



# HIV-Specific B Cells Are Enriched in a Dysfunctional Memory B-Cell Compartment in HIV-Infected Viremic Individuals

Susan Moir<sup>1</sup>, Jason Ho<sup>1</sup>, Angela Malaspina<sup>1</sup>, Wei Wang<sup>1</sup>, Angela DiPoto<sup>1</sup>, Marie O'Shea<sup>2</sup>, Gregg Roby<sup>1</sup>, James Arthos<sup>1</sup>, Tae-Wook Chun<sup>1</sup>, Anthony S. Fauci<sup>1</sup>

<sup>1</sup>Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, and <sup>2</sup>Critical Care Medicine Department, National Institutes of Health, Bethesda, Maryland, USA



## Abstract

**Background:** Several B-cell subsets are over-represented in the peripheral blood of HIV-infected viremic individuals. Among these subsets are mature/activated B cells that likely arise from HIV-induced immune activation. The present study investigated a unique subset of memory B cells with immunoregulatory properties that has recently been described in the lymphoid tissue (tonsils) of normal individuals.

**Methods:** Mature B cells were isolated from the peripheral blood of HIV-viremic individuals and fractionated into subsets by magnetic bead approaches. The replication history and immunoglobulin (Ig) diversity of each fraction were assessed by kappa-deletion recombination excision circle (KREC) and restriction enzyme-based hotspot assays, respectively. Proliferative properties and antigen-specific frequencies of each fraction were assessed by thymidine incorporation and ELISPOT, respectively, following *ex vivo* stimulation.

**Results:** We observed in the peripheral blood of HIV-viremic individuals a memory B-cell subset that has been recently described in tonsils of normal individuals and is defined by the expression of the inhibitory receptor, Fc-receptor-like-4 (FcRL4). FcRL4-expressing B cells were significantly more prevalent in the blood of HIV-viremic compared to HIV-aviremic and HIV-negative individuals. FcRL4 was enriched on B cells with a tissue memory phenotype (CD27<sup>hi</sup>/CD21<sup>lo</sup>) but not on B cells with a classic memory (CD27<sup>+</sup>) or naïve (CD27<sup>-</sup>/CD21<sup>hi</sup>) B-cell phenotype. Tissue memory B cells expressed increased levels of tissue homing receptors CXCR3 and CD11c, and inhibitory receptors CD22, CD85j, LAIR-1, and CD72. Ig diversities and replication histories were lower in tissue compared to classic memory B cells. Proliferation in response to various B-cell stimuli was lower for tissue memory B cells compared to classic memory and naïve B cells. Remarkably, HIV-specific antibody-secreting cells (ASC) were enriched in tissue memory B cells whereas total and influenza-specific ASCs were enriched in classic memory B cells.

**Conclusions:** HIV-specific B-cell responses in the peripheral blood of HIV-infected individuals are enriched in a memory B-cell subset formerly described in the lymphoid tissue (tonsils) of normal individuals that is characterized by reduced replicative potential, reduced affinity maturation, and increased inhibitory properties. Premature senescence of B cells may help explain why antibody responses against HIV are largely ineffective.

## Background and Rationale

HIV infection is associated with a number of abnormalities in the B-cell compartment that likely result from HIV-induced immune activation. These abnormalities include hypergammaglobulinemia, increased expression of B-cell activation markers, increased terminal differentiation, increased B-cell turnover, increased activation-induced B-cell apoptosis, and increased susceptibility to B-cell malignancies (1-4).

HIV and other viral infections have been associated with activation-induced exhaustion of virus-specific T cells, with features that include over-expression of multiple inhibitory receptors and alterations in the expression of homing receptors (5).

In a recent study (6), a new subset of memory B cells expressing high levels of the inhibitory receptor Fc receptor-like 4 (FcRL4) was described in human tonsillar tissues. Several phenotypic features of these B cells were similar to those we have observed in the peripheral blood of HIV-viremic individuals (CD27<sup>hi</sup>/CD27<sup>-</sup>/CD21<sup>lo</sup>). We have named these B cells tissue-like memory B cells.

The objective of the current study was to characterize the tissue-like memory B cells found in the peripheral blood of HIV-viremic individuals.

