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

Susan Moir,[1] Jason Ho,[1] Angela Malaspina,[1] Wei Wang,[1] Angela C. DiPoto,[1] Marie A. O'Shea,[1] Gregg Roby,[1] Shyam Kottilil,[1] James Arthos,[1] Michael A. Proschan,[2] Tae-Wook Chun,[1] and Anthony S. Fauci[1]

[1]Laboratory of Immunoregulation and [2]Biostatistics Research Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892

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 ($CD20^{hi}/CD27^{lo}/CD21^{lo}$) 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 ($CD20^{hi}/CD27^{-}/CD21^{lo}$) when compared with B cells with a classical memory ($CD27^{+}$) or naive ($CD27^{-}/CD21^{hi}$) 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.

CORRESPONDENCE
Susan Moir:
smoir@niaid.nih.gov

The typical course of HIV infection for a majority of untreated individuals is persistent viral replication and a gradual loss of $CD4^{+}$ T cells. One of the consequences of ongoing HIV replication is increased immune activation, affecting all major cell populations of the immune system (1–3). Within the B cell population, HIV infection has been associated with numerous perturbations (4), many of which have been attributed to changes in the distribution of B cell subpopulations found in the peripheral blood. These changes include increased frequencies of activated and terminally differentiated B cells expressing low levels of CD21 that have been associated with ongoing viral replication (5, 6), a decreased frequency of memory B cells that is not reversed by antiretroviral therapy (7), and an increased frequency of immature/transitional B cells that has been associated with $CD4^{+}$ T cell lymphopenia (8, 9).

The effects of immune activation in persistent viral infections have recently been shown to include virus-specific T cell exhaustion. After the original description in chronic lymphocyte choriomeningitis virus (LCMV) infection in mice (10), observations of virus-specific $CD4^{+}$ and $CD8^{+}$ T cell exhaustion have recently been extended to HIV-viremic individuals (11, 12). Although PD-1 was the first inhibitory receptor associated with virus-specific T cell exhaustion, recent findings suggest that exhaustion may result

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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 tonsillar 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 CD21^{lo} B cells in HIV-viremic individuals. We observed that tissuelike CD21^{lo} 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 CD21^{lo} B

