B-lymphocyte Dysfunction in Chronic HIV-1 Infection Does Not Prevent
Cross-clade Neutralization Breadth

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ABSTRACT

Aberrant expression of regulatory receptors PD-1 and BTLA are linked with dysregulation and exhaustion of T-lymphocytes during chronic HIV-1 infection; however, less is known about whether a similar process impacts B-lymphocyte function during HIV-1 infection. We reasoned that disruption of the peripheral B cell compartment might be associated with decreased neutralizing antibody activity. Expression of markers that indicate dysregulation (BTLA and PD-1), immune activation (CD95), and proliferation (Ki-67) were evaluated in B cells from HIV-1 infected viremic and aviremic subjects and healthy subjects, in conjunction with immunoglobulin production and CD4 T cell count. Viral load and cross-clade neutralizing activity in plasma from viremic subjects was also assessed. Dysregulation of B-lymphocytes was indicated by a marked disruption of peripheral B cell subsets, increased levels of PD-1 expression, and decreased levels of BTLA expression in viremic subjects, compared to aviremic subjects and healthy controls. PD-1 and BTLA were correlated in a divergent fashion with immune activation, CD4 T cell count, and the total plasma IgG level, a functional correlate of B cell dysfunction. Within viremic subjects, the total IgG level correlated directly with cross-clade neutralizing activity in plasma. The findings demonstrate that even in chronically infected subjects where B-lymphocytes display multiple indications of dysfunction, antibodies that mediate cross-clade neutralization breadth continue to circulate in plasma.
INTRODUCTION

Infection with human immunodeficiency virus type-1 (HIV-1) leads to widespread dysfunction of the immune system, including B-lymphocytes. One sign of B cell dysfunction in HIV-1 infection is an increase in the production of IgG, or hypergammaglobulinemia (8, 21, 29). B-lymphocytes of HIV-1 infected persons also exhibit signs of polyclonal activation and auto-reactivity (46) and impaired responses to both T-dependent and -independent antigenic stimuli or immunization (19, 20, 36, 39). These dysfunctions have been attributed, in part, to an imbalance of four major subsets within the B cell compartment (31, 32). Combination antiretroviral therapy (cART) only partially restores the balance, even after 12 months of treatment (31).

Since first introduced by Ascher and Sheppard in the late 1980s, the concept of immune activation as a causative mechanism of HIV-1 pathogenesis/AIDS has garnered immense consideration and experimental evaluation (1). The degree of immune activation has been implicated in disease progression pace (15). Normally, delicate interplay among several regulatory receptors tightly governs activation of the immune system. Recently, the importance of programmed death-1 (PD-1, CD279) has been emphasized in the development of hyper immune activation and exhaustion within T-lymphocytes during chronic viral infections, including HIV-1 (2, 6, 7, 17, 18, 52). Less is known about the role of PD-1 in the maintenance of B cell function, but a recent study demonstrated that PD-1 expression on activated memory B cells in SIV infection was associated with rapid disease progression (49). Similar to PD-1, B and T lymphocyte attenuator (BTLA, CD272) is another member of the B7/CD28 superfamily (51). This regulatory receptor is decreased on CD4 and CD8 T cells during chronic HIV-1 infection, and its expression is inversely correlated with disease progression (53). Thus, aberrant expression of PD-1 and BTLA on T cells in HIV-1 infection has been associated with disease progression.

Antibodies that can mediate neutralization of heterologous HIV-1 viruses are desirable from a vaccine perspective, but it is unclear how they arise or if they provide any benefit to the patient. Furthermore, these types of neutralizing antibodies (nAbs) are detected only after several years of infection, and in...
only a subset of infected individuals (3, 5, 10, 13, 14, 35, 42, 47, 50). Factors that have been suggested to promote the development of neutralization breadth include prolonged exposure to antigen, higher envelope diversity, and plasma viral load (9, 12, 14, 34, 37, 42). Nevertheless, neutralization breadth does not delay disease progression (13, 14, 37). Others have demonstrated that peripheral B cell decline and other perturbations do not necessarily impede nAb activity as measured in vitro (4, 35), but to date no one has measured neutralization breadth in a cohort of HIV-1 infected subjects where multiple aspects of B cell dysfunction have been evaluated in parallel.

