Evidence for HIV-associated B cell exhaustion in a dysfunctional memory B cell compartment in HIV-infected viremic individuals

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Human immunodeficiency virus (HIV) disease leads to impaired B cell and antibody responses through mechanisms that remain poorly defined. A unique memory B cell subpopulation (CD20hi/CD27lo/CD21lo) in human tonsillar tissues was recently defined by the expression of the inhibitory receptor Fc-receptor-like-4 (FCRL4). In this study, we describe a similar B cell subpopulation in the blood of HIV-viremic individuals. FCRL4 expression was increased on B cells of HIV-viremic compared with HIV-aviremic and HIV-negative individuals. It was enriched on B cells with a tissuelike memory phenotype (CD20hi/CD27lo/CD21lo) when compared with B cells with a classical memory (CD27hi) or naive (CD27lo/CD21hi) B cell phenotype. Tissuelike memory B cells expressed patterns of homing and inhibitory receptors similar to those described for antigen-specific T cell exhaustion. The tissuelike memory B cells proliferated poorly in response to B cell stimuli, which is consistent with high-level expression of multiple inhibitory receptors. Immunoglobulin diversities and replication histories were lower in tissuelike, compared with classical, memory B cells, which is consistent with premature exhaustion. Strikingly, HIV-specific responses were enriched in these exhausted tissuelike memory B cells, whereas total immunoglobulin and influenza-specific responses were enriched in classical memory B cells. These data suggest that HIV-associated premature exhaustion of B cells may contribute to poor antibody responses against HIV in infected individuals.
from the combined effect of increased expression of multiple inhibitory receptors (13, 14).

The recent characterization of the inhibitory receptor Fc-receptor-like-4 (FCRL4), expressed on a unique subpopulation of memory B cells in human tonsil tissues, led to the suggestion of an immunoregulatory role for this receptor (15). The phenotypic features of these tissue memory B cells included increased expression of CD20 and reduced expression of CD21 and CD27. Given that reduced expression of CD21 on peripheral blood B cells is associated with ongoing HIV replication (5, 6), we sought to further investigate the nature of CD21lo B cells in HIV-viremic individuals. We observed that tissue-like CD21lo memory B cells expressing high levels of FCRL4 and other inhibitory receptors circulate in the blood of HIV-viremic individuals. Furthermore, we find that HIV-specific B cells are enriched within this B cell subpopulation that exhibits many properties associated with virus-specific exhaustion. We thus provide evidence for HIV-associated exhaustion in the B cell compartment that may, in part, explain the inadequacy of the anti-HIV antibody response in HIV-infected individuals (16).

RESULTS AND DISCUSSION

Low expression of CD21 on peripheral blood B cells of HIV-infected individuals identifies distinct B cell subpopulations that are associated with HIV viremia and lymphopenia (5, 6, 8, 9). We previously demonstrated that immature/transitional B cells (CD10+/CD27−) account for a fraction of CD21lo B

Figure 1. Phenotypic characterization of tissue-like memory B cells in the peripheral blood of HIV-viremic individuals. (A) Color-coded gating on CD20/CD21-stained B cells of a representative HIV-viremic individual was used to identify CD20hi/CD21lo activated (CD27int) and tissue-like (CD27+/H11002−) memory B cells (green); CD20int/CD21hi resting memory (CD27int) and naive (CD27−/H11002−) B cells (red); and CD20−/CD27+/H11002−/CD21lo plasmablasts (blue). (B) Percentage of expression of FCRL4 on peripheral blood B cells of HIV-viremic (n = 16), HIV-aviremic (n = 12), and HIV-negative individuals (n = 12). Horizontal bars indicate medians. (C–E) Color-coded gating on CD27/CD21-stained B cells (C and E) of a representative HIV-viremic individual was used to establish expression of FCRL4 (C), frequency of FCRL4 on B cell subpopulations of 12 HIV-viremic individuals (D), and other inhibitory/homing receptors and adhesion molecules (E) on CD20hi/CD27+/CD21lo tissue-like memory (black), CD27− classical memory (blue), and CD20−/CD27−/CD21lo naive (red) B cells. MFI, mean fluorescence intensity. All stains included CD19, which was used to establish the B cell gate. Immature/transitional B cells were excluded by performing stains on CD10-depleted B cells or on PBMCs gated on CD19+/CD10− cells.
cells in CD4+ T cell–lymphopenic individuals (8, 9), whereas plasmablasts (CD20+−) account for another fraction of CD21hi B cells in HIV-viremic individuals (Fig. 1A) (5, 6). Few of these B cells are present in the peripheral blood of HIV-aviremic and HIV-negative individuals (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20072683/DC1). However, aside from plasmablasts and immature/transitional B cells, we observed another population of CD21hi B cells in the peripheral blood of HIV-viremic individuals; these CD21hi B cells express high levels of CD20 and low-to-intermediate levels of CD27 (Fig. 1A). Such a profile is similar to that of tonsillar tissue memory B cells, defined by the expression of the inhibitory receptor FCRL4 (15).