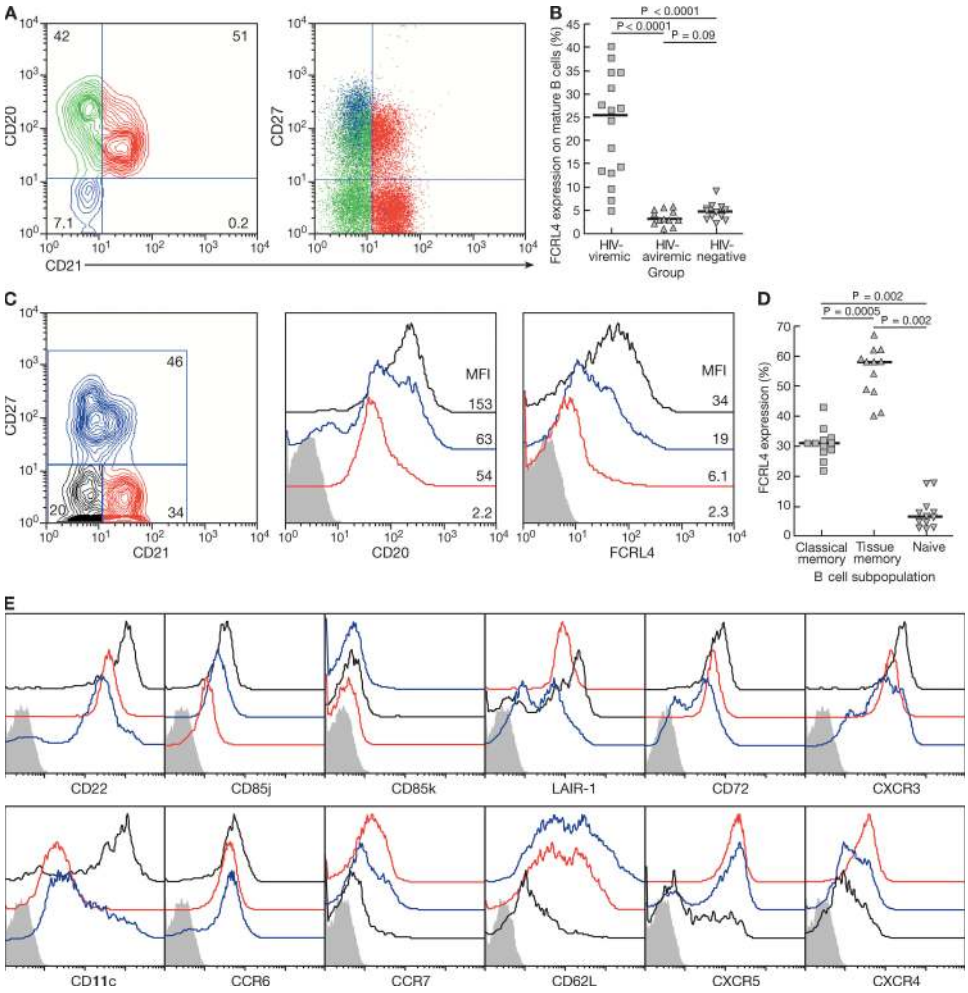

Figure 1. Phenotypic characterization of tissuelike memory B cells in the peripheral blood of HIV-viremic individuals. (A) Color-coded gating on CD20/CD21-stained B cells of a representative HIV-viremic individual was used to identify CD20^{hi}/CD21^{lo} activated (CD27^{int}) and tissuelike (CD27$^-$) memory B cells (green); CD20^{int}/CD21^{hi} resting memory (CD27^{int}) and naive (CD27$^-$) B cells (red); and CD20$^-$/CD27^{hi}/CD21^{lo} 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 CD20^{hi}/CD27$^-$/CD21^{lo} tissuelike memory (black), CD27$^+$ classical memory (blue), and CD20^{int}/CD27$^-$/CD21^{hi} 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 $CD21^{lo}$ B cells in HIV-viremic individuals (Fig. 1 A) (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 $CD21^{lo}$ B cells in the peripheral blood of HIV-viremic individuals; these $CD21^{lo}$ B cells express high levels of CD20 and low-to-intermediate levels of CD27 (Fig. 1 A). 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 $CD20^{hi}/CD27^-/CD21^{lo}$ 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. 1 B). 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 tissuelike memory B cells, defined here by the expression profile $CD20^{hi}/CD27^-/CD21^{lo}$, compared with classical memory ($CD27^+$) and naive ($CD20^{int}/CD27^-/CD21^{hi}$) B cells (Fig. 1, C and D, and Table I). Of note and illustrated in Fig. 1 A and C, $CD27^+$ B cells of HIV-viremic individuals are comprised of resting memory ($CD20^{int}/CD27^{int}/CD21^{hi}$), activated memory B cells ($CD20^{hi}/CD27^{int}/CD21^{lo}$), and plasmablasts ($CD20^-/CD27^{hi}/CD21^{lo}$) (5, 6). The latter two subpopulations are responsible for the FCRL4 expression within the $CD27^+$ B cell compartment (Fig. 1 C 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, tissuelike 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. 1 E and Table I). Levels of FCRL4, CD22, and CD85j were significantly higher on tissuelike memory compared with classical memory and naive B cells, whereas the expression of other inhibitory receptors on tissuelike memory B cells was similar or intermediate to that of either naive or classical memory B cells (Table I). Furthermore, these tissuelike memory B cells in the blood expressed a profile of trafficking receptors similar to that described for tonsillar tissue memory B cells (15) (see Ehrhardt et al. [19] on p. 1807 of this issue), namely $CXCR3^{hi}/CD11c^{hi}/CCR6^{hi}/CCR7^{lo}/CXCR5^{lo}/CXCR4^{lo}$ (Fig. 1 E 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 tissuelike memory compared with both classical memory and naive B cells (Table I). Expression of CXCR4 was significantly lower on both tissuelike and classical memory compared with naive B cells (Table I). Collectively, tissuelike 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

