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B-Lymphocyte Dysfunction in Chronic HIV-1 Infection Does Not Prevent Cross-Clade Neutralization Breadth

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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 autoreactivity (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, a delicate interplay among several regulatory receptors tightly governs activation of the immune system. Recently, the importance of programmed death-1 (PD-1) and B- and T-lymphocyte attenuator (BTLA) is linked with dysregulation and exhaustion of T lymphocytes during chronic human immunodeficiency virus type 1 (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) was 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 were 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 in which B lymphocytes display multiple indications of dysfunction, antibodies that mediate cross-clade neutralization breadth continue to circulate in plasma.
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 not on ART and had plasma viral loads greater than 1,000 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 was collected from each participant in acid-citrate-dextrose (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 (−180°C) until needed for flow cytometry.

**Flow-cytometric analysis of peripheral B cells.** PBMCs were thawed and washed twice with phosphate-buffered saline (PBS) and then resuspended in fluorescence-activated cell sorter (FACS) buffer (PBS with 1% bovine serum albumin [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-CD4 V500 (M5E2), anti-PD-1 APC (EH12.2H7), anti-BTLA PE (J168-fluorescent reactive dye (live/dead stain), anti-CD3 V500 (SP34-2), anti-CD14 V500 (M5E2), anti-CD19 Qdot655 (SJ25C1), anti-CD10 APC-Cy7 (HI10a), anti-CD21 PE-Cy5 (B-ly4), anti-CD27 PE-Cy7 (1AA4CD27), 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 red blood cells (RBC) was removed by incubation in 1 × lysing solution (BD Bioscience) for 10 min at room temperature. For intracellular staining, PBMCs were further washed twice with FACS buffer and permeabilized with 1 × 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 resuspended 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 versus side scatter profile, and B lymphocytes were gated as CD19− cells after exclusion of dead, CD3+, and CD14+ cells. Data were analyzed using FlowJo software (version 9.3.1; TreeStar Inc., USA).

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

**ELISA 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 h. Plates were washed 3 times, and 100 μl of horseradish peroxidase (HRP)-conjugated goat anti-human IgG was added to each well. After a 1-h incubation at 37°C, plates were washed, and 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added. After 10 min, reactions were stopped with 4 N H2SO4, endpoint absorbance was measured at 450 nm with a BioTek Synergy multidetection microplate reader, and data were 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 50% inhibitory concentration (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 for each given plasma-Env combination by the median IC50 for that pseudovirus against all plasma samples and (ii) adding the scores for each plasma sample, as described in reference 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, SS196.1, TRO.11, AC10.0.29, and PVO.4 are from the standard Env reference panel for subset B HIV-1 Env clones (23); ZM197MP7, Du172.17, Du156.12, ZM109PB4, 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 Q789ENV22 (25) were contributed by 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, the 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. The plasma HIV-1 RNA level was measured using the Cobas AmpliC Abbott 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.** Nonparametric one-way analysis of variance (1-way ANOVA, Kruskal-Wallis with Dunn's posttest) and Spearman's 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) (Fig. 1). In HC, only a minor proportion of B cells expressed PD-1 (Fig. 1A), consistent with what is observed in T lymphocytes (7, 52). In VI, expression of PD-1 was significantly increased (Fig. 1A, *P* < 0.001). AV subjects had PD-1 expression levels that were significantly lower than those of VI (Fig. 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, which is alleviated by cART. Despite a greater percentage of B cells expressing PD-1 in VI, the level of receptor expression per CD19<sup>+</sup> B cell was not different from that of HC or AV (Fig. 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 (Fig. 1C). However, VI showed a significant decline in BTLA expression compared to HC (Fig. 1C, *P* < 0.001). AV individuals had intermediate levels of BTLA expression that were significantly different from that of both HC and VI (Fig. 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<sup>+</sup> BTLA<sup>+</sup> cells were significantly decreased.

**FIG 1** Expression of PD-1 and BTLA by B lymphocytes. (A and C) Percentages of total B cells (CD19<sup>+</sup>) that express PD-1 (A) and BTLA (C) in HC, AV, and VI subjects. (B and D) Mean fluorescence intensity (MFI) for PD-1 (B) and BTLA (D) expression by individual PD-1<sup>+</sup> or BTLA<sup>+</sup> CD19<sup>+</sup> B cells. 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: *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001. (E) Correlation between percentages of total B cells that express PD-1 and BTLA. Spearman's rank correlation coefficient (r) and level of significance (p) are indicated within the graph. Open diamonds, HC; closed triangles, AV; closed circles, VI.
lower in VI and AV than in HC (Fig. 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 (Fig. 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 levels 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^hiCD27^+), and classical memory (CD10^-CD21^hiCD27^-), 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 (Fig. 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 between the proportions seen in HC and in VI.

The B cell subsets differed in their respective PD-1 and BTLA expression patterns (Fig. 2C and D, respectively). In HC, low PD-1 expression was observed in all subsets, but particularly within naïve and memory B cells (Fig. 2C). In VI, PD-1 expression was significantly increased in the naïve and immature subpopulations of B cells compared to HC (Fig. 2C, P < 0.01 and P < 0.001, respectively). However, PD-1 expression on naïve and immature B cells in AV was not different from HC, indicating some partial restoration (Fig. 2C). BTLA expression in VI was significantly decreased in the naïve, immature, and memory B cell subsets compared to HC and AV (Fig. 2D, P < 0.001 and at least P < 0.05, respectively). BTLA expression in AV was not different from that of HC except in immature B cells, where it remained significantly lower than in HC (Fig. 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 (Fig. 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 is 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 was 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) (Fig. 3A and B, respectively), whereas BTLA exhibited an inverse correlation with these markers (Fig. 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 (Fig. 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 (Fig. 4C and D, respectively, P < 0.0001). 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 is 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 ex-
pression 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 (Fig. 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 between total IgG level and PD-1 or BTLA expression on B cells were identified (Fig. 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 these data were used to calculate a breadth (how many Envs were neutralized) and potency (the strength of neutralization) score for each plasma sample, as described in reference 37. Infectivity curves for each plasma sample are shown in Fig. S1 in the supplemental material. 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) (Fig. 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 (Fig. 6B and C, P = 0.009 and P = 0.02, respectively). We next quantitated the level of antibodies that bind 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 toward significance (Fig. 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 and 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 simian-human immunodeficiency virus (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 a 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 pretreatment 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 that of 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). Similarly in our study, peripheral blood CD4 T cell count was also inversely and directly correlated with PD-1 and BTLA expression, respectively, on B lymphocytes. 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 HIV-1 strains.
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 ab-
errant 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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