To further characterize the peripheral blood-derived CD20hi/CD27−/CD21hi B cells of HIV-viremic individuals, which comprise a median of 19% of B cells compared with <4% in HIV-aviremic and -negative individuals, we measured cell surface expression of FCRL4. Peripheral blood mature B cells of HIV–viremic individuals expressed significantly higher levels of FCRL4 compared with B cells of HIV-aviremic and -negative individuals (Fig. 1B). The term mature B cells, applied here and throughout the manuscript, is used to indicate that CD10+ immature/transitional B cells, which do not express FCRL4 (not depicted), were excluded from this study. In HIV–viremic individuals, FCRL4 expression was increased on tissue-like memory B cells, defined here by the expression profile CD20hi/CD27hi/CD21hi, compared with classical memory (CD27hi) and naive (CD20hi/CD27−/CD21hi) B cells (Fig. 1, C and D, and Table I). Of note and illustrated in Fig. 1A and C, CD27hi B cells of HIV–viremic individuals are comprised of resting memory (CD20+−/CD27hi/CD21hi), activated memory B cells (CD20hi/CD27hi/CD21hi), and plasmablasts (CD20hi/CD27hi/CD21hi) (5, 6). The latter two subpopulations are responsible for the FCRL4 expression within the CD27hi B cell compartment (Fig. 1C and not depicted). However, for clarity and consistency with the functional data in the following paragraphs, B cells expressing CD27 are henceforth collectively referred to as classical memory B cells.

In addition to FCRL4, tissue-like memory B cells in the blood of HIV-viremic individuals expressed relatively high levels of other potentially inhibitory receptors (17, 18), including CD22, CD85j, CD85k, LAIR–1, and CD72 (Fig. 1E and Table I). Levels of FCRL4, CD22, and CD85j were significantly higher on tissue-like memory compared with classical memory and naive B cells, whereas the expression of other inhibitory receptors on tissue-like memory B cells was similar or intermediate to that of either naive or classical memory B cells (Table I). Furthermore, these tissue-like memory B cells in the blood expressed a profile of trafficking receptors similar to that described for tonsillar tissue memory B cells (15) (see Ehhardt et al. [19] on p. 1807 of this issue), namely CXCR3hi/CD11c+/CCR6hi/CCR7hi/CXCR5hi/CXCR4lo (Fig. 1E and Table I). CXCR3, CD11c, and CCR6 were expressed at significantly higher levels, whereas CCR7, CD62L, and CXCR5 were expressed at significantly lower levels on tissue-like memory compared with both classical memory and naive B cells (Table I). Expression of CXCR4 was significantly lower on both tissue-like and classical memory compared with naive B cells (Table I). Collectively, tissue-like memory B cells in the blood of HIV-viremic individuals express high levels of inhibitory receptors and a profile of trafficking receptors that is consistent with migration to chronically inflamed tissues and away from lymphoid tissues that favor B–T cell interactions (20–23). Of note, increased expression of multiple inhibitory receptors and alterations in the expression

