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BLyS-Mediated Modulation of Naive B Cell Subsets Impacts HIV Env-Induced Antibody Responses

Pia Dosenovic,* Martina Soldemo,* Jean L. Scholz,† Sijy O’Dell,§ Emilie K. Grasset,§ Nadège Pelletier,§ Mikael C. I. Karlsson,§ John R. Mascola,‡ Richard T. Wyatt,§ Michael P. Cancro,† and Gunilla B. Karlsson Hedestam*

Neutralizing Abs provide the protective effect of the majority of existing human vaccines. For a prophylactic vaccine against HIV-1, broadly neutralizing Abs targeting conserved epitopes of the viral envelope glycoproteins (Env) are likely required, because the pool of circulating HIV-1 variants is extremely diverse. The failure to efficiently induce broadly neutralizing Abs by vaccination may be due to the suboptimal immunogens or immunization regimens, or it may indicate that B cells specific for broadly neutralizing Env determinants are selected against during peripheral checkpoints, either before or after Ag encounter. To investigate whether perturbation of B cell subsets prior to immunization with recombinant Env protein affects the vaccine-induced Ab response in mice, we used B lymphocyte stimulator (BLyS), a cytokine that regulates survival and selection of peripheral B cells. We show that the transient BLyS treatment used in this study substantially affected naïve B cell populations; in particular, it resulted in more B cells surviving counter-selection at the transitional stages. We also observed more mature naïve B cells, especially marginal zone B cells, in BLyS-treated mice. Intriguingly, provision of excess BLyS prior to immunization led to a consistent improvement in the frequency and potency of HIV-1 Env vaccine-induced neutralizing Ab responses, without increasing the number of Env-specific Ab-secreting cells or the Ab-binding titers measured after boosting. The results presented in this article suggest that an increased understanding of BLyS-regulated processes may help the design of vaccine regimens aimed at eliciting improved neutralizing Ab responses against HIV-1.

Efforts to elicit broadly neutralizing Abs (bNAb) against HIV-1 through envelope glycoprotein (Env) vaccination have been unsuccessful, despite robust Ab titers to multiple epitopes on Env stimulated by current vaccine candidates. Even during chronic HIV-1 infection, bNAb are elicited in only a subset of infected individuals, usually only after years of active viral replication (1). This suggests that effective Ab cell responses against bNAb epitopes on Env are infrequent and subject to limitations imposed by extensive immune-selection pressure for resistant isolates during infection. The barriers to achieving appropriate Ab specificity and affinity maturation following vaccination are substantial and may be reflective of a variety of factors, including suboptimal presentation of bNAb epitopes on candidate Env immunogens, insufficient affinity maturation of critical Ab specificities, as well as potential limitations in the B cell repertoire caused by events that occur either before or after B cell exposure to Ag.

Developing B cells undergo counter-selection at multiple checkpoints during maturation, resulting in the loss of most emerging BCR reactivities. At the transitional developmental stages (2), about two thirds of newly formed B cells migrating from the bone marrow (BM) die before entering mature pre-immune pools. These losses reflect selection based on BCR signal strength (3, 4) and mediate the elimination of autoreactive and polyreactive specificities in both mice and humans (5, 6). Accordingly, if clonotypes capable of broadly neutralizing activity against HIV-1 are prone to deletion at the transitional stage, their frequency in the preimmune repertoire may be low to nil. Indeed, some HIV-1 infection-elicited bNAb share features with specificities prone to elimination during transitional differentiation, such as long H chain CDR3 (7) or polyspecificity (8, 9). Alternatively, broadly neutralizing clonotypes or their precursors may survive to populate the preimmune pools and respond to Ag exposure, yet fail to persist as the immune response evolves and peripheral tolerance mechanisms come into play (10). After Ag activation and costimulation, B cells enter the germinal center (GC) reaction where novel specificities are generated through somatic hypermutation (SHM). Among these newly arising specificities, those that most effectively compete for Ag and survival signals selectively persist and differentiate into memory and Ab-secreting plasma cells (11). Thus, if bNAb specificities are rarely generated by SHM, or if these clones are poor competitors within the GC, their entrance into memory or Ab-forming pools may occur at a very low frequency.

