Published in final edited form as:

AIDS Res Hum Retroviruses. 2008 April ; 24(4): 621–633. doi:10.1089/aid.2007.0186.

A Splenic Marginal Zone-Like Peripheral Blood CD27⁺B220⁻ B Cell Population Is Preferentially Depleted in HIV Type 1-Infected Individuals

Matthew Morrow¹, Antonio Valentin¹, Richard Little², Robert Yarchoan², and George N. Pavlakis¹

¹Human Retrovirus Section, Vaccine Branch, Center for Cancer Research, National Cancer Institute-Frederick, Frederick, Maryland 21702.

²HIV and AIDS Malignancy Branch, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland 20895.

Abstract

Peripheral blood CD27⁺ B cells are reduced in HIV-1-infected individuals. In healthy individuals, the human peripheral blood CD27⁺ B cell pool consists of two subsets defined by the expression, or lack thereof, of the CD45 isoform B220. We investigated the presence of circulating B220⁺ and B220⁻ memory B cells in HIV⁺ individuals and found that the reduction in CD27⁺ memory B cells occurs primarily among CD27⁺B220⁻ B cells. Studies conducted using healthy controls indicate that CD27⁺B220⁻ B cells have a splenic marginal zone like the immunophenotype IgM^{hi}IgD^{lo}CD21⁺CD23⁻, express TLR9, and proliferate and secrete IgG and IgM in response to B cell-specific ODN. CD27⁺B220⁺ B cells have the immunophenotype IgM^{lo}IgD^{hi}CD21⁺CD23⁺, express activation-induced cytidine deaminase, and proliferate in response to SAC but do not secrete immunoglobulin. The AICD expression, along with CD86 expression, by CD27⁺B220⁺ suggests these cells are of germinal center origin. The preferential depletion of CD27⁺B220⁻ B cells mirrors alterations in spleen morphology and resident B cell populations due to HIV infection reported by other investigators and may play an important role in the defective B cell immunity against T-independent pathogens such as pneumococcus observed in HIV-1-infected individuals.

INTRODUCTION

Peripheral blood CD27⁺ memory B cells are significantly depleted early in the course of HIV-1 infection and are not restored to pre-HIV infection levels when plasma viremia becomes undetectable and peripheral blood CD4⁺ T cell counts rebound with successful antiretroviral therapy (ART).^{1–3} Although the reason behind the loss of memory B cells in HIV-infected individuals has not been clearly established, increased apoptosis and increased plasmacytosis without replenishment of the memory B cell pool have been implicated as causes.^{4–6} The loss of circulating memory B cells corresponds to decreased serum antigen-specific immunoglobulin and maintenance of serological memory, and diminished responses to challenge with recall antigens.^{7–10} This phenomenon is in fact part of a larger HIV-induced impairment of B cell immunity including hypergammaglobulinemia with spontaneous Ig secretion *in vitro*, altered antigen expression, and decreased T cell costimulatory function.⁹ As

with the loss of memory B cells, the cause of these changes has not been definitively established; however cytokine imbalance, decreased T cell function, and direct exposure to virus and viral antigens have been shown to play significant roles.^{11–22}

The peripheral blood memory B cell pool in healthy individuals consists of two subsets defined by the presence or lack of a high-molecular-weight CD45 isoform known as CD45RABC and commonly referred to as B220.²³ Unlike murine B cells, which continually express B220 as early as pre-pro-B cells and throughout maturation to memory B cells, histological analyses of human germinal center follicles suggests naive mature CD27⁺B220⁺ B cells downregulate B220 expression at some point after or during migration from follicular mantle zones to germinal centers (GCs) and prior to reentering the circulation.²⁴ This is reflected in the peripheral blood as approximately two-thirds of circulating CD27⁺ B cells lack B220. Further histological studies indicate that splenic white pulp marginal zone CD27⁺ B cells, a specific CD27⁺ B cell population recognizing T-independent antigens, also lack B220.²⁴

Peripheral blood CD27⁺B220[−] memory B cells are preferentially depleted in individuals with X-linked hyper-IgM syndrome (X-HIGM), a primary immunodeficiency in which defective CD40 ligand (CD40L, CD154) expression by activated T cells prevents normal T cell–B cell interaction.^{23,25,26} This molecular defect results in an abortive GC reaction, thereby preventing the development of the cellular microenvironment in which molecular events driving immunoglobulin (Ig) class switch recombination (CSR), somatic hypermutation, and B cell differentiation to memory B cells occur.^{27,28} HIV-1 infection may disrupt the same CD40–CD40L signaling pathway responsible for the immunological defects observed in patients with X-HIGM via diverse molecular mechanisms: viral gp120 binding to CD4 on target T cells inhibits CD154 expression during T cell activation inhibiting interleukin (IL)-12 production *in vitro*; and HIV nef protein suppresses Ig CSR in bystander B cells by inducing transcription of the inhibitor of κ B (IkB α) and SOCS proteins that block CD40L and cytokine-induced NF- κ B and STAT transcription factors, respectively, *in vitro*.^{17,29}

Because of some similarities in clinical presentation observed in X-HIGM and HIV-1 infection, we examined circulating memory B cells in HIV-1⁺ individuals in order to determine whether the B220[−] memory B cell subset is the primary depleted peripheral blood memory B cell population as it is in X-HIGM. We found that CD27⁺B220[−] memory B cells are in fact preferentially depleted compared to the B220⁺ memory B cell subset. Subsequently, we characterized B220⁺ and B220[−] memory B cells in healthy donors by cell surface markers, Ig production, and gene expression in order to determine whether these subsets represent either different stages of memory B cell maturation or distinct memory B cell populations. Our analysis indicates that B220[−] memory B cells are a distinct B cell pool and that their reduction is not a function of blocked memory B cell maturation but rather the result of a yet unknown HIV-related mechanism of depletion.