### Table I. Expression of inhibitory and trafficking receptors on B cell subpopulations of HIV-viremic individuals

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Classical memory</th>
<th>Tissue-like memory</th>
<th>Naive</th>
<th>1 vs. 2</th>
<th>1 vs. 3</th>
<th>2 vs. 3</th>
<th>P values</th>
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<tr>
<td><strong>Inhibitory</strong></td>
<td></td>
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<tr>
<td>FCRL4</td>
<td>12 (7.7–26)</td>
<td>25 (16–40)</td>
<td>3.8 (1.5–9.7)</td>
<td>0.002</td>
<td>0.0005</td>
<td>0.0005</td>
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<tr>
<td>CD85j</td>
<td>23 (14–40)</td>
<td>49 (24–85)</td>
<td>12 (4.4–26)</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
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<tr>
<td>CD22</td>
<td>197 (81–388)</td>
<td>593 (258–997)</td>
<td>442 (258–655)</td>
<td>0.0005</td>
<td>0.0005</td>
<td>0.028</td>
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<tr>
<td>CD85k</td>
<td>3.2 (0.8–11)</td>
<td>3.4 (0.3–12)</td>
<td>1.1 (0.2–4.1)</td>
<td>0.25</td>
<td>0.002</td>
<td>0.002</td>
<td></td>
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<tr>
<td>CD72</td>
<td>20 (7.7–29)</td>
<td>62 (20–85)</td>
<td>57 (32–72)</td>
<td>0.002</td>
<td>0.002</td>
<td>0.270</td>
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<tr>
<td>LAIR–1</td>
<td>23 (12–42)</td>
<td>66 (21–162)</td>
<td>159 (84–250)</td>
<td>0.0005</td>
<td>0.0005</td>
<td>0.002</td>
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<tr>
<td><strong>Trafficking</strong></td>
<td></td>
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<tr>
<td>CXCR3</td>
<td>73 (19–153)</td>
<td>222 (65–384)</td>
<td>86 (23–207)</td>
<td>0.0005</td>
<td>0.136</td>
<td>0.0005</td>
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<tr>
<td>CD11c</td>
<td>64 (38–228)</td>
<td>190 (71–365)</td>
<td>38 (8.7–185)</td>
<td>0.0005</td>
<td>0.001</td>
<td>0.0005</td>
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<tr>
<td>CCR6</td>
<td>26 (11–49)</td>
<td>54 (24–165)</td>
<td>33 (15–101)</td>
<td>0.0005</td>
<td>0.003</td>
<td>0.003</td>
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<tr>
<td>CCR7</td>
<td>9.3 (3.0–30)</td>
<td>4.4 (1.4–12)</td>
<td>9.8 (2.5–30)</td>
<td>0.0005</td>
<td>0.207</td>
<td>0.001</td>
<td></td>
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<tr>
<td>CD62L</td>
<td>114 (32–325)</td>
<td>13 (7.1–66)</td>
<td>70 (25–370)</td>
<td>0.002</td>
<td>0.176</td>
<td>0.0005</td>
<td></td>
</tr>
<tr>
<td>CXCR5</td>
<td>70 (16–147)</td>
<td>40 (8.7–126)</td>
<td>177 (96–281)</td>
<td>0.021</td>
<td>0.0005</td>
<td>0.002</td>
<td></td>
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<tr>
<td>CXCR4</td>
<td>8.2 (2.9–14)</td>
<td>7.5 (2.9–18)</td>
<td>29 (8.7–49)</td>
<td>0.478</td>
<td>0.0005</td>
<td>0.0005</td>
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*Refer to Fig. 1C for gating used to identify each subpopulation.

*Values are median and range of mean fluorescence intensities (n = 12) after subtraction of background staining with isotype control.
of homing receptors CXCR3, CD11c, CCR7, and CD62L similar to those described here are signatures of virus-specific CD8⁺ T cell exhaustion in chronic LCMV infection (13). Given that exhaustion of virus-specific T cells similar to that observed in LCMV has been described in HIV-viremic individuals (11, 12), we considered that exhaustion could also be occurring in HIV-specific B cells.