Abbreviations used in this article: ALP, alkaline phosphatase; ASC, Ab-secreting cell; BLyS, B lymphocyte stimulator; bNAb, broadly neutralizing Ab; BM, bone marrow; BR3, B lymphocyte stimulator receptor 3; CD4bs, CD4 binding site; Env, envelope glycoprotein; FO, follicular; GC, germinal center; LN, lymph node; MZ, marginal zone; ON, overnight; PC, phosphorylcholine; RT, room temperature; SHM, somatic hypermutation.
The B lineage-specific survival factor, B lymphocyte stimulator (BLyS; also termed BAFF), plays key roles in peripheral B cell development, homeostasis, and selection. Although BLyS binds three different receptors, its most profound effects are mediated by signaling through BLyS receptor 3 (BR3, also termed BAFF-R), which is expressed by transient, mature naive, and GC B cells (12). There is ample evidence that the BLyS/BR3 axis modulates selection at the transitional stages, because mice and humans deficient in either BLyS or BR3 show severely compromised transitional and mature naive B cell pools (13, 14), whereas BLyS overexpression yields B cell hyperplasia and signs of autoimmunity (15, 16). Furthermore, in studies using transgenic mice, exogenous BLyS treatment rescues specificities normally lost at the transitional stage, allowing them to enter the mature preimmune pools (5). There is evidence for a similar role of the BLyS/BR3 axis in GC evolution (17), although the mechanisms have not been determined. For example, GCs can be initiated, but are not sustained, in mice deficient in BLyS (18).

In this study, we investigated whether potential counter-selection of bNAb specificities, particularly at the transitional checkpoint, might be overcome by transient manipulation of BLyS levels. We treated mice with BLyS immediately prior to immunization and observed a marked increase in transitional and mature naive B cell numbers, indicating a significant increase in cells surviving selection at the transitional checkpoint. Upon termination of BLyS treatment, these subsets returned to pretreatment levels in less than a week. When BLyS- and control-treated mice were immunized with recombinant Env trimers (19), we found a consistent increase in the frequency of animals displaying HIV-1-neutralizing activity among the BLyS-treated group. These results intriguingly suggest that manipulation of selective processes in the peripheral B cell compartment may be used to qualitatively improve vaccine-elicted neutralizing Ab responses against HIV-1.

Materials and Methods
Recombinant BLyS and HIV-1 Env glycoproteins
Recombinant human BLyS was kindly provided by Human Genome Sciences (Rockville, MD). Recombinant soluble HIV-1 Env gp140 trimers based on the YU2 isolate of HIV-1, gp140-F (19), were produced by transient transfection, as previously described (20), and used for immunizations. In brief, cells were transfected at a density of 1.1 × 10⁶/ml in GIBCO FreeStyle 293 expression media using 293Fectin, according to the manufacturer’s instructions (Invitrogen). Supernatants were collected 4 d after transfection. Following collection, all supernatants were centrifuged at 3500 × g to remove cells or cell debris, filtered through a 0.22-μm filter, and supplemented with complete, EDTA-free protease inhibitor mixture (Roche) and penicillin-streptomycin (Invitrogen). Proteins were captured via glycans by lentil–lectin affinity chromatography (GE Healthcare). After extensive washing with PBS, supplemented with 0.5M NaCl, the proteins were eluted with 1 M methyl imidazole and 0.5 M NaCl in PBS, proteins were eluted with 300 mM imidazole in PBS. Biotinylated Env probes used in the B cell ELISPOT assay (21) were produced in a similar fashion.

Treatment of mice and preparation of cells and tissue for analysis
Adult BALB/c mice (Taconic A/S) were injected i.p. with 10 μg recombinant BLyS or PBS once daily for 10 d. At the indicated time points after treatment, mice were either sacrificed for analysis or immunized s.c. with 10 μg HIV-1 Env in 10 μg of the adjuvant AbSciCO-100 (Isonova). Animals were boosted once or twice with the same regimen 14 d apart. In some experiments (Fig. 5C, 5D), a third boost was given 60 d after the second boost. Animals were sacrificed at 4 or 21 d after the last immunization. Single-cell suspensions were prepared from spleen, BM (two femur and two tibia/mouse), or inguinal lymph node (LN), as described (21). For immunostaining of spleen, tissue was immersed in Optimal Cutting Medium (Histolab) and snap-frozen in 2-methyl butane that was kept cold in liquid nitrogen. All animal experiments were approved by the Committee for Animal Ethics (Stockholm, Sweden), and performed according to specified guidelines.

Flow cytometry
Single-cell suspensions from spleen and LN were stained with the following Abs: PerCP anti-B220 (RA3-62B), biotinylated anti-CD23 (B2B4), PE anti-IGM (R6-60.2), PE anti-CD95 (Jo2), alkaliphycocyanin anti-CD8 (53-6-7) all from BD Biosciences, as well as alkaliphycocyanin anti-CD93 (AA4.1), Alexa Fluor 488 anti-GL7, PE anti-CD1d (1B1), PE anti-CD3 PE (145-2C11) and FITC anti-CD4 (RM4-4), all from eBiosciences. Biotinylated Ab was visualized with Alexa Fluor 488-conjugated streptavidin (Invitrogen). Stained cells were fixed in fixation buffer (BD Biosciences) and analyzed on a FACS Calibur (BD Biosciences).