	1	2	3	P values		
Receptor	Classical memory[a]	Tissuelike memory	Naive	1 vs. 2	1 vs. 3	2 vs. 3
Inhibitory						
FCRL4	12 (7.7–26)[b]	25 (16–40)	3.8 (1.5–9.7)	0.002	0.0005	0.0005
CD85j	23 (14–40)	49 (24–85)	12 (4.4–26)	0.002	0.002	0.002
CD22	197 (81–388)	593 (258–997)	442 (258–655)	0.0005	0.0005	0.028
CD85k	3.2 (0.8–11)	3.4 (0.3–12)	1.1 (0.2–4.1)	0.25	0.002	0.002
CD72	20 (7.7–29)	62 (20–85)	57 (32–72)	0.002	0.002	0.270
LAIR-1	23 (12–42)	66 (21–162)	159 (84–250)	0.0005	0.0005	0.002
Trafficking						
CXCR3	73 (19–153)	222 (55–384)	86 (23–207)	0.0005	0.136	0.0005
CD11c	64 (38–228)	190 (71–365)	38 (8.7–185)	0.0005	0.001	0.0005
CCR6	26 (11–49)	54 (24–165)	33 (15–101)	0.0005	0.003	0.003
CCR7	9.3 (3.0–30)	4.4 (1.4–12)	9.8 (2.5–30)	0.0005	0.207	0.001
CD62L	114 (32–325)	13 (7.1–66)	70 (25–370)	0.002	0.176	0.0005
CXCR5	70 (16–147)	40 (8.7–126)	177 (96–281)	0.021	0.0005	0.002
CXCR4	8.2 (2.9–14)	7.5 (2.9–18)	29 (8.7–49)	0.478	0.0005	0.002

[a]Refer to Fig. 1 C for gating used to identify each subpopulation.
[b]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, $CD27^-/CD21^{lo}$ tissuelike memory, and $CD27^-/CD21^{hi}$ naive B cells. First, we evaluated the in vivo replication history of each fraction using a recently described PCR-based assay for measuring κ-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 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 plasmablasts, which underwent the highest number of cell divisions (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20072683/DC1). The KREC assay was then performed on the three B cell subpopulations illustrated in Fig. 1 C; the results for one representative individual are depicted in Fig. 2 A. 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. 2 B). 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. 1 C, and the results for one representative individual are depicted in Fig. 2 C. The median Ig VH3 diversity index of tissuelike memory B cells was significantly lower and higher compared with that of classical memory and naive B cells, respectively (Fig. 2 D). Collectively, these data suggest that the tissuelike memory B cells found in the blood of HIV-viremic individuals have reached a stage of differentiation and a degree of somatic hypermutation that are intermediate to those of naive and classical memory B cells.

