Normalization of B Cell Counts and Subpopulations after Antiretroviral Therapy in Chronic HIV Disease

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Background. Untreated human immunodeficiency virus (HIV) disease leads to abnormalities in all major lymphocyte populations, including CD4+ T cells, CD8+ T cells, and B cells. However, little is known regarding the effect of antiretroviral therapy (ART)–induced decrease in HIV viremia on B cell numbers and subpopulations.

Methods. We conducted a longitudinal study to evaluate changes in B cell numbers and subpopulations that occur during the course of 12 months of effective ART in a group of individuals with chronic HIV infection.

Results. ART-induced decrease in HIV viremia was associated with a significant increase in B cell counts, similar to increases in CD4+ T cell counts yet distinct from the lack of increase in CD8+ T cells. The increase in B cell counts was accompanied by a significant decrease in the frequency of apoptosis-prone B cell subpopulations, namely mature activated and immature transitional B cells, which are overrepresented in untreated HIV disease. The increase in B cell counts was reflected by a significant increase in naive and resting memory B cells, both of which represent populations that are essential for generating adequate humoral immunity.

Conclusions. Normalization of B cell counts and subpopulations may help to explain the improvement in humoral immunity reported to occur after an ART-induced decrease in HIV viremia.

Untreated HIV disease is associated with perturbations of all major lymphocyte populations, including B cells, NK cells, and CD4+ and CD8+ T cells [1–3]. Given that CD4+ T cells are the only major lymphocyte population targeted by HIV and that only a small fraction of circulating CD4+ T cells carry replication-competent HIV at any given time [4], it is reasonable to assume that the bulk of HIV-induced perturbations in lymphocyte populations reflects indirect effects of HIV replication. Although most of these observations have been made on cells isolated from peripheral blood, the majority of HIV activity occurs in lymphoid and mucosal tissues [5]. Nonetheless, similarities between these compartments exist, including evidence of alterations in tissues that are mediated by indirect effects of viral replication [6, 7]. Generalized immune activation is regarded to be a main consequence of ongoing HIV replication that leads to disruption of immune function [5, 8]. B cells exhibit numerous effects of HIV-induced immune activation, including increased expression of markers of activation and proliferation [9, 10], induction of terminal differentiation with increased immunoglobulin secretion [2, 11], and increased incidence of B cell neoplasms likely associated with B cell hyperactivation [12].

Evidence of increased lymphocyte turnover in HIV and simian immunodeficiency virus infection is well documented for CD4+ and CD8+ T cells [13–15] and is documented to a lesser extent for B cells [16–18]. If increased rate of cellular turnover were the only factor associated with HIV replication, these observations would predict that a reduction in HIV plasma viremia by antiretroviral therapy (ART) would decrease cell turnover and thus might actually decrease the absolute number of circulating lymphocytes. However, HIV infection is also associated with increased cell death as a result of direct and indirect effects of ongoing viral replication, including the generation of short-lived lymphocyte subpopulations [14, 19–21]. Further confounding effects of HIV
replication on lymphocytes include redistribution of cells between tissues and blood, which is thought to occur after ART [22, 23], and perturbations in the regeneration of lymphocytes [24].

In a previous study, we found overrepresentation of 2 apoptosis-prone B cell subpopulations in the peripheral blood of HIV-infected individuals with active disease [25]. One overrepresented B cell subpopulation, described as mature activated B cells, was characterized by reduced expression of the complement receptor CD21 and increased expression of the proliferation marker Ki-67 and the death receptor CD95, and it likely arose as a result of HIV-induced immune activation [10]. These B cells were highly susceptible to extrinsic apoptosis mediated by CD95 ligand (CD95L), and this susceptibility to CD95L-mediated apoptosis was directly correlated with HIV plasma viremia [10, 25]. The second overrepresented B cell subpopulation, described as immature transitional B cells, was characterized by the expression of the lymphocyte precursor marker CD10 and was associated with the effects of HIV-induced CD4+ T cell lymphopenia [26, 27]. These B cells expressed low levels of prosurvival members of the Bcl-2 family, including Bcl-2 and Bcl-xL, rendering them highly susceptible to intrinsic apoptosis [25]. These findings, along with other indications of decreased life span in HIV-viremic individuals [2, 10], would predict a loss of B cells in the setting of untreated HIV disease.

