Local BLyS production by T follicular cells mediates retention of high affinity B cells during affinity maturation

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We have assessed the role of B lymphocyte stimulator (BLyS) and its receptors in the germinal center (GC) reaction and affinity maturation. Despite ample BLyS retention on B cells in follicular (FO) regions, the GC microenvironment lacks substantial BLyS. This reflects IL-21–mediated down-regulation of the BLyS receptor TACI (transmembrane activator and calcium modulator and cyclophilin ligand interactor) on GC B cells, thus limiting their capacity for BLyS binding and retention. Within the GC, FO helper T cells (T FO helper cells) provide a local source of BLyS. Whereas T cell–derived BLyS is dispensable for normal GC cellularity and somatic hypermutation, it is required for the efficient selection of high affinity GC B cell clones. These findings suggest that during affinity maturation, high affinity clones rely on T FO helper–derived BLyS for their persistence.

The efficacy of antibodies involves competition among B cells expressing novel specificities generated by somatic hypermutation (SHM). This process occurs in germinal centers (GCs), transient structures formed during T cell–dependent immune responses that enable the preferential survival of B cells producing higher affinity antibodies. Ultimately, this competitive selection process preserves GC B cells with improved antigen affinity and eliminates those that lose specificity or gain autoreactivity. The mechanisms responsible for differential survival remain uncertain but involve tripartite interactions between the GC B cells, FO DCs (FDCs), and T FO helper (T FO helper cells) that provide a local source of BLyS. How the B cell receptor (BCR) drives this affinity–dependent selection process is debated. Although loss of BCR–associated signals disrupt GC kinetics (Wang and Carter, 2005; Huntington et al., 2006), recent findings suggest that antigen capture may be its primary function because BCR signaling is damped in most GC B cells by negative regulatory mechanisms (Khalil et al., 2012). This is consistent with models whereby GC B cell compete for antigen displayed on FDCs to mediate effective MHCI–restricted antigen presentation, thereby fostering sustained T FO helper interactions, which in turn promote GC B cell survival (Allen and Cyster, 2008; McHeyzer-Williams et al., 2009; Victora and Nussenzweig, 2012). This idea is further supported by observations indicating that cognate T FO helper interactions are a limiting factor in affinity maturation (Schwickert et al., 2011). Thus, higher affinity GC B cells can capture and present antigen more effectively, enabling their preferential access to T FO helper cells and facilitating positive selection (Victora et al., 2010; Schwickert et al., 2011).

Despite mounting evidence for this model, the mechanism whereby T FO helper interactions mediate selective survival of higher affinity GC B cells remains unclear. T–B interactions via receptors such as co-stimulatory molecules, death receptor ligands, and soluble survival factors are probably involved. However, the precise...
identities and relative roles of these molecules remain obscure because most potential candidates also play roles in GC initiation or maintenance on their own. Therefore, separating these functions from direct roles in the preferential selection of high affinity clones has proven difficult. For example, the initiation and maintenance of GCs rely on sustained CD40/CD40L signals, and death receptors such as Fas/FasL interactions act to limit GC responses (Foy et al., 1993; Han et al., 1995; Hao et al., 2008). Similarly, soluble mediators such as IL-21 are essential for maintenance of GC B cell character as well as fate choices (Linterman et al., 2010; Zotos et al., 2010).

The B lineage survival cytokine, B lymphocyte stimulator (BLyS, also termed B cell activating factor [BAFF]), plays a key role in setting thresholds for BCR-mediated selection among naive B cells (Cancro, 2004), making it an attractive candidate for mediating analogous processes in the GC. Consistent with this notion, GC responses prematurely terminate in mice with either global BLyS deficiency or defects in BLyS receptor 3 (BR3, also known as BAFFR) signaling (Rahman et al., 2003). Straightforward interpretation of these findings is difficult, because both BLyS-deficient and BR3 mutant mice are severely B lymphopenic (Moore et al., 1999; Schneider et al., 1999; Yan et al., 2001a). Thus, deficits in naive B cell numbers might explain an inability to sustain GC reactions because GCs are resupplied from the naive pools (Schwickert et al., 2007). Moreover, defects in FDC network maturation and T<sub>FH</sub> function also occur in B lymphopenic environments (Rahman et al., 2003; Johnston et al., 2009). Thus, whether BLyS plays a direct role in GC B cell selection and affinity maturation has remained unclear.