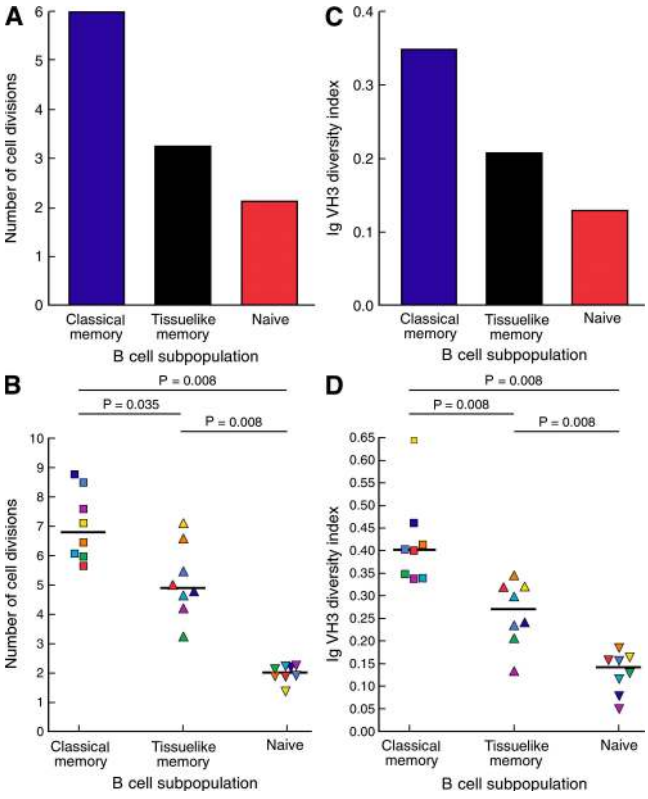

Figure 2. Distinct properties of tissuelike memory B cells isolated from the peripheral blood of HIV-viremic individuals. Evaluation of the number of cell divisions undergone in vivo by KREC analysis on mature ($CD10^-$) B cells of a representative (A) and a group of HIV-viremic individuals (B; $n = 8$) after fractionation into classical memory ($CD27^+$), tissuelike memory ($CD27^-/CD21^{lo}$), and naive ($CD27^-/CD21^{hi}$) B cells. Evaluation of Ig VH3 diversity by restriction enzyme-based hotspot analysis on mature B cells of a representative (C) and a group of HIV-viremic individuals (D; $n = 8$) after fractionation into classical memory, tissuelike memory, and naive B cells. Each individual is identified by a different color in B and D.

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. 3 A), 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. 1 C). 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. 3 B). These latter data contrasted with those reported

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 tissuelike memory B cells (Fig. 3 B) (15). Nonetheless, these data indicate that tissuelike 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 Igs 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 tissuelike memory and naive B cells (Fig. 4 A). There was no difference in total Ig ASC frequencies between tissuelike 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 tissuelike 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;

