroup of 290 HIV-infected men enrolled in the Multicenter AIDS Cohort Study (MACS).

Methods—Serum levels of immune activation-associated molecules were measured by ELISA and multiplexed immunometric assays. Reference values were determined by the 5th to 95th percentiles from a sample of 109 HIV-uninfected MACS men.

Results—HAART use was associated with a reduction, but not normalization, of most biomarkers tested. Serum levels of IL-6 and C-reactive protein appeared to be unaffected by HAART.

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Correspondence to Otoniel Martínez-Maza, PhD, UCLA AIDS Institute, 173 BSRB, 615 Charles Young Drive South, Los Angeles, CA 90095-7363, USA. Tel: +1 310 825 2542; fax: +1 310 206 5387; omartinez@mednet.ucla.edu.

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Conclusions—These results suggest a partial normalization of serum cytokine levels post HAART. However, a chronic state of B-cell hyperactivation continues 2–3 years after HAART initiation. These findings may explain, in part, the excess incidence of lymphoma still occurring in HIV-infected persons in the post-HAART era.

Keywords

activation; AIDS; antiretroviral therapy; B lymphocytes; highly active; HIV; non-Hodgkin's lymphoma

Introduction

With HIV infection, there is a quantitative decrease and dysfunction of T-lymphocyte (T cell) activity, as well as an increase in B-cell activation [1–3] and an overproduction of B-lymphocyte (B cell) stimulatory cytokines [4–6]. This cytokine overproduction, together with the B-cell activation driven by antigen exposure and the direct stimulation of B cells by HIV [7–12], can result in chronic B-cell hyperactivation. Previous epidemiological studies have shown that cell surface and soluble markers of B-cell activation, as well as serum levels of B-cell-stimulatory cytokines, are elevated in HIV-infected persons [13–15]. In addition, progressive increases in levels of many of these molecules are seen as persons progress to AIDS, and even higher levels in those who develop AIDS-related non-Hodgkin lymphoma (AIDS-NHL) [13, 14, 16–24]. In addition to their effects in promoting B-cell activation, many of these cytokines and immune stimulatory molecules also have broader effects, and can promote inflammation and contribute to generalized immune system activation. For example, interleukin 6 (IL-6) is a proinflammatory cytokine with pleiotropic biological effects, in addition to being a potent B-cell-stimulatory cytokine [25, 26].

Whereas multiple mechanisms may contribute to lymphomagenesis in HIV-infected persons, there are two major mechanisms that appear to be involved in the development of these cancers: loss of immunoregulatory control of Epstein–Barr virus (EBV) and/or HHV8, and chronic B-cell activation due to the immune dysfunction resulting from HIV infection [14, 27]. Many forms of AIDS-NHL are characterized by the presence of recurrent genetic alterations, which may be due, in part, to errors in normal processes that occur in activated B cells, which involve modification of somatic DNA, such as immunoglobulin gene (*Ig*) class-switch recombination (CSR) and somatic hypermutation (SHM). Activation-induced cytidine deaminase (AID) is a DNA-editing enzyme normally active during B-cell activation, which plays a central role in both *Ig* SHM and CSR [28]. AID also promotes *c-MYC/IgH* translocations, and is required for the genesis of lymphomas of GC origin [29]. Moreover, AID is able to produce DNA double-strand breaks, resulting in widespread genome instability, which may contribute to some of the non-*Ig*-related genetic modifications that are present in AIDS-NHL [29]. Notably, *AID* expression is elevated in peripheral blood mononuclear cells (PBMCs) of HIV-positive individuals prior to the diagnosis of NHL [30]. Therefore, changes in HIV infection that result in chronic B-cell hyperactivation have the potential to contribute to the genesis of AIDS-NHL, particularly those forms that are EBV-negative, and are therefore not driven by the expression of EBV-encoded oncogenes.

It is not known whether effective HAART, which results in profound plasma HIV RNA suppression and consequent partial restoration of immune competence, allows normalization of serum levels of these markers of immune activation and inflammation, or of B-cell activity. It has been suggested by Biggar [31] that the continued increased risk for NHL among HAART-treated HIV-infected persons may be a reflection of incomplete immune reconstitution, and possibly a chronic state of B-cell hyperactivation. The continuation of

immune activation and inflammation following HAART initiation may contribute to ongoing B-cell hyperactivation, accounting, in part, for a persistent incidence in the post-HAART era of those AIDS-lymphoma subtypes associated with chronic B-cell hyperactivation (mainly EBV-negative, systemic NHL) [14, 31, 32].