MATERIALS AND METHODS

Blood samples from HIV-1-infected patients and healthy donors

All blood samples were collected in acid-citrate dextrose (ACD) tubes after informed consent and under protocols approved by the National Cancer Institute Investigational Review Board. Samples were obtained from 22 HIV-seronegative individuals (healthy donors) participating in the NCI-Frederick registered donor program in Frederick, MD. Samples from 27 different HIV⁺ individuals were obtained at the National Institutes of Health.

Materials

Fluorochrome-labeled antibodies to CD19, CD21, CD23, CD27, CD40, CD80, CD86, CD95, IgM, IgD, and IgG were purchased from BD Biosciences (San Diego, CA). Fluorochrome-labeled antibodies to B220 were purchased from Caltag (San Diego, CA). Carboxyfluorescein ester was purchased from Sigma (St. Louis, MO), recombinant human IL-2 was purchased from Roche (Indianapolis, IN), *Staphylococcus aureus* Cowan's strain SAC was purchased from Calbiochem (San Diego, CA), and ODN2006 was purchased from Coley Pharmaceuticals (Ottawa, Ontario). Premixed primer and probe cocktails for activation-induced cytidine deaminase, Toll-like receptor 9, and β -actin for use in quantitative real-time PCR were purchased from Applied Biosystems (Foster City, CA).

Cell culture

Peripheral blood mononuclear cells (PBMCs) were obtained from whole blood collected in ACD tubes by centrifugation over Histopaque (Sigma) gradient. Lymphocytes were cultured in RPMI-1640 media supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μ g/ml), L-glutamine (2 mM), and IL-2 (5 U/ml). Cells were incubated at 37°C with 5% CO₂.

Cell sorting and flow cytometry

Human PBMCs were enriched for CD19⁺ B cells by negative selection using immunomagnetic beads (Miltenyi, Auburn, CA). The enriched B cells were labeled with fluorochrome-conjugated antibodies to CD19, CD27, and B220 and sorted on a FACS Aria flow cytometer (BD Biosciences). The purity of the sorted populations was verified using FlowJo Software (Treestar, Ashland, OR). Analytical cytometry was performed on whole blood using a BD LSR with CellQuest software, and on PBMCs when appropriate using a BD FACS Aria. The following fluorochrome-conjugated monoclonal antibodies were used: CD27-FITC, CD27-PE, CD19-PECy7, B220-PECy5.5, IgM-APC, IgD-PE, IgG-PE, CD21-APC, CD23-PE, CD40-APC, CD95-PE, CD86-APC, and CD80-PE. The antibodies used are listed in the corresponding figure legends. All analytical data generated on the FACS Aria, including CFSE proliferation, were analyzed with FlowJo Software.

Real-time quantitative PCR

Total RNA was obtained from sorted cell populations using RNA-Bee (Tel-Test, Inc., Friendswood, TX) according to the manufacturer's protocols. Reverse transcription was performed with a Superscript III reverse transcription kit (Invitrogen, Carlsbad, CA) using oligo(dT) primers. Quantitative polymerase chain reaction (PCR) was performed using commercially available kits specific for human TLR9 and AICD containing premixed primers and probes and Platinum TAQ-UDG premixed PCR reagents (Invitrogen). Amplification was performed for 40 cycles on an ABI Prism 7700 (15 sec at 95°C followed by 1 min at 60°C).

Statistical analysis

Statistics were performed using SigmaStat (Systat Software, Inc., Point Richmond, CA) or Prism (Graphpad Software, Inc.) statistical analysis software. Comparisons of two groups were done by the Mann-Whitney test. One-way ANOVA was used for comparison of more than two groups.

HIV-1 infected patients

We examined 40 peripheral blood samples obtained from 27 different HIV-infected individuals. These patients were coinfecting with Kaposi's sarcoma-associated herpes virus (KSHV). Eleven of these patients had active KS at the time of sampling. All the patients were

receiving combination ART. Some patients had previously received agents for KS therapy including IL-12, liposomal doxorubicin, and/or thalidomide, though not within 30 days of sampling.

RESULTS

CD27⁺ B220[−] B cells are preferentially depleted in HIV-1 infection

Peripheral blood (PB) B cells were identified among gated lymphocytes by the expression of CD19 in 22 whole blood samples from healthy donors (HDs) and 40 samples from HIV-1-infected individuals (HIV⁺), then examined for the expression of the memory B cell marker CD27 using flow cytometry (Fig. 1A). Similar to previously published reports, we found that the frequency of CD27⁺ cells among circulating B cells was reduced in HIV⁺ samples (mean: 10.2%) compared to HDs (mean: 29.5%). However, the addition of B220 expression to the CD27 analysis indicated this reduction predominantly occurred among CD27⁺B220[−] B cells: 20.0% of B cells from HDs are CD27⁺B220[−] compared to 4.1% in HIV⁺ (Fig. 1B). The frequency of CD27⁺B220⁺ B cells was only slightly reduced (9.5% of B cells in HDs, 6.1% of B cells in HIV⁺). On average, there was a 3-fold decrease in memory B cells in HIV⁺ individuals compared to HDs (10.2% versus 29.5%, Fig. 1). This decrease occurred preferentially within the B220[−] set, since it decreased by ~5-fold, compared to less than 2× in the B220⁺ population. As a result, the ratio of the B220⁺ and B220[−] CD27⁺ memory B cells was reversed in HIV-infected individuals (from 0.5 in HDs to 2.2, $p < 0.0001$, Mann–Whitney test).

The relative frequency of PB B cells among circulating lymphocytes in HIV⁺ individuals was marginally increased compared to healthy controls (HDs, 8.1%; HIV⁺, 9.1%, $p > 0.05$) (Fig. 1C). Taken together with the decrease of circulating CD27⁺ B cells, this suggests the CD27[−] mature naive B cell compartment in HIV⁺ individuals is expanded, which is in agreement with previous reports.³⁰ The reduction of total CD27⁺ B cells, CD27⁺B220⁺ B cells, and CD27⁺B220[−] B cells among all B cells is not the result of expansion of CD27[−] B cells, but rather a decrease of these cells in the circulation, as indicated by their reduction among total circulating lymphocytes [CD27⁺: 2.4% (HDs), 0.8% (HIV⁺), $p < 0.001$; CD27⁺B220⁺: 0.8% (HDs), 0.5% (HIV⁺), $p < 0.05$; CD27⁺B220[−]: 1.6% (HDs), 0.3% (HIV⁺), $p < 0.001$].