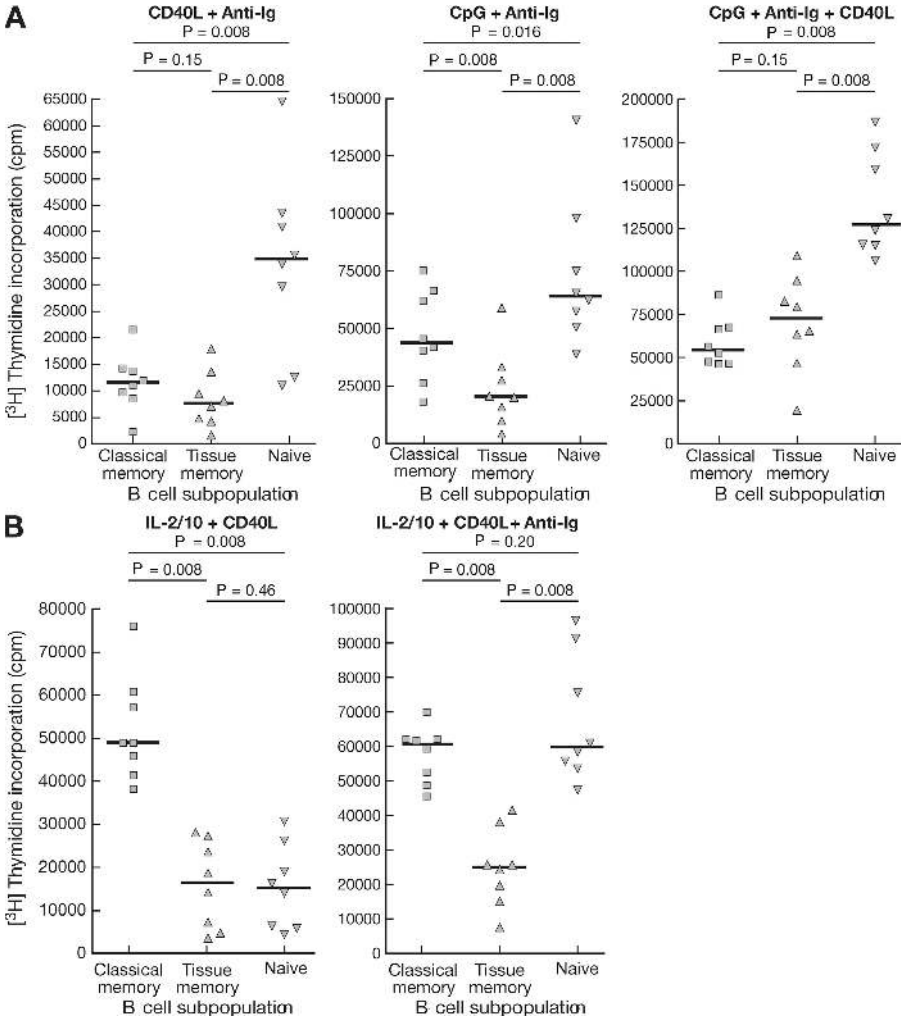

Figure 3. Low proliferative capacity of tissuelike memory B cells isolated from the peripheral blood of HIV-viremic individuals. (A and B) Proliferation was measured in response to various B cell stimuli in the absence (A) or presence (B) of cytokines on mature B cells that had been fractionated into classical memory, tissuelike 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 tissuelike memory.