The primary objective of this study was to determine the effect of HAART on serum levels of selected immune system molecules that are associated with B-cell hyperactivation and which are known to be elevated preceding the diagnosis of AIDS-associated lymphoma, specifically IL-6, a B-cell-stimulatory and proinflammatory, sCD30 and sCD27, soluble cytokine receptor molecules that are in the TNF receptor superfamily and are biomarkers associated with both T and B-cell activation [33, 34], and immunoglobulins IgG and IgA, products of activated B cells. In addition, we examined the effect of HAART on CXCL13 (BLC), a B-lymphocyte chemokine involved in B-cell trafficking and stimulation of B-cell activation that is produced by CD4-positive follicular helper T cells (T_{FH}) and by follicular dendritic cells [35], and is elevated in HIV infection and preceding AIDS-NHL diagnosis [15, 24], and C-reactive protein (CRP), an acute-phase protein and a marker of inflammation, the production of which is driven by IL-6 and related cytokines [36–39]. Many of these molecules can enhance B-cell activation, resulting in the induction of potentially oncogenic molecular lesions in B cells. The results presented here suggest that HAART is associated with a reduction, but not normalization, of some, but not all, of these biomarkers for B-cell activation and/or inflammation.

Methods

We retrospectively examined a cohort of men enrolled in the Multicenter AIDS Cohort Study (MACS), measuring serum biomarker levels at up to four time points pre-HAART and post-HAART initiation: T1 (3.0–1.5 years pre-HAART, $n = 270$), T2 (1.5–0.5 years pre-HAART, $n = 236$), T3 (0.5–1.5 years post-HAART, $n = 261$), and T4 (1.5–3.0 years post-HAART, $n = 266$). Detailed information on the MACS cohort has been reported elsewhere [40]. Established in 1984 to study the natural history of HIV disease, the MACS is a prospective cohort of 6972 high-risk adult men who have sex with men. Data and blood samples are collected at semi-annual visits. Archival serum specimens, collected at bi-annual MACS study visits, were stored frozen at -70°C in the MACS national central repository, and were stored in this manner until thawed for testing.

Patient eligibility

Eligible patients had to be HIV-infected and to have reported HAART use for at least two of three consecutive semi-annual MACS visits. HIV seroconverters were eligible if the interval between the date of seroconversion and HAART initiation was at least 2 years. Participants were required to have at least one serum sample prior to and after HAART initiation, within ± 3 years from HAART initiation. There were 467 men who met eligibility requirements. Of the 467, we included men who developed AIDS-related NHL ($n = 12$) and all eligible seroconverters ($n = 105$). From the remaining 350 eligible participants, we selected 183 men by random sampling, to achieve a study size of 300. Elimination of 10 men who were later determined to be ineligible resulted in a final sample size of 290.

A convenience sample of 109 HIV-seronegative men from the MACS was used to determine reference ranges within this population.

Data collection

Serum samples for this study were obtained from the MACS national repository, and kept frozen at -70°C until used for testing. Other demographic and clinical information was

obtained from the MACS database. Most biomarkers were measured by enzyme-linked immunosorbent assay (ELISA): IL-6 (Biosource), CRP (Hemagen), sCD30 (Bender MedSystems), sCD27 (Sanquin PeliKine), and CXCL13 (R&D Systems). IgG and IgA were determined using Luminex-based multiplexed bead immunosorbent assay (Millipore), and analyzed using a Bio-Rad Bioplex 200 analyzer.

The definition of HAART was guided by the DHHS/Kaiser Panel (DHHS/Kaiser 2006) guidelines [41]. We defined HAART exposure as use that was reported on at least two of three consecutive visits after HAART initiation.

Baseline CD4 and HIV viral load were defined at the visit prior to HAART initiation. CD4 slope was determined by regressing CD4 levels on time, using data within the interval beginning two visits after either study entry for seroprevalent or seroconversion for seroincident men to the last visit at which no HAART use was reported. Viral response was defined as demonstrating a nondetectable plasma/serum HIV RNA (viral load) level within 1.5 years after HAART initiation (T3), with evidence of sustained viral suppression in the subsequent measurement (T4), if available. A nondetectable viral load was designated as 50 copies/ml, the lower limit of detection (Roche, TriCore Reference Laboratories). Virologic nonresponse was defined as HIV viral load greater than 400 copies/ml within 1.5 years after HAART initiation (T3), with nonsuppression sustained in the subsequent measurement (T4), if available. Patients who had no reported viral load within 1.5 years after HAART initiation and those with viral loads of 50–400 copies/ml were excluded in the subgroup analysis of viral response.

Statistical methods

Using the one-sample *t* test, we determined that a sample size of 290 had at least 80% power to detect expected differences in biomarker levels over time (NCSS/PASS evaluation version 2004).

