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Bone Marrow Plasma Cells Are a Primary Source of Serum HIV-1–Specific Antibodies in Chronically Infected Individuals

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Several potent and broadly neutralizing Abs to HIV-1 have been isolated recently from peripheral blood B cells of infected individuals, based on prescreening of Ab activity in the serum. However, little is known regarding the cells that make the Abs that circulate in the blood. Accordingly, we investigated the most likely source, the bone marrow, of chronically HIV-1–infected individuals who were not receiving antiretroviral therapy. Increased frequencies of plasma cells, as well as B cell precursors, namely preB-I and preB-II, and decreased frequencies of mature B cells were observed in bone marrow aspirates of these individuals compared with HIV-negative counterparts. Increased frequencies of bone marrow plasma cells are consistent with known hallmarks of HIV-1 infection, namely hypergammaglobulinemia and increased frequencies of peripheral blood plasmablasts. Levels of HIV-1 envelope (Env)-binding and HIV-1–neutralizing Abs were measured in serum, and corresponding frequencies of Ab-secreting or Env-binding cells were measured in the blood (plasmablasts and memory B cells) and in the bone marrow (plasma cells). A strong correlation was observed between serum HIV-1–specific Abs and Env-specific bone marrow–derived plasma cells, but not circulating plasmablasts or memory B cells. These findings demonstrate that, despite HIV-1–induced phenotypic and functional B cell dysregulation in the peripheral blood and secondary lymphoid tissues, bone marrow plasma cells remain a primary source for circulating HIV-1–specific Abs in HIV-1–infected individuals.   *The Journal of Immunology*, 2015, 194: 2561–2568.

D espite the effectiveness and scale-up of antiretroviral therapy (ART) in the treatment of HIV-1 infection, development of an Ab-based HIV-1 vaccine is a critical element in strategies to end this pandemic (1). Such an endeavor has remained an elusive goal for over two decades, largely due to the inadequacy of the natural immune response to HIV-1 infection and difficulty in establishing a correlate of immunity upon which to model a vaccine. However, over the past 5 y, there has been a rapid succession of advances in the isolation of broadly neutralizing Abs (bnAbs) from memory B cells in the peripheral blood of HIV-1–infected individuals (2–6). These bnAbs target a variety of different epitopes within HIV-1 envelope (Env) proteins gp120 and gp41, described as sites of vulnerability of the virus, and have been derived by a number of different methods (7, 8). However, most methods begin with the same approach, that of screening serum for the presence of HIV-1–specific bnAbs, which arise in ~10–25% of individuals after several months to years of infection (8). These approaches are premised on an assumption that has not been widely validated, with only two known examples (3), that HIV-1–specific circulating memory B cells from which bnAbs are cloned are closely related to the Abs in the serum from which neutralization screens are performed. There is also evidence for recapitulation of serum neutralization breadth by a small number of Abs derived from memory B cells (4, 9), although the individuals in these studies were selected on the basis of potent HIV-1–neutralizing activity in their serum. It remains unclear whether this phenomenon applies to the vast majority of individuals whose serum does not show potent HIV–neutralizing capacity. Other studies have described large numbers of specificities, either from B cell clones or in serum of each individual (10, 11), although, in these cases, the link between cellular and serologic sources of Abs was not investigated. However, another study reported discordance between HIV-1 Env-specific memory B cell responses and circulating Abs in infected individuals who naturally control viremia (12).

Abs are produced by B cells that have undergone partial differentiation, referred to as plasmablasts (PBs), or have completed

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Abbreviations used in this article: aMLV, amphotropic murine leukemia virus; ART, antiretroviral therapy; ASC, Ab-secreting cell; BMMC, bone marrow mononuclear cell; bnAb, broadly neutralizing Ab; BV, Brilliant Violet; Env, HIV-1 envelope; I/T, immature/transitional; PB, plasmablast; PC, plasma cell.

the differentiation process, and are referred to as plasma cells (PCs). Several other features distinguish these two populations of Ab-secreting cells (ASCs). Both populations in humans express high levels of CD27 and CD38 while having lost expression of CD20; PBs have recently cycled (Ki-67^{+}) and maintain expression of CD19 more than do PCs, whereas PCs express CD138, a marker of differentiation rarely observed on PBs (13, 14). PBs arise during the early stages of an immune response in secondary lymphoid tissues and can circulate between tissues and into the peripheral blood (14–16). PBs may arise directly from naive B cells in extrafollicular sites following antigenic stimulation; however, they can accumulate relatively high levels of somatic hypermutation, as has been shown in acute HIV-1 infection (17), a process more consistent with having undergone affinity maturation in germinal centers. Furthermore, those PBs, which were directed against gp41 of the HIV-1 Env, most likely arose from pre-existing memory B cells (17), suggesting there may exist multiple routes of B cell differentiation, and not necessarily linear relationships between naive and memory B cells, as well as PBs and PCs.

In contrast to the high turnover/short-lived properties of PBs, PCs are by definition long-lived and sessile, residing primarily in the bone marrow, and to a lesser and possibly more short-lived extent, in secondary lymphoid tissues such as spleen, lymph nodes, tonsils, and mucosal-associated lymphoid tissues (15, 16). The processes that dictate the survival of PCs and their homing to the bone marrow are not completely understood, although CXCR4 and its ligands are essential for homing and survival is maintained by a "bone marrow niche" comprised of reticular cells, stromal cells, fibroblasts, eosinophils, and macrophages and the factors they secrete, including IL-6, IL-5, APRIL, BAFF, and TNF (16, 18).

Relatively little is known regarding the cellular/tissue origin of HIV-specific Abs present in serum of infected or even vaccinated individuals, although, as with other pathogens and immunogens, the bone marrow PCs are thought to be a major source (3, 15, 16). In addition, there are several unique factors to consider in HIV-1 infection, and, in particular, in the setting of chronic viremia in which hypergammaglobulinemia and increased frequencies of circulating PBs are observed, although only a relatively small fraction of PBs is HIV-1 specific (19). Given the uncertainty of the origin of Abs that circulate in the blood, together with their importance in the screening process in the generation of HIV-neutralizing Abs from B cells, we investigated HIV-1–specific B cells in the peripheral blood and bone marrow of chronically HIV-1–infected individuals. We found an increased frequency of PCs in the bone marrow from HIV-1–infected individuals compared with HIV-negative counterparts and, more importantly, a strong correlation between HIV-1–specific circulating Abs and HIV-1 Env-specific PCs in the bone marrow, but no correlation with circulating PBs or memory B cells. Our results demonstrate that, despite the strong evidence of B cell hyperactivity in the blood and secondary lymphoid tissues in HIV-1–infected individuals, the bone marrow remains the major source of HIV-1–specific Abs in the blood.