ELISA
The HIV-1 Env-specific ELISA was performed by coating 96-well ELISA plates (Nunc) with 100 μl soluble HIV-1 Env protein diluted to a concentration of 2 μg/ml in PBS. After overnight (ON) incubation at 4°C, plates were washed in PBS with 0.05% Tween (wash buffer). The plates were then blocked for 1.5 h at room temperature (RT) in PBS with 2% dry milk (blocking buffer). Serum was added in fresh blocking buffer and incubated for 1.5 h at RT. Plates were washed six times in wash buffer, and the secondary Ab, goat anti-mouse IgG-HRP, was added in wash buffer. Plates were incubated for 1.5 h at RT. The plates were washed six times in wash buffer, and the assay was developed using the SigmaFAST OPD kit (Sigma-Aldrich). The reaction was stopped by the addition of 1 M H₂SO₄, and the OD was read at 492 nm using an Assys Expert 96 ELISA reader (Biochrom).

For detection of anti-dsDNA Abs, ELISA plates were precoated with 50 μl met-BSA (Sigma-Aldrich) diluted to a concentration of 5 μg/ml in PBS and incubated for 6 h at 4°C. Plates were then washed four times in PBS with 0.05% Tween (wash buffer) and coated with 50 μg DNA (Sigma-Aldrich) diluted to a concentration of 50 μg/ml in PBS. For detection of anti-phosphorylcholine (PC) Abs, ELISA plates were coated with 50 μl PC-BSA diluted to a concentration of 2.5 μg/ml in PBS at 4°C ON. After washing four times in wash buffer, both sets of plates were blocked with PBS containing 1.5% BSA, 0.1% gelatin, and 3 mM EDTA (blocking buffer) for 2 h at RT. Serum was then added in fresh blocking buffer and incubated for 2 h at RT prior to washing four times in wash buffer. Secondary Abs, anti-mouse IgM-Alkaline phosphatase (ALP) (Mabtech) or anti-mouse IgM-HRP (Southern Biotech), were added in blocking buffer and incubated for 1 h at RT. Depending on the secondary Ab used, the ELISA was developed using either SIGMAFAST p-Nitrophenyl phosphate mix (Sigma-Aldrich), and the reaction was stopped by adding 3 N NaOH, or SureBlue TMB Microwell Peroxidase Substrate (KPL), and the reaction was stopped by adding TMB Stop Solution (KPL). The OD was measured at 405 or 450 nm, respectively, using an Assys Expert 96 ELISA reader (Biochrom).

The cardiolipin ELISA was performed by coating 96-well ELISA plates (Nunc) with 50 μl cardiolipin (Sigma-Aldrich) diluted to a concentration of 50 μg/ml in 99.5% EtOH. The plates were allowed to evaporate completely at 4°C ON. The plates were blocked in PBS with 10% FCS (blocking buffer) for 1 h at 37°C. Serum was added in fresh blocking buffer and incubated for 1 h at 37°C. The plates were then washed in PBS only. Secondary Ab anti-mouse IgM-HRP (Southern Biotech) was added in blocking buffer, and the plates were washed in fresh blocking buffer and incubated for 1 h at 37°C, washed in PBS only, and developed as described above.

To measure total IgM levels in serum, 96-well ELISA plates (Nunc) were coated with 100 μl polyclonal goat anti-mouse IgM (Southern Biotech) diluted to a concentration of 2 μg/ml in 0.05 M carbonate buffer. Plates were incubated ON at 4°C. Next, ELISA plates were washed six times in PBS with 0.05% Tween (wash buffer) and blocked for 1.5 h in PBS with 5% FCS (blocking buffer) at RT. Serum was added to fresh blocking buffer and incubated for 2 h at RT. The plates were washed six times in wash buffer and secondary Ab, anti-mouse IgM-ALP (Mabtech) or anti-mouse IgM-HRP, was added in blocking buffer. After 1.5 h of incubation at RT the plates were washed six times in wash buffer. The assay was developed as described above.