We used a paired *t* test for descriptive analyses, comparing pre-HAART and post-HAART mean biomarker values. A linear mixed model was used to model each biomarker, by HAART and time, using T1 as reference. We then controlled for other covariates, first individually and then in a multiple variable model. SAS V8.2 (SAS Institute, Cary, North Carolina, USA) was used for statistical analyses. Informed consent was obtained from all participants in compliance with the appropriate ethical committee at each study site.

Results

Among the original 300 patient study cohort, 186 (62%) participants contributed four samples, 92 (31%) contributed three samples and 22 (7%) contributed two samples. After eliminating ineligible participants, the final analysis included 290 participants with 1033 samples.

Table 1 describes the characteristics of the study population at the first MACS visit in which HAART use was reported, defined as the date of HAART initiation. The median date of HAART initiation was July 1997, the mean age was 43.8 years, and the median CD4 and HIV RNA levels were 296 cells/ μ l and 21 906 copies/ml, respectively. Sixteen percent ($n = 47$) of the study group had been diagnosed with AIDS, and the most common first AIDS-defining illness was *Pneumocystis carinii* (*jiroveci*) pneumonia (PCP). The NHL subgroup tended to be older, with higher baseline HIV RNA levels, and lower CD4. In this group, the mean age was 48.5 years, and the median CD4 and HIV RNA levels were 167 cells/ μ l and 88 080 copies/ml, respectively.

Table 2 describes selected population characteristics at each measurement time point. The majority of measurements occurred between 1995 (T1) and 1999 (T4). Median pre-HAART CD4 and HIV RNA levels at the measurement closest to HAART initiation were 322 cells/ μ l and 27 276 copies/ml, respectively, with the median CD4 cell count increasing to 474 cells/ μ l and nondetectable HIV RNA levels apparent 1.5–3 years after HAART initiation. For persons who were antiretroviral therapy (ART) experienced prior to HAART initiation, nondetectable HIV RNA levels were achieved in 49% (T3) and 53% (T4) of persons after starting HAART, compared with approximately 80% of those study participants who were ART-naïve prior to HAART. About half of the participants were not receiving any antiretroviral therapy at the measured pre-HAART visits.

As shown in Fig. 1a–g, we observed a heterogeneous pattern of change in crude biomarker levels over time. Compared with the HIV-seronegative controls, after HAART initiation there was a reduction, but not normalization, of all biomarkers assayed with the exception of IL-6 and CRP. IL-6 remained elevated, and CRP marginally elevated, across all four time points, and the serum levels of these molecules appeared to be unaffected by HAART use.

Similar results were seen in the subgroup of men who developed NHL. Pre-HAART biomarker levels tended to be higher than in the total study group. However, their response to HAART was similar to that seen in the larger group: comparing results from the latest post-HAART (T4) to the earliest pre-HAART (T1) time-point, median [interquartile range (IQR)] sCD27 decreased from 581 (382–697) to 320 (276–525) U/ml; sCD30 from 92 (80–123) to 48 (28–88) U/ml; CXCL13 from 272 (133–489) to 121 (72–228) pg/ml; IgG from 34 257 (20 629–32 323) to 22 845 (20 780–24 564) ng/ml; and IgA from 984 (736–1651) to 870 (408–1114) ng/ml. IL-6 remained relatively stable and CRP showed an increase over time: IL-6 from 3.23 (2.03–3.82) to 2.82 (2.00–5.13) pg/ml, and CRP from 1.80 (1.22–2.31) to 3.13 (2.02–5.84) μ g/ml.

Subgroup analyses of 100 men with effective viral responses and post-HAART CD4 cell count at least 350 cells/ μ l showed a similar heterogeneous pattern: at 2–3 years after HAART initiation, 29% of sCD27, 48% of CXCL13, and 51% of IgG analyte levels continued to exceed reference limits, whereas sCD30 approached normalization.

To determine if the biomarkers tested displayed distinct patterns of co-expression, we assessed the correlation of serum levels of these molecules. Levels of sCD27 and sCD30 were strongly correlated with each other ($r = 0.44$, $P < 0.001$). Both of these molecules are members of the TNF receptor superfamily, are associated with immune activation, and demonstrate functional similarities [33, 34], so the strong correlation noted is consistent with their biological properties. Additionally, both sCD27 and sCD30 were moderately correlated with IgG ($r = 0.32$, $P < 0.001$ and $r = 0.29$, $P < 0.001$, respectively) and with IgM ($r = 0.20$, $P < 0.001$ and $r = 0.30$, $P < 0.001$, respectively), consistent with these soluble receptors being associated with enhanced B-cell activity [33, 34]. Serum levels of CXCL13 were seen to correlate with both sCD27 ($r = 0.18$, $P < 0.001$) and sCD30 ($r = 0.42$, $P < 0.001$), as well as with IgG ($r = 0.22$, $P < 0.001$) and IgM ($r = 0.19$, $P < 0.001$), consistent with the B-cell-stimulatory properties of this chemokine [15, 42]. There was a moderate correlation between IgG and IgA ($r = 0.26$, $P < 0.001$), and a strong correlation between IgG and IgM ($r = 0.47$, $P < 0.001$). As expected, there was a moderate correlation between IL-6 and CRP ($r = 0.21$, $P < 0.001$). This is consistent with the known role of IL-6 in the induction of the acute-phase response [26], which includes the elevated production of CRP.