Materials and Methods
Study participants

Bone marrow aspirates and core biopsies were obtained from HIV-1–infected individuals after providing informed consent and in accordance with the Institutional Review Board of the National Institute of Allergy and Infectious Diseases, National Institutes of Health, and the Declaration of Helsinki. Eight individuals were recruited, as detailed in Table I. HIV-1–infected participants were not receiving ART at time of study, and all were classified as chronically infected. Specimens from HIV-negative individuals were obtained from the Department of Laboratory Medicine, Clinical Research Center, National Institutes of Health. Bone marrow biopsies and

aspirates were obtained from posterior iliac crests under conscious sedation and local anesthesia. In addition, cryopreserved bone marrow mononuclear cells (BMMCs) were obtained from commercially available sources (Allcells or StemCell Technologies).

Morphologic analyses

Sections from decalcified formalin-fixed paraffin-embedded core bone marrow biopsies were stained with CD138 Ab using an autostaining system (Ventana). Immunohistochemistry stains were digitally scanned and analyzed to determine the percentage of CD138^{+} cells in the cellular bone marrow.

Processing of bone marrow aspirates and peripheral blood

Three serial bone marrow aspirate pulls were obtained from each participant. Quality assessment of each pull was performed by establishing their hemodilution, based on the presence of mature neutrophils (CD13^{+}/CD16^{+}/CD45^{+}), which should be <30% within the myeloid cell gate, as previously described (20). BMMCs from each bone marrow aspirate pull and PBMCs from blood draws or leukapheresis were obtained by Ficoll-Hypaque density gradient centrifugation. BMMCs corresponding to the pull with the lowest hemodilution were used for further processing and analyses. CD38^{high}/CD138^{+} PCs were sorted from BMMCs using a FACSAria cell sorter (BD Biosciences), with a median purity of 95%. For functional and certain phenotyping analyses, mature (CD10^{−}) B cells were enriched to ~95% purity from PBMCs by negative selection (StemCell Technologies), as described previously (21).

Phenotypic analyses

Neutrophil staining for analysis of hemodilution was performed with the following mAbs: CD13-PE, CD16-Brilliant Violet (BV) 510, and CD45-FITC from BioLegend. Freshly processed BMMCs were used for phenotypic analyses. Cryopreserved BMMCs were also included in the HIV-negative group after verifying that there were minimal differences between fresh and thawed samples. Cryopreserved PBMCs were used to measure the frequency of PBs among HIV-1–infected individuals and the frequency of gp140-specific memory B cells. The following mAbs were used for cell surface staining: CD3-BV510, CD19-PE-Cy7, CD27-PerCP-Cy5.5, and CD38-BV421 from BioLegend; CD10-allophycocyanin, CD20-allophycocyanin-H7, and IgG-PE-Cy7 from BD Biosciences; and CD138-PE from eBioscience. For Ig isotype staining of PBs and PCs, cells were permeabilized for intracellular staining (Permeabilizing Solution 2; BD Biosciences), and the following mAbs were used: IgM-BV510 and IgG-allophycocyanin from BD Biosciences, and IgA-FITC from Dako. Frequencies of HIV-1 Env-specific memory B cells were measured with a trimeric HIV-1 Env gp140 probe (YU2-gp140-F), as previously described (22). In brief, the probe containing an Avitag biotinylation motif at the C termini was generated, as previously described (23), and fluorescently labeled with streptavidin-allophycocyanin (Invitrogen). Flow cytometry was performed on a FACSCanto flow cytometer (BD Biosciences), with data analyses on FlowJo Version 9.6 software (Tree Star).

Functional analyses

Frequencies of PBs and PCs secreting IgG, IgA, and IgM, as well as those specific for gp140, were measured by ELISPOT, as previously described (24). Briefly, Immobilon-P polyvinylidene difluoride membrane plates (MAIPSWU10; Millipore) were coated with 5 µg/ml anti-Ig L chain Abs (Rockland Immunochemicals), followed by addition of sorted PCs, incubation overnight, and detection with biotinylated Abs against each of the Ig classes or biotinylated YU2-gp140-F. Unlabeled or biotinylated keyhole limpet hemocyanin served as negative control Ag.

ELISA

Maxisorp plates (Nunc) were coated with 2.5 µg/ml goat anti-human IgG (H+L; Jackson ImmunoResearch Laboratories) in PBS overnight at 4°C. Serum samples from study participants and a HIV-negative control individual were diluted 5-fold, starting from a 1:50 dilution in PBS–0.02% azide, mixed 1:1 with blocking buffer (PBS containing 2% skim milk and 5% FBS), and added to the plate. Consecutive incubations of 1 h interspaced with vigorous washing (PBS–0.02% Tween 20) were as follows: serum sample mix at room temperature; biotinylated YU2-gp140-F (2.5 µg/ml) at 37°C; and streptavidin-HRP (Sigma-Aldrich; 1/1000) at room temperature. Plates were developed 5 min with tetramethylbenzidine peroxidase substrate (Bio-Rad), stopped with an equal volume of 3% HCl, and the OD was measured at 450 nm. An OD of 2 SDs above that of HIV-negative serum was considered positive, and OD values at 1/50 dilution of end-point dilution curves (Supplemental Fig. 1) were used in data analyses.

Serum neutralizing activity

Serum samples were tested for HIV-1–neutralizing activity using a pseudovirus assay with a tier 2 panel developed and performed by Monogram Biosciences (25). The panel consisted of viruses pseudotyped with HIV-1 Env from strains 92BR020, MGRM-C-026, 92TH021, 93IN905, 94UG103, and JRCSF. A tier 1 pseudovirus, NL4/3, was also included, as well as a negative control, virus pseudotyped with the envelope of amphotropic murine leukemia virus (aMLV). Neutralization titers were reported as the reciprocal serum dilution that inhibited virus infection by 50% (IC_{50}).