To further investigate the hypothesis of B cell exhaustion in HIV infection, we fractionated peripheral blood mature B cells from HIV-viremic individuals into CD27⁺ classical memory, CD27⁻/CD21hi tissuelike memory, and CD27⁻/CD21lo naive B cells. First, we evaluated the in vivo replication history of each fraction using a recently described PCR-based assay for measuring k-deletion recombination excision circles (KRECs) (24). Given that these circles, which are generated during the final stages of Ig gene rearrangements in the bone marrow, become diluted by a factor of two each time a B cell divides, this approach provides an estimate of the number of cell divisions a B cell has undergone after exiting the bone marrow. Initially, we validated the assay on several well-defined B cell subpopulations that were sorted from the peripheral blood of HIV-viremic individuals. The median number of cell divisions was lowest in immature/transitional B cells, followed by an increasing number in naive; tissuelike memory; resting memory; activated memory; and finally in plasma blasts, which underwent the highest number of cell divisions (Fig. 2A). The number of cell divisions that tissuelike memory B cells underwent was significantly lower than that of classical memory B cells and significantly higher than that of naive B cells (Fig. 2B). In addition, we evaluated the degree of somatic hypermutation in each fraction using a restriction enzyme–based hotspot assay that measures Ig VH3 diversity (8, 24, 25). Similar to the KREC assay, the Ig VH3 diversity assay was performed on the three B cell subpopulations illustrated in Fig. 1C, and the results for one representative individual are depicted in Fig. 2A. The number of cell divisions that tissuelike memory B cells underwent was significantly lower than that of classical memory B cells and significantly higher than that of naive B cells (Fig. 2D).

Next, we evaluated the proliferative capacities of each B cell fraction. Tissuelike memory B cells proliferated significantly less than naive B cells in response to B cell receptor (BCR) triggering and CD4⁺ T cell help (CD40 ligand; CD40L), and/or Toll-like receptor (TLR) 9-triggering with type B synthetic CpG oligodeoxynucleotides (CpG-B). Proliferative deficiencies in tissuelike memory B cells were not as extensive when compared with classical memory B cells, especially in the presence of all three stimuli (Fig. 3A), suggesting that inhibitory pressures on tissuelike memory B cells may be overridden. Of note, we have demonstrated that classical memory B cells of HIV-viremic individuals are themselves deficient, especially in response to BCR and CD40 triggering, when compared with classical memory B cells of HIV-aviremic and HIV-negative individuals (6, 26). This deficiency in proliferation was explained in part by the presence of CD20⁻ plasmablasts among classical memory B cells of HIV-viremic individuals (Fig. 1C). Analysis of CFSE-labeled cells indicated that the majority of plasmablasts were short-lived ex vivo, regardless of culture conditions (not depicted). Furthermore, in the presence of B cell stimulatory cytokines IL-2 and -10 and CD40L, tissuelike memory and naive B cells proliferated at significantly lower levels compared with classical memory B cells, whereas BCR triggering increased the proliferation of naive, but not tissuelike, memory B cells to levels similar to those observed for classical memory B cells (Fig. 3B). These latter data contrasted with those reported...
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for tonsil-derived FCRL4+B cells, which responded robustly to IL-2 and -10 (15). This difference may be explained by the presence of IL-2/-10-responsive CD27+B cells among the tonsil-derived FCRL4+, but not among our blood-derived tissue-like memory B cells (Fig. 3 B) (15). Nonetheless, these data indicate that tissue-like memory B cells found in the blood of HIV-viremic individuals exhibit proliferative deficiencies when compared with naive and classical memory B cells. The low proliferative capacity of these B cells may be caused by the overexpression of inhibitory receptors, which is consistent with their reduced replication history in vivo (Fig. 2, A and B). These data are also consistent with the low proliferative capacity that has been shown for exhausted HIV-specific T cells in HIV-viremic individuals (11, 12).

Next, we measured frequencies of antibody-secreting B cells (ASCs), including total Ig and antigen-specific ASCs, in each fraction after polyclonal stimulation ex vivo. Of note, these culture conditions, whereas unfavorable to the short-lived plasmablasts that are responsible for the spontaneous secretion of IgGs in HIV disease (not depicted) (for review see [4]), are required for the induction of ASCs from all other B cell subpopulations. Total Ig ASC frequencies were significantly higher in classical memory compared with tissue-like memory and naive B cells (Fig. 4 A). There was no difference in total Ig ASC frequencies between tissue-like memory and naive B cells (Fig. 4 A). However, as one would predict, frequencies of IgM ASCs were significantly higher than IgG/A ASCs in the naive B cell fraction (Fig. 4 B; P = 0.002), whereas there was no difference between isotypes in the tissue-like memory B cell fraction (Fig. 4 B). Also as expected, frequencies of IgG/A ASCs were significantly higher than IgM ASCs in the classical memory B cell fraction (Fig. 4 B;