Here we evaluated the state of the peripheral B cell compartment in chronically HIV-1 infected individuals, infected but aviremic subjects treated with cART, and healthy controls by evaluating levels of PD-1 and BTLA expression on total B cells, and within peripheral B cell subsets. Aberrant expression of these receptors was observed in viremic individuals and was correlated with increased levels of immune activation, proliferation, IgG production, and CD4 T cell decline. We also investigated whether individuals experiencing these signs of B cell dysfunction possessed antibody-mediated neutralization capacity against pseudotyped heterologous HIV-1 envelope (Env) glycoproteins. Strong cross-clade neutralizing antibody activity was detected in the plasma of a subset of these infected individuals, even though the B cell compartment was perturbed.

**MATERIALS AND METHODS**

**Study subjects.** In compliance with procedures approved by the Emory University Institutional Review Board (IRB), 41 individuals were enrolled with informed consent for this study. Participants were categorized into three groups: healthy controls (HC, n=12) included persons without HIV-1 infection or any clinical symptoms at the time of enrollment; viremic subjects (VI, n=16) had clinical records of HIV-1 infection, but were cART naïve and had plasma viral loads greater than 1000 copies/ml; and aviremic subjects (AV, n=13) were HIV-1 infected and currently on cART with a plasma viral load of fewer than 100 copies/ml. Median age, CD4 T cell count, and viral load, as well as the gender and ethnicity of the study participants, are listed in Table 1.
PBMC isolation. Approximately 50 ml blood were collected from each participant in ACD containing BD-vacutainer® blood collection tubes with informed consent from the donor. Peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood by standard Ficoll-Paque density gradient centrifugation (Ficoll-Paque Plus, GE Healthcare). PBMCs were then aliquoted and cryopreserved in liquid nitrogen (-160°C) until needed for flow cytometry.

Flow cytometric analysis of peripheral B cells. PBMCs were thawed and washed twice with PBS and then re-suspended in FACS buffer (PBS with 1% BSA and 0.1% sodium azide). Two million cells were used for surface staining with the following antibodies: yellow fluorescent reactive dye (live/dead stain), anti-CD3 V500 (SP34-2), anti-CD14 V500 (M5E2), anti-PD-1 APC (EH12.2H7), anti-BTLA PE (J168-540), anti-CD19 Qdot655 (SJ25C1), anti-CD10 APC-Cy7 (HI10a), anti-CD21 PE-Cy5 (B-ly4), anti-CD27 PE-Cy7 (1A4CD27), and anti-CD95 FITC (DX2). Following live/dead cell staining, PBMCs were incubated with antibodies at 4°C for 30 min; cells were fixed, and any contamination of RBC was removed by incubation in 1X lysing solution (BD Bioscience) for 10 min at room temperature. For intracellular staining, PBMCs were further washed twice with FACS buffer and permeabilized with 1X permeabilizing solution (BD Bioscience) for 30 min at room temperature. Anti-Ki-67 Alexa Fluor 700 (B56) antibody was used for the intracellular staining at room temperature for 30 min. After washing twice, cells were re-suspended in 400 µl FACS buffer containing 1% paraformaldehyde. Fluorescence minus one (FMO) negative controls were included for staining. An LSR-II cell analyzer (BD Bioscience) was used to acquire data. Lymphocytes were gated based on forward vs. side scatter profile, and B-lymphocytes were gated as CD19+ cells after exclusion of dead, CD3+, and CD14+ cells. Data was analyzed using FlowJo software (version 9.3.1, TreeStar Inc., USA).