Statistical analyses

Statistical analyses were performed using Prism software (version 6.0 for Mac). Two-group comparisons were performed using the Mann–Whitney *U* test (Figs. 2–5). The Spearman rank method was used to test for correlation (Fig. 6). A *p* value ≤0.05 was considered significant.

Results

B cell subsets in the bone marrow

In humans, the bone marrow is the primary site of B cell development as well as the major repository of long-lived PCs. Given the paucity of current studies on bone marrow B cells in HIV-1 infection, we first sought to analyze all B cell subsets among aspirate BMMCs of study participants (Table I). All participants had detectable HIV-1 viremia and were not receiving ART at time of study. Of note, six of the participants were therapy naive, whereas two had discontinued ART several years prior to participation (Table I). A panel of markers was chosen, based on our preliminary analyses (data not shown) and published studies (26–29), to highlight the different stages of B cell development in the bone marrow (Fig. 1A). The earliest B cells to express CD19 are PreB-I, followed by PreB-II, immature/transitional (I/T), and mature B cells. With CD19 as the pan B cell marker, subsets were defined as follows: PreB-I cells, CD10^{high}/CD45^{low}/CD20$^-$/CD38^{high}; preB-II, CD10^{int}/CD45^{int}/CD20$^-$/CD38^{int}; I/T B cells, CD10^{low}/CD45^{high}/CD20$^+$/CD38^{low}; mature B cells, CD10$^-$/CD45^{high}/CD20$^+$/CD38^{low}; and PCs, CD10$^-$/CD45^{low}/CD20$^-$/CD138$^+$/CD38^{high}. As shown for the representative plots in Fig. 1B, all five B cell subsets were clearly delineated with distinct patterns of expression for CD10, CD20, CD38, and CD45. Of note, the profile for CD10 by CD20 was similar to the one that is commonly observed for B cell subsets in the human bone marrow (Fig. 1C) (27). The PCs in the bone marrow were also distinguished by the expression of CD38 and CD138 (Fig. 1B). In group analyses, the frequencies of PCs and PreB-I and PreB-II cells, as a percentage of total B cells, were significantly increased in the bone marrow of HIV-1–infected compared with HIV-negative individuals (Fig. 2A). The frequency of mature B cells was significantly decreased in HIV-1–infected compared with HIV-negative individuals (Fig. 2A), and, based on available subanalyses, this difference

was due to lower frequencies of CD27$^+$ memory B cells (data not shown). There was no significant difference in the frequency of I/T B cells between the two groups (Fig. 2A). Finally, we assessed the Ig isotype distribution among the PCs in the bone marrow with a staining strategy that included cell permeabilization, a necessary step because Igs produced by PCs are predominantly intracellular with little or no expression on the cell surface. As shown in Fig. 2B, the majority of bone marrow PCs of both HIV-1–infected and HIV-negative individuals expressed IgG, fewer expressed IgA, and only a fraction expressed IgM. There was no significant difference in the Ig isotype distribution in bone marrow PCs between the two groups. Collectively, these findings reveal that there exist differences in the frequencies of B cells at early and later stages of development and differentiation in the bone marrow of HIV-1–infected individuals compared with uninfected individuals.

Morphologic analyses of bone marrow core biopsies

Bone marrow core biopsies from eight HIV-1–infected and four HIV-negative individuals were available for hematopathologic review and analysis. Evidence of progressive trilineage (erythroid, myeloid, and lymphoid) hematopoiesis indicative of adequate maturation was observed in all specimens. Enumeration of PCs, based on morphology and expression of CD138, was performed on core biopsies by immunohistochemistry combined with a digital quantification method. As shown in Fig. 3, the frequency of PCs was significantly increased in the core biopsies of HIV-1–infected (median of 6.2%) compared with HIV-negative (median of 2.2%) individuals. These findings confirmed the increased frequency of PCs observed by flow cytometry in the bone marrow aspirates of HIV-1–infected individuals.

Frequencies of HIV-1–specific PCs in the bone marrow of infected individuals

Analyses relating to HIV-1–specific PC responses in the bone marrow of HIV-1–infected individuals have been conducted in a limited number of studies and study subjects (3, 17, 30). To gain a better understanding of the contribution of the bone marrow to the Ab response against HIV-1, we measured frequencies of HIV-1 Env-specific (hereafter referred to as Env-specific) bone marrow PCs using an ELISPOT assay that was previously described for similar measurements among PBs in the peripheral blood (19). PCs were sorted from BMMCs based on the expression of CD19, CD138, and CD38 (Fig. 1B), and both total and Env-specific frequencies of ASCs were measured. The distribution of Ig isotype among total ASCs was similar to that determined by flow cytometry on unfractionated BMMCs (Fig. 2B), in that IgG represented the major isotype, followed by IgA and, lastly, IgM (Fig. 4). One caveat to the ASC analysis was that we expected our isotype analysis to account for 100% of sorted cells given that they

Table I. Demographic and immunologic profile of HIV-1–infected individuals

Patient No.	Age (y)	Gender	Ethnicity	Estimated[a] Time of Infection at Biopsy (mo)	Plasma HIV-1 RNA at Time of Biopsy[b] (Copies/ml)	CD4 Count at Time of Biopsy (Cells/µl)	Serum gp140-Binding ELISA (OD)
1	26	Male	Hispanic	14	1,118	777	0.428
2	34	Male	Hispanic	16–28	14,200	460	0.816
3	31	Male	Black	>120	164	714	2.461
4	26	Female	Black	19	2,549	479	0.95
5	44	Male	White	>96	3,739	403	2.593
6	29	Female	Hispanic	>101	1,562	434	2.091
7	28	Male	Hispanic	27	20,964	397	3.189
8	23	Male	Hispanic	23	19,156	875	2.989

[a]Estimated from clinical history taken at screening visit.
[b]All were ART naive, except patients 5 and 6 discontinued ART 35 and 75 mo prior to study, respectively.