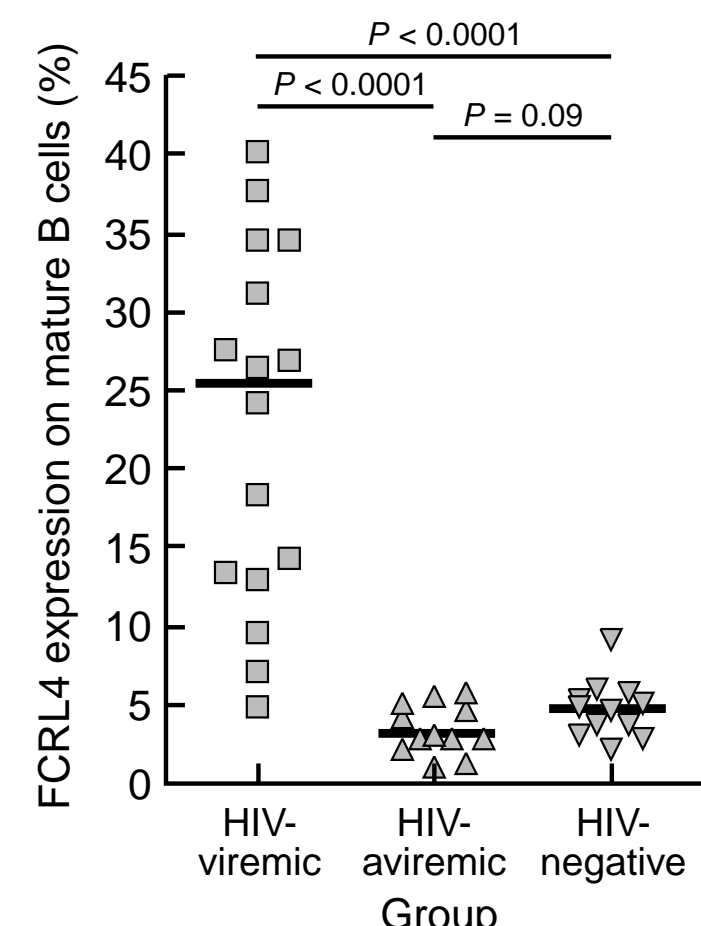
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## Results