In the present study, we investigated changes in B cell counts in parallel with changes in CD4+ and CD8+ T cell counts after 12 months of ART in a group of individuals with chronic HIV infection. In addition, we characterized changes in the frequency of B cell subpopulations after the administration of ART. Our findings demonstrate that ART leads to a significant increase in B cell numbers and a normalization of B cell subpopulations, thus providing a possible explanation for improved B cell responses to both T cell–independent and T cell–dependent immunogens after ART [28–30].

SUBJECTS, MATERIALS, AND METHODS

Study design and participants. The study included 29 HIV-infected individuals who were about to start effective ART. Of these individuals, 27 were naive to ART, and 2 had received short courses of ineffective ART before effective ART. Peripheral blood samples were obtained at months 0, 3, 6, and 12 after initiation of effective ART. HIV plasma viremia was measured by branched DNA assay (Bayer Diagnostics), with a lower limit of detection of 50 copies/mL [31]. All individuals achieved a virologic response to below the limit of detection of HIV plasma viremia within 12 months of ART. Thirty-four HIV-negative individuals were included as control subjects. The 2 groups of participants were matched for age (with medians of 39 and 36 years in the control and HIV groups, respectively) and for sex (with male participants representing 80% and 83% of the control and HIV groups, respectively). All study participants provided informed consent, in accordance with the Institutional Review Board of the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

Immunophenotyping. Lymphocyte counts were measured in whole blood by standard laboratory methods that included measurement of pan-lymphocyte surface markers CD4, CD8, and CD19. Normal ranges for each population were established with whole blood from the HIV-negative individuals. Phenotypic analyses of B cell subpopulations were performed on peripheral blood mononuclear cells (PBMCs) isolated from whole blood or leukapheresis products. Four-color stains were performed with the combination of markers CD19/CD21/CD10/CD27 using monoclonal antibodies (MAbs) directly conjugated to the following fluorochromes, listed in the same order as the markers: phycoerythrin–cyanine 7 tandem/fluorescein isothiocyanate/allophycocyanin/phycoerythrin. The anti-CD19, anti-CD10, and anti-CD27 MAbs and corresponding isotype controls were obtained from BD Biosciences, and the anti-CD21 MAb was obtained from Beckman Coulter. Data were acquired on a FACS Calibur flow cytometer (BD Biosciences) and analyzed using FlowJo software (version 8; Tree Star).

Statistical analyses. Differences between groups were evaluated using the Mann Whitney U test, and differences within a group between baseline and follow-up were evaluated using the Wilcoxon signed rank test or the paired t test. Differences were considered to be significant at P < .05. Mixed models incorporating an overall linear plus quadratic trend (the fixed effects) plus patient-specific linear and quadratic trends (the random effects) were used to assess and compare patterns in cell counts over time. A significant and positive linear fixed effect coupled with a significant and negative quadratic fixed effect would indicate more leveling off than a straight linear increase. A permutation test was used to compare the set of percentages of immature transitional, mature activated, resting memory, and naive B cells at baseline (P I, P M, P R, P N) with the set of percentages at 1 year (P I, P M, P R, P N), where these percentages were averaged over the 14 participants for whom data were available. The distance between the baseline and 1-year percentages was defined to be the sum of the squared differences: D = (P I – P I)² + (P M – P M)² + (P R – P R)² + (P N – P N)². D should be large if the 2 sets of percentages differ in a consistent way among participants and small if random variability causes some of the participant percentages to change one way and others to change in a different way. The actual value of D was compared with a reference distribution obtained by considering all possible permutations of the baseline and 1-year labels and recomputing D for each permutation. The P value was the proportion of permuted D values that were at least as large as the actual D value.

RESULTS

Kinetic analyses of lymphocyte counts after ART. Twenty-nine individuals with chronic HIV infection were recruited for
longitudinal analyses before and for up to 1 year after the initiation of effective ART. Before the initiation of ART, the geometric mean of the plasma viral load was 138,330 HIV RNA copies/mL, and the median lymphocyte counts in the peripheral blood were 247 CD4+ T cells/μL, 171 CD19+ B cells/μL, and 804 CD8+ T cells/μL. Corresponding median lymphocyte counts for uninfected control subjects were 878 CD4+ T cells/μL, 270 CD19+ B cells/μL, and 429 CD8+ T cells/μL. These counts were significantly higher than the CD4+ T cell (P < .0001) and CD19+ B cell (P < .0001) counts and were significantly lower than the CD8+ T cell (P < .0001) counts of the HIV-infected individuals before initiation of ART.