Results of multivariable analyses, controlling for baseline CD4 and HIV RNA levels, CD4 slope, and AIDS status pre-HAART are shown in Table 3 and illustrated in Fig. 2. In addition, as age was shown to be independently associated with IL-6 and CRP, we

controlled for age in the analyses of these two biomarkers. Results are depicted for the total population (Fig. 2a), stratified by HIV viral response (Fig. 2b), and stratified by ART status at HAART initiation (Fig. 2c). Separate models were run for each immune activation biomarker. There was a statistically significant effect of HAART on all biomarkers except IL-6, CRP and IgA. These ranged from a post-HAART decrease of 17.5 (10.7–24.3)% for IgG to 41.6 (31.7–51.5)% for CXCL13 in the total population, 19.6 (7.2–31.9)% for IgG to 55.8 (38.7–72.9)% for CXCL13 in viral responders, and 29.3 (14.2–44.3)% for IgG to 62.5 (45.0–79.9)% for CXCL13 in those naïve to ART prior to HAART initiation.

Highly active antiretroviral therapy was seen to be associated with a significant decrease in serum levels of sCD27 and sCD30, even in the absence of an apparent viral response. This observation may be explained either by a direct effect of antiretroviral drugs on the immune cells that produce these molecules, or more likely, by a HAART-mediated restoration of immunity resulting from virological suppression, even when this suppression was suboptimal. In support of this, a substantial rebound in CD4 numbers was seen, in the absence of adequate virologic response (i.e. even among persons who achieve only partial viral suppression on HAART), as shown in Fig. 3. Among participants who were considered viral nonresponders post-HAART CD4 cell count, compared with pre-HAART levels, increased by 61 (21–101) cells/ml to a median (IQR) of 364 (230–537) cells/ μ l 2–3 years post HAART ($P = 0.003$).

Baseline CD4 cell count appears to modify the effect of HAART use on CXCL13 level, where higher CD4 was associated with a greater biomarker response to HAART. When stratifying by baseline CD4, a decrease of 34.8 (14.8–54.8) % was seen in CXCL13 in those with baseline CD4 cell count below 200 cells/ μ l; in contrast, a decrease of 48.2 (34.1–62.3)% was seen in those with baseline CD4 cell count at least 350 cells/ μ l.

As shown in Table 2 HAART was most effective among persons who had been ART-naïve prior to HAART initiation. Consistent with this observation, there was a statistically significant interaction between pre-HAART status and HAART effect upon most of the biomarkers (Table 3c), with a greater responses noted among those persons who were ART-naïve prior to HAART.

Discussion

Serum levels of the biomarkers associated with immune activation and inflammation, with the exception of IL-6 and CRP, were seen to be decreased, but not normalized, following the initiation of HAART. It is assumed that these decreased levels of serum biomarkers of inflammation and B-cell activation result primarily from the virological suppression mediated by HAART. IL-6 levels were elevated prior to HAART initiation and appeared to be unaffected by HAART. CRP levels were modestly elevated prior to HAART and increased over time, a pattern seen in a previous longitudinal study of untreated HIV-infected men from the MACS [37].

Only one-third of study participants achieved immediate and sustained virologic responses. In the subgroup that did not achieve optimal HIV suppression, increases in CD4 cell number were still apparent, with significant reductions in serum sCD27 and sCD30 levels. Results of subgroup analyses suggest that the effects of HAART on serum biomarkers was most pronounced in those persons who were naïve to ART prior to HAART initiation, consistent with previous studies [43]. Therefore, persons who have experienced therapeutic failure with prior nonfully suppressive ART regimens, with resultant accumulation of drug resistance, may have a decreased probability of immune normalization with subsequent regimens.