The reduction of CD27⁺ B220[−] B cells in HIV⁺ individuals is not related to plasma viremia or CD4⁺ T cell counts

Several previous reports describe a strong correlation between the B cell abnormalities associated with HIV infection and plasma viremia.^{6,9,20,31} Therefore, we analyzed CD27 and B220 expression in our HIV⁺ cohort with respect to plasma viremia (>50 copies RNA/ml plasma) and aviremia (<50 copies RNA/ml plasma), and also with respect to absolute CD4 counts (high CD4, >200 CD4⁺ T cells/μl; low CD4, <200 CD4⁺ T cells/μl), and compared the total CD27, CD27⁺B220⁺, and CD27⁺B220[−] B cell frequencies of these groups to those of HDs (Fig. 2). Plasma viral load and CD4⁺ T cell counts were available for 37 of the 40 HIV⁺ samples. The frequency of total CD27⁺ B cells was significantly reduced among circulating B cells in both viremic ($n = 15$) and aviremic ($n = 22$) HIV⁺ subgroups (means: 10.8% and 8.5%, respectively), and in both high CD4 ($n = 22$) and low CD4 ($n = 15$) subsets (means: 9.7% and 10.2%, respectively) compared to HDs (mean: 29.5%, $n = 22$) (Fig. 2A). There were no significant differences in either the frequency of CD27⁺ B cells between viremic or aviremic HIV⁺ subgroups, or high CD4 or low CD4 subsets ($p > 0.05$ in both cases). The frequency of CD27⁺B220⁺ B cells was similarly reduced with regard to either plasma viremia (aviremic: 6.2%, viremic: 5.3%) or CD4 high/low subsets (high CD4: 6.0%, low CD4: 5.7%) compared to HDs (mean: 9.5%, $p < 0.05$ for each comparison) (Fig. 2B). There was no significant difference in the frequency of CD27⁺B220⁺ B cells between aviremic and viremic

HIV⁺ groups, or in high CD4 and low CD4 groups ($p > 0.05$ for both). The frequency of CD27⁺B220⁻ B cells among HIV⁺ aviremic/viremic and CD4 high/low subsets was similarly affected (aviremic mean: 4.6%, viremic mean: 3.2%; high CD4 mean: 3.7%, low CD4 mean: 4.5%) compared to HDs (mean: 20.0%, $p < 0.001$ for each comparison). Differences in the frequency of CD27⁺B220⁻ B cells among aviremic/viremic and high CD4/low CD4 HIV⁺ subsets were not significant ($p > 0.05$). In summary, there was no correlation between either virus loads or CD4 counts and the degree of depletion of any of the B cell subsets.

CD27⁺B220⁻ memory B cells have a distinct phenotype

At present, human B220⁺ and B220⁻ memory B cell subsets have not been characterized. Therefore, we examined B220⁺ and B220⁻ memory B cells in healthy individuals in order to determine if these cell subsets represent either functionally unique memory B cell subsets, the latter of which may be more susceptible to depletion by HIV-1 infection, or if B220⁻ memory B cells represent a “more mature” memory B cell subset and infection with HIV-1 prevents full memory B cell development to this phenotype. We used 5-color flow cytometry to immunophenotype CD27⁺ memory B cell B220⁺ and B220⁻ subsets in PBMCs isolated from healthy donors for the expression of common B cell makers (CD21, CD23, CD40), immunoglobulin (Ig) (IgD, IgM, and IgG), and B cell-associated activation markers (CD80, CD86, and CD95) (Fig. 3). The frequency of cells within each subset expressing these markers is summarized in Table 1.

Mature naive CD27⁻B220⁺ B cells are typically CD21⁺CD23⁺CD40⁺IgD^{hi}IgM^{lo} and lack IgG, CD80, CD86, and CD95. B220⁺ memory B cells are typically CD21⁻CD23⁺CD40⁺IgD^{hi}IgM^{hi} and contain small populations of cells expressing IgG, CD80, CD86, or CD95. B220⁻ memory B cells are typically CD21⁺CD23⁻CD40⁺IgD^{dim}IgM^{hi}. Furthermore, while small populations of CD80⁺, CD95⁺, and IgG⁺ B cells are present in this subset, CD86⁺ cells are notably absent unlike the B220⁺ memory B cells subset. Thus, major differences were found in these two memory B cell populations in the expression of CD23 and IgD.

B220⁺ and B220⁻ memory B cells differentially express the memory B cell associated genes AICD and TLR9, respectively

Activation-induced cytidine deaminase (AICD) and Toll-like receptor 9 (TLR9) are expressed by circulating CD27⁺ memory B cells and are detectable *ex vivo*. AICD is a member of the APOBEC family of RNA-editing enzymes and initiates B cell Ig CSR. TLR9 is an innate immunity receptor, which recognizes bacterial CpG oligodinucleotide sequences and is rapidly expressed upon B cell receptor engagement. We investigated whether the expression of these genes correlates with a particular memory B cell subset. We sorted immunomagnetically enriched CD19⁺ B cells obtained from freshly prepared HD PBMCs into CD27⁻B220⁺ (>95% purity), CD27⁺B220⁺ (>92% purity), and CD27⁺B220⁻ (>95% purity) B cell populations (Fig. 4A, representative experiment, one of three) and analyzed the RNA content of each population for TLR9 and AICD expression using real-time quantitative PCR (RT-qPCR). Gene expression was standardized to the expression of the housekeeping gene β -actin. Our results indicated that each subset expressed TLR9. However, CD27⁺B220⁻ B cells expressed 2- to 3-fold more TLR9 than either CD27⁺B220⁺ B cells or mature naive CD27⁻B220⁺ B cells (Fig. 4B). In contrast, for AICD we observed a reciprocal pattern of expression among the CD27⁺ B cell subsets: CD27⁺B220⁺ B cells expressed approximately 40-fold more AICD than either CD27⁺B220⁻ memory B cells or CD27⁻B220⁺ naive B cells (Fig. 4C, average of four determinations from different donors, $p = 0.0143$, Mann–Whitney test).