Each of these parameters was measured at 3, 6, and 12 months after the initiation of ART. As shown by kinetic analysis in 24 individuals with data available for all time points (figure 1), the reduction in HIV plasma viremia was accompanied by rapid increases in CD4+ T and CD19+ B cell counts, whereas CD8+ T cell counts decreased slightly. When the overall change in each lymphocyte population was analyzed at baseline and after 12 months of ART, CD4+ T cell counts increased significantly by 76% (P < .0001), with a median absolute increase of 205 cells/μL (figure 2A). Similar to the rise in CD4+ T cell numbers, CD19+ B cell counts increased significantly by 69% (P < .0001), with a median absolute increase of 118 cells/μL (figure 2B). In contrast, the 10% overall decrease in CD8+ T cell counts after 12 months of ART was not significant (figure 2C). Furthermore, a mixed model test was used to establish that there was a significant leveling off of counts after 12 months of ART for CD19+ B cells and CD4+ cells, whereas there was no statistically significant relationship between CD8+ T cells and time. Taken together, these data indicate that the effect of ART on CD19+ B cells was similar to that on CD4+ T cells and was dissimilar to that on CD8+ T cells.

Figure 1. Changes in lymphocyte counts and HIV plasma viremia after the initiation of antiretroviral therapy (ART). Plotted lines represent the median lymphocyte count or the geometric mean HIV plasma viremia for 24 participants with data available for all time points. Measurements were made at time 0 and at 3, 6, and 12 months after the initiation of ART. The dotted horizontal line indicates the lower limit of detection of HIV plasma viremia.

Figure 2. Individual changes in lymphocyte counts before and after 12 months of antiretroviral therapy (ART). Counts of CD4+ T cells (A), CD19+ B cells (B), and CD8+ T cells (C) were determined in 29 participants at time 0 and at 12 months after ART; for 2 individuals, month 9 was used as the second time point. Horizontal bars indicate median values, and dotted horizontal lines indicate median lymphocyte counts for uninfected control subjects.
Changes in B cell subpopulations after ART. HIV infection is associated with a number of perturbations in the B cell compartment, including the overrepresentation of subpopulations of B cells in the blood that are thought to arise as a result of HIV-induced immune activation and CD4+ T cell lymphopenia. To investigate changes in B cell subpopulations after ART, PBMCs were stained for a series of markers that would identify changes in the frequency of the 4 major B cell subpopulations that circulate in the peripheral blood of HIV-uninfected and HIV-infected individuals [2, 10, 26]. The 4 B cell subpopulations include immature transitional B cells, defined by the expression of CD10 on CD19+ B cells that do not express CD27 (CD10+CD27-); mature activated B cells, defined by a reduced expression of CD21 on CD19+ B cells that do not express CD10 (CD10-CD21lo); resting memory B cells, defined by the expression of CD27 on CD19+ B cells that express high levels of CD21 (CD27+CD21hi); and naive B cells, defined by the absence of CD10 and CD27 on CD19+ B cells that express high levels of CD21 (CD10-CD27+CD21hi). On the basis of these markers, profiles of B cell subpopulations in the PBMCs of 14 of the 29 participants were determined before and 12 months after the initiation of ART. As shown in figure 3 for a representative individual, the composition of the CD19+ B cell population before ART was 26% CD10+CD27 immature transitional, 34% CD10-CD21hi mature activated, and 9.8% CD27+CD21hi resting memory, with the remaining 30% being naive B cells (CD10-CD27-CD21hi). After 12 months of ART, these frequencies were dramatically shifted, with immature transitional and mature activated B cells decreasing to 10% and 6.8% of the B cell population, respectively, compared with increases to 12% for resting memory and 71% for naive B cells.

When data on all 14 individuals were compiled and analyzed relative to the 4 B cell subpopulations before and 12 months after ART, the percentages of immature transitional and mature activated B cells were significantly decreased, whereas the percentages of resting memory and naive B cells were significantly increased, with ART (P = .0002, for simultaneous comparison of subpopulations before and 12 months after ART) (figure 4).

Figure 3. Representative profiles of B cell subpopulations before (top panels) and after 12 months of (bottom panels) antiretroviral therapy (ART). Dot plots show the expression of CD10, CD21, and CD27 on CD19-gated cells. Lymphocyte counts are given in cells per microliter. Viral loads (VLs) are given in HIV RNA copies per milliliter.