There is a paucity of data examining the effect of HAART on the biomarkers measured in this study, and no published study has measured this group of biomarkers simultaneously. Atlas *et al.* [44] and De Milito *et al.* [45] examined the effect of HAART on plasma sCD27 levels. Atlas *et al.* reported results from a study of 64 persons (primarily of African ethnicity living in Sweden) with mixed HIV-1 subtypes, who were ART-naïve at HAART initiation and experienced effective viral responses to HAART. When comparing results from a subgroup infected with HIV-1 subtype B, the predominant viral subtype in North America, to our results, Atlas *et al.* showed a more modest 9% decrease in sCD27 levels. De Milito *et al.* found that HAART-induced significant and progressive reductions, but not normalization, of plasma sCD27 levels in 26 HIV-infected persons after 27 months.

The results of our study are consistent with a previous pilot study of 34 MACS participants that examined the effect of HAART on CXCL13 serum levels [15], which reported a 21% decrease in CXCL13 serum levels after 1 year of HAART. Our results, in a larger study population, demonstrated greater than 40% decreases in CXCL13 after 2–3 years of HAART. Interestingly, baseline CD4 cell count appeared to modify the effect of HAART use on CXCL13 level, with higher CD4 being associated a greater decrease in CXCL13 levels following HAART initiation. CXCL13 is produced by follicular helper T cells (T_{FH}), which is the T-cell subset that interacts with activated germinal-center B cells in secondary lymphoid organs [35, 42]. Little is known about the effect of HIV infection on T_{FH} cells, or about how these cells are normally regulated. A recent study identified a novel regulatory $CD8^{+}$ T-cell subset in mice, which can suppress the activity of T_{FH} cells [46]. When these $CD8^{+}$ regulatory T cells were disrupted, this resulted in the enhanced production of auto-antibodies and in autoimmunity, and in a 10-fold increase in the size of germinal-center area [46]. It is interesting to speculate that more advanced HIV disease, for which low CD4 number can act as a marker, results in a more pronounced loss of such regulatory T-cell subsets, making it more difficult for HAART to restore the activity of such cells and dampen T_{FH} activity, thereby resulting in a less marked decrease in CXCL13 levels post HAART. Clearly, additional research is needed to better define the effect of HIV infection, and of HAART, on T_{FH} cells and on potential regulatory T-cell subsets that may act on those cells.

Several studies have examined the association between HAART and hypergammaglobulinemia [47–50]. Overall, these studies report a reduction in IgG with HAART use, and in some cases, normalization. Our results are consistent with these prior studies, but show much more modest decreases in serum IgG levels. In a case–control study, Chong *et al.* [47] reported 65% lower IgG levels among virologic responders who were compared with an ART-untreated group. Notermans *et al.* [48] reported complete normalization of IgG in 13 persons with virologic responses to HAART after 1.5 years of therapy, using a population-based reference range determined for the specific methodology in the testing hospital. Jacobson *et al.* [50] and Redgrave *et al.* [49] reported findings that were fairly consistent with ours, noting significant decreases, but not normalization, of IgG levels in persons who achieved effective HAART responses, with 45% (Jacobson *et al.*) and 37% (Redgrave *et al.*) of IgG values continuing to exceed reference limits.

Our results indicate that serum CRP levels were unaffected by HAART, and in fact appear to increase over time following HAART initiation. A similar pattern was seen in a study by Lau *et al.* [37], who reported a general increase in CRP over time in untreated HIV-infected men in the MACS, with a 4.5 and 8% increase per year for men who remained AIDS-free and for men who progressed to AIDS, respectively.

Overall, these findings suggest that HAART resulted in near normalization of some biomarkers, whereas others continue to be markedly elevated post-HAART. It is possible that such sustained immune system alterations occurring among HAART recipients may

lead to ongoing or new pathological processes. It is also possible that immune dysfunction is resolving slowly, and will eventually normalize over time, with resultant normalization in markers of immune activation and inflammation. However, our findings do not support this, in that we saw fairly consistent results between the two post-HAART measurements. Perhaps more extended longitudinal study of immune activation biomarkers among successfully treated HAART recipients is warranted, to determine whether and when full immune function is restored. It would be expected that the longer a state of chronic B-cell hyperactivation exists, the greater the risk for accumulation of genetic errors that can lead to the development of AIDS-related NHL. Therefore, even if immune normalization is ultimately achieved, it would be expected that the excess risk of NHL among HIV-infected persons would be reduced, but not eliminated, with HAART use. This is consistent with the results of epidemiologic studies, which have noted whereas HAART is associated with a decreased incidence of AIDS-NHL, a significant elevation in NHL risk remains in HIV-positive persons receiving HAART [31, 32, 51].