Figure 1. GCs are largely devoid of BLyS. (A) Mice were immunized with NP-CGG, and 14 d later spleen sections were stained with PNA and anti-BLyS antibody, as well as antibodies against IgD (top), Igα [middle], or CD35 (bottom). Igα-rich and CD35-rich regions demarcate the LZ and DZ, respectively. Bars, 50 µm. (B) Histogram analysis (bottom) of fluorescence pixel intensity of BLyS, PNA, and IgD staining through a cross section of a representative GC shown in A, top (from top to bottom along the white arrow). (C) Splenocytes from NP-CGG immunized mice were harvested at 14 d after immunization and stained with various antibodies against non-B and B lineage cells, as well as PNA to detect GC B cells. We gated on CD4<sup>+</sup>-CD8<sup>-</sup>-F4/80<sup>-</sup>-Gr1<sup>-</sup>-CD19<sup>+</sup> cells that were further subdivided into PNA<sup>-</sup>IgD<sup>-</sup>Fas<sup>-</sup> GC B and PNA<sup>-</sup>IgD<sup>-</sup>Fas<sup>-</sup> FO B cells. (D) Splenocytes from NP-CGG immunized mice were harvested at 14 d after immunization and stained with anti-BLyS antibody or corresponding isotype control (IC) as well as other cell surface antigens to identify GC and FO B cells (outlined in C). Histogram overlays and MFI of BLyS bound to GC and FO B cell surfaces (n = 3 mice). Representative results are from more than three independent experiments. (E) Splenocytes were harvested at 14 d after immunization with NP-DVA. (Left) CD4<sup>+</sup>-CD8<sup>-</sup>-F4/80<sup>-</sup>-Gr1<sup>-</sup>-CD19<sup>+</sup>-PNA-IgD<sup>-</sup>-Fas<sup>-</sup> GC B cells (left histogram) were further sub-divided into CXCR<sub>4</sub><sup>+</sup>-CD68<sup>-</sup> DZ and CXCR<sub>4</sub><sup>-</sup>-CD68<sup>-</sup> LZ (right histogram). (Right) BLyS expression on FO B and GC B as well as DZ and LZ GC B cells was assessed by flow cytometry using anti-BLyS antibody (n = 4 mice). Horizontal bars represent the mean of the points shown. * denotes statistical significance at 0.01 < P < 0.05 and ** denotes statistical significance at P < 0.01 using a two-tailed Student’s t test when compared with FO B cells.
To better understand how BLyS influences GC function, we investigated the distribution and expression of BLyS and its receptors during GC responses in normal mice. We find that BLyS is spatially segregated between the follicles and GCs, as well as within the GCs, where it is found mainly in the light zone (LZ). Thus, in contrast to FO B cells, GC B cells lack appreciable surface-bound BLyS. This results from profound down-regulation of the BLyS receptor, transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI), which occurs as FO B cells adopt GC B character after IL-21 signals in the context of BCR cross-linking and CD40 co-stimulation. However, in the LZ, BLyS is highly expressed by and associated with FO T cells, both helper (T(H1)) and regulatory (T(H2)). Mixed BM chimeras that lack T cell-derived BLyS have normal GC cellularity and low-affinity IgM and IgG1 antibodies but exhibit significant reductions in high affinity antibody. Moreover, although SHM occurs under these conditions, the patterns of mutation are broadly distributed and show a lower strength of positive selection. Together, these findings indicate that T(H1)-derived BLyS is required to preserve high affinity clones among antigen binding GC B cells.

**RESULTS**

**GC B cells lack receptor-bound BLyS**

Neither BLyS distribution nor BLyS receptor expression within GCs has been extensively examined in situ. Accordingly, we used confocal fluorescence microscopy and flow cytometry to assess these parameters in splenic GCs 14 d after immunization with the nitrophenylacetate (NP) hapten conjugated to chicken gamma globulin (NP-CGG). Consistent with prior studies indicating that the BLyS binding capacity of FO B cells is nearly saturated under steady-state conditions with prior studies indicating that the BLyS binding capacity of FO B cells is nearly saturated under steady-state conditions in vivo (Lesley et al., 2004), cells in the peanut agglutinin (PNA) IgD+ FO regions were consistently decorated with BLyS (Fig. 1, A and B). In contrast, PNA-IgD+ GC B cells displayed minimal BLyS staining. Indeed, little if any BLyS was visible in the GC dark zone (DZ), and only a fine network of staining was seen in the IgM+ or CD35+ LZ (Fig. 1 A). This distribution was confirmed by quantitative analyses of staining intensity through GC cross sections, where the green pixels corresponding to BLyS positivity had higher intensity staining intensity through GC cross sections, where the green pixels corresponding to BLyS positivity had higher intensity. This distribution was confirmed by quantitative analyses of staining intensity through GC cross sections, where the green pixels corresponding to BLyS positivity had higher intensity.