B cell receptor and TLR9 stimulation induce B220⁺ and B220⁻ memory B cell proliferation, respectively

High TLR9 expression by CD27⁺B220⁻ memory B cells suggests these cells are likely to be more susceptible to stimulation with B cell-specific CpG ODNs than CD27⁺B220⁺ and CD27⁻B220⁺ B cells. We labeled PBMCs obtained from HDs with 10 μ M carboxyfluorescein succinate ester (CFSE) and cultured them for 5 days in the presence of either class B ODN, SAC + IL-2, or nothing (control). Viable (DAPI⁻) CD19⁺ B cells were analyzed using flow cytometry for CD27 and B220 expression, and proliferation indicated by decreased CFSE fluorescence. The frequency of CD27⁺B220⁻ memory B cells decreased in nonstimulated (14.3%) and SAC + IL-2-stimulated (16.0%) cultures compared to the *ex vivo* frequency (23.4%), but increased with ODN stimulation (35.3%) (Fig. 5A, representative experiment). Although CFSE analysis of ODN-stimulated cultures indicated that each CD27/B220 B cell subset contained proliferating cells, the ODN-stimulated CD27⁺B220⁻ memory B cells displayed a higher frequency of division than CD27⁺B220⁺ B cells. The average values for % proliferation and proliferation index (PI) for 10 independent experiments are shown in Fig. 5C. An increase in CD27 expression was observed among CD27⁺B220⁻ B cells as well. SAC + IL-2 stimulation, which activates B cells via their B cell receptor (BCR), resulted in an increase in the relative frequency of CD27⁺B220⁺ memory B cells (23.4%, *ex vivo*: 9.6%). SAC + IL-2 stimulation increased CD27 expression in both memory subsets. In contrast to ODN, no significant difference was found in the proliferative responses between the B220⁺ and B220⁻ memory B cells upon SAC + IL-2 stimulation (Fig. 5C).

Ig secretion correlates to a B220⁻ memory B cell phenotype

B cells stimulated *in vitro* through TLR9 with B cell-specific CpG ODN differentiate into Ig-secreting cells (ISCs). Therefore, we analyzed B cells for changes in IgM and IgG expression induced by ODN and SAC + IL-2 stimulation (Fig. 6A, representative plot) in addition to Ig secretion in cell culture supernatants (Fig. 6B, mean concentration of 10 HD cultures). The frequency of IgG⁺(IgM⁻) and IgM⁺(IgG⁻) cells among all B cells in nonstimulated PBMCs was 4% and 36.5%, respectively. Stimulation with ODN, which resulted in a predominantly CD27⁺B220⁻ memory B cell population, increased the relative frequency of IgG⁺(IgM⁻) B cells to 26.4%, while the frequency of IgM⁺(IgG⁻) B cells remained unchanged (36.6%). Stimulation with SAC + IL-2, which resulted in a predominantly CD27⁺B220⁺ memory B cell population, also increased the frequency of IgG⁺(IgM⁻) B cells (30.2%); however the frequency of IgM⁺(IgG⁻) B cells decreased to 18.9%.

Regarding secreted Ig, nonstimulated culture supernatants contained 201 pg/ml of IgG and 18.2 pg/ml of IgM. As anticipated, the expansion of CD27⁺B220⁻ B cells in response to ODN stimulation coincided with large amounts of Ig secretion [IgG: 3451.3 pg/ml (17-fold increase) and IgM: 2469.3 pg/ml (135-fold increase)]. Although the expansion of CD27⁺B220⁺ B cells by SAC + IL-2 stimulation was accompanied by increased IgG expression, significantly less IgG (467 pg/ml, 2-fold increase) and IgM (115 pg/ml, 6-fold increase) was secreted.

DISCUSSION

The circulating CD27⁺ memory B cell pool is reduced in individuals infected with HIV-1. We show here that the reduction of this population is primarily attributed to a phenotypically and functionally distinct memory B cell subset defined as CD27⁺B220⁻ B cells. Both B220⁺ and B220⁻ memory B cells express CD40 and contain subsets of cells expressing the activation markers CD80 and CD95. Additionally, both subsets contain Ig class-switched non-IgM/non-IgD cells, of which at least half have detectable IgG. However, the B220⁻ memory B cell pool contains a unique subpopulation of cells that express the immunophenotype IgM^{hi}+IgD^{lo}+CD21⁺CD23⁻, express TLR9, and lack AICD. These cells expand in response to

stimulation with class B ODN *in vitro* and differentiate into Ig-secreting cells, increasing both production and secretion of IgG and IgM. B220⁺ memory B cells differ in that they have the immunophenotype IgM^{lo}+IgD^{hi}+CD21⁺CD23⁺, express significantly less TLR9, and express AICD. This population increases Ig expression in response to BCR-specific stimulation with SAC, but does not secrete Ig *in vitro*.

The immunophenotype and gene expression profile of circulating CD27⁺B220⁻ B cells is similar to that of CD27⁺ splenic marginal zone B cells, and some evidence suggests CD27⁺B220⁺ B cells are recent GC escapees. High CD21 expression, the receptor for the complement fragment C3d, which binds bacteria in the absence of prebound Ig and which is also known as complement receptor 2, by B220⁻ memory B cells is an important link to an SMZ origin. The presence of CD21 among a complex of B cell proteins, including CD19, Leu-13, TAPA-1, and an IgM BCR, lowers the activation threshold for B cells, which allows B cells to respond to antigens with low affinity for the BCR.³² In agreement with previous reports, these cells did not express AICD; however, CD27⁺B220⁻ memory B cells did express the innate immune receptor TLR9, which recognizes unmethylated bacterial DNA sequences and promotes B cell proliferation and differentiation into Ig-secreting cells (ISCs).^{33–35} Activation through TLR9 results in polyclonal B cell activation and may help sustain serological immunity against various pathogens.³⁶ The high concentration of IgM-CD21 surface complexes and TLR9 expression allows these cells to operate in a T-independent manner. In contrast to these SMZ-like features, B220⁺ memory B cells express less surface IgM, making them less likely to have the lower activation thresholds needed to respond to low-affinity T-independent antigens, and express more of the low-affinity IgE receptor CD23, a B cell receptor that is downregulated with SHM. These cells did express CD86, an essential T cell costimulatory molecule required for SHM and memory B cell maturation.³⁷ B220⁺ memory B cells also expressed AICD, which, as other investigators have demonstrated previously, is restricted to follicular GC B cells and suggests a T-dependent mechanism of transcription and/or activation.^{33,38} Taken together, the CD86 and AICD expression and decreased CD21/IgM expression implicate B220⁺ memory B cells as GC-origin CD27⁺ B cells.