B cell ELISPOT
Env-specific and total IgG Ab-secreting cells (ASCs) were enumerated in a B cell ELISPOT assay, as previously described (21). Briefly, 96-well MultiScreen-IP filter plates (Millipore) were pretreated with 70% ethanol and washed three times in sterile PBS before being coated with 1 μg/well (10 μg/ml) a polyclonal goat anti-mouse IgG Ab (Mabtech). The plates were incubated ON at 4°C. Two hours before addition of the cells, the plates were washed five times in sterile PBS and blocked with complete

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Exogenous BLyS treatment increases primary B cell numbers but does not alter splenic architecture

To characterize the effect of transient BLyS treatment on B cell compartments, we injected mice with recombinant BLyS or PBS (controls) daily for 10 d prior to analysis. Consistent with prior reports (27), spleen size and weight increased in BLyS-treated mice (Fig. 1A), reflecting expanded B cell numbers without significant effects on T cells (Fig. 1B, left panel). A similar effect on B cell populations was seen in LN (Fig. 1B, right panel). The

**FIGURE 1.** BLyS treatment increases B cell numbers but leaves the splenic architecture unaffected. Mice were injected with PBS or BLyS once daily for 10 d and were sacrificed on day 11 for analysis of lymphocytes in spleen and LN. (A) Representative image of spleens (left panel) and measurements of spleen weight (right panel). (B) Effect of BLyS treatment on B (B220+) and T cell (CD3+) numbers in splenic LN (left panel) and the two inguinal LNs (right panel). (C) Representative flow cytometry plots of B cell subsets in PBS- and BLyS-treated mice; left panel, immature naive B cells (B220+, CD93+); middle panel, T1 (IgMhi, CD23+), T2 (IgMlo, CD23+); right panel, FO (CD23hi, CD1dlo) and MZ B cells (CD23lo, CD1dhi). (D) Effect of BLyS treatment on splenic B cell subsets: transitional (T1, T2, T3), FO, MZ, and ratio of FO/MZ B cells in PBS- and BLyS-treated mice. Black bars: PBS treated, white bars: BLyS treated. (E) Confocal microscopy images of spleen sections: TCRβ (T cells, green), B220 (B cells, blue), and metallophilic macrophages (red). The MZ area is present outside of metallophilic macrophages, as indicated. Stains used: allophycocyanin-conjugated B220, Alexa Fluor 488-conjugated TCRβ, and biotinylated MOMA-1 in combination with Cy3-conjugated streptavidin from Jackson Immunoresearch. Slides were scanned on a Leica TCS SP5X, using a 10×/0.30 magnification objective: Leica 506505 HCX FLUOTAR. Leica Application Suite Advanced Fluorescence software was used to prepare images, and ImageJ was used for final adjustment of brightness and contrast.

Neutralization assays

Neutralization assays were performed using a single round of infection of HIV-1 Env pseudoviruses and TZM-bl target cells, as previously described (22, 23). In total, we had enough sera from individual mice to analyze neutralization against four tier 1 viruses (MN, HXBc2, SF162, and BaL) and one tier 2 virus (6535). Results are reported as the serum neutralization ID50, the reciprocal of the serum dilution producing 50% virus neutralization without subtracting prebleed values. For competition-neutralization assays, test or control ligand was added to the serum 30 min prior to the addition of virus. V3 sp. act. was mapped using a YU2-derived V3 peptide and a scrambled control peptide, as previously described (24). CD4 binding site (CD4bs)-directed neutralizing activity was mapped using a pair of probes referred to as TriMut and TriMut368/70 (25, 26).

Statistical analysis

Data that passed the D’Agostino and Pearson omnibus normality test were analyzed by unpaired, two-tailed Student t tests; data that did not pass were analyzed by the Mann–Whitney U test. GraphPad Prism software was used for all analyses: data were considered significant at *p < 0.05, **p < 0.01, and ***p ≤ 0.001.

Results

Exogenous BLyS treatment increases primary B cell numbers but does not alter splenic architecture

To characterize the effect of transient BLyS treatment on B cell compartments, we injected mice with recombinant BLyS or PBS (controls) daily for 10 d prior to analysis. Consistent with prior reports (27), spleen size and weight increased in BLyS-treated mice (Fig. 1A), reflecting expanded B cell numbers without significant effects on T cells (Fig. 1B, left panel). A similar effect on B cell populations was seen in LN (Fig. 1B, right panel). The
numbers of transitional (T2 and T3) B cells increased significantly in BLyS-treated mice, and there was an accumulation of both follicular (FO) and marginal zone (MZ) B cells, especially MZ B cells, as shown by the decreased FO/MZ B cell ratio (gated populations are shown in Fig. 1C, calculated as absolute numbers in Fig. 1D). Immunofluorescence analysis of frozen spleen sections from PBS- and BLyS-treated mice showed normal splenic architecture, suggesting that trafficking and homing of cells were largely unaffected by BLyS treatment (Fig. 1E).