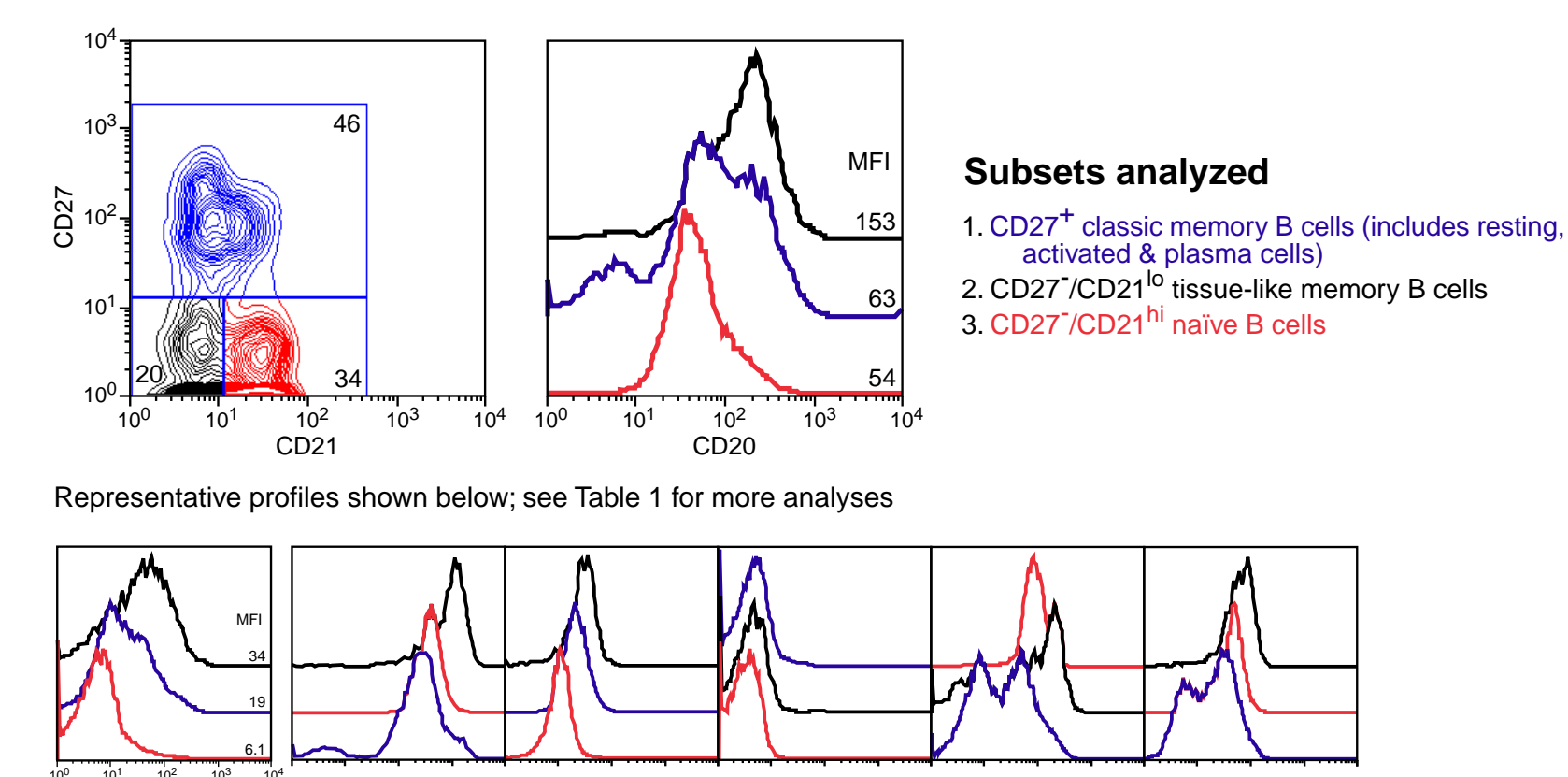
**Figure 1**

**Higher expression of the inhibitory receptor FCRL4 on blood-derived B cells of HIV-viremic compared to HIV-aviremic and HIV-negative individuals**



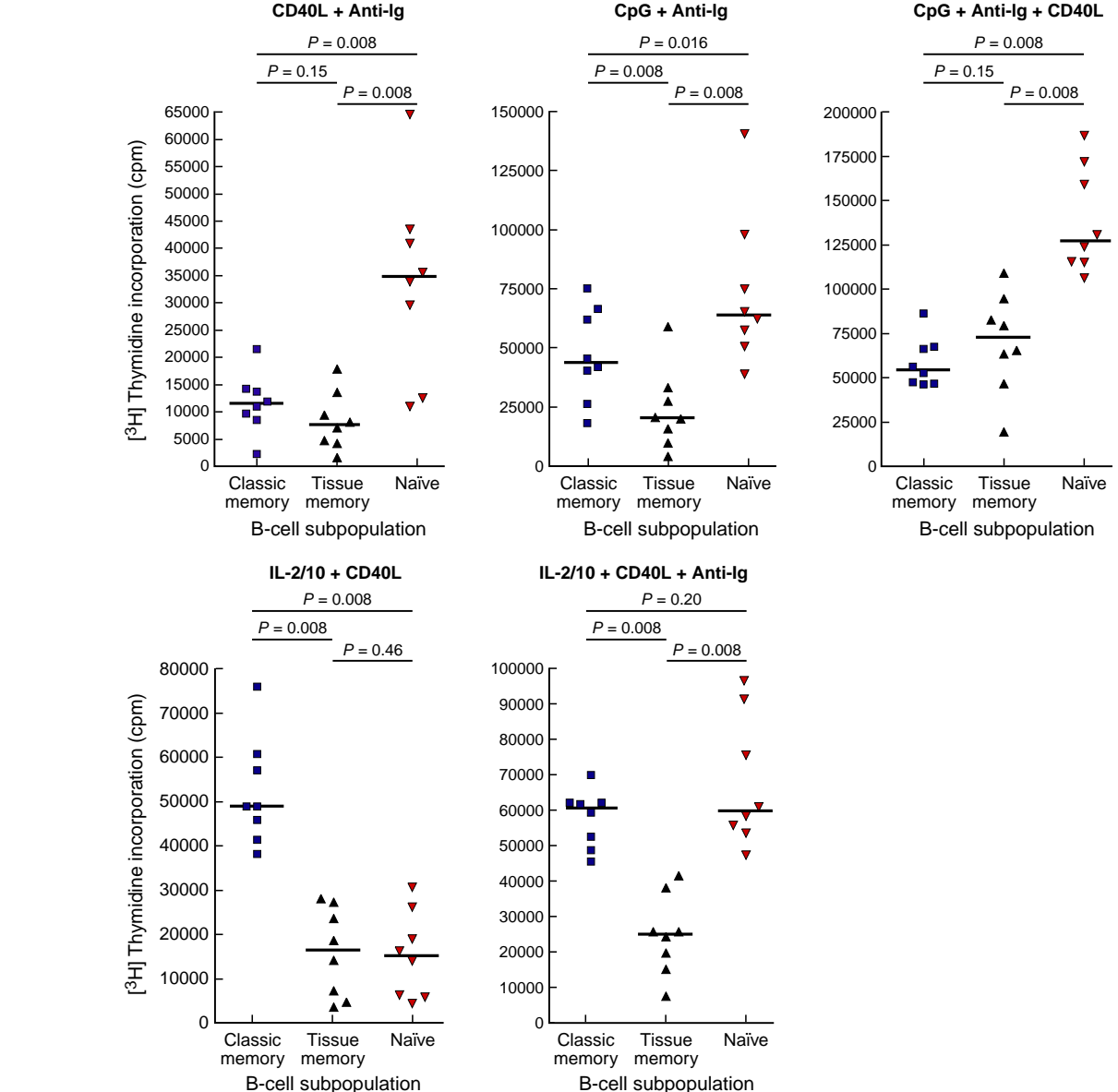
**Figure 3**

**Increased expression of multiple inhibitory receptors on tissue-like memory B cells in the blood of HIV-viremic individuals**