ELISA assay for plasma IgG. Total IgG concentration in plasma was measured by using a human IgG ELISA quantitation set (Bethyl Laboratories Inc.) following the manufacturer’s directions. Plasma was heat inactivated (56°C for 60 min) and then diluted to 1:100,000 for the experiments. Endpoint
absorbance was measured at 450nm with a BioTek Synergy multi-detection microplate reader, and data was analyzed with KC4 v3.4 software. A human reference serum was used to normalize total IgG concentrations in plasma.

**ELISA assay for binding to monomeric gp120.** Immulon microtiter 96 well plates were coated with 100 μl of HIV-1 BaL gp120 diluted to 5 μg/ml in coating buffer (Institute of Human Virology, μQuant Facility). Plates were washed 3 times and then blocked for 30 min at 37°C. Following washing, 100 μl of heat-inactivated plasma was added to each well and incubated for 1 hour. Plates were washed 3 times and 100 μl of HRP-conjugated goat anti-human IgG was added to each well. After a 1 hour incubation at 37°C, plates were washed, and TMB substrate was added. After 10 min, reactions were stopped with 4N H₂SO₄, endpoint absorbance was measured at 450nm with a BioTek Synergy multi-detection microplate reader, and data was analyzed with KC4 v3.4 software.

**Neutralization assay.** The ability of plasma from 16 viremic individuals to neutralize a cross-clade panel of 13 HIV-1 envelope (Env) pseudotyped virions was measured using the Tzm-bl luciferase assay as described previously (22, 27, 40, 41). Each plasma-Env combination was analyzed independently at least two times with duplicate wells. The neutralization IC₅₀ for each plasma-Env combination was calculated using linear regression analysis in GraphPad Prism version 5.0. IC₅₀ values that were less than the highest dilution of plasma tested (1:100) were assigned a score of 1:50. Neutralization breadth was calculated as the number of pseudoviruses neutralized with an IC₅₀ of greater than 1:100, and potency was defined by (i) dividing the IC₅₀ value for each given plasma-Env combination by the median IC₅₀ value for that pseudovirus against all plasma samples and (ii) adding the scores for each plasma sample, as described by (26). Higher scores indicate greater breadth and potency. All Env clones were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: 6535.3, TRO.11, AC10.0.29, and PVO.4 are from the Standard Reference Panel for Subtype B HIV-1 Env Clones (23); ZM197M.PB7, Du172.17, Du156.12, ZM109F.PB4, CAP45.2.00.G3, and
ZM214M.PL15 are from the Subtype C HIV-1 Reference Panel of Env Clones (24); subtype A Env clones Q23ENV17 (38) and Q769ENVd22 (25) were contributed by Dr. Julie Overbaugh.

**Blood CD4 T cell count and plasma HIV-1 viral load.** Blood CD4 T cell count was measured by the Emory University CFAR Immunology core, and plasma viral load was quantified by the Virology core.

Briefly, absolute number of peripheral blood lymphocytes was calculated from the total white blood cell (WBC) count determined with an automated hematology analyzer, and the percentage of CD4 T-lymphocyte population was determined by flow cytometry. Plasma HIV-1 RNA level was measured using the Cobas® Amplicor HIV Monitor test (version 1.5, Roche) or the Abbott Real Time HIV-1 Assay on an automated m2000 system, according to the manufacturer’s directions.

**Statistical analysis.** Non-parametric one-way analysis of variance (1-way ANOVA, Kruskal-Wallis with Dunn’s post-test) and Spearman rank correlation tests were performed with GraphPad Prism version 5.0 to evaluate the data. A p-value of less than 0.05 (95% confidence level) was considered significant.