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**Figure 3.** Low proliferative capacity of tissue-like memory B cells isolated from the peripheral blood of HIV-viremic individuals. (A and B) Proliferation was measured in response to various B cell stimuli in the absence (A) or presence (B) of cytokines on mature B cells that had been fractionated into classical memory, tissue-like memory, and naive B cells (n = 8). Anti-Ig refers to goat anti-human IgG/A/M antibodies, and CpG refers to CpG type B, which is described in the Materials and methods. Horizontal bars indicate medians, and tissue memory in the graphs refers to tissue-like memory.
There were no differences between IgG/A and IgM ASC frequencies against HIV within fractions (Fig. 4 F), except for naive B cells (IgG/A > IgM; P = 0.004). The reason for this difference is unclear, but may be explained by the presence of recently described IgG+/CD21+/CD27+ memory B cells in this fraction (27), or by HIV-induced in vivo priming of CD21+ B cells (28, 29), leading to preferential expansion and class switching ex vivo. Nonetheless, these data clearly indicate that only IgG was tested on this individual. Horizontal bars indicate medians, and tissue memory in the graphs refers to tissue-like memory.

Finally, we measured ASC frequencies against the HIV envelope gp120 and influenza hemagglutinin, a recall antigen chosen for its widespread representation in our HIV-infected cohort as a result of annual vaccination campaigns. As expected, the frequency of influenza-specific ASCs was significantly higher in classical memory B cells compared with tissue-like memory and naive B cells (Fig. 4 C). There were no differences between IgG/A and IgM ASC frequencies against influenza within fractions (Fig. 4 D). When the HIV response was evaluated, a significantly higher frequency of HIV-specific ASCs was observed in tissue-like memory B cells compared with classical memory and naive B cells (Fig. 4 E).

Figure 4. Enrichment of HIV-specific ASCs in tissue-like memory B cells contrasted by enrichment of total and influenza-specific ASCs in classical memory B cells. Total Ig ASCs (A) and Ig isotypes of total ASCs (B) in classical memory, tissue-like memory, and naive B cells after in vitro polyclonal stimulation (n = 13). Total influenza-specific ASCs (C) and Ig isotypes of influenza-specific ASCs (D) in classical memory, tissue-like memory, and naive B cells after in vitro polyclonal stimulation (n = 12). Total HIV-specific ASCs (E) and Ig isotypes of HIV-specific ASCs (F) in classical memory, tissue-like memory, and naive B cells after in vitro polyclonal stimulation (n = 13). V01-V15 along the x axis of B, D, and F identifies each individual tested, and the asterisk indicates that only IgG was tested on this individual. Horizontal bars indicate medians, and tissue memory in the graphs refers to tissue-like memory.
but not tissue-like, memory B cells were largely responsible for these increases (Table SI and SII, available at http://www.jem.org/cgi/content/full/jem.20072683/DC1). Of note, plasmablasts, which represent a median of 5.5% of B cells in the peripheral blood of HIV-viremic individuals, expressed the highest levels of CD86, Ki-67, and CD38 (Table SII). Collectively, our current and previous findings demonstrate heterogeneous effects of HIV disease on B cells. These include lymphopenia associated with the appearance in blood of immature/transitional B cells (8, 9), and increased cell turnover/activation/differentiation (CD27⁺/CD21lo B cells) and exhaustion (CD27⁻/CD21hi B cells) associated with viremia.

In summary, we provide evidence for HIV-associated B cell exhaustion during HIV viremia. We have identified a unique population of tissue-like memory B cells in the blood of HIV-viremic individuals that are enriched with HIV-specific B cells and bear several of the same features that have been associated with virus-induced exhaustion of T cells (10–14). Evidence of exhaustion include increased expression of multiple inhibitory receptors; altered expression of homing receptors; reduced proliferative potential; and stunted replication history and receptors; altered expression of homing receptors; reduced exhaustion include increased expression of multiple inhibitory receptors on tissue-like memory B cells.paragraph

Furthermore, it is conceivable that it is the overall increase in multiple inhibitory receptors on tissue-like memory B cells that may contribute to their premature exhaustion, as has been discussed for exhausted LCMV-specific CD8⁺ T cells (13). Without better tools to analyze virus-specific B cells at the single-cell level, many of these concepts regarding dysfunction of B cells by exhaustion will remain somewhat speculative. Nonetheless, our findings suggest that HIV-specific responses are enriched in a compartment of tissue-like memory B cells in HIV-viremic individuals that exhibit many features of premature exhaustion. These findings may help explain in part the relatively ineffective HIV-specific antibody response in viremic, infected individuals.