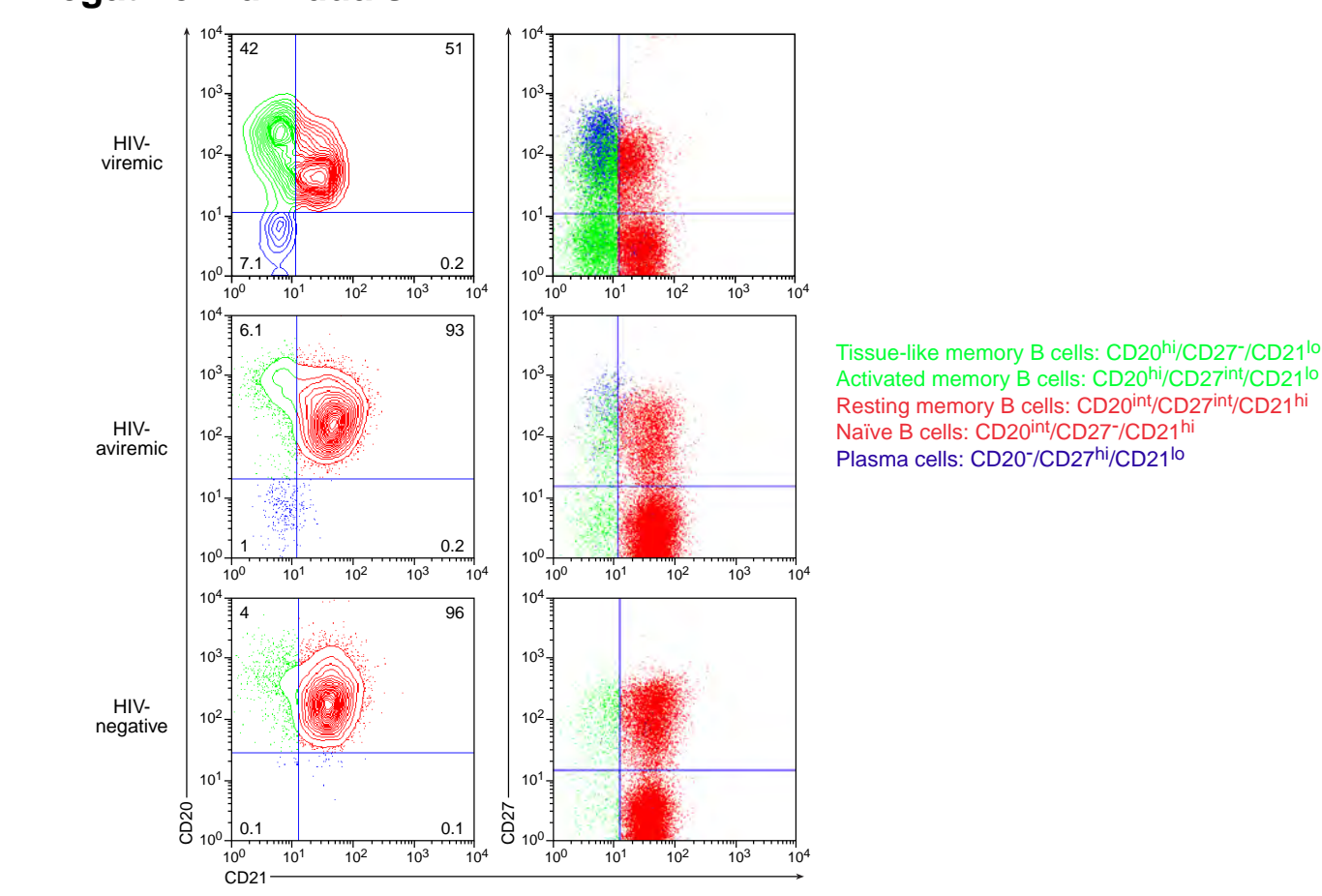
**Figure 6**

**Tissue-like memory B cells of HIV-viremic individuals have a lower proliferative capacity compared to classic memory and naïve B cells**