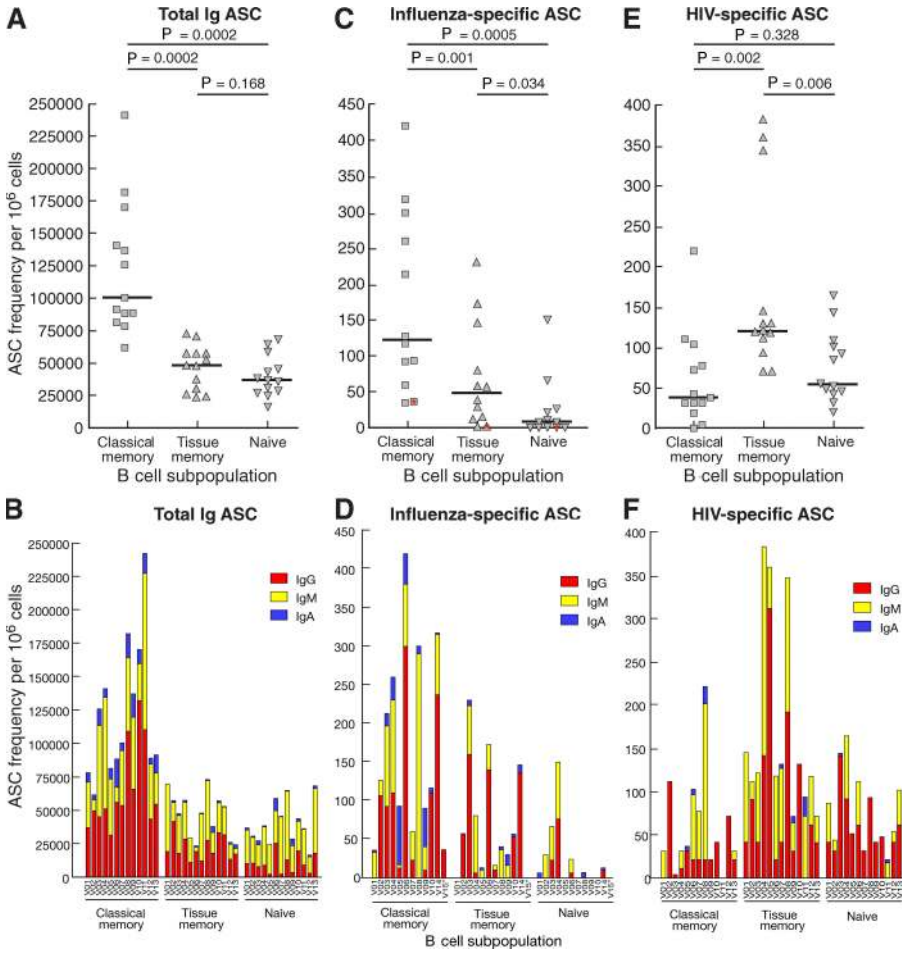

Figure 4. Enrichment of HIV-specific ASCs in tissuelike memory B cells contrasted by enrichment of total and influenza-specific ASCs in classical memory B cells. Total Ig ASCs (A) and Ig isotypes of total ASCs (B) in classical memory, tissuelike memory, and naive B cells after in vitro polyclonal stimulation (n = 13). Total influenza-specific ASCs (C) and Ig isotypes of influenza-specific ASCs (D) in classical memory, tissuelike 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, tissuelike 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 tissuelike memory.

P = 0.02). Collectively, these data indicate that tissuelike memory B cells fall between naive and classical memory B cells with regard to the extent of class switching. These data are consistent with the levels of somatic hypermutation shown in Fig. 2 (C and D).

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 tissuelike 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 tissuelike memory B cells compared with classical memory and naive B cells (Fig. 4 E). 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 although total Ig and recall antigen ASCs were most prominent in classical memory B cells, HIV-specific ASCs were enriched in tissuelike memory B cells.

We previously demonstrated that $CD21^{lo}$ B cells of HIV-viremic individuals expressed increased levels of markers of activation, turnover, and differentiation, including CD80, CD86, CD95, Ki-67, and CD38 (6). Phenotypic analyses of the three subpopulations investigated in the current study demonstrated that the $CD21^{lo}$ B cells within the classical,

but not tissuelike, 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 ($CD27^+/CD21^{lo}$ B cells) and exhaustion ($CD27^-/CD21^{lo}$ 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 tissuelike 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 Ig diversity. Alternatively, these stunted features may reflect reduced $CD4^+$ T cell help and other factors required for productive B cell responses, and/or overriding effects of increased inhibitory receptor expression. Given that overexpression of FCRL4 on tissuelike and, to a lesser extent, classical memory B cells in the blood is a feature unique to HIV infection, it is tempting to speculate that FCRL4 is key to the inhibitory properties suggested by our findings. However, FCRL4 remains a putative inhibitory receptor with no known ligand. Furthermore, it is conceivable that it is the overall increase in multiple inhibitory receptors on tissuelike 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 tissuelike 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 antiretroviral-treated HIV-aviremic 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, CXCR3, 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 Univeristy School of Medicine. Atlanta, GA) (15), and its secondary was anti–mouse IgG2a (Invitrogen). FACS analyses were performed on a FACSCalibur flow cytometer (BD Biosciences) using FlowJo software (Tree Star, Inc.).

B cell fractionation. Mature B cells were separated into $CD27^+$, $CD27^-/CD21^{hi}$, and $CD27^-/CD21^{lo}$ 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 $CD27^-$ fractions were typically >85% and >95%, respectively. The $CD27^-$ 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 FACSAria instrument (BD Biosciences). Mature B cells were fractionated with anti–CD20-FITC (BD Biosciences) for plasmablast analyses and with anti–CD21-biotin (Ancell) for CFSE-based analyses, each followed by appropriate Microbead-based selection.

KREC assay. The ratio of KREC joints (signal joint) to the Jκ-Cκ recombination 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 PCR detecting 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 (RNeasy; 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 uncut PCR product in the presence of Alu I to uncut PCR product in the absence of Alu I.

Proliferation assay. This assay was performed as previously described (5). In brief, cells were plated at 1×10^5 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 Laboratories); 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 × 10^6 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 λ 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 Immunospot 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 Igs 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 predicating 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. Fig. S2 shows the replicative histories of various B cell subpopulations isolated from the peripheral blood of two HIV-viremic individuals. Tables S1 and S2 depict differences in the expression of activation markers and Ki-67 on and in the various B cell subpopulations, with Table S1 comparing expression between classical memory, tissuelike memory, and naive B cells and Table S2 comparing expression within subpopulations of the classical memory B cell compartment. The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20072683/DC1.

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