FIGURE 1. Phenotypic characterization of B cells in the bone marrow. (**A**) Combination of cell surface markers used to identify B cell developmental stages and PCs in the bone marrow. Intensity of color indicates level of expression. (**B**) Phenotypic and subset analyses of B cells in the bone marrow of an HIV-1–infected individual, identified based on their expression of CD45 and CD10, CD20 and CD10, and CD38. In the *upper panels*, CD19+CD3− cells were gated from all live cells and PCs were identified from CD19+ cells based on the expression profile CD38high/CD138+. (**C**) Cartoon of B cell subsets in bone marrow reflecting the expected B cell pattern delineated by their expression of CD10 and CD20.
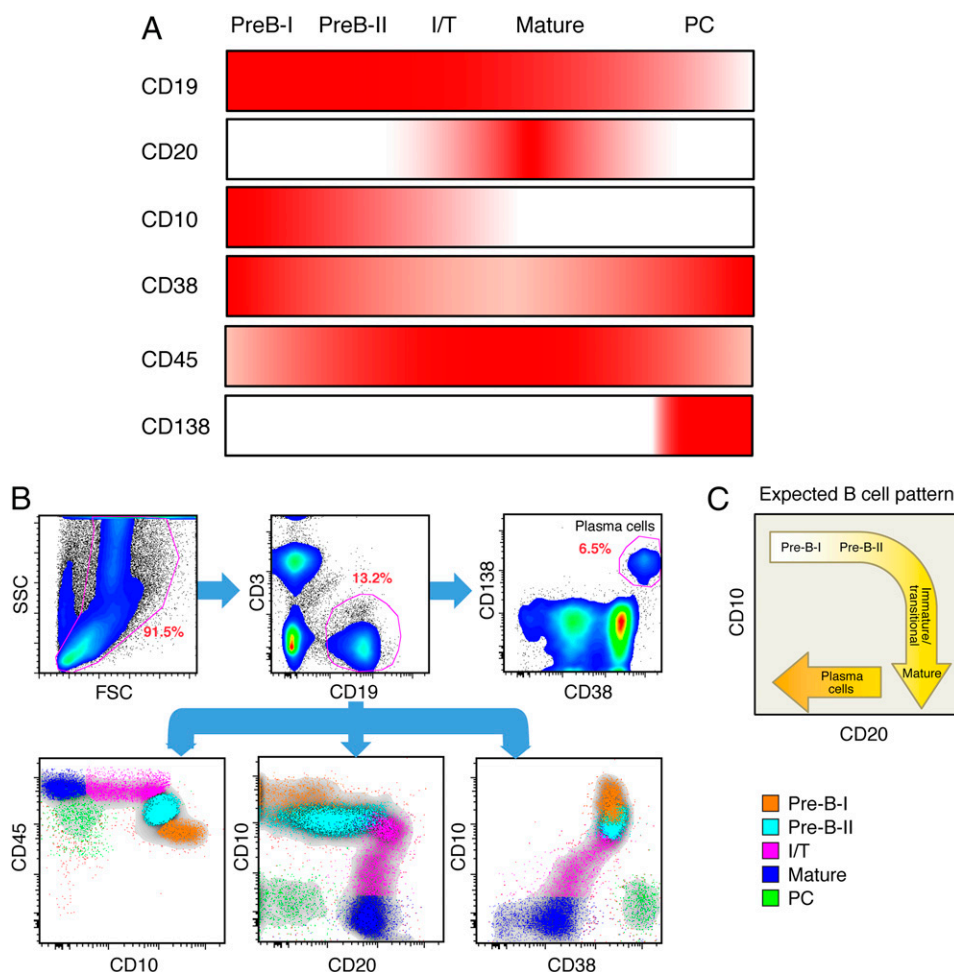
were all PCs. We do not have an explanation for the variations observed among individuals relative to total ASCs given that purity of sorted cells was similar, although the lower frequencies were observed in individuals with the lowest percentage of PCs in the aspirate and loss of viability associated with a longer sorting period may have been a factor. Nonetheless, the sorting step was deemed essential to accurately measure the relatively low frequencies of Env-specific ASCs. In this regard, the frequencies of Env-specific BMMC-derived PCs ranged from 0.1 to 1.3% (Fig. 4). Although this may be considered a low frequency, the

median of 0.7% for this group is nonetheless ~10-fold higher than frequencies of tetanus toxoid–specific PCs reported following immunization with tetanus toxoid (31). Of note, the approach taken to measure the Env-specific ASC frequencies does not provide information on Env specificity per Ig isotype, and obtaining such information would be technically difficult and require twice or more the number of cells used in the current assay. Collectively, these data demonstrate that ELISPOT is an assay that can provide information on the Ig isotype distribution of BMMC-sorted PCs that is comparable to that observed by flow cytometry,
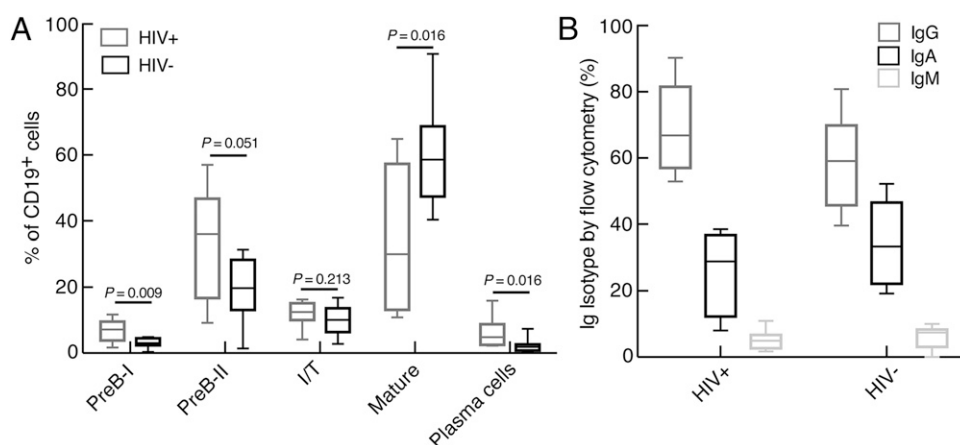
FIGURE 2. Comparison of B cell subsets in the bone marrow of HIV-1–infected and HIV-negative individuals. (**A**) Frequencies of B cell subsets were measured, as defined by markers shown in Fig. 1. (**B**) Distribution of Ig isotypes among bone marrow PCs was determined following permeabilization of cells. Median (horizontal bar) and scatter boxes shown represent analyses of eight HIV-1–infected and eight HIV-negative individuals.
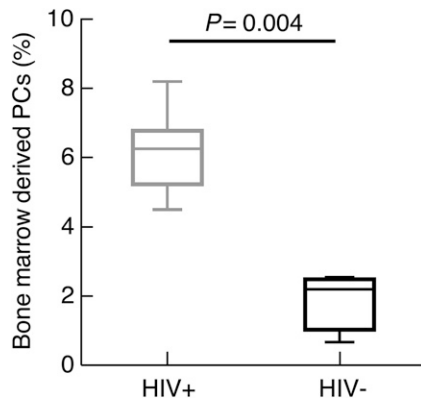
FIGURE 3. Frequencies of bone marrow core biopsy-derived PCs calculated from immunohistochemistry. Frequencies were determined by immunohistochemistry following staining with CD138 and quantification by digital analysis. Median (horizontal bar) and scatter boxes shown represent analyses of eight HIV-1–infected and four HIV-negative individuals.

and, furthermore, this approach provides a means for measuring HIV-1–specific responses among these PCs.