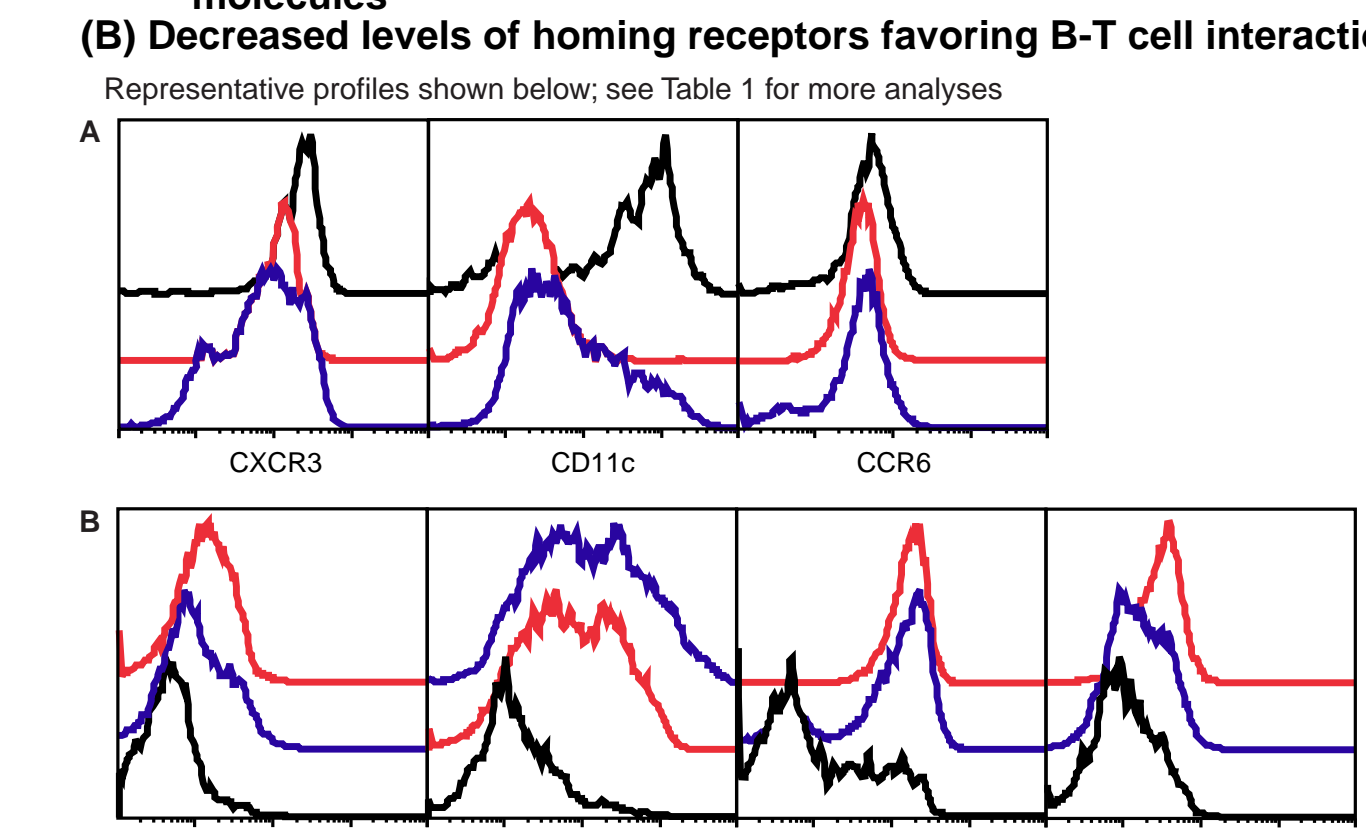
**Figure 2**

**Increased frequency of tissue-like memory (CD20<sup>hi</sup>/CD27<sup>hi</sup>/CD21<sup>lo</sup>) B cells in the blood of HIV-viremic compared to HIV-aviremic and HIV-negative individuals**