**RESULTS**

**Regulatory receptors PD-1 and BTLA are aberrantly expressed on B-lymphocytes during chronic HIV-1 infection.** To investigate dysregulation within the B cell compartment during chronic HIV-1 infection, expression of the inhibitory receptors PD-1 and BTLA was assessed by flow cytometry and compared among healthy controls (HC), aviremic subjects (AV), and viremic subjects (VI) (Figure 1). In HC, only a minor proportion of B cells expressed PD-1 (Figure 1A), consistent with what is observed in T-lymphocytes (7, 52). In VI, expression of PD-1 was significantly increased (Figure 1A, p<0.001). AV subjects had PD-1 expression levels that were significantly lower than VI (Figure 1A, p<0.001), but were not significantly different from HC. Thus, active viral replication in VI is associated with a significant increase in PD-1 expression on B cells that is alleviated by cART. Despite a greater percentage of B cells expressing PD-1 in VI, the level of receptor expression per CD19+ PD-1+ cell was not different from
HC or AV (Figure 1B). Instead, comparable mean fluorescence intensity (MFI) values were observed across the three groups.

The majority of B cells in HC expressed BTLA on their surface (Figure 1C). However, VI showed a significant decline in BTLA expression compared to HC (Figure 1C, p<0.001). AV individuals had intermediate levels of BTLA expression that were significantly different than both HC and VI (Figure 1C, p<0.05) representing only partial restoration of normal BTLA levels. In addition to the decrease in percentage of BTLA-expressing B cells in VI and AV, the MFIs of individual CD19\(^+\) BTLA\(^+\) cells were significantly lower in VI and AV compared to HC (Figure 1D, p<0.001 and p<0.05, respectively). Thus, modulation of BTLA expression by HIV-1 infection occurred at both the population and single cell level and remained depressed even when viral replication was suppressed by cART. HIV-1 infection exerts a differential effect on B cell expression of PD-1 and BTLA, as evidenced by the strong inverse correlation between the two receptors (Figure 1E, p<0.0001). The aberrant expression of these receptors in VI indicates that homeostasis within the B cell compartment is significantly disrupted.

Peripheral B cell subsets are dysregulated during chronic HIV-1 infection. We next examined whether altered PD-1 and BTLA expression in the total B cells of VI were reflected in specific B-lymphocyte subsets. Figure 2A displays the strategy used for separating total B cells (CD19\(^+\)) into 4 phenotypic subsets: immature (CD10\(^+\)CD27\(^-\)), mature (CD10\(^-\)CD21\(^lo\)), naïve (CD10\(^-\)CD21\(^hi\)CD27\(^-\)), and classical memory (CD10\(^-\)CD21\(^hi\)CD27\(^+\)), as described previously (31). Similar to a published study by Moir et al. (31), a decrease in the proportion of naïve and memory B cells and an increase in the immature and mature populations were observed in VI compared to HC (Figure 2B). The change in the proportion of mature B cells was dramatic, increasing from 8% in HC to 43% in VI. Likewise, a substantial decline in the memory B cell subset, from 36% in HC to 8% in VI, was observed. Thus, mature B cells came to dominate the peripheral B cell compartment in VI. The balance within B cell subsets in AV was partially restored, falling somewhere in between HC and VI.
The B cell subsets differed in their respective PD-1 and BTLA expression patterns (Figure 2C and D, respectively). In HC, low PD-1 expression was observed in all subsets, but particularly within naive and memory B cells (Figure 2C). In VI, PD-1 expression was significantly increased in the naive and immature subpopulations of B cells compared to HC (Figure 2C, p<0.01 and p<0.001, respectively). However, PD-1 expression on naive and immature B cells in AV was not different from HC, indicating some partial restoration (Figure 2C). BTLA expression in VI was significantly decreased in the naive, immature, and memory B cell subsets compared to HC and AV (Figure 2D, p<0.001 and at least p<0.05, respectively). BTLA expression in AV was not different from HC except for in immature B cells, where it remained significantly lower than HC (Figure 2D, p<0.05). In the mature B cell subset, significant differences in the expression of PD-1 and BTLA were not detected among HC, AV, and VI. However, because the mature B cell subset is expanded in VI (Figure 2B), these B cells may contribute disproportionately to the overall increase in PD-1 and decrease in BTLA expression.