Figure 4. Changes in the frequency of B cell subpopulations before and after 12 months of antiretroviral therapy (ART). The mean frequency for each B cell subpopulation was determined in 14 participants at time 0 and 12 months after initiation of ART; for 2 individuals, month 9 was used as the second time point.
Analysis of the B cell subpopulations revealed the following mean frequencies before and 12 months after ART: immature transitional B cells, 31% and 16% (figure 5A); mature activated B cells, 29% and 12% (figure 5B); naive B cells, 31% and 55% (figure 5C); and resting memory CD19$^{+}$/CD11001$^{+}$B cells, 8.8% and 17% (figure 5D); each of these changes was significant. Of note regarding CD27$^{+}$/CD11001$^{+}$B cells, although the percentage of resting memory (CD27$^{+}$/CD21$^{lo}$) B cells increased with ART, the percentage of activated (CD21$^{lo}$) B cells expressing CD27 significantly decreased with ART (figure 3 and data not shown), with the overall percentage of B cells expressing CD27 not significantly different after 12 months of ART (data not shown).

Taken together, these data demonstrate that, as the absolute number of B cells increases with reduction of HIV plasma viremia by ART, the 2 apoptosis-prone subpopulations of B cells—namely, CD10$^{+}$/CD27$^{+}$ immature transitional and CD10$^{+}$/CD21$^{lo}$ mature activated B cells, which represented $>$50% of the total B cell population before ART—were substantially decreased and replaced by naive and resting memory B cells after 12 months of ART. It should be noted, however, that the mean of 17% of resting memory B cells after 12 months of ART (figure 4) was nonetheless far below the average of 40% that has been reported for the peripheral blood of HIV-negative healthy individuals [32]. This underrepresentation of memory B cells even after initiation of ART is consistent with previous studies of HIV-infected individuals [33–35].

**DISCUSSION**

In the present study, we investigated the changes in B cell numbers and in B cell subpopulations that occur in the blood of individuals with chronic HIV infection after control of viremia by ART. The reduction of HIV plasma viremia by ART was associated with a significant increase in B cell numbers and a significant reduction in subpopulations of B cells that have previously been associated with HIV-induced immune activation and CD4$^{+}$ T cell lymphopenia [10, 25, 26]. These B cell subpopulations included CD10$^{+}$/CD27$^{+}$ immature transitional and CD10$^{+}$/CD21$^{lo}$ mature activated B cells, both of which have been shown in a previous study to be highly prone to intrinsic and extrinsic modes of apoptosis, respectively [25]. Thus, the disappearance of apoptosis-prone and likely short-lived B cell subpopulations concomitant with the normalization of B cell numbers and control of HIV plasma viremia suggest that ongoing HIV replication leads to loss of B cells that may be due in part to the overrepresentation of B cells that rapidly die by apoptosis. The accumulation associated with chronic immune activation of abnormal and nonresponsive subpopulations of CD4$^{+}$ and CD8$^{+}$ T cells that are highly prone to apoptosis has recently been described in untreated HIV-infected individuals [36] and is consistent with our present and past findings for similar subpopulations of B cells [2, 10, 26].

HIV infection, when left untreated, is associated with a gradual depletion of CD4$^{+}$ T cells, whereas CD8$^{+}$ T cells are maintained at high levels until late in disease [37]. The mechanisms that dictate changes in CD4$^{+}$ and CD8$^{+}$ T cells in the setting of HIV infection remain a matter of intense debate and speculation [5, 38, 39], and the picture is even less clear regarding B cells. The effects of ART on CD4$^{+}$ and CD8$^{+}$ T cells have been addressed in numerous studies [13–16], which have consistently revealed that, although markers of cellular activation and turnover decrease with ART in both populations, only CD4$^{+}$ T cells undergo a significant increase in numbers with ART. The differences in response to ART between these 2 T lymphocyte populations have been explained at least in part by direct effects of HIV on CD4$^{+}$ T but not CD8$^{+}$ T cells [20] and by differences between naive and memory compartments of both lymphocyte populations [14, 40, 41]. By extension, changes in B cell numbers after
ART would be expected to mirror more closely those observed in CD8+ but not CD4+ T cells, because B cells are not direct targets of HIV. However, in the present study we demonstrate that the increases in B cell counts were similar, in terms of both percentage increase and kinetics, to those in the CD4+ T cell compartment and were very distinct from those in the CD8+ T cell compartment. Similar increases in B cell numbers after ART have been reported in other, less comprehensive studies [42, 43].