Previous studies have shown that although HAART results in viral suppression to undetectable levels in peripheral blood, viral eradication is not achieved, even after years of continuous therapy [52], and there is quick rebound of plasma viremia with therapy interruption. Viral persistence is thought to be the result of long-term survival of infected memory CD4 cells, permitting a steady-state low level of viral replication in lymphoid tissue that is not detectable in the peripheral blood [53]. This low-level HIV replication may contribute to persisting B-cell hyperactivation in those on HAART. The progressive increases in serum CRP levels seen in this study suggest ongoing immune activation may continue post-HAART.

We found no apparent effect of HAART upon IL-6 levels. The SMART study, a study of HAART administration, with one arm based on minimizing drug use using target CD4 number (drug conservation arm) and another aiming at maximal suppression of HIV plasma levels (viral suppression arm), found that IL-6 levels increased by 30% over the first month of the trial in the drug conservation arm but were unchanged in the viral suppression arm, and also that the risk of death was associated with higher IL-6 levels at study entry [36]. In another study, plasma IL-6 levels did not seem to change in most HAART recipients following initiation of treatment, except in those who developed immune restoration and inflammatory syndrome (IRIS), who showed an increase in plasma IL-6 post-HAART [54]. IL-6 is a B-cell-stimulatory cytokine that is involved in promoting *IgH* class switching and also has antiapoptotic effects on B cells, effects that have the potential to promote the development of B-cell cancers. In addition, a persistent excess of IL-6 may promote nascent lymphoma clones through its anti-apoptotic effects. Therefore, even if levels of other cytokines are normalized in HAART recipients, there still may be increased risks for those forms of AIDS-related NHL that are associated with errors in *IgH* class switch DNA recombination, which may be promoted by IL-6. If this is true, one would expect to observe excess levels of those forms of AIDS-NHL associated with this genetic error among HAART-treated persons. In light of this, it is informative to note that whereas the incidence of EBV-associated AIDS-related NHLs has decreased in the first decade of the HAART era, the incidence of Burkitt's lymphoma has not decreased significantly [32].

Because this study did not include concurrent HAART-naïve HIV-infected controls, we cannot adequately account for temporal changes that may have confounded this analysis (i.e. changes in patient management strategies over time, and better management of comorbidities including other opportunistic illnesses). However, our results were generally consistent within each time period studied, and significant changes in biomarker levels were seen within the fairly brief time periods closest to HAART initiation, often as short as a 1-year interval. Results from historical controls selected from the MACS cohort suggest that

these biomarker levels are relatively stable over a period of three to five years (data not shown).

Most of the HIV-infected men in our study sample are long-term survivors. In this group of 467 participants, 81% were HIV-infected by early 1988, and may have been infected as early as 1978. Hence, this is a population that survived between 8 (seroincident participants) and 14 years (seroprevalent participants), respectively, prior to the introduction of HAART. These survivors are healthier and slower progressors than other MACS participants, who progressed to AIDS and died prior to HAART availability, and therefore could not have participated in this study. Obviously, the results obtained in this study cannot provide information on HAART's impact upon serum B-cell activation associated molecules in these faster progressors.

Another potential source of error can occur with spurious events that cause transient increases in B-cell activation, such as responses to acute infection. Since effective HAART will potentially decrease the likelihood of acute infections, it is probable that acute infections will more likely occur prior to exposure to HAART.

Ultimately we would like to have been able to evaluate most closely those persons who developed AIDS-related NHL during the course of their infection and HAART treatment. However, with only 12 men this group developing AIDS-NHL, it was not possible to provide a formal analysis of this subgroup. Descriptive analyses suggest the NHL subgroup had a similar HAART response to that seen in the larger group of HIV-infected persons, with substantial decreases, but not normalization, of biomarker levels following HAART. Further studies in a larger NHL group are needed to support these findings.

Overall, our study indicates that HAART is associated with reductions in, but not normalization of, some markers of B-cell activation. None of the biomarkers were completely normalized in all individuals; 65% of individuals continued to have at least one elevated biomarker 2–3 years after HAART initiation. It is clear that good virologic and CD4 responses to HAART do not necessarily equate with normalization of these B-cell activation-associated molecules. Perhaps, markers of immune recovery should include routine assessment of markers of both B-cell and T-cell function. Finally, our results suggest that an altered immune environment still exists 2–3 years after HAART initiation, which has the potential to contribute to the increased risk of AIDS-related NHL that persists in those receiving effective antiretroviral treatment.