MATERIALS AND METHODS

Study subjects. Leukapheresis and blood draw products were obtained from study subjects. We recruited 40 untreated HIV-viremic individuals (median plasma viremia: 18,606 [range 106–264,747] copies HIV RNA/ml), 12 anti-retroviral-treated HIV-viremic individuals (plasma viremia <50 copies HIV RNA per ml), and 12 HIV-negative individuals. HIV plasma viremia was measured by branched DNA assay (Bayer Diagnostics), with a lower limit of detection of 50 copies per ml. All study subjects provided informed consent, in accordance with the Institutional Review Board of the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

Phenotypic analysis. Peripheral blood mononuclear cells (PBMCs) were obtained by density-gradient centrifugation. Mature (CD10⁻) B cells were isolated from PBMCs by negative magnetic bead-based selection using a B cell enrichment cocktail that was supplemented with tetrameric anti-CD10 mAb ( StemCell Technologies). This approach excluded immature/transitional B cells, which are overrepresented in HIV-infected individuals with active disease (8), and would have confounded the results in the current study. Phenotypic analyses were performed with anti-human mAbs mostly obtained from BD Biosciences, with the following exceptions: anti-human CD21 was obtained from Beckman Coulter; anti-human CD85j, CD85k, CXCX3, CCR6, CCR7, CXCR4, and CXCR5 were obtained from R&D Systems; anti-human CD11c was obtained from Invitrogen; and anti-human FCRL4 was obtained from M.D. Cooper (Emory University School of Medicine, Atlanta, GA) (15), and its secondary was anti-mouse IgG2a (Invitrogen). FACs analyses were performed on a FACS Calibur flow cytometer (BD Biosciences) using FlowJo software (Tree Star, Inc.).

B cell fractionation. Mature B cells were separated into CD27⁺, CD27⁻/CD21hi, and CD27⁻/CD21lo fractions using a two-step magnetic bead-based selection process. Cells were first fractionated by CD27 with biotinylated anti-CD27 (BioLegend), followed by anti-biotin Microbeads (Miltenyi Biotech), and recovery of fractions according to manufacturer specifications. Purities of CD27⁺ and CD27⁻/CD21hi fractions were typically >85% and >95%, respectively. The CD27⁻/CD21lo fraction was further fractionated with anti-CD21-FITC, followed by anti-FITC Microbeads (Miltenyi Biotech), and recovered according to manufacturer specifications. Purities after CD21 fractionation were typically >85%. Alternatively, in some individuals where fractions were only to be used for molecular analyses, fractionation was done by cell sorting on a FACS Aria instrument (BD Biosciences). Mature B cells were fractionated with anti-CD20-FITC (BD Biosciences) for plasmablast analyses and with anti-CD21-biotin (AnceII) for CFSE-based analyses, each followed by appropriate Microbead-based selection.

KREC assay. The ratio of KREC joints (signal joint) to the Jκ-Cκ recombinogenic genomic joints (coding joint) was determined as previously described (24). In brief, genomic DNA was isolated from each B cell fraction by lysing cell pellets in 10 mM Tris-HCl pH 8.0, containing 100 μg/ml proteinase K (Roche), incubating for 1 h at 56°C, and heat inactivating the enzyme at 95°C for 10 min. Two separate PCR reactions were performed on ~50 ng DNA (each) (based on the approximation of 6 pg DNA per cell), one reaction to amplify the signal joint and the other to amplify the coding joint, as previously detailed (24). The number of cell divisions was calculated by subtracting the cycle threshold of the PCR detecting the coding joint from that of the signal joint.

Somatic hypermutation analysis. A restriction enzyme-based hotspot assay was used to evaluate Ig VH3 diversification as previously described (25), with recently described modifications (8). In brief, cell pellets from B cell fractions were lysed in RNA buffer (RNAeasy; QIAGEN) and the RNA was reverse transcribed with a JH consensus reverse primer. The cDNA was amplified with a Cy5-labeled VH3 consensus forward primer and the JH consensus reverse primer. The PCR products were digested with Alu I and separated on a polyacrylamide gel, and band intensities were measured using a PhosphorImager (Molecular Dynamics). The Ig VH3 diversity index was defined as the ratio of uncct PCR product in the presence of Alu I to uncct PCR product in the absence of Alu I.