PD-1 and BTLA expression on B-lymphocytes are correlated with markers of immune activation, proliferation, and disease progression. Because generalized immune activation is an important factor in determining the course of HIV-1 infection, we also investigated whether PD-1 and BTLA expression were associated with Ki-67 or CD95 on total B cells. A strong positive correlation was found between PD-1 expression and Ki-67 (p<0.0001) and CD95 (p=0.0004) (Figure 3A and B, respectively), whereas BTLA exhibited an inverse correlation with these markers (Figure 3C and D, p<0.0001 and p=0.006, respectively). These results suggest a direct link between dysregulation and immune activation in the B cell compartment.

The relationship between PD-1 and BTLA expression on total B cells and two indicators of disease progression, plasma viral load and blood CD4 T cell count, was also assessed. PD-1 or BTLA expression on B-lymphocytes was not significantly associated with plasma viral load in VI (Figure 4A and B, respectively). However, a significant correlation was observed between CD4 T cell count and PD-1 or BTLA expression, including data from the three subject groups (Figure 4C and D, respectively,
PD-1 expression on total B cells was inversely correlated with CD4 T cell count, while the correlation for BTLA expression and CD4 T cell count was direct.

**PD-1 and BTLA expression on B-lymphocytes are correlated with plasma IgG level.**

Hypergammaglobulinemia is a direct manifestation of B cell dysfunction during HIV-1 infection. We therefore examined the relationship between PD-1 and BTLA expression on B cells and plasma total IgG level for each group of subjects. Concurrent with previous reports, a significant increase in the plasma total IgG level was observed in VI compared to both AV and HC (Figure 5A, p<0.05 and p<0.001, respectively). Viral suppression mediated by cART resulted in lower levels of total IgG production. In addition, highly significant direct and indirect correlations were identified between total IgG level and PD-1 or BTLA expression on B cells (Figure 5B and C, p=0.0005 and p<0.0001, respectively). Thus, regulatory receptor expression is linked with this functional anomaly of the B cell compartment.

**Total IgG level in plasma but not immune dysregulation is associated with HIV-1 neutralization breadth in viremic individuals.** We next investigated if heterologous neutralizing activity was present in VI with established B cell dysfunction and if nAb breadth was dependent upon the level of B cell activation or dysfunction. Plasma samples from VI were tested for their ability to neutralize a panel of 13 HIV-1 envelope (Env) pseudotyped virions from clades A, B, and C, which included three tiers of sensitivity, as determined by Seaman et al. (45). While HIV-1 subtypes were not determined, our cohort of viremic subjects was most likely infected with subtype B, as this viral clade predominates in the southeastern United States. The neutralization IC₅₀ was calculated for each plasma-Env combination, and this data was used to calculate a breadth (how many Envs were neutralized) and potency (the strength of neutralization) score for each plasma sample, as described in (37). Infectivity curves for each plasma sample are shown in Supplementary Figure 1. A range of neutralization breadth was observed in these 16 subjects: three plasma samples (19%) demonstrated widespread neutralizing activity against this panel of Envs while five subjects (31%) exhibited a complete lack of detectable neutralization at the lowest dilution of plasma tested (1:100) (Figure 6A). No correlation was observed between neutralization...
breadth or potency and parameters of B cell dysfunction (PD-1, BTLA), immune activation (Ki-67, CD95),
or disease progression (CD4 T cell count, plasma viral load) (data not shown). However, the level of total
IgG in each VI plasma sample was significantly correlated with both neutralization breadth and potency
(Figure 6B and C, p=0.009 and p=0.02, respectively). We next quantitated the level of antibodies that
binds to the monomeric form of a subtype B Env gp120 (HIV-1 BaL) in each VI plasma sample, and
determined whether antibodies with this specificity were correlated with nAb breadth or potency. Like
total IgG, anti-gp120 antibodies were positively correlated with nAb breadth and potency, but in this case
the correlations only trended towards significance (Figure 6D and E, respectively, p=0.09 for both). Anti-
gp120 antibodies did not correlate with parameters of B cell dysfunction, immune activation, disease
progression, or total IgG level. These findings suggest that gp120 binding as well as other IgG antibody
specificities contribute to nAb breadth, but neither is overtly influenced by perturbations in the B cell
compartment during chronic HIV-1 infection.