Because most systemic BLyS is secreted as free trimers (Mackay and Schneider, 2009), and there are no physical barriers between the follicle and the GC, we reasoned that the disparity in bound BLyS likely reflects changes in the expression or proportions of BLyS receptors on GC versus FO B cells. We therefore analyzed the expression of all three BLyS receptors on GC B and FO B cells by both qPCR and FACs (gating shown in Fig. 1 C). As expected, GC B cells were enriched for the transcription factor B cell lymphoma 6 (Bcl6) and maintained Pax5 expression when compared with FO B cells (Fig. 2 A; Shaffer et al., 2002). In accord with prior studies, FO B cells express both BR3 and TACI (Hsu et al., 2002; O’Connor et al., 2004; Stadanlick et al., 2008). In contrast, although GC B cells continue to express BR3 message and protein (Fig. 2, A and B), TACI expression is profoundly down-regulated (Fig. 2, A and C). Interestingly, BR3 surface levels assessed by MFI are slightly higher on GC B cells than on FO B cells, despite similar message levels. The basis for this...
Moreover, because much less bound BLyS is retained on GC B cells owing to the loss of TACI, overall BLyS binding may be less avid, yielding less frequent or sustained BR3 occupation.

IL-21 extinguishes TACI expression in activated B cells

The negligible TACI expression among GC B cells prompted us to ask whether stimuli that foster a GC B cell transcriptional profile regulate TACI expression. Enriched FO B cells were stimulated for 3 d with combinations of F(ab')2 anti-Ig, anti-CD40, and IL-21, a GC fate-promoting cytokine (Linterman et al., 2010; Zotos et al., 2010). As previously reported, in the context of BCR and CD40 ligation, IL-21 engenders GC character by up-regulating Bcl6 and activation-induced deaminase (AID), maintaining Pax5, and down-regulating B lymphocyte–induced maturation protein 1 (Blimp1; Fig. 3 A; Goenka et al., 2011). Although BCR and CD40 ligation modestly down-regulate surface TACI expression, TACI mRNA is not affected (Fig. 3, B and C). Importantly, TACI deficiency eliminated the difference in surface-bound BLyS between GC and FO B cells (Fig. 2, D and F). Therefore, TACI is critical for the retention of BLyS on FO B cells, suggesting that the disparate TACI receptor density between FO B and GC B cells is necessary to partition systemic BLyS into the FO regions and out of GCs, yielding BLyS-rich and BLyS-poor microenvironments, respectively.

is unclear but may reflect altered surface turnover or recycling. Neither FO nor GC B cells express B cell maturation antigen (BCMA; unpublished data).

The paucity of bound BLyS on GC B cells correlates with TACI down-regulation, suggesting that TACI is responsible for most retained BLyS on FO B cells, and that TACI down-regulation yields the disparate distribution of BLyS between follicles and GCs. These possibilities were investigated through ex vivo staining of FO and GC B cells from control WT and TACI-deficient mice (Mantchev et al., 2007). Our analyses revealed that TACI-deficient FO B cells have less surface-bound BLyS than FO B cells from WT mice (Fig. 2 D). Consistent with this reduction in FO B cell BLyS binding capacity, TACI-deficient mice had significantly higher levels of free serum BLyS than did WT mice (Fig. 2 E; Bossen et al., 2008). Importantly, TACI deficiency eliminated the difference in surface-bound BLyS between GC and FO B cells (Fig. 2, D and F). Therefore, TACI is critical for the retention of BLyS on FO B cells, suggesting that the disparate TACI receptor density between FO B and GC B cells is necessary to partition systemic BLyS into the FO regions and out of GCs, yielding BLyS-rich and BLyS-poor microenvironments, respectively.
signaling abrogates TACI expression, we repeated the experiment with IL-21R–deficient cells. Activated WT and IL-21R−/− cells had comparable viability and proliferation profiles as assessed by exclusion of TOPRO-3 and by dilution of CFSE, respectively (Fig. 3 D). As expected, IL-21–mediated TACI down-regulation was not observed in IL-21R–deficient B cells (Fig. 3, E and F). Thus, TACI down-regulation is a fundamental component of the overall GC B cell transcriptional profile induced by IL-21 signals received in the context of BCR and CD40 ligation.