Proliferation assay. This assay was performed as previously described (5). In brief, cells were plated at 1 x 10⁵ cells per well of a 96-well flat-bottom plate with various combinations of the following reagents: 20 U/ml IL-2 (Roche); 100 ng/ml IL-10 (R&D Systems); 10 μg/ml goat anti-human IgG/A/M (Jackson Immunoresearch Laboratones); 500 ng/ml CD40 ligand (5); and phosphorothiolated CpG oligodeoxynucleotide type B (CpG-B; 2.5 μg/ml; Operon). Cells were incubated for 72 h and pulsed for 16 h with tritiated thymidine. Proliferation was also assessed by FACs analysis of cells labeled with CFSE (Invitrogen).
ELISpot assay. HIV-viremic individuals who were likely to have a B cell response to the influenza vaccine formulation of 2006–2007, either from vaccination or natural influenza infection, were selected for this assay. The ELISpot assays were performed as previously described (30), with the following modifications. Each B cell fraction was plated at 1–2 × 10⁶ cells/well in 24-well plates and incubated in the presence of 1/10,000 Staphylococcus aureus Cowan (EMD Biosciences) and 2.5 μg/ml CpG-B. 96-well nitrocellulose filtration plates (MAHAS45; Millipore) were coated overnight at 4°C with the following: 5 μg/ml each anti-human α and anti-human κ (Rockland); 5 μg/ml ancestral HIV gp120 (31); 5 μg/ml influenza vaccine formulation for 2006–2007 (Sanofi-Pasteur); and 5 μg/ml keyhole limpet hemocyanin control antigen (EMD Calbiochem). The HIV gp120 protein was produced and purified as previously described (32). Wells were blocked with RPMI containing 5% FCS for 2 h at room temperature before use. At day 4, cells were collected, counted, and transferred to coated plates at two different dilutions, ranging from 3,000–300,000 cells/well depending on the coating antigen or antibody. Cells were incubated at 37°C for 5 h, after which plates were washed and incubated overnight at 4°C with biotinylated anti-human IgG (1:20,000; Jackson), IgA (1:5,000; BD Biosciences), or IgM (1:5,000; BD Biosciences). Plates were washed, incubated with substrate (ELISpot Blue; R&D Systems), dried, and spots were enumerated with an automated Immunoscan Series 3A analyzer and software (Cellular Technology). The number of antigen-specific spots was adjusted for background by subtracting spots in the keyhole limpet hemocyanin wells from those in the test wells and adjusting for the number of input cells. All washes were performed with PBS containing 0.25% Tween-20 (PBS-T) and anti-human Fcγ receptors were diluted in PBS-T containing 1% FCS. For analyses of spontaneous antibody secretion, freshly isolated cells were plated directly onto antigen-coated plates, as previously described (30).

Statistical analyses. The three groups of individuals (Fig. 1 B) were compared simultaneously using the Kruskall–Wallis test, which, if significant at level 0.05, prompted pairwise comparisons by Wilcoxon rank sum tests. This approach of predating pairwise comparisons on a significant result for the simultaneous test provides strong control of the family-wise error rate when there are three comparisons. B cell subpopulations (Table I; Table S1; and Table S2; Fig. 1 D; Fig. 2, B and D; Fig. 3; and Fig. 4, A, C, and E) were compared simultaneously by the Friedman test which, if significant, prompted pairwise comparisons by Wilcoxon signed rank tests. Comparisons between switched and unswitched immunoglobulin isotypes (Fig. 4, B, D, and F) were made by Wilcoxon signed rank tests.

Online supplemental material. Fig. S1 shows the B cell subpopulations that are present in the peripheral blood of HIV-aviremic and -negative individuals. Online supplemental material. Fig. S2 shows the replicative histories of various B cell subpopulations present in the peripheral blood of HIV-aviremic and -negative individuals. Microarray data on gene modulation by HIV–1 in immune cells: 2000–2006. J. Leukoc. Biol. 80:1031–1043.

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