DISCUSSION

An effective humoral immune response, in concert with cell-mediated immunity, may contribute to the
control of HIV-1 replication. Several lines of evidence from SIV and SHIV infection of nonhuman primates
and from studies of HIV-1 infection support the importance of B-lymphocytes. A suboptimal antibody
response can influence disease progression and even lead to fatal outcome during SIV/SHIV infections
(11, 43, 44, 48, 49, 54). Furthermore, studies of HIV-1 infection have shown that B-lymphocyte
dysfunction correlates with markers of disease progression (28, 30, 33). In one HIV-1 infected individual,
monoclonal antibody-mediated depletion of B cells resulted in a decrease in neutralizing antibody titer
and an increase in plasma viral load, which was reversed when the neutralizing antibody titer recovered
to the pre-treatment level (16). Thus, strategies to reverse or limit B cell dysfunction during HIV-1
infection could potentially limit disease progression.

Here we have demonstrated that PD-1 and BTLA, previously recognized mainly for their effects on T
cells, are also aberrantly expressed on B-lymphocytes during chronic HIV-1 infection. Our data
demonstrate that expression of PD-1 was increased and BTLA decreased on B-lymphocytes during persistent HIV-1 viremia, and that alteration in PD-1 and BTLA expression on B cells is comparable to the patterns observed in T cells (7, 52, 53). Expanded analysis into the four major subsets of B-lymphocytes revealed that PD-1 expression was notably higher in naive and immature B cells, and BTLA was lower in naive, immature, and memory B cells in VI. Interestingly, the mature B cell subset exhibited the least quantifiable differences in expression of these regulatory markers among VI, AV, and HC but was the most affected with respect to the peripheral B cell subset distribution.

Plasma viral load in VI was not significantly correlated with either PD-1 or BTLA expression on B cells. In contrast, other studies have reported correlations between PD-1 or BTLA expression on T cells and plasma viral load (7, 53). These studies also demonstrated that the CD4 T cell count was inversely correlated with PD-1 expression and directly correlated with BTLA expression on T cells (7, 53). Similarly in our study, peripheral blood CD4 T cell count was also indirectly and directly correlated with PD-1 and BTLA expression on B-lymphocytes, respectively. Thus, an imbalance in immune homeostasis, rather than simply the presence of persistent viral antigen, could be reflected in the aberrant expression of these regulatory receptors on B cells. A strong correlation was also observed between PD-1 and BTLA expression on B cells and markers of cell proliferation and activation. These findings suggest a possible role for aberrant PD-1 and BTLA expression in driving increased B cell activation. Finally, this report is among the first to link B cell dysregulation with the extent of hypergammaglobulinemia, a functional measure of B cell dysfunction in HIV-1 infection.

Having established multiple tiers of disruption within the B cell compartment in the VI cohort, we investigated whether plasma from these individuals contained nAbs with cross neutralizing capacity. Broad and potent neutralization was observed in 3 of the 16 subjects analyzed here. This frequency of 19% is consistent with that reported for individuals possessing greater nAb breadth in other cohorts. These three individuals did not systematically differ from the others exhibiting less nAb activity with regard to measures of immune activation, dysregulation, CD4 T cell count, or plasma viral load. Instead,
in this cohort of typical progressor patients, nAb breadth and potency were associated directly with the level of hypergammaglobulinemia and gp120 binding antibodies, even though the latter did not reach statistical significance. A recent study from Oballah et al. demonstrated that the absolute B cell count in a subtype A HIV-1 infected cohort in Uganda was inversely correlated with neutralizing activity against heterologous Envs (35). In our study, we did not find a correlation between total B cell count and nAb breadth or potency (data not shown). However, consistent with their results, we did observe that relatively strong and broad nAbs are present in individuals that exhibit B cell dysregulation and hypergammaglobulinemia. Others have reported that the time since infection (14, 42) and plasma viral load or CD4 T cell count were associated with nAb breadth (9, 13, 37, 42). It is likely that these associations did not emerge in our study because of the smaller cohort size, which was targeted toward facilitating an extensive flow cytometric analysis of B cells in addition to measuring nAb breadth.