**TFH cells are a local BLyS source in the GC**

Because our flow cytometric analyses revealed that GC B cells have uniformly less BLyS bound to their surface and lack TACI (Fig. 1, D and E; Fig. 2 C), we reasoned that BLyS+ cells in the GC LZ are unlikely to be a subset of GC B cells that retain robust BLyS binding capacity. Instead, BLyS might be produced by other GC resident cells such as FDCs, TFH cells, or TFR cells (Hase et al., 2004; Chung et al., 2011; Linterman et al., 2011). Accordingly, we evaluated the BLyS expression among GC B, TFH, and TFR cells. Each of these cell populations, as well as naive FO B cells, were sorted from FoxP3-eGFP knockin mice at day 7 after immunization with NP-OVA, according to the gating strategy shown in Fig. 4 A. GC B cells expressed little if any BLyS, whereas both TFH and TFR cells were enriched for BLyS transcripts (Fig. 4 B). To determine if FO T lineage cells were unique in their ability to produce BLyS, we measured BLyS transcripts in non-TFH effector and regulatory T cells (Treg cells). We find that although non-TFH cells produce BLyS, albeit at lower levels than FO T cells, Treg cells do not express BLyS (Fig. 4 B). To more precisely track BLyS expression by TFH cells in an ongoing immune response, we transferred CD45.2+ OVA-specific OTII T cells into B6.SJL (CD45.1+) hosts. At 10 d after NP-OVA immunization, we sorted the OTII-derived TFH cells and found that they expressed ~10-fold higher levels of BLyS transcripts than did naive CD4+ T cells (Fig. 4 C). Moreover, although some T cell subsets express TACI (von Bülow and Bram, 1997), OVA-specific TFH cells did not (Fig. 4 C), arguing against the possibility that TFH sequester BLyS in the LZ through TACI.

To confirm that BLyS protein is made by GC-resident T cells, we performed immunohistological analyses on spleens from mice at day 14 after NP-CGG immunization. CD3ε positivity (red) colocalized with BLyS + pixels (green) in the GC, and thus appeared yellow in the merged image (Fig. 4, D and E). The boxed cross section of the GC shown in Fig. 4 D was imaged at a higher magnification and shown in Fig. 4 E. Pixel intensity was determined along the white arrow (from left to right, Fig. 4 E) and depicted as a histogram. Intense BLyS staining is largely congruent with CD3ε in the GC

white arrow drawn across a cross section of the GC shown in D (dashed box). Representative results are from more than three independent experiments.

**Figure 4.** TFH and TFR cells produce BLyS in the GC. (A and B) FoxP3-eGFP knockin mice were immunized with NP-OVA and splenocytes were isolated at day 7 after immunization. (A) B220+TCR-β+CD4+CD62L− cells were gated and further subsetted into non-TFH (PD1+CXCR5−), TFH (PD1+CXCR5+FoxP3+), or TFR (PD1+CXCR5+FoxP3+). Gating strategy for B220+TCR-β+CD4+CD62L+FoxP3+ (TFH cells) is not shown. Representative results are from more than two independent experiments. (B) CD11b+ myeloid (positive control), FO B, GC B, non-TFH, TFH, and TFR cells were FACS sorted and BLyS expression was measured by qPCR. Expression was normalized to FO B cells (n > 3 mice). Horizontal bars represent the mean of the points shown. (C) CD45.2+ OVA-specific OTII T cells were transferred into B6.SJL (CD45.1−) mice and the hosts mice were immunized with NP-OVA. After 10 d, splenic naive endogenous CD4 T cells (CD45.2−CD62L+CXCR5−), as well as ova-specific non-TFH (CD45.2−CD62L+PD1−CXCR5−) and TFH (CD45.2−CD62L+PD1−CXCR5−) cells, were FACs sorted, and Bcl6, BLyS, and TACI expression was measured by qPCR. Expression was normalized to naive T cells. Data are presented as mean ± SD and representative of more than two independent experiments. (D and E) WT mice were immunized with NP-CGG, and 14 d later spleen sections were stained with PNA, anti-BLyS, and anti-CD3ε antibodies. Bar, 50 µm. (E) Histogram analysis (bottom) of fluorescence pixel intensity of BLyS, PNA, and CD3ε along the
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or to the establishment and maintenance of primary B cell pools (Gorelik et al., 2003). Thus, reconstitution of the primary B cell pools in WT and BLyS−/− chimeras was comparable, achieving normal steady-state numbers by 11 wk after BM transfer (Fig. 5 A). Furthermore, naive T cells numbers were also normal and similar in both types of chimeras (Fig. 5 A).