It remains to be determined whether CD27⁺ SMZ B cells actually recirculate into the peripheral blood. CD27⁺ B cells account for up to 40% of all peripheral blood B cells, and of these 50% express IgM. Given the high density of similar cells in the splenic white pulp and the organization of human splenic marginal zones, which differ from both mouse and rat spleen models, it is not unreasonable to assume that recirculation occurs.³⁹ However, this suggests that a reduction in the circulating SMZ B cell population might be reflected in the actual SMZ B cell compartment. All of the HIV-infected individuals in our study were receiving ART at the time blood samples were obtained, and the frequency of CD27⁺ B cells was reduced regardless of CD4⁺ count and whether or not plasma viremia was detectable. These observations correlate with similar observations reported separately by other investigators in which peripheral blood CD27⁺ B cells remained suppressed despite being on HAART.^{1–3} A histological analysis of splenic tissue from deceased HIV-1 infected individuals by Wilkins *et al.* demonstrated that splenic B cell compartments, including the marginal zone, are severely atrophied even early in the course of HIV-1 infection.⁴⁰ Taken together, these observations support the idea that SMZ B cells are closely linked to the SMZ-like peripheral blood B cell pool we define as CD27⁺B220⁻.

The importance of the CD27⁺ SMZ B cell pool is underscored by the increased susceptibility to encapsulated bacteria exhibited by individuals with compromised splenic function. Young children are naturally susceptible: although the SMZ is clearly established by 4 months of age, CD27⁺CD21⁺ B cells do not inhabit the SMZ until after 2 years of age despite the apparent ability to produce CD27⁺ memory B cells as early as 4–6 months of age.^{32,41} HIV-1-infected individuals have an increased susceptibility to pneumococcal infection even in the presence of

increased CD4⁺ T cell counts >200 cells/ μ l and HAART.⁴² Although a direct link has not been established with splenic function, the increased susceptibility to pneumococcus by HIV⁺ patients corresponds to the observations described above. The increased susceptibility to pneumococcal infection by HIV⁺ patients has led other investigators to study the efficacy of polyvalent pneumococcal vaccines in HIV-infected individuals.^{43–45} These studies mostly demonstrated improved ag-specific Ig production in the presence of HAART, while pre-HAART trials showed mixed ag-specific responses.^{46–51}

While the impact of HIV infection on the circulating CD27⁺B220[−] B cell population is obvious, the CD27⁺B220⁺ B cell population is not unscathed. Our conclusion that this population may be GC-related contributes to defects in serological memory and vaccine response observed in both HIV⁺ adults and children. Specifically, Malaspina *et al.* demonstrated that HIV-infected adults produced a weak influenza-specific Ig response to flu vaccination, and Bekker *et al.* demonstrated that pre-HAART HIV⁺ children who received measles–mumps–rubella vaccination did not maintain high viral-specific IgG levels and revaccination with live-attenuated or wild-type virus after HAART-induced immune reconstitution did not lead to sustainable viral-specific IgG.^{7,10} In the case of the flu study, there was a direct correlation between CD4⁺ and plasma viral load at the time of vaccination. However, the MMR study clearly indicates that T-dependent B cell immunity remains damaged despite CD4⁺ T cell reconstitution.

Although we have concluded that CD27⁺B220[−] B cells represent a circulating splenic marginal zone-related B cell population, it should be noted that we observed a discrepancy in this population in both healthy donors and HIV⁺ individuals compared to the circulating SMZ pool in patients with X-HIGM identified by Weller *et al.* The original paper by Bleesing described the circulating CD27⁺ B cells in these patients as CD27⁺B220⁺; however, we characterize these cells as CD27⁺B220[−]. Although the immunopathogenesis of HIV-1 infection and molecular defect of X-HIGM intersect at the CD40–CD40L signaling pathway, which, at first, might suggest a similar SMZ phenotype, it is likely that the life-long X-HIGM molecular defect blocks the direct or indirect signals required to decrease SMZ B cell B220 expression. While CD40–CD40L signaling is clearly disrupted in HIV-1 infection, it is not completely inhibited. This explanation requires that SMZ B cells are T cell dependent early in their development, an idea that fits the description of human SMZ development described by Zandvoort *et al.*³² and Agematsu *et al.*⁴¹

In conclusion, our data supplement previously published reports focused on the reduction of peripheral blood CD27⁺ memory B cells among HIV⁺ individuals. SMZ-like CD27⁺B220[−] B cells may play a vital role in immunity against encapsulated bacteria and therefore should be monitored during attempts at protective immunization with pneumococcal vaccines, and during attempts at humoral immune reconstitution with HAART and other future strategies. We encourage monitoring this population in HIV⁺ individuals in order to determine if it has predictive value toward susceptibility to infection with encapsulated bacteria such as pneumococcus.

ACKNOWLEDGMENTS

We thank Kathleen M. Wyvill and the other clinical staff members of HAMB for their assistance. This research was supported by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.