A number of reasons, most of which remain speculative, may explain why B cells and CD4+ T cells may respond similarly to ART and distinctly from CD8+ T cells. On the one hand, there are indications that CD8+ T cell numbers increase in HIV disease as a compensatory mechanism to CD4+ T cell lymphopenia [37], although homeostatic effects between CD4+ T cells and B cells remain speculative [44]. On the other hand, all 3 lymphocyte compartments respond to ART-induced decrease in HIV viremia and its consequent decrease in immune activation with a decrease in the expression of activation markers and a decrease in cell turnover. Both CD4+ T and CD8+ T cells have been shown to undergo redistribution between tissues and blood after ART [22, 23], and B cells are likely to undergo similar changes. It is possible that the redistribution effect is stronger in the B cell than in the CD8+ T cell compartment; however, there is no direct evidence for such a difference. It is also possible that the residual HIV persistence observed in lymphoid tissues after ART [45] could have differential effects on B cells and CD8+ T cells.

U ntreaiti d HIV dis ease almiost inevit aly leads to 2 interlinked consequences, namely, high HIV plasma viremia and CD4+ T cell lymphopenia, which together lead to immune cell activation and homeostatic compensation. In addition, treatment of HIV disease with ART almost always leads to both a reduction in HIV plasma viremia and an increase in CD4+ T cell numbers. Of the 29 individuals investigated longitudinally in this study before and after ART, only 1 achieved undetectable HIV plasma viremia without an increase in CD4+ T cell numbers. Thus, it is difficult to distinguish between the effects of ongoing HIV replication and those of CD4+ T cell lymphopenia on other lymphocyte populations. In this regard, we have recently shown that CD10+CD27- immature transitional B cells are also overrepresented in patients suffering from non–HIV-induced idiopathic CD4+ T cell lymphopenia (ICL) [27], suggesting that this subpopulation of B cells is most likely to arise in HIV disease as a result of CD4+ T cell lymphopenia. It remains to be determined whether CD10+CD27- immature transitional B cells arise in the peripheral blood as a direct effect of increased serum levels of interleukin-7 observed in both HIV disease and ICL [27] or whether they arise in direct response to CD4+ T cell lymphopenia. Irrespective of the underlying mechanism, ART induces a significant reduction in the frequency of CD10+CD27- immature transitional B cells in the blood. Of note, the appearance of CD10+CD27- immature transitional B cells in the blood of HIV-infected individuals with active disease and their subsequent disappearance after ART is similar to the sequence of changes that occur in B cell subpopulations after bone marrow transplantation [46] and after treatment with the B cell–depleting antibody rituximab [47].

Several studies have demonstrated that HIV infection is associated with reduced frequencies of memory B cells [33–35] and that ART-induced suppression of HIV viremia does not appear to normalize this compartment completely [33]. However, expression of CD27 was the sole defining marker for memory B cells in all of these studies; no distinctions were made between levels of CD27 expression for activated versus resting B cells. In the present study, we demonstrate that ART leads to a significant decrease in the frequency of CD27+ activated B cells and a significant increase in the frequency of CD27+ resting B cells, which we consider (as have others [48]) to represent the true memory B cell compartment. Our findings indicate that, although the frequency of CD27+ resting B cells was significantly increased after 12 months of ART, it had not reached the average 40% reported for healthy individuals [32]. It remains to be determined whether normalization of the memory B cell compartment can eventually occur in HIV-infected individuals in whom ART is initiated during chronic disease or whether it may be feasible only in those whose therapy was initiated during the acute or early phase of infection, as suggested by others [49]. Answers to these questions may help explain why individuals with chronic HIV infection mount lower-than-normal B cell memory responses to vaccination irrespective of ART [50] and may provide indications as to whether individuals treated early during the course of HIV infection may retain such responses.

In summary, the present study demonstrated a significant recovery of B cell numbers concomitant with a reduction in HIV plasma viremia by ART. This increase was accompanied by a normalization of B cell subpopulations, although the percentage of resting memory B cells remained below normal levels even after 12 months of ART. The reduction in the frequency of apoptosis-prone B cell subpopulations associated with ART may in part explain the increase in B cell numbers. The improved B cell profile may also explain the benefits of ART in improving the response of B cells to specific immunogens, including T cell–independent antigens [28–30].

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References