In summary, this paper is among the first to demonstrate aberrant expression profiles of the regulatory receptors PD-1 and BTLA on peripheral B cells, as well as within individual B cell subsets, during HIV-1 infection. These receptors were associated with activation, proliferation, and dysfunction in B cells in viremic subjects. Despite this, broad and potent nAbs were produced in some individuals, and their activity was possibly augmented through increased IgG production. The observations reported here provide new insight into peripheral B cell dysfunction in chronic HIV-1 infection, supporting its impact on immune activation and disease progression but revealing a less dramatic effect on nAb activity and breadth.

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REFERENCES


FIGURE LEGENDS

Figure 1. Expression of PD-1 and BTLA by B-lymphocytes. The percentage of total B cells (CD19+) that express PD-1 and BTLA in HC, AV, and VI subjects is shown in panels (A) and (C), respectively. The mean fluorescence intensity (MFI) for PD-1 and BTLA expression by individual PD-1+ or BTLA+ CD19+ B cells is shown in panels (B) and (D), respectively. Each point represents data from a single subject. Horizontal bars within the point plots indicate the median percentage for each group. Significance between groups determined by 1-way ANOVA is indicated above the groups, depicted as * = p<0.05, ** = p<0.01, and *** = p<0.001. The correlation between percentage of total B cells that express PD-1 and BTLA is presented in the graph in panel (E). Spearman correlation coefficient (r) and level of significance (p) are indicated within the graph (open diamonds = HC; closed triangles = AV; closed circles = VI).

Figure 2. Distribution of B-lymphocyte subsets and expression of PD-1 and BTLA. The flow cytometry gating strategy for separating B cell subsets is shown in panel (A). Within the B-lymphocytes (CD19+), cells were further gated into four subsets, defined as: immature (CD10+CD27-), mature (CD10-CD21lo), naive (CD10-CD21hiCD27-), and memory (CD10-CD21hiCD27+). The mean proportions of naive, immature, mature, and memory subsets within the total B cell population in HC, AV, and VI subjects are shown in panel (B). The percentage of naive, immature, mature, and memory B cells that express PD-1 (C) and BTLA (D) in HC, AV, and VI is presented in each graph. Each point represents data from a single subject. Horizontal bars within the point plots indicate median percentage for each group. Significance between groups determined by 1-way ANOVA is indicated below the groups, depicted as * = p<0.05, ** = p<0.01 and *** = p<0.001.

Figure 3. Correlation of PD-1 and BTLA expression on B-lymphocytes with Ki-67 and CD95. The correlations between percentage of total B cells (CD19+) that express PD-1 and percentage expressing Ki-67 or CD95 are shown in panels (A) and (B), respectively. The correlations between BTLA and Ki-67...
or CD95 expression are shown in panels (C) and (D), respectively. Spearman correlation coefficient (r) and level of significance (p) are indicated within each graph. Each point represents data from a single subject (open diamonds = HC; closed triangles = AV; closed circles = VI).

**Figure 4. Correlation of PD-1 and BTLA expression on B-lymphocytes with markers of HIV-1 disease progression.** The correlations between plasma viral load (HIV-1 RNA copies/ml) and percentage of total B cells (CD19+) that express PD-1 or BTLA are shown in panels (A) and (B), respectively. The correlations between blood CD4 count (CD4 T cells/μl) and percentage of total B cells (CD19+) that express PD-1 or BTLA are shown in panels (C) and (D), respectively. Spearman correlation coefficient (r) and level of significance (p) are indicated in each graph. Each point represents data from a single subject (open diamonds = HC; closed triangles = AV; closed circles = VI).