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Fig. 1. Biomarker levels pre and post-HAART

Levels of cytokines and molecules associated with inflammation and immune activation were assessed as described in the Methods section, in sera collected at two MACS study visits prior to, and two visits after, the initiation of HAART. The pre-HAART study visits were at -3.0 to -1.5 years (T1) ($n = 270$), or -1.5 to -0.5 years (T2) ($n = 236$), preceding HAART initiation. Post-HAART visits were at 0.5 to 1.5 years (T3) ($n = 261$), or 1.5 to 3.0 years (T4) ($n = 266$), post-HAART initiation. The characteristics of these participants and visits are outlined in Table 3. Serum levels of these same biomarkers, assessed in a reference group of HIV-negative MACS participants also is provided (HIV-) ($n = 109$). Average levels of all of the biomarkers shown in this figure were significantly decreased post-

HAART ($P < 0.001$), except IL-6 and CRP, which were not significantly decreased post-HAART. CRP, C-reactive protein; HAART, highly active antiretroviral therapy; MACS, Multicenter AIDS Cohort Study.

Fig. 2. Effect of HAART on biomarkers of inflammation and immune activation
 (a) Total population, (b) stratified by HIV viral response, and (c) stratified by ART exposure at HAART initiation. HAART, highly active antiretroviral therapy.

Fig. 3. Distribution of CD4 lymphocytes over time by HIV viral response

T1: -3.0 to -1.5 years pre-HAART ($n = 270$), T2: -1.5 to -0.5 years pre-HAART ($n = 236$), T3: 0.5 to 1.5 years post-HAART ($n = 261$), T4: (1.5 to 3.0 years post-HAART) ($n = 266$).

Table 1

Characteristics of the study population and NHL subgroup.

Characteristic	Total study cohort (n = 290)	NHL subgroup (n = 12)
Age at HAART start (years)	43.8 ±6.9	48.5 ±7.5
Race/ethnicity (%)		
White	86%	83%
Black	9%	17%
Hispanic	4%	0
Asian	<1%	0
Education (%)		
Less than high school	2%	0
High school/some college	40%	8%
College degree	25%	33%
Graduate school	33%	59%
Serostatus ^a (%)		
Seroprevalent at cohort entry	67%	83%
Seroconverter	33%	17%
Date of HAART Initiation	1997.6 ±1.4	1997.2 ±1.0
Duration of HIV disease at initiation of HAART (years) ^b	12.0 ±3.7	12.0 ±4.0
CD4 at HAART (per mm ^c)	296 (164–459)	167 (84–269)
VL at HAART (copies/ml)	21 906 (4623–84 512)	88 080 (30 362–158 363)
AIDS diagnosis ^c at HAART initiation (%)	16%	50%
First pre-HAART AIDS diagnosis (%) ^d		
PCP	30%	16%
Kaposi sarcoma	17%	0
Wasting syndrome	13%	0
Other	40% ^e	84% ^f

Count data are in percentage and continuous values are in mean ± SD if normally distributed or median (25th, 75th percentile) if skewed.

^a Study participant was classified as sero-prevalent if he was HIV sero-positive at the time of entry into the MACs cohort. Men who were HIV sero-negative at entry into the MACs who became HIV sero-positive were considered sero-convertors.

^b For seroprevalent men from the first MACS recruit, date of seroconversion was approximated by the method of Munoz [55].

^c AIDS defined by CDC case definition (1993) (from MACS dossier updates, CAMACS, May 2007).

^d Percent of those with AIDS diagnosis prior to HAART initiation (n = 47).

^e Includes toxoplasmosis (n = 1), cryptosporidiosis (n = 2), CMV (n = 3), brain lymphoma (n = 1), NHL (n = 3), Candida esophagitis (n = 3), mycobacteria (n = 1), cryptococcal meningitis (n = 1), cryptococcal septicemia (n = 1), chronic mucocutaneous Herpes simplex (n = 2).

^f Includes toxoplasmosis (n = 1), brain lymphoma (n = 1) and NHL (n = 3).

Table 2

Characteristics of the study population, stratified by time.

Characteristic	Pre-HAART		Post-HAART	
	T1 (−3.0 to −1.5 years pre-HAART) (n = 270)	T2 (−1.5 to −0.5 years pre-HAART) (n = 236)	T3 (0.5 to 1.5 years post-HAART) (n = 261)	T4 (1.5 to 3.0 years post-HAART) (n = 266)
Date of visit (year)	1995.2 (1994.6–1996.1)	1996.3 (1995.6–1997.1)	1998.3 (1997.8–1999.1)	1999.2 (1998.7–2000.1)
CD4 (per µl) ^a	346 (225–491)	322 (175–466)	427 (284–617)	474 (297–644)
HIV viral load (copies/ml) ^a	30 190 (7054–92 933)	27 276 (5544–85 015)	66 (<50–5294)	<50 (<50–4086) ^b
ART effectiveness (% of those reporting ART at visit) ^c				
	n = 64	n = 129	n = 239	n = 243
Fully effective	3%	9%	49%	53%
Partially effective	8%	6%	14%	12%
Not effective	89%	85%	37%	35%
ART effectiveness (% of those naive to ART pre-HAART)				
	n = 49	n = 45	n = 45	n = 43
Fully effective	0	0	76%	82%
Partially effective	0	0	18%	16%
Not effective	100%	100%	6%	2%
Therapy at visit (%)				
No therapy	54%	42%	5%	5%
Monotherapy	25%	19%	<1%	<1%
Combotherapy	21%	39%	7%	7%
HAART	0	0	88%	88%
Therapy at visit by drug class (%) ^d				
NRTI	78%	73%	52%	48%
NNRTI	<1%	<1%	8%	12%
NTRTI	0	0	<1%	<1%
PI	1%	5%	34%	34%
Other antiviral, not anti retroviral	20%	21%	6%	6%