Under conditions in which the BLyS level is permanently increased and the homeostatic regulation of B cells is defective, such as in BLyS transgenic mice, immature self-reactive B cells survive peripheral selection checkpoints and progress to mature naive pools as in BLyS transgenic mice, immature self-reactive B cells survive peripheral selection checkpoints and progress to mature naive pools (5, 15, 16, 28, 29). To investigate whether BLyS treatment promoted survival of autoreactive B cells, we measured Ab titers against dsDNA, PC, and cardiolipin. Immediately after cessation of BLyS treatment, we found elevated levels of anti-dsDNA, anti-PC, and anti-cardiolipin Abs (Fig. 2A, left panels). However, the total Ab levels at this time point were also increased; when normalized to these, there was no difference between BLyS- and control-treated animals (Fig. 2A, right panels). Furthermore, when anti-dsDNA and anti-PC Ab responses were measured 19 d after termination of BLyS treatment, the levels were no longer increased in BLyS-treated mice (Fig. 2B). Within 5 d of the last BLyS treatment, spleen weights (Fig. 3A), B lymphocyte numbers (Fig. 3B), and the number of IgM-secreting cells/spleen (Fig. 3C) also approached pretreatment levels. Accordingly, we chose to initiate Env immunizations 1 d after the last day of BLyS treatment, when peripheral B cells were maximally affected.

**BLyS treatment prior to Env immunization does not affect the magnitude of the Env-specific B cell response**

Codelivery of Ag fused to BLyS or the use of BLyS as an adjuvant component administered at the same time as the Ag were reported previously (30–32). In this study, we investigate the magnitude and quality of Env vaccine-elicited B cell responses after BLyS pretreatment, using HIV-1 gp140-F trimers in adjuvant. The gp140-F trimers possess a heterologous trimerization motif (F) appended to the Env ectodomain (gp140) and are derived from the primary HIV-1 isolate, YU2 (19) (Fig. 4A). Immunized mice were sacrificed for analysis 4 or 21 d after the boost, and the presence of mature splenic B cells with a GC phenotype was investigated. We detected no significant difference in the number of cells with this phenotype between BLyS- and control-treated mice at either time point after the boost, suggesting that GC formation is not sensitive to BLyS treatment, when peripheral B cells were maximally affected.

**Envi subdeterminants, with the exception of an increase in the non-gp41, non-V123 fraction referred to as “Other” in BLyS-treated animals. However, this difference was not consistently observed (Fig. 4D). We also examined the Env-specific B cell memory compartment after in vitro LPS stimulation of splenocytes collected 21 d after the second immunization. We observed an increase in the number of memory B cells in BLyS-treated animals compared with controls but no difference in the number of Env-specific or Env-subspecific memory B cells (Fig. 4E). Taken together, these data demonstrate that BLyS treatment prior to im-
munization did not affect the magnitude of the total Env-specific B cell response.

BLyS treatment prior to immunization increases the frequency and potency of neutralizing Ab responses against a subset of viruses

To investigate the quality of the elicited B cell response at a functional level, we next focused on the serological Ab response. We first assessed Ab-binding titers against YU2 Env in BLyS- and control-treated mice immunized twice with the HIV-1 Env trimers. Despite the profound difference in B cell subsets observed at the time of priming, we detected similar titers of Env-specific Abs in the two groups (Fig. 5A), in agreement with the B cell ELISPOT results. Next, we sought to examine the capacity of the sera to neutralize diverse HIV-1 strains, a powerful assay to detect qualitative or quantitative differences in elicited Ab functional activity. We used a panel of five heterologous tier 1A and 1B HIV-1 Env pseudotyped viruses and a negative control virus pseudotyped with SIV Env, which was the maximum number of viruses that we could include based on the available serum volumes. When these viruses were used in a well-standardized neutralization assay (22), we detected neutralizing activity against the tier 1A clade B viruses (MN, HXBc2, and SF162) and the clade B tier 1B virus (6535) in sera from some BLyS-treated animals; this was not

FIGURE 3. The effect of BLyS treatment on lymphocyte populations is transient. At different times after day 10 of BLyS or PBS treatment, the weight of the spleen (A), flow cytometric analysis showing the total numbers of B220+, CD3+, CD4+, and CD8+ cells in spleen (B), and B cell ELISPOT analysis of the number of total IgM-secreting cells in spleen (C) were measured. Mean values ± SD are shown in all diagrams. The data are from one experiment with 3–6 mice/group.