**Figure 5. Correlation of PD-1 and BTLA expression on B-lymphocytes with total plasma IgG levels.** The concentrations of total IgG (mg/ml) in the plasma of HC, AV, and VI subjects are presented in panel (A). Horizontal lines within the boxes indicate the median value for each group. Boxes represent the 25th to 75th percentile, and brackets represent the minimum to maximum values in each group. Significance between groups by 1-way ANOVA is indicated above the groups, depicted as * = p<0.05 and *** = p<0.001. The correlations between total IgG concentration (mg/ml) in plasma and percentage of total B cells (CD19+) that express PD-1 or BTLA are presented in panels (B) and (C), respectively. Spearman correlation coefficient (r) and level of significance (p) are indicated within each graph. Each point represents data from a single subject (open diamonds = HC; closed triangles = AV; closed circles = VI).

**Figure 6. Neutralization breadth of plasma from VI subjects correlates with total IgG level.** Sixteen plasma samples from VI were evaluated for their neutralization breadth and potency against a cross-clade panel of 13 HIV-1 Env pseudotypes. The neutralization IC_{50} was calculated for each plasma-Env combination using linear regression, and these values are shown in panel (A). IC_{50} values of less than 1:100 were assigned a value of 1:50. Color shading indicates the potency of neutralization: red > dark
orange > light orange > green. HIV-1 Envs are listed along the top and are grouped by subtype. The tier designation for each Env (1B, 2, or 3) is shown and represents overall neutralization phenotype, as described by (45). Tier 1B viruses are ‘easy’ to neutralize; tier 2 are representative of most patient viruses; tier 3 are ‘difficult’ to neutralize. Higher breadth and potency scores shown in panel (A) indicate greater neutralization capacity. Panels (B) and (C) show the correlations between neutralization breadth (the number of Env pseudotypes neutralized) or potency (the sum of IC₅₀ values for each plasma-Env combination divided by the median IC₅₀ value for that virus against all plasma samples) and total IgG level for each plasma sample. Panels (D) and (E) show the correlations between nAb breadth and potency and the level of anti-gp120 binding antibodies in plasma, detected by ELISA (plotted as the optical density reading at 450 nm). The Spearman correlation coefficient (r) and level of significance (p) are indicated within each graph. Panels D and E showed positive r values and trended toward significance, but did not reach a level of p<0.05.
A

$r = 0.14$
$p = 0.62$

HIV-1 RNA copies/ml

B

$r = -0.25$
$p = 0.35$

HIV-1 RNA copies/ml

C

$r = -0.81$
$p < 0.0001$

CD4 T cells per μl

D

$r = 0.74$
$p < 0.0001$

CD4 T cells per μl
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### A

**B**

\[ r = 0.63 \]
\[ p = 0.009 \]

**C**

\[ r = 0.59 \]
\[ p = 0.02 \]

### D

\[ r = 0.44 \]
\[ p = 0.09 \]

### E

\[ r = 0.43 \]
\[ p = 0.09 \]
## TABLE 1. Characteristics of the study participants

<table>
<thead>
<tr>
<th></th>
<th>Healthy Control (HC)</th>
<th>Aviremic (AV)(^{b,c})</th>
<th>Viremic (VI)(^{c})</th>
<th>P &lt; 0.05</th>
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<tr>
<td>Number of subjects</td>
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<td>Age, years(^{a})</td>
<td>32 (20-56)</td>
<td>46 (33-65)</td>
<td>37 (22-50)</td>
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<tr>
<td>CD4 count, (cells/µl)</td>
<td>632 (375-1,094)</td>
<td>329 (31-988)</td>
<td>104 (4-465)</td>
<td>HC vs VI</td>
</tr>
<tr>
<td>Viral load (copies/ml)</td>
<td>NA</td>
<td>&lt;100</td>
<td>129,092 (4,189-676,811)</td>
<td>AV vs VI</td>
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</tbody>
</table>

\(^{a}\) the median value is shown, with the range in parentheses

\(^{b}\) the duration of cART regimen was greater than 6 months for all aviremic subjects

\(^{c}\) all HIV-1 infected subjects (aviremic and viremic) were classified as CDC Stage C3