REFERENCES

1. De Milito A, Morch C, Sonnerborg A, Chiodi F. Loss of memory (CD27) B lymphocytes in HIV-1 infection. *AIDS* 2001;15:957–964. [PubMed: 11399977]

2. Titanji K, Chiodi F, Bellocchio R, et al. Primary HIV-1 infection sets the stage for important B lymphocyte dysfunctions. *AIDS* 2005;19:1947–1955. [PubMed: 16260900]
3. Chong Y, Ikematsu H, Kikuchi K, et al. Selective CD27+ (memory) B cell reduction and characteristic B cell alteration in drug-naïve and HAART-treated HIV type 1-infected patients. *AIDS Res Hum Retroviruses* 2004;20:219–226. [PubMed: 15018710]
4. Titanji K, Nilsson A, Morch C, et al. Low frequency of plasma nerve growth factor detection is associated with death of memory B lymphocytes in HIV-1 infection. *Clin Exp Immunol* 2003;132:297–303. [PubMed: 12699420]
5. Nagase H, Agematsu K, Kitano K, et al. Mechanism of hypergammaglobulinemia by HIV infection: Circulating memory B-cell reduction with plasmacytosis. *Clin Immunol* 2001;100:250–259. [PubMed: 11465955]
6. Moir S, Malaspina A, Pickeral OK, et al. Decreased survival of B cells of HIV-viremic patients mediated by altered expression of receptors of the TNF superfamily. *J Exp Med* 2004;200:587–599.
7. Malaspina A, Moir S, Orsega SM, et al. Compromised B cell responses to influenza vaccination in HIV-infected individuals. *J Infect Dis* 2005;191:1442–1450. [PubMed: 15809902]
8. Titanji K, De Milito A, Cagigi A, et al. Loss of memory B cells impairs maintenance of long-term serologic memory during HIV-1 infection. *Blood* 2006;108:1580–1587. [PubMed: 16645169]
9. De Milito A. B lymphocyte dysfunctions in HIV infection. *Curr HIV Res* 2004;2:11–21. [PubMed: 15053337]
10. Bekker V, Westerlaken G, Scherpbier H, et al. Varicella vaccination in HIV-1-infected children after immune reconstitution. *AIDS* 2006;20:2321–2329. [PubMed: 17117018]
11. Kehr JH, Rieckmann P, Kozlow E, Fauci AS. Lymphokine production by B cells from normal and HIV-infected individuals. *Ann NY Acad Sci* 1992;651:220–227. [PubMed: 1376041]
12. Moir S, Ogwaro KM, Malaspina A, et al. Perturbations in B cell responsiveness to CD4+ T cell help in HIV-infected individuals. *Proc Natl Acad Sci USA* 2003;100:6057–6062. [PubMed: 12730375]
13. Chirmule N, Oyaizu N, Kalyanaraman VS, Pahwa S. Inhibition of normal B-cell function by human immunodeficiency virus envelope glycoprotein, gp120. *Blood* 1992;79:1245–1254. [PubMed: 1346976]
14. Kacani L, Stoiber H, Dierich MP. Role of IL-15 in HIV-1-associated hypergammaglobulinaemia. *Clin Exp Immunol* 1997;108:14–18. [PubMed: 9097905]
15. Kacani L, Sprinzl GM, Erdei A, Dierich MP. Interleukin-15 enhances HIV-1-driven polyclonal B-cell response in vitro. *Exp Clin Immunogenet* 1999;16:162–172. [PubMed: 10394054]
16. Wolthers KC, Otto SA, Lens SM, Van Lier RA, Miedema F, Meyaard L. Functional B cell abnormalities in HIV type 1 infection: Role of CD40L and CD70. *AIDS Res Hum Retroviruses* 1997;13:1023–1029. [PubMed: 9264289]
17. Zhang R, Fichtenbaum CJ, Hildeman DA, Lifson JD, Chougnet C. CD40 ligand dysregulation in HIV infection: HIV glycoprotein 120 inhibits signaling cascades upstream of CD40 ligand transcription. *J Immunol* 2004;172:2678–2686. [PubMed: 14764743]
18. Chougnet C. Role of CD40 ligand dysregulation in HIV-associated dysfunction of antigen-presenting cells. *J Leukoc Biol* 2003;74:702–709. [PubMed: 12960257]
19. Patke CL, Shearer WT. gp120- and TNF- α -induced modulation of human B cell function: Proliferation, cyclic AMP generation, Ig production, and B-cell receptor expression. *J Allergy Clin Immunol* 2000;105:975–982. [PubMed: 10808179]
20. Malaspina A, Moir S, Ho J, et al. Appearance of immature/transitional B cells in HIV-infected individuals with advanced disease: Correlation with increased IL-7. *Proc Natl Acad Sci USA* 2006;103:2262–2267. [PubMed: 16461915]
21. Rodriguez B, Valdez H, Freimuth W, Butler T, Asaad R, Lederman MM. Plasma levels of B-lymphocyte stimulator increase with HIV disease progression. *AIDS* 2003;17:1983–1985. [PubMed: 12960832]
22. Rieckmann P, Poli G, Fox CH, Kehr JH, Fauci AS. Recombinant gp120 specifically enhances tumor necrosis factor- α production and Ig secretion in B lymphocytes from HIV-infected individuals but not from seronegative donors. *J Immunol* 1991;147:2922–2927. [PubMed: 1918999]