FIGURE 4. GC and Env-specific B cell numbers are similar in PBS- and BLyS-treated mice. (A) Schematic diagram of the gp140-F Env immunogen and the treatment/immunization regimen. (B) Representative flow cytometry plots showing splenic GC B cells (B220+, CD93\(^{hi}\), GL7\(^{hi}\), and CD95\(^{hi}\)) in PBS-treated mice (upper panels), and BLyS-treated mice (lower panels) 21 d after boosting. Right panel, Splenic GC cells in animals terminated at 4 or 21 d after boosting. (C–E) Enumeration of Env-specific B cells by B cell ELISPOT analysis; total IgG- and gp140-specific cells (left panels) and subregion-specific cells (gp41, V123, and Other, right panels). (C) ASCs in spleen 4 d after boost. (D) ASCs in BM 21 d after boost. (E) ASCs from LPS-cultured splenocytes collected 21 d after boost. Mean values ± SD are shown in all panels. Data in (B) (4 d) are representative of two independent experiments with 7–12 mice/group; data in (C, left panel) are representative of three independent experiments with 4–12 mice/group. The remaining results in (B)–(E) are from one experiment with 12 mice/group.
observed in control-treated mice (Fig. 5B). To confirm this effect, we performed two independent immunization experiments with additional boosts (3 × Env and 4 × Env), but otherwise replicated the same conditions that we used in the first experiment (2 × Env).

Overall, the frequency of mice displaying detectable neutralization titers was increased in these repeat experiments compared with the first experiment, likely reflecting higher Env-binding titers elicited by the additional boosts (Fig. 5C). Consistent with the first experiment, we saw no difference in total Env-binding Ab titers between BLyS- and control-treated animals, yet the neutralizing activity against MN, HXBc2, SF162, and 6335 was more frequent and more potent in BLyS-treated animals; almost all mice displayed neutralizing activity, some at relatively high titers (Fig. 5D).

When the number of mice responding with positive neutralizing activity against MN, HXBc2, SF162, and 6335 in the control- and BLyS-treated groups was compared, we observed a statistically significant difference (Fig. 5E). We also observed statistically significant differences between the PBS- and BLyS-treated mice when the average ID50 neutralization titers against MN and SF162, but not against HXBc2 and 6535, were compared (Fig. 5F). There was no correlation between YU2 Env-binding titers and virus-neutralizing activity (Supplemental Fig. 1A), suggesting that the improved neutralizing activity of the serum Abs was not simply due to an increase in overall Ab titers against the Ag used for immunization. Because YU2 Env is heterologous to the viruses used in the neutralization assay, we also measured serum-binding titers against recombinant MN and SF162 Env proteins. These

**FIGURE 5.** Sera from mice immunized twice (2 ×) with Env in adjuvant collected 21 d after the second immunization were analyzed for total Env-binding Abs. OD50 values are shown for total Env-specific Ab titers. Each point in the ELISA analysis represents data from one mouse and the bar indicates the mean of all values ± SD. (B) The sera in (A) were also analyzed for neutralizing activity against a panel of heterologous HIV-1 isolates. Neutralization data are shown as the reciprocal dilution giving 50% neutralization (ID50 titer). (C) Sera from animals immunized three (3 ×) or four times (4 ×) with Env in adjuvant collected 4 d after the last immunization were analyzed for Env-binding Abs as in (A). (D) The sera from 3 × and 4 × Env-immunized animals described in (C) were analyzed for neutralization activity in the same way as described in (B). (E) The number of mice with detectable neutralization activity against MN, HXBc2, SF162, or 6535, combining all experiments (two sequential injections [2 × Env], three sequential injections [3 × Env], and four sequential injections [4 × Env] of Env), was plotted and analyzed by a nonparametric Mann–Whitney test. To have an equal number of mice in each group, one mouse was removed from the 4 × Env BLyS group. This mouse (number 46) displayed the highest ID50 neutralization titer against several of the viruses. Thus, by removing this mouse from the comparison, we underestimate rather than overestimate the effect caused by the BLyS treatment. (F) The potency of neutralization was also analyzed by calculating the average ID50 neutralization titers against MN, HXBc2, SF162, and 6535 among PBS- and BLyS-treated mice, combining the three experiments (2 × Env, 3 × Env, and 4 × Env). Statistical significance was analyzed using the nonparametric Mann–Whitney test. The ELISA data from 2 × are from one experiment with 12 mice per group; the results from 3 × are representative data from two independent experiments with 5–7 mice per group, and the data from 4 × are from one experiment with 4–5 mice per group. The neutralization data are from one experiment each for the 2 × (12 mice per group), 3 × (7 mice per group) and 4 × (4–5 mice per group) groups.
analyses showed increased binding titers against MN, providing a possible explanation for the improved MN-neutralizing activity in BLyS-treated animals. However, a similar relationship between binding and neutralization was not observed for SF162 (Supplemental Fig. 1B).