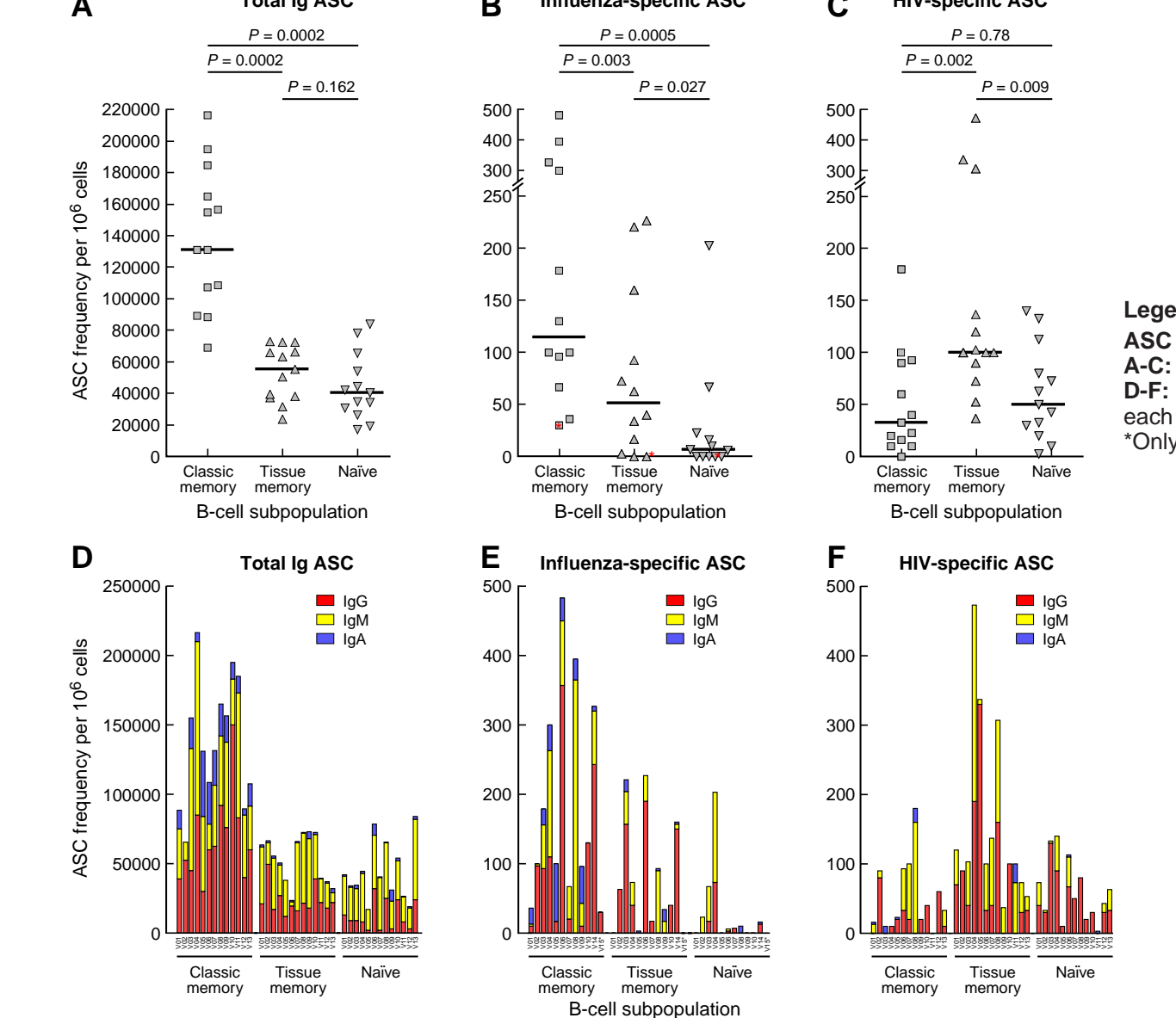
**Figure 4**

**Tissue-like memory B cells in the blood of HIV-viremic individuals express (A) increased levels of inflammatory homing receptors and adhesion molecules (B) decreased levels of homing receptors favoring B-T cell interactions**



**Figure 7**

**HIV-specific B cells are enriched in tissue-like memory B cells whereas influenza-specific and total Ig-secreting B cells are enriched in classic memory B cells**