23. Bleesing JJ, Fleisher TA. Human B cells express a CD45 isoform that is similar to murine B220 and is downregulated with acquisition of the memory B-cell marker CD27. *Cytometry* 2003;51B:1–8. [PubMed: 12500291]
24. Rodig SJ, Shahsafaei A, Li B, Dorfman DM. The CD45 isoform B220 identifies select subsets of human B cells and B-cell lymphoproliferative disorders. *Hum Pathol* 2005;36:51–57. [PubMed: 15712182]
25. Aruffo A, Farrington M, Hollenbaugh D, et al. The CD40 ligand, gp39, is defective in activated T cells from patients with X-linked hyper-IgM syndrome. *Cell* 1993;72:291–300. [PubMed: 7678782]
26. Allen RC, Armitage RJ, Conley ME, et al. CD40 ligand gene defects responsible for X-linked hyper-IgM syndrome. *Science* 1993;259:990–993. [PubMed: 7679801]
27. Facchetti F, Appiani C, Salvi L, Levy J, Notarangelo LD. Immunohistologic analysis of ineffective CD40-CD40 ligand interaction in lymphoid tissues from patients with X-linked immunodeficiency with hyper-IgM. Abortive germinal center cell reaction and severe depletion of follicular dendritic cells. *J Immunol* 1995;154:6624–6633. [PubMed: 7539026]
28. Agematsu K, Nagumo H, Shinozaki K, et al. Absence of IgD-CD27(+) memory B cell population in X-linked hyper-IgM syndrome. *J Clin Invest* 1998;102:853–860. [PubMed: 9710455]
29. Qiao X, He B, Chiu A, Knowles DM, Chadburn A, Cerutti A. Human immunodeficiency virus 1 Nef suppresses CD40-dependent immunoglobulin class switching in bystander B cells. *Nat Immunol* 2006;7:302–310. [PubMed: 16429138]
30. Chong Y, Ikematsu H, Yamamoto M, et al. Increased frequency of CD27-(naive) B cells and their phenotypic alteration in HIV type 1-infected patients. *AIDS Res Hum Retroviruses* 2004;20:621–629. [PubMed: 15242538]
31. Malaspina A, Moir S, Kottlil S, et al. Deleterious effect of HIV-1 plasma viremia on B cell costimulatory function. *J Immunol* 2003;170:5965–5972. [PubMed: 12794123]
32. Zandvoort A, Lodewijk ME, de Boer NK, Dammers PM, Kroese FG, Timens W. CD27 expression in the human splenic marginal zone: The infant marginal zone is populated by naive B cells. *Tissue Antigens* 2001;58:234–242. [PubMed: 11782274]
33. Willenbrock K, Jungnickel B, Hansmann ML, Kuppers R. Human splenic marginal zone B cells lack expression of activation-induced cytidine deaminase. *Eur J Immunol* 2005;35:3002–3007. [PubMed: 16180254]
34. He B, Qiao X, Cerutti A. CpG DNA induces IgG class switch DNA recombination by activating human B cells through an innate pathway that requires TLR9 and cooperates with IL-10. *J Immunol* 2004;173:4479–4491. [PubMed: 15383579]
35. Bernasconi NL, Onai N, Lanzavecchia A. A role for Toll-like receptors in acquired immunity: Up-regulation of TLR9 by BCR triggering in naive B cells and constitutive expression in memory B cells. *Blood* 2003;101:4500–4504. [PubMed: 12560217]
36. Bernasconi N, Traggiai A, Lanzavecchia A. Maintenance of serological memory by polyclonal activation of human memory B cells. *Science* 2002;298:2199–2202. [PubMed: 12481138]
37. Han S, Hathcock K, Zheng B, Kepler T, Hodes R, Kelsoe G. Cellular interaction in germinal centers: Roles of CD40 ligand and B7-2 in established germinal centers. *J Immunol* 1995;155:556–567. [PubMed: 7541819]
38. Cattoretto G, Buttner M, Shaknovich R, Kremmer E, Alobeid B, Niedobitek G. Nuclear and cytoplasmic AID in extrafollicular and germinal center B cells. *Blood* 2006;107:3967–3975. [PubMed: 16439679]
39. Steiniger B, Timphus EM, Jacob R, Barth PJ. CD27+ B cells in human lymphatic organs: Re-evaluating the splenic marginal zone. *Immunology* 2005;116:429–442. [PubMed: 16313357]
40. Wilkins BS, Davis Z, Lucas SB, Delsol G, Jones DB. Splenic marginal zone atrophy and progressive CD8+ T-cell lymphocytosis in HIV infection: A study of adult post-mortem spleens from Cote d'Ivoire. *Histopathology* 2003;42:173–185. [PubMed: 12558750]
41. Agematsu K, Nagumo H, Yang FC, et al. B cell subpopulations separated by CD27 and crucial collaboration of CD27+ B cells and helper T cells in immunoglobulin production. *Eur J Immunol* 1997;27:2073–2079. [PubMed: 9295047]

42. Jordano Q, Falco V, Almirante B, et al. Invasive pneumococcal disease in patients infected with HIV: Still a threat in the era of highly active antiretroviral therapy. *Clin Infect Dis* 2004;38:1623–1628. [PubMed: 15156452]
43. Falco V, Jordano Q, Cruz MJ, et al. Serological response to pneumococcal vaccination in HAART-treated HIV-infected patients: One year follow-up study. *Vaccine* 2006;24:2567–2574. [PubMed: 16423429]
44. Tangsinmankong N, Kamchaisatian W, Day NK, Sleasman JW, Emmanuel PJ. Immunogenicity of 23-valent pneumococcal polysaccharide vaccine in children with human immunodeficiency virus undergoing highly active antiretroviral therapy. *Ann Allergy Asthma Immunol* 2004;92:558–564. [PubMed: 15191025]
45. Rodriguez-Barradas MC, Alexandraki I, Nazir T, et al. Response of human immunodeficiency virus-infected patients receiving highly active antiretroviral therapy to vaccination with 23-valent pneumococcal polysaccharide vaccine. *Clin Infect Dis* 2003;37:438–447. [PubMed: 12884170]
46. Ballet JJ, Sulcebe G, Couderc LJ, et al. Impaired anti-pneumococcal antibody response in patients with AIDS-related persistent generalized lymphadenopathy. *Clin Exp Immunol* 1987;68:479–487. [PubMed: 3652522]
47. Janoff EN, Fasching C, Ojoo JC, O'Brien J, Gilks CF. Responsiveness of human immunodeficiency virus type 1-infected Kenyan women with or without prior pneumococcal disease to pneumococcal vaccine. *J Infect Dis* 1997;175:975–978. [PubMed: 9086163]
48. Klein RS, Selwyn PA, Maude D, Pollard C, Freeman K, Schiffman G. Response to pneumococcal vaccine among asymptomatic heterosexual partners of persons with AIDS and intravenous drug users infected with human immunodeficiency virus. *J Infect Dis* 1989;160:826–831. [PubMed: 2572650]
49. Pakker NG, Kroon ED, Roos MT, et al. Immune restoration does not invariably occur following long-term HIV-1 suppression during antiretroviral therapy. INCAS Study Group. *AIDS* 1999;13:203–212. [PubMed: 10202826]
50. Loeliger AE, Rijkers GT, Aerts P, et al. Deficient antipneumococcal polysaccharide responses in HIV-seropositive patients. *FEMS Immunol Med Microbiol* 1995;12:33–41. [PubMed: 8580899]
51. Unsworth DJ, Rowen D, Carne C, Sonnex C, Baglin T, Brown DL. Defective IgG2 response to Pneumovax in HIV seropositive patients. *Genitourin Med* 1993;69:373–376. [PubMed: 8244356]

FIG. 1.