The specificity of the HIV-1-neutralizing activity was confirmed by two independent assays, the first of which used SIV Env, which is not cross-reactive with HIV-1 Env, resulting in no activity above background in any of the sera (Fig. 5B, 5D). To further confirm the specificity of the neutralizing activity elicited in BLyS-treated mice, as well as to map the response to distinct antigenic regions on Env by ligand-competition analysis, we tested selected sera from BLyS-treated mice displaying the highest neutralization titers. The ligands included in these analyses were designed to differentially detect neutralizing Ab activity directed against CD4bs, TriMut, and TriMut-368/70, described in (26), as well as the V3 region (V3 peptide and scrambled V3 peptide). To validate this mapping approach, we show that neutralization of HXBc2 by the CD4bs-directed mAb IgG1b12 was effectively absorbed by TriMut, but not by TriMut-368/70, whereas no neutralization of HXBc2 was detected with the V3-directed mAb 447-52D (Fig. 6A, left panels). Neutralization of MN by 447-52D was absorbed by the V3 peptide, but not by the scrambled V3 control peptide, and no neutralization was detected of MN with IgG1b12 (Fig. 6B, left panels). When analyzing the sera from BLyS-treated mice, we found that CD4bs-directed Abs were responsible for a significant fraction of the neutralizing activity against HXBc2 in mouse #13 (Fig. 6A, upper right panel), whereas V3-specific Abs were responsible for a significant fraction of the neutralizing activity against MN in mouse #22 and part of the neutralizing activity in mouse #13 (Fig. 6B, right panels). These analyses confirm that the Ab response in these responding mice was Ag-specific and directed against distinct neutralizing determinants of HIV-1 Env.

**Discussion**

Current knowledge suggests that HIV-1 has evolved to render conserved Env determinants poorly immunogenic to escape recognition by bNAbs. Consistent with this, it was suggested that some bNAb epitopes on Env display self-like properties and necessitate autoreactive Abs for efficient epitope recognition (8, 10, 33). Such regions include the membrane proximal external region of gp41, recognized by the prototypic membrane proximal external region-directed MAbs 2F5 and 4E10, which were shown to cross-react with cardiolipin (8). Although the role of cardiolipin cross-reactivity and the relationship of that property with regard to efficient virus neutralization has been a subject of some debate (34), it was shown that 4E10 depends on the presence of aromatic amino acids in its H chain CDR3 region for efficient recognition of its epitope, perhaps through lipophiliic interactions (35). Furthermore, polyreactivity appears to be a common feature of neutralizing Abs elicited during chronic HIV-1 infection (9, 36, 37). Thus, the possibility exists that B cells specific for some bNAb targets are subject to counter-selection at one or several stages during the path toward an ASC. It follows that if such processes are at play, they may diminish the likelihood of eliciting desired neutralizing B cell responses by vaccination. To investigate this possibility experimentally, we established a system to perturb B cell homeostasis and selection by transient provision of recombinant BLyS, prior to immunization with HIV-1 Env. We show that the BLyS treatment markedly increased the proportion of B cells surviving through the transitional stages, resulting in substantially increased numbers of follicular and MZ B cells and a reduced ratio of follicular: MZ B cells, consistent with previous reports (38). The effect of BLyS was short-lived, with B cell numbers already returning to near-baseline levels 5 d after treatment termination.

When mice were primed with soluble Env trimers in adjuvant, on 1 d after termination of the BLyS treatment when the B cell populations were maximally affected, a substantial and consistent increase in the frequency of mice displaying neutralizing activity was observed in the BLyS-treated group at the time of serum analysis, ≥18 d after priming. We did not observe an improvement in terms of breadth of neutralization, but neutralizing activity in the BLyS-treated animals was more frequent and, against some viruses, more potent and could be assigned to distinct Env subreactivities by serological mapping analysis of samples with high neutralizing titers. Interestingly, the frequency of Env-specific ASCs, as measured by B cell ELISPOT analysis in multiple B cell compartments, including splenic plasma cells (Fig. 4C), BM plasma cells (Fig. 4D), and in vitro-cultured splenic memory B cells (Fig. 4E) after boosting, were comparable be-
tween control and BLyS-treated mice. We also did not observe a difference between the groups in terms of the total binding Ab titers to the YU2 Env immunogen, suggesting that, despite a larger naive B cell pool at the time of priming, the magnitude of the vaccine-induced response was not augmented. This may be consistent with effective feedback mechanisms, such as FCRyII signaling, regulating the magnitude of the elicited B cell response. When the relationship between YU2 Env binding and neutralizing activity was analyzed for individual mice, we observed no correlation for any of the viruses against which neutralizing activity was detected (r² = 0.20 for MN, r² = 0.21 for HXBc2, r² = 0.040 for SF162, and r² = 0.085 for 6435) (Supplemental Fig. 1A). Instead, several mice displaying high Env-binding titers did not possess detectable neutralizing activity; conversely, neutralizing activity was occasionally detected in serum displaying modest Env-binding titers. When binding titers were measured against the matched Env glycoprotein, improved neutralization of MN could be explained by an increased binding titer to the homologous (MN) Env in some mice. However, a similar effect was not observed for SF162-directed neutralization, because there was no difference between BLyS-treated and control mice in terms of binding Abs against SF162 Env, and the individual mice in the BLyS group displaying neutralizing activity against SF162 (#19 and #22) were not the same as those with the highest binding titers to SF162 Env (Supplemental Fig. 1B). Collectively, this analysis suggests that BLyS pretreatment had a qualitative effect on the Env-specific B cell response.