Frequencies of HIV-1–specific PBs and memory B cells in the peripheral blood

We previously evaluated Env-specific PBs and memory B cells in the peripheral blood of large cohorts of HIV-1–infected individuals (19, 22). For the purpose of comparisons with the bone marrow compartment, we extended these analyses to the current study. Mature (CD10$^-$) B cells were isolated from PBMCs in seven of the eight individuals listed in Table I on whom there were stored cells available within 6 mo of the bone marrow biopsy. Frequencies of total Ig and Env-specific PB ASCs were measured among mature B cells by ELISPOT (Fig. 5), as performed on the BMMC PCs, although without prior enrichment by cell sorting. Similar to bone marrow PCs, IgG was the main isotype contributor to the total ASCs, followed by IgA and then IgM (Fig. 5). When calculated as a percentage of total Ig ASCs, the frequencies of Env-specific PBs ranged from 0.2 to 1.2% (data not shown), with a median of 0.6% that was similar to that previously reported (19).

Whereas ELISPOT remains the assay of choice for evaluating Ag-specific responses among PBs and PCs, HIV-1 specificity among memory B cells can be assessed by flow cytometry with HIV-1 Env probes because these cells express high levels of surface Ig (22, 32, 33). Of note, the same probe was used for both ELISPOT and flow cytometry, namely an Avitag-based biotinylated form of trimerized YU2 gp140 that was used directly for detection of Env-specific ASCs, as well as a fluorochrome-conjugated form that was used for evaluation of Env-specific memory B cells. As shown in Fig. 5, the frequencies of Env-specific memory B cells among PBMCs ranged from 0.1 to 1.46% of total IgG-expressing B cells, with a median of 0.8%, which was similar to that described previously for a similar group of HIV-1–infected individuals (22). Collectively, the frequencies of Env-specific memory B cells and PBs in the peripheral blood of individuals who underwent a bone marrow biopsy were comparable to previous studies and thus representative of larger similar cohorts.

Contribution of PCs, PBs, and memory B cells to the HIV-1–specific B cell immunity

Finally, we wished to evaluate the association between the different sources of Env-specific B cells and titers of Env-binding Abs in the serum. Accordingly, we measured reactivities by ELISA of serum Abs to HIV-1 gp140 from the eight individuals on whom we had

BMMC B cell data at a time point that was within 6 mo of the bone marrow biopsy. Of note, the same biotinylated gp140, used to measure cellular specificities by flow cytometry and ELISPOT, was used to measure serologic specificities by ELISA, with the latter two assays also set up in the same orientation, namely gp140 was added in the detection phase of both assays. The individual OD values are shown in Table I and were used to assess correlations with various Env-specific cellular measurements made in this study. As shown in Fig. 6A, levels of Env-specific serum Abs were strongly and significantly correlated with frequencies of Env-specific bone marrow–derived PCs, whereas there was no significant correlation between serum Env-binding Abs and frequencies of peripheral blood Env-specific PBs or memory B cells. Of note, eight individuals were included in the correlation between serum and bone marrow, whereas only seven were included in the correlations involving peripheral blood cells. Nonetheless, the bone marrow correlation remained significant when only those seven individuals were included in the analysis ($r = 0.8829$; $p = 0.015$). With regard to the bone marrow ASCs, the correlation with serum Abs was strongest when Env-specific PCs were reported as a percentage of sorted cells, whereas for the peripheral blood ASCs, there was no significant correlation whether the values were reported as an absolute count or as a fraction of total ASCs or as a percentage of PBs among B cells (data not shown). However, in extended analyses of values shown in Figs. 4 and 5, we found one additional significant correlation between frequencies of Env-specific and total ASCs in the peripheral blood ($r = 0.9643$, $p = 0.003$; data not shown). This was consistent with our previous findings that PBs in the blood are associated with HIV-1 viremia, which in turn drives HIV-1–specific responses (19, 22). Finally, we also evaluated the HIV-1–neutralizing activity of the serum of our participants using a panel of five tier 2 and one tier 1 pseudoviruses described in *Materials and Methods*. As shown in Supplemental Table I, neutralization against tier 2 viruses was weak, rarely 2-fold above negative control aMLV, whereas neutralization against the tier 1 NL4/3 strain was above this threshold in seven of the eight individuals. When IC$_{50}$ values were correlated with the Env-specific cellular responses shown in Fig. 6A, we found significant correlations between serum-neutralizing activity against NL4/3 and bone marrow Env-specific ASCs, but not peripheral blood Env-specific ASCs or memory B cells (Fig. 6B). Furthermore, when the contribution of the negative control, aMLV,
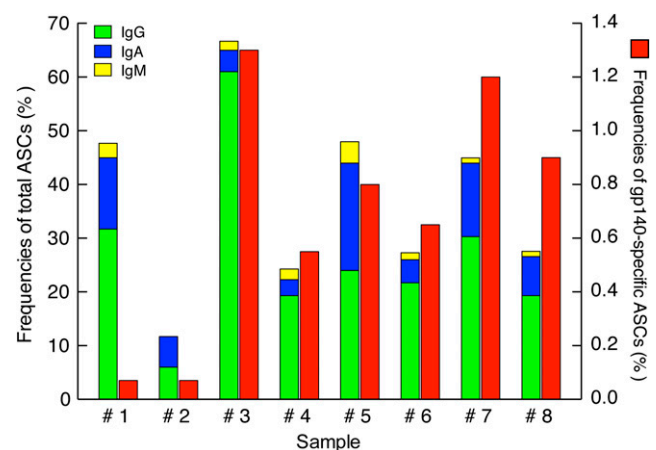
FIGURE 4. Frequencies of total and Env-specific PCs in bone marrow of HIV-1–infected individuals. CD38^{++}/CD138$^+$ PCs were sorted from bone marrow aspirates and evaluated for total Ig (*left y*-axis) and HIV-1 gp140-specific (*right y*-axis) ASC frequencies by ELISPOT. Sample numbers refer to individuals described in Table I.
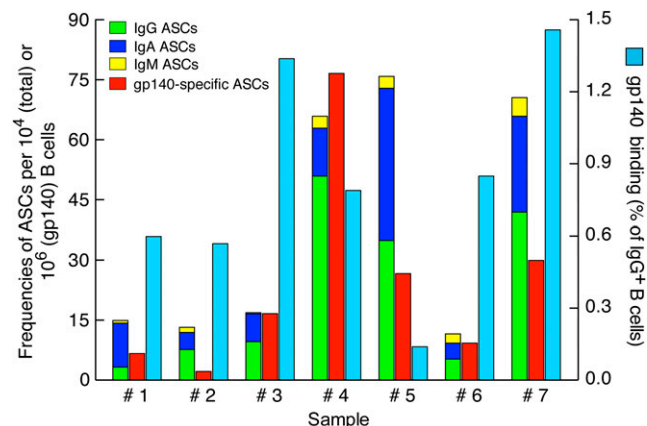
FIGURE 5. Frequencies of total Ig and Env-specific ASCs and memory B cells in the peripheral blood of HIV-1–infected individuals. Mature B cells were enriched from PBMCs and evaluated for total Ig (*left y*-axis) and HIV-1 gp140-specific ASC frequencies by ELISPOT (*left y*-axis), as well as for binding to HIV-1 gp140 probe by flow cytometry (*right y*-axis). Sample numbers refer to individuals described in Table I; analyses were performed on seven individuals.