Note: count data are in percentage and continuous values are reported as median (25th,75th percentile). NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; NTRTI, nucleotide reverse transcriptase inhibitor; PI, protease inhibitor.

^a $P < 0.001$ for difference between time-points.

^b Lowest detectable limit=50 copies/ml.

^c Fully effective: viral load nondetectable. Partially effective: viral load: 50–399 copies/ml; not effective: viral load ≥ 400 copies/ml.

^d Percent of those on therapy.

Table 3

Effect of HAART on biomarkers of immune activation.

Biomarker	% Change (95% CI)	P value	% Change (95% CI)	P value
(a) Total population (<i>n</i> = 290)				
	Crude analysis ^a		Multivariate analysis ^b	
sCD27	-23.0 (-28.1, -18.0)	<0.001	-26.3 (-32.2, -20.5)	<0.001
sCD30	-30.7 (-39.3, -22.1)	<0.001	-38.1 (-48.1, -28.2)	<0.001
CXCL13	-40.2 (-49.9, -30.4)	<0.001	-41.6 (-51.5, -31.7)	<0.001
IL-6	-8.9 (-20.1, +2.3)	0.12	-8.6 (-19.8, +2.6)	0.13
CRP	+10.4 (-5.3, +26.0)	0.19	+10.7 (-5.0, +26.3)	0.18
IgG	-15.8 (-22.5, -9.1)	<0.001	-17.5 (-24.3, -10.7)	<0.001
IgA	-6.4 (-12.3, -0.4)	0.04	-5.2 (-11.2, +0.8)	0.09
(b) Stratified by HIV viral response (<i>n</i> = 181)				
	Viral responders ^b (<i>n</i> = 100)		Viral non-responders ^b (<i>n</i> = 81)	
sCD27	-26.8 (-38.4, -15.2)	<0.001	-20.8 (-29.8, -11.8)	<0.001
sCD30	-43.6 (-61.6, -25.5)	<0.001	-26.7 (-42.7, -10.8)	0.001
CXCL13	-55.8 (-72.9, -38.7)	<0.001	-10.9 (-26.7, +4.8)	0.17
IL-6	+0.6 (-20.7, +21.8)	1.0	-7.9 (-27.9, +12.1)	0.4
CRP	+9.4 (-19.8, +38.6)	0.5	+18.0 (-8.7, +44.6)	0.19
IgG	-19.6 (-31.9, -7.2)	0.002	-10.3 (-23.1, +2.5)	0.11
IgA	-2.8 (-13.6, +7.9)	0.6	-3.2 (-15.3, +8.8)	0.6
(c) Stratified by ART status at HAART initiation (<i>n</i> = 290)				
	ART-naïve ^b (<i>n</i> = 53)		ART exposed ^b (<i>n</i> = 237)	
sCD27	-44.3 (-58.4, -30.1)	<0.001	-23.0 (-29.5, -6.5)	<0.001
sCD30	-61.2 (-83.5, -38.9)	<0.001	-33.5 (-44.8, -2.2)	<0.001
CXCL13	-62.5 (-79.9, -45.0)	<0.001	-38.1 (-49.4, -6.9)	<0.001
IL-6	-27.4 (-57.0, +2.9)	0.08	-4.2 (-16.2, +7.6)	0.5
CRP	-8.9 (-45.1, +27.2)	0.6	15.1 (-2.3, +32.5)	0.09
IgG	-29.3 (-44.3, -14.2)	<0.001	-13.5 (-20.9, -6.0)	<0.001
IgA	-5.4 (-18.8, 8.1)	0.4	-3.7 (-10.4, 3.0)	0.3

There was a statistically significant interaction between ART-naïve pre-HAART and HAART on all markers with the exception of IgA.

^aControlling for time.

^bControlling for time, baseline CD4 and HIV viral load, CD4 slope, AIDS status pre-HAART, and age for IL-6 and CRP.