Peripheral blood CD27⁺B220⁻ B cells are preferentially reduced in HIV-1-infected individuals. (A) CD19⁺ B cells in whole blood samples were analyzed for CD27 expression (gray: a representative healthy control; black line: an HIV⁺ individual). (B) CD19⁺ B cells were analyzed for CD27 and B220 expression. Numbers in (A) and (B) represent mean data (frequency of gated B cells) of HDs ($n = 22$) and HIV⁺ ($n = 40$). (C) The frequency of total B cells, CD27⁺ memory B cells, and CD27⁺ memory B cell subsets among circulating lymphocytes for HDs and HIV⁺, represented as box plots.

FIG. 2.

The reduction of circulating CD27⁺ B cells and its subsets does not correlate with plasma viremia or absolute T cell count. Whole blood CD27⁺, CD27⁺B220⁺, and CD27⁺B220⁻ B cell populations from HDs were compared to HIV⁺ samples separated on the basis of plasma viremia (>50 copies RNA/ml, $n = 14$) or aviremia (<50 copies RNA/ml, $n = 22$), and absolute CD4⁺ T cell counts (>200 CD4⁺ T cells/ μ l, $n = 21$, and <200 CD4⁺ T cells/ μ l, 15). One-way ANOVA and Dunn's multiple comparison test were used to compare the different groups.

FIG. 3.

Surface antigen and Ig expression varies among naive B cells and memory B cell subsets. CD27⁻B220⁺ (red line), CD27⁺B220⁺ (blue line), and CD27⁺B220⁻ (green line) CD19⁺ B cells in PBMCs isolated from HDs were analyzed using flow cytometry for various markers. The following antibody panels were used: CD27-FITC/CD19-PECy7/B220-PECy5.5 together with IgM-APC, IgD-PE, CD21-APC, CD23-PE, CD40-APC, CD95-PE, CD86-APC, CD80-PE, and IgG-PE. Histogram overlays display data from one representative experiment (CD21, CD23, CD40, CD80, CD86, IgD, IgM: $n = 16$; CD95: $n = 11$; IgG: $n = 13$).

FIG. 4.

Expression of memory B cell-associated genes by memory B cell subsets. (A) Cells were immunomagnetically enriched by negative selection from PBMCs obtained from healthy donors. CD27⁻B220⁺ (green), CD27⁺B220⁺ (blue), and CD27⁺B220⁻ (red) B cell subsets were separated after labeling with CD19, CD27, and B220 (see Materials and Methods) using a fluorescence-activated cell sorter to >95%, >92%, and >95%, respectively. Each fraction was analyzed for the expression of TLR9 (B) and AICD (C) mRNA and the values were normalized to the expression of β-actin.

FIG. 5.

The pattern of CD27 and B220 expression by proliferating B cells is signal-pathway specific. CFSE-labeled PBMCs (1×10^6 cells/ml) from healthy donors were stimulated with either CpG2006 (3 g/ml) or SAC (1:10,000 dilution) + IL-2 (50 U/ml), or nonstimulated. After 5 days of culture, the cells were stained with CD19-PECy7, CD27-PE, and B220-PECy5.5 and viable (DAPI⁺) CD19⁺ B cells were analyzed for CD27 and B220 expression (A), and CD27⁻B220⁺, CD27⁺B220⁺, and CD27⁺B220⁻ populations were analyzed for proliferation as indicated by CFSE fluorescence (B). Numbers in dot plots represent the proportion (%) of gated B cells and numbers in histograms represent the frequency (%) of proliferating cells. A representative experiment of 10 different healthy donors is shown. (C) Average proliferative

responses and proliferation indices of 10 determinations for B220⁺ and B220⁻ memory B cells in response to either ODN or SAC.

FIG. 6.

B cell differentiation to an Ig-secreting cell is signal pathway dependent. PBMCs (1×10^6 cells/ml) from healthy donors were stimulated with either CpG2006 (3 μ g/ml) or SAC (1:10,000 dilution) + IL-2 (50 U/ml), or left nonstimulated for 5 days. **(A)** CD19⁺ B cells were analyzed for IgM and intracellular IgG by flow cytometry. Numbers in plots represent the frequency of gated B cells. Note the differences in IgG staining intensity. PBMC culture supernatants were analyzed for secreted IgM **(B)** and IgG **(C)** by ELISA (cumulative data for 10 different samples).

TABLE 1
B Cell-Associated Marker Expression by Circulating CD27/B220-Defined B Cell Subsets from Healthy Controls^a

	CD21	CD23	CD40	CD80	CD86	CD95	IgD	IgM	IgG
CD19 ⁺	96.0	69.2	94.3	8.3	2.3	7.8	84.6	68.5	9.0
CD27 [−] B220 ⁺	98.2	85.9	95.8	1.4	0.6	1.0	97.6	75.6	1.6
CD27 ⁺ B220 ⁺	84.2	38.3	90.9	23.8	14.5	27.0	57.4	49.0	20.1
CD27 ⁺ B220 [−]	94.1	16.8	92.4	21.4	2.5	21.6	50.3	48.9	25.0

^aResults represent the mean expression (%) from multiple HC analyzed with 5-color flow cytometry. CD21, CD23, CD40, CD80, CD86, IgD, IgM: *n* = 16. CD95, *n* = 11. IgG, *n* = 13. B cells were identified by CD19 expression.