was subtracted from test values in Supplemental Table I, significant correlations between bone marrow Env-specific ASCs and serum-neutralizing activity were observed for four of the seven strains (Supplemental Fig. 2), whereas none of the correlations

were significant between serum activity and peripheral blood Env-specific ASCs or memory B cells (data not shown). Collectively, these data suggest that PCs in the bone marrow are the main contributors to the Abs, including those with neutralizing activity, that circulate in the blood, at least with regard to Env-specific responses in infected individuals.

Discussion

In the current study, we demonstrate that bone marrow–derived PCs are the main source of circulating HIV-1–specific Abs in HIV-1–infected individuals. The past few years have seen extraordinary progress in understanding and harnessing Ab responses to HIV-1 in infected individuals (8, 34–36). However, circulating Abs remain poorly understood in terms of cellular origin and relationships among the various B cell populations from which bnAbs can potentially arise. It has been assumed, largely based on murine models (37, 38), that long-lived PCs in the human bone marrow are the source of serum Abs, providing long lasting immunity against infectious agents. However, Ag-specific PCs in the bone marrow compartment and their contribution to the pool of Ag-specific circulating Abs in serum have not been previously ascertained in humans. In the setting of HIV-1 infection, in which hypergammaglobulinemia is common and increased frequencies of PBs and/or PCs have been observed in the peripheral blood and secondary lymphoid tissues (19, 39), the contribution of bone marrow to circulating
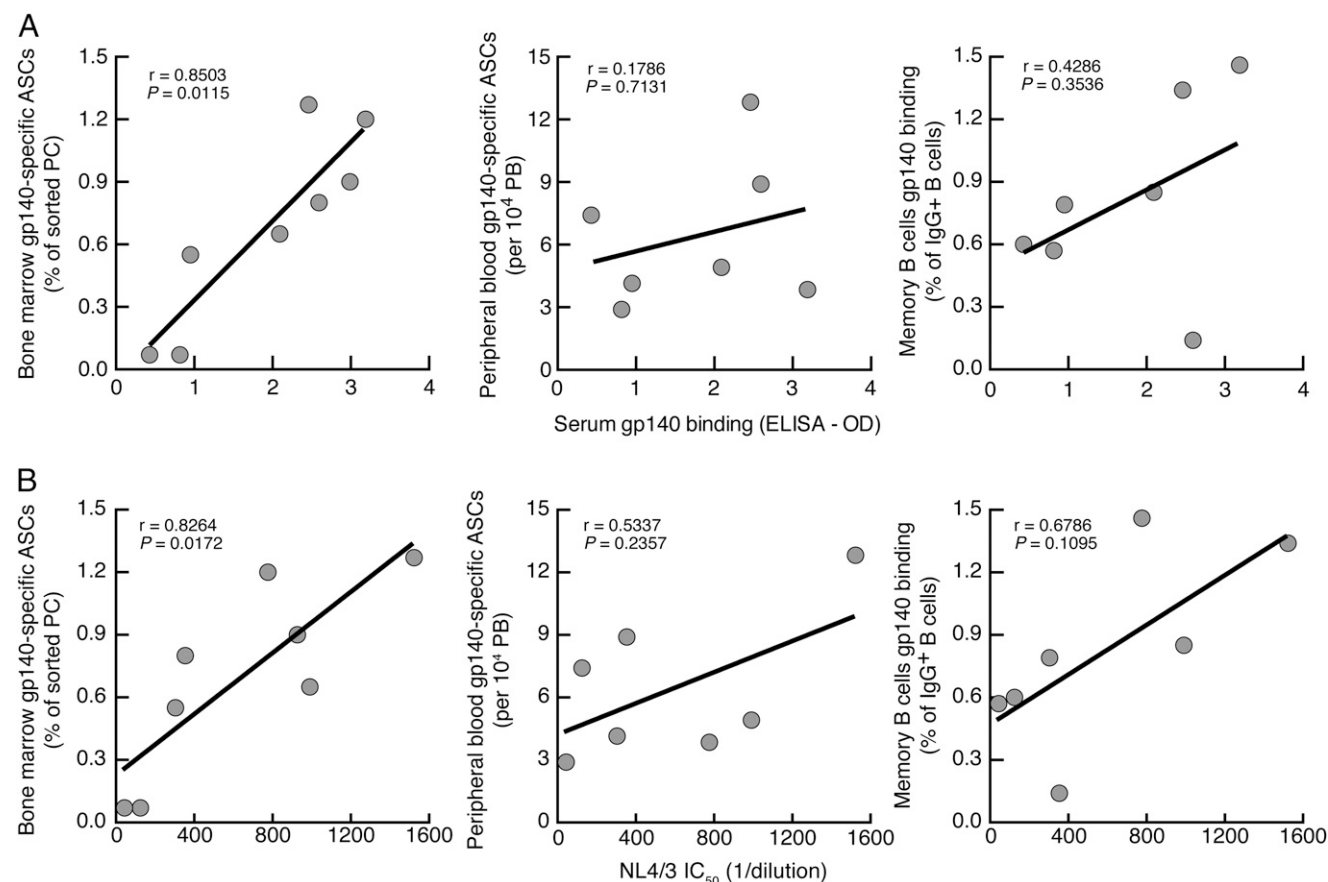
FIGURE 6. Relationship between HIV-1–specific Abs in the serum of HIV-1–infected individuals and corresponding frequencies of Env-specific B cells in bone marrow and peripheral blood. Graphs showing correlation of serum (**A**) Env-binding Abs and (**B**) HIV-neutralizing activity (reported as IC$_{50}$) against NL4/3 pseudovirus with the frequency of Env-specific cells among bone marrow PCs (*left*), PBMC PBs (*center*), and IgG$^+$ PBMC (memory) B cells (*right*). For PCs and PBs, the assay was ELISPOT, and for IgG$^+$ B cells the assay was flow cytometry, and data are those shown in Figs. 4 and 5, except Env-specific peripheral blood ASCs are reported relative to total frequencies of PBs among B cells, as measured by flow cytometry.