There are several possible explanations for the observed effects, because BLyS can affect B cell survival at multiple stages. The best-established activity of BLyS is at the transitional checkpoint, where, under normal circumstances, immature B cells compete for available BLyS before surviving clones mature and populate the B cell follicles (14, 39). Recent evidence suggests that BLyS also modulates GC evolution (reviewed in Ref. 40). In the studies presented in this article, we believe that it is most likely that BLyS improved survival of B cells prior to BCR-specific activation, thereby affording increased representation of Ag-responsive neutralizing clonotypes in BLyS-treated mice. We consider it less likely that the effect arose from BLyS modulating the GC reaction, because we only administered BLyS prior to the first Env immunization, and the effects of the BLyS treatment that we measured were very short-lived. In fact, we limited the time frame during which exogenous BLyS was given to minimize the possibility that the exogenous BLyS would affect the fate of B cells after they had encountered Ag. Thus, the experimental conditions used in this study were chosen to modulate the B cell pools prior to immunization rather than to affect processes at later time points. This is a distinctly different approach from that used in a recent study by Melchers et al. (31), in which BLyS (BAFF) was coadministered with an HIV-1 Env Ag through plasmid DNA vaccination, thus the effect of BLyS in that system is expected to be different from the experimental set-up described in this article.

We have not shown directly that naive B cells capable of producing neutralizing Abs were rescued by the exogenous BLyS treatment and responded to the vaccination. However, our cellular analyses strongly suggest that excess BLyS levels substantially affected the composition of transitional and mature naive B cell pools, consistent with an altered naive repertoire. This is reflected by the disproportionate enlargement of the MZ pool, as well as the shift in the transitional B cells to a greater proportion and number of later transitional subsets (T2 and T3 cells; CD23+ CD93+) in BLyS-treated mice compared with PBS-treated mice. Although we have not analyzed the B cell repertoire at the molecular level, the MZ is well established as a pool that contains a different repertoire of B cells (41, 42). Furthermore, an enlargement of the late transitional pool indicates a relaxation of negative selection that normally occurs at this checkpoint (5, 28, 43, 44). Thus, our cellular analysis strongly supports the statement that we have altered the conditions under which the Env-specific B cell response is elicited, which is reflected in a higher frequency of mice responding with detectable neutralizing activity and, in several cases, higher neutralizing titers (Fig. 4). A molecular characterization of the preimmune B cell repertoire in BLyS-treated mice will help to elucidate this question and is the focus of our future efforts. Similarly, an analysis of the Env-specific B cell repertoire in control and BLyS-treated mice is needed to fully dissect the effects observed in this study.

It is interesting to note that increased levels of transitional B cells were reported in individuals chronically infected with HIV-1 (45). Along with CD4+ Th cell destruction caused by the infection, HIV-1-infected individuals often present multiple alterations in peripheral B cell subsets, including decreased stringency at peripheral B cell selection checkpoints, which may be a common consequence of chronic immune activation (46). The observation that a subset of chronically infected individuals develop potent and broad serum-neutralizing activity after several years of infection (47–49) suggests that the human immune system is capable of generating such Ab responses. Consistent with this, bNAbs elicited by natural infection display extensive SHM and, when back-mutated to their germ-line sequence, lose broadly neutralizing activity (49). Thus, activation of rare B cell clones and successive rounds of GC selection to enhance otherwise poorly competitive specificities may be required before broadly neutralizing activity is achieved.

In summary, we established a system that can be used to improve our understanding of HIV-1 Env vaccine-induced B cell responses. We show that manipulation of naive B cell subsets by exogenous BLyS treatment prior to vaccination with recombinant Env trimers in adjuvant resulted in an improvement in the elicited HIV-1–specific neutralizing Ab response. These studies provide a foundation for mechanistic investigations of BLyS-regulated peripheral B cell selection processes and their roles for vaccine-induced neutralizing Ab responses.

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Disclosures
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