**Table 1**

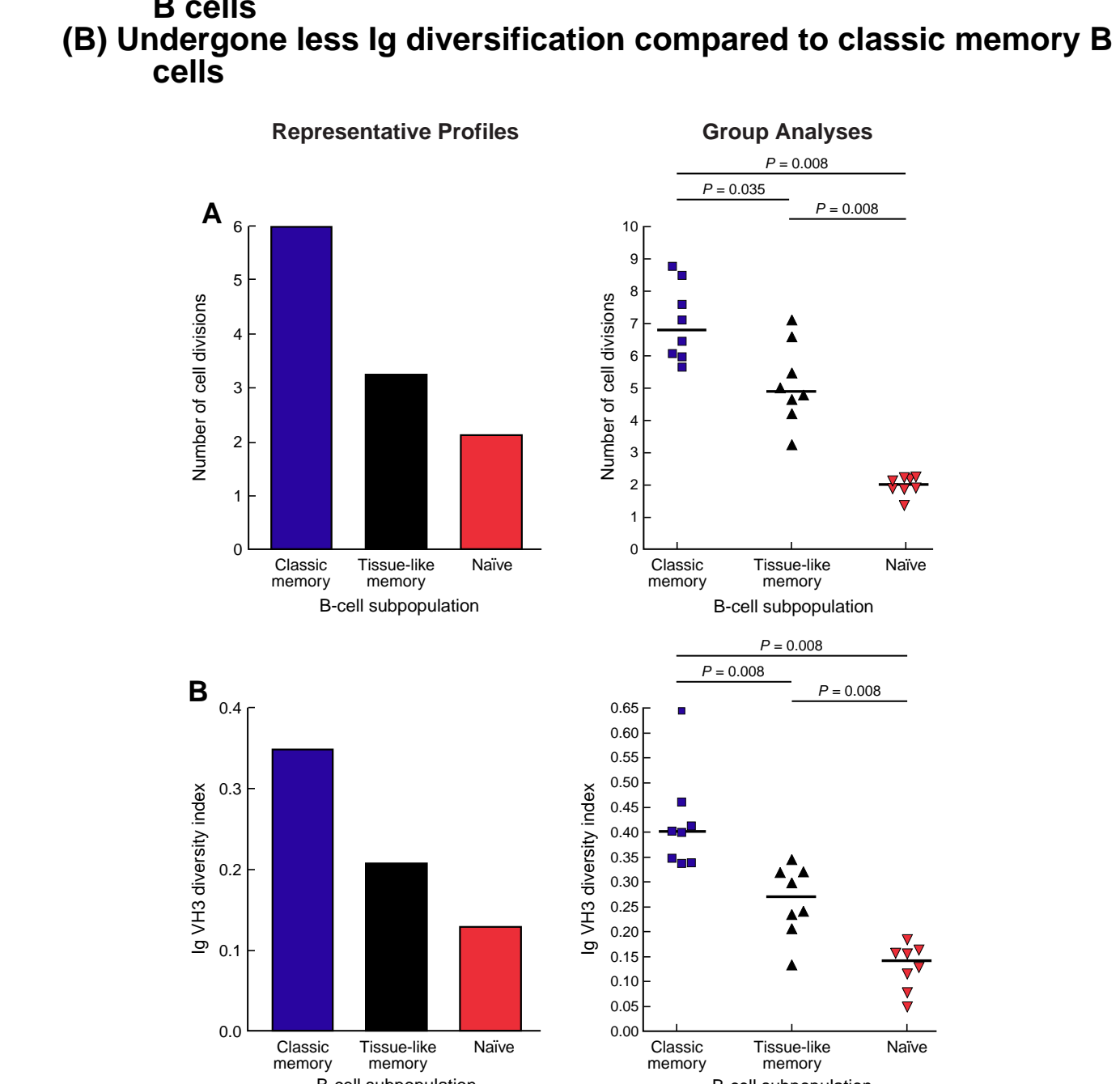
**Expression of inhibitory and trafficking receptors on B-cell subpopulations of HIV-viremic individuals**

Receptor	1 Classic Memory <sup>a</sup>	2 Tissue-like Memory	3 Naïve	1 vs 2	1 vs 3	2 vs 3
<b>Inhibitory:</b>						
FCRL4	14 (10-28) <sup>b</sup>	28 (18-42)	6.2 (3.9-12)	0.002	0.0005	0.0005
CD85j	26 (16-42)	52 (26-87)	14 (6.6-28)	0.002	0.002	0.002
CD22	199 (83-390)	596 (260-657)	444 (260-657)	0.0005	0.0005	0.028
CD85k	5.5 (3.1-13)	5.6 (2.6-14)	3.4 (2.5-6.3)	0.266	0.002	0.028
CD72	22 (9.9-31)	64 (22-87)	60 (34-74)	0.002	0.002	0.287
LAIR-1	26 (14-44)	68 (23-164)	161 (86-252)	0.0005	0.0005	0.002
<b>Trafficking:</b>						
CXCR3	75 (21-155)	224 (57-386)	88 (25-209)	0.0005	0.136	0.0005
CD11c	66 (40-230)	192 (73-367)	40 (11-187)	0.0005	0.001	0.0005
CCR6	28 (13-51)	56 (26-167)	36 (17-103)	0.002	0.003	0.003
CCR7	12 (5.3-32)	6.6 (3.7-14)	12 (4.8-32)	0.0002	0.326	0.003
CD62L	118 (34-327)	16 (10-68)	72 (27-273)	0.002	0.176	0.0005
CXCR5	72 (18-149)	42 (11-128)	179 (98-283)	0.021	0.0005	0.002
CXCR4	10 (5.2-16)	9.7 (5.2-20)	31 (11-51)	0.478	0.0005	0.002

<sup>a</sup>Refer to Figure 2 for gating used to identify each subpopulation. <sup>b</sup>Values are median and range of mean fluorescence intensities (n = 12).