Abs could come from many different cellular sources. Despite the fact that there are multiple potential cellular sources of HIV-1–specific serum Ab in the blood, secondary lymphoid tissues, and bone marrow, we demonstrate a strong correlation between HIV-1–specific Abs circulating in the serum of HIV-1–infected individuals and the frequencies of Env-specific PCs in the bone marrow. These findings suggest that HIV-1–specific bone marrow–derived PCs are the main source of HIV-1–specific serum Abs.

The HIV-1–infected individuals we studied were chronically infected, and all were viremic to varying degrees. However, they were also relatively healthy, with a median CD4+ T cell count of 470 cells/µl. These demographics most likely explain the absence of gross hematologic abnormalities in this group, contrasting with studies performed on individuals with more advanced HIV-1 disease in which bone marrow pathology was reported (40–42). Nonetheless, we did observe several abnormalities within B cell subsets of the bone marrow in our cohort that are perhaps indicative of systemic effects of chronic HIV-1 infection on lymphoid cells, including homeostatic dysregulation. On the one hand, we found increased frequencies of PCs in the aspirates and core biopsies, consistent with chronic antigenic stimulation in general and, in this case, HIV-1–induced B cell terminal differentiation (43, 44). In contrast, we also found evidence of perturbations of B cell development in the bone marrow. Frequencies of B cell precursors were elevated, whereas those of mature B cells were reduced. The increased precursor frequency could be a response to HIV-1–induced B cell depletion, similar to, although not as profound as, CD4+ T cell depletion (45). A lower frequency of mature B cells could reflect either a block in maturation due to limiting factors needed for this process, or a premature exit from the bone marrow of I/T B cells. In this regard, HIV-1 infection is associated with an increased frequency of I/T B cells in the periphery (46). Whether these changes in the bone marrow also contribute to the paucity of memory B cells in the periphery in HIV-1 infection is a possibility that warrants further investigation, especially given the importance of the memory B cell compartment in responding to HIV and other pathogens (47, 48).

Finally, the important contribution of HIV-1–specific bone marrow PCs to the circulating HIV-1–specific Abs raises several points for discussion and future consideration relative to bnAbs. One study has shown a link between bone marrow PCs and HIV-1–specific bnAbs present in the serum in two patients (3). In addition, in that study, the serum Ab was shown to be linked to the Ab reconstituted from memory B cells in the peripheral blood and Ab gene sequences in the bone marrow PCs. A link between HIV-1–neutralizing serum Abs and memory B cells has also been established in studies of individuals with potent HIV-1–neutralizing serum activity (4, 9), and our data indicate a similar trend in individuals who do not have strong neutralizing responses (Fig. 6). However, these potentially important relationships need to be further explored in larger, more diverse cohorts. Nonetheless, given that bone marrow PCs are likely the durable repository of Abs responsible for maintaining long-term immunity, based on our study of the relationship between bone marrow PCs and HIV-1–specific Abs, this compartment should be included as part of the evaluation of responses to immunogens being considered in HIV vaccine strategies aimed at inducing broadly reactive HIV-specific Abs.

Acknowledgments

Disclosures

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Supplemental Table I. Serum neutralization activitity against HIV-1 and aMLV pseudoviruses

Patient number	Pseudovirus							
	94UG103	92TH021	93IN905	92BR020	MGRM-C-026	JRCSF	NL43	aMLV
1	37	43	41	41	43	32	125	49
2	16	23	31	26	24	17	44	26
3	78	82	144	160	97	139	1523	65
4	24	18	25	38	23	28	304	26
5	21	42	37	36	31	65	354	21
6	61	48	68	237	59	40	991	43
7	37	28	68	287	57	72	776	25
8	30	34	76	48	41	26	927	36

Values represent reciprocal dilution of IC_{50}.
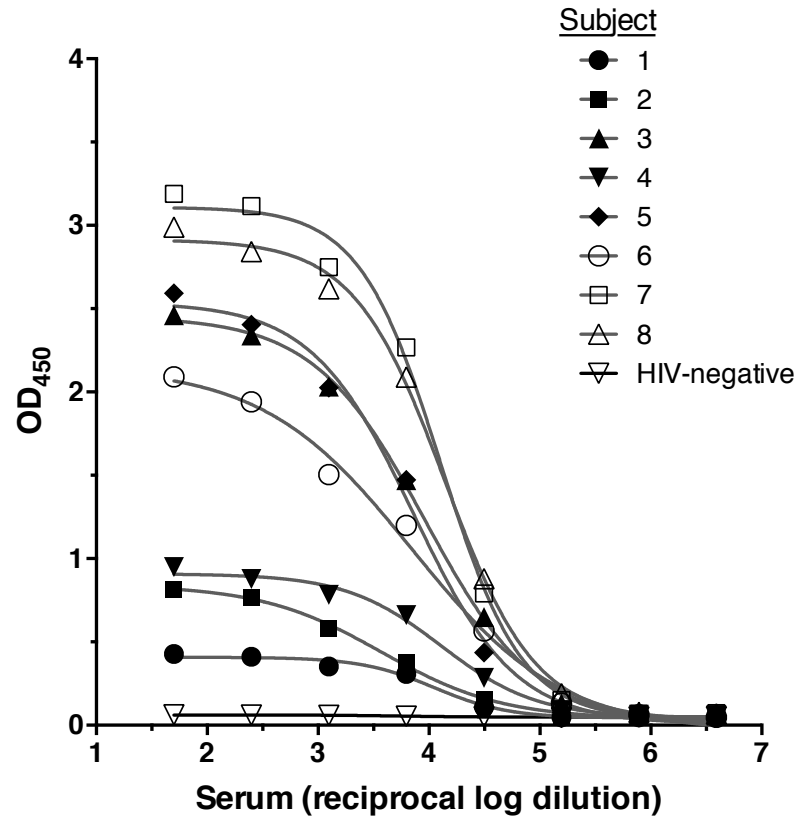
Supplemental Figure 1. Measurement of Env-binding antibodies in serum by ELISA. Titration curves of serum samples obtained from the eight HIV-1-infected participants and one HIV-negative individual were generated. Wells of 96-well plates were coated with anti-Ig light chain mAbs, followed by addition of serum samples that were serially diluted five-fold, beginning at 1:50. Biotinylated HIV-1 YU2 gp140 was used to detect captured Env-binding antibodies and OD was read at 450 nm following incubation with substrate.
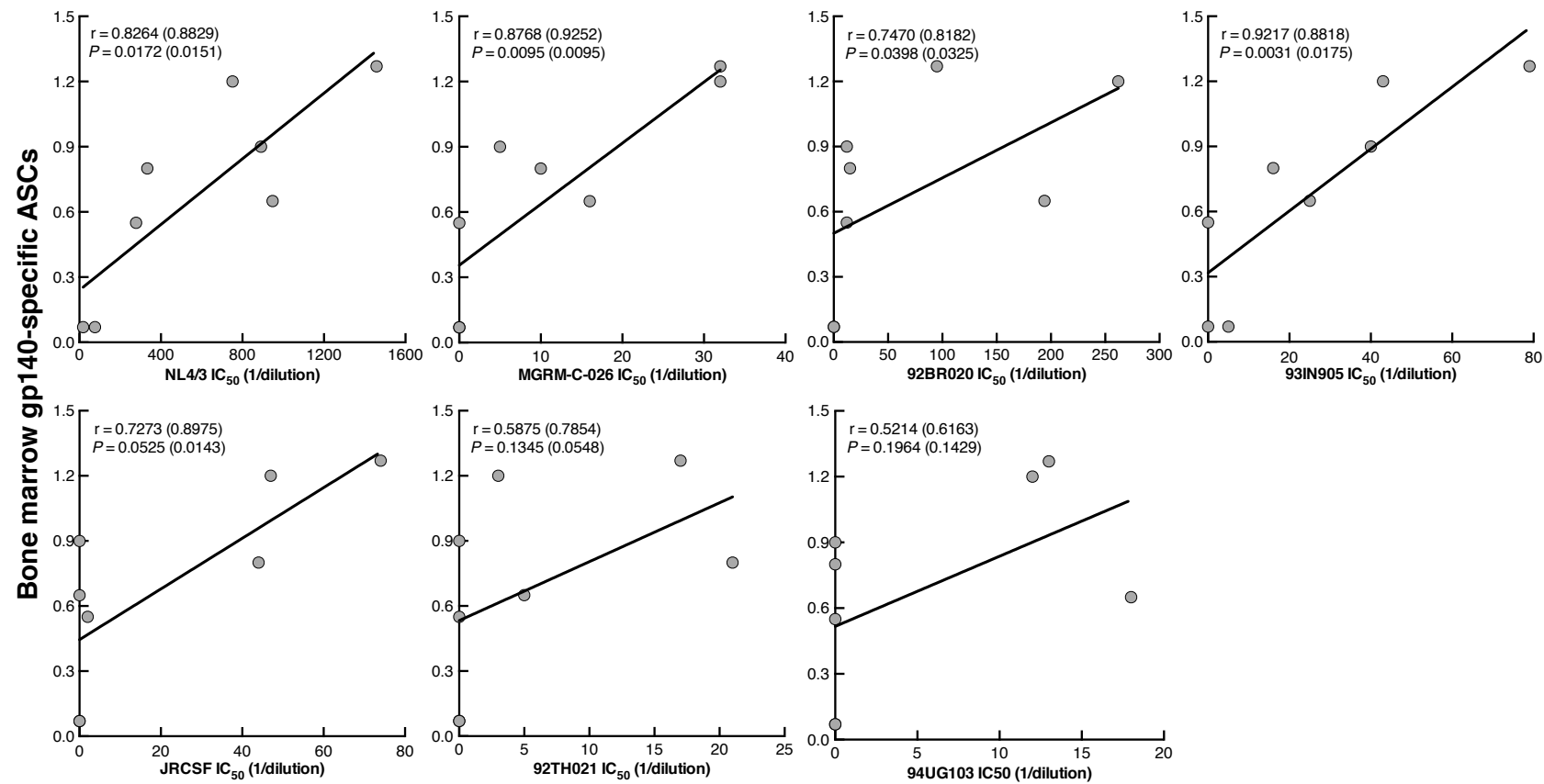
Supplemental Figure 2. Graphs showing correlation of serum HIV-neutralizing activity (reported as IC_{50}) against various HIV enveloped

pseudoviruses with the frequency with of Env-specific cells among bone marrow PCs. Reciprocal IC_{50} values shown represent test values in

Supplemental Table 1 from which respective negative control aMLV values were subtracted (reporting 0 if subtracted number was negative).

The r and *P* values shown in each graph were calculated using the eight data points shown whereas the values in parentheses were calculated

using seven data points, excluding the Patient 8 whose peripheral blood B cells were not evaluated.