**Figure 5**

**Tissue-like memory B cells of HIV-viremic individuals have (A) undergone fewer cell divisions *in vivo* compared to classic memory B cells (B) undergone less Ig diversification compared to classic memory B cells**



**Legend:**  
ASC = antibody-secreting cells  
A-C: total ASC frequencies  
D-F: IgG/IgA/IgM ASC frequencies for each individual  
<sup>\*</sup>Only tested IgG on this individual

## Conclusions

- Ongoing HIV replication is associated with the over-expression of tissue-like memory B cells in the peripheral blood of infected individuals
- Tissue-like memory B cells show evidence of HIV-induced exhaustion, including:
  - Increased expression of multiple inhibitory receptors
  - Altered expression of homing receptors and adhesion molecules similar to that of exhausted LCMV-specific CD8<sup>+</sup> T cells (5)
  - Stunted replication histories and somatic hypermutation
  - Poor replicative responses to various B-cell stimuli
  - Enrichment of HIV-specific but not other antigen-specific B cells within this B-cell compartment
- Taken together, these findings provide evidence for HIV-induced B-cell exhaustion that may help explain the relatively ineffective antibody response in HIV-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 per 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 (PBMC) were obtained by density-gradient centrifugation. Mature (CD10<sup>+</sup>) B cells were isolated from PBMC 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 over-represented in HIV-infected individuals with active disease (7), and would have confounded the results in the current study. Phenotypic analyses were performed with anti-human mAbs obtained mostly 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; anti-human CD11c was obtained from Invitrogen; and anti-human FCRL4 was obtained from M.D. Cooper (U. Alabama at Birmingham) and its secondary was anti-mouse IgG2a (Invitrogen). FACS analyses were performed on a FACSCalibur flow cytometer (BD Biosciences) using FlowJo software (Tree Star).

**B-cell fractionation.** Mature B cells were separated into CD27<sup>+</sup> and CD27<sup>hi</sup>/CD21<sup>hi</sup> and CD27<sup>hi</sup>/CD21<sup>lo</sup> 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), and recovery of fractions according to manufacturer specifications. Purities of CD27<sup>+</sup> and CD27<sup>hi</sup> fractions were typically > 85% and > 95%, respectively. The CD27<sup>+</sup> fraction was further fractionated with anti-CD21-FITC, followed by anti-FITC Microbeads (Miltenyi), and recovered according to manufacturer specifications. Purities following 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).

**KREC assay.** The ratio of kappa (k)-deletion recombination circle joints (signal joint) to the Jk-Ck recombination genomic joints (coding joint) was determined as described (6). Briefly, genomic DNA was isolated from each B-cell fraction by lysing cell pellets in 10mM Tris-HCl pH 8 containing 100 µg/ml proteinase K (Roche Applied Science), incubating for one hour at 56°C, and heat inactivating the enzyme at 95°C for 10 min. Two separate PCR reactions were performed on approximately 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 described in detail (6). 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 (7). Briefly, 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, 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.** Cells were plated at 1 X 10<sup>5</sup> cells per well of a 96-well flat-bottom plate with various combinations of the following reagents: IL-2 (20 U/ml; Roche); IL-10 (100 ng/ml; R&D Systems); goat anti-human IgG/A/M (10 µg/ml; Jackson); CD40 ligand (500 ng/ml); and CpG-B (2.5 µg/ml; Operon). Cells were incubated for 72 h and pulsed for 16 h with thymidine.

**ELISPOT assay.** Individuals who had a B-cell response to the influenza vaccine formulation of 2006-2007 were selected for this assay. Each B-cell fraction was plated at 1 X 10<sup>6</sup> cells/well in 24-well plates and incubated in the presence of 1/10,000 Staphylococcus aureus Cowan (SAC; 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: anti-human  $\lambda$  and (5 µg/ml each; Rockland); ancestral HIV gp120 (5 µg/ml); influenza vaccine formulation for 2006-2007 (5 µg/ml; Sanofi-Pasteur); and keyhole limpet hemocyanin (KLH) control antigen (5 µg/ml; EMD Calbiochem). Wells were blocked with RPMI containing 5% FCS for 2 h at room temperature prior to 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  $\lambda$  and  $\kappa$  IgG (1:20,000; Jackson), IgA (1:5000; BD Biosciences), or IgM (1:5000; BD Biosciences). Plates were washed, incubated with substrate (ELISPOT Blue; R&D Systems), dried, and spots enumerated with an inverted microscope fitted with a counting grid (Nova). The number of antigen-specific spots was adjusted for background by subtracting spots in the KLH wells from those in the test wells and adjusting for 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.

**Statistical analyses.** The three groups of individuals were compared simultaneously using the Kruskal-Wallis test which, if significant at level .05, prompted pair-wise comparisons by Wilcoxon rank sum tests. This approach of predicated pair-wise 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 were compared simultaneously by the Friedman test which, if significant, prompted pair-wise comparisons by Wilcoxon signed rank tests. Comparisons between switched and unswitched immunoglobulin isotypes were made by Wilcoxon signed rank tests.