Finally, in BLyS−/− T cell chimeras, FDCs should be BLyS-sufficient because they are radioresistant and originate from nonhematopoietic precursors (Kapasi et al., 1998; Krautler et al., 2012), making TFR and TFH the only BLyS-producing, GC-resident cells that lack BLyS. Although some myeloid cells may also be derived from the BLyS-deficient donor, they are not known to influence GC B cells directly (Grouard et al., 1996); moreover, TFR cells likely do not interact directly with GC B cells (Sage et al., 2013).

Once full reconstitution was achieved, the chimeras were immunized with NP-CGG. At 2 wk after immunization, (Fig. 4 E, right) and not with PNA (Fig. 4 E, left). Thus, BLyS in the GC LZ is primarily attributed to T cells, the majority of which are T11 cells (Chung et al., 2011; Linterman et al., 2011).

T cell–restricted BLyS deficiency yields normal GC formation but impairs affinity maturation

These findings indicated that FO T cells are a local source of BLyS within the otherwise BLyS-poor GC microenvironment and, coupled with continued BR3 expression but low BLyS retention capacity by GC B cells, led us to ask whether T cell–derived BLyS is important for GC initiation, maintenance, or selection. By reconstituting lethally irradiated hosts with TCR−β−/− BM combined with either BLyS−/− or WT BM, we generated mixed BM radiation chimeras in which the T lineage lacks BLyS. Importantly, systemic BLyS levels are unperturbed in such chimeras because hematopoietic sources of BLyS contribute minimally to systemic BLyS production or to the establishment and maintenance of primary B cell pools (Gorelik et al., 2003). Thus, reconstitution of the primary B cell pools in WT and BLyS−/− chimeras was comparable, achieving normal steady-state numbers by 11 wk after BM transfer (Fig. 5 A). Furthermore, naive T cells numbers were also normal and similar in both types of chimeras (Fig. 5 A). Finally, in BLyS−/− T cell chimeras, FDCs should be BLyS-sufficient because they are radioresistant and originate from nonhematopoietic precursors (Kapasi et al., 1998; Krautler et al., 2012), making TFR and T11 the only BLyS-producing, GC-resident cells that lack BLyS. Although some myeloid cells may also be derived from the BLyS-deficient donor, they are not known to influence GC B cells directly (Grouard et al., 1996); moreover, TFR cells likely do not interact directly with GC B cells (Sage et al., 2013).

Once full reconstitution was achieved, the chimeras were immunized with NP-CGG. At 2 wk after immunization,
GC formation and architecture were intact in both groups of chimeric mice; GCs formed near the T-B border and were similar in size (Fig. 5 B). Similarly, key GC architectural features were preserved because LZs were populated with T cells (Fig. 5, B and F), and the numbers of PD1hiCXCR5+ GC TFH cells did not significantly differ between the chimeras (Fig. 5, C and D). When compared with control chimeras, there was a modest but consistent (~1.5-fold) reduction in NP-specific GC B cells in BLyS-deficient T cell chimeras, but this did not achieve statistical significance (Fig. 5 E). Importantly, immunohistochemistry confirmed that BLyS co-localized with GC LZ T cells in WT control but not BLyS−/− T cell chimeras (Fig. 5 F), thus substantiating the origin of T cells in each chimera type, and further confirming the specificity of BLyS staining in histological sections. Moreover, Tfh cells appear to be a major source of BLyS within the GC, as the disparity in BLyS distribution between LZ and DZ was minimal in the BLyS−/− T chimera mice (Fig. 5 F).

Having established that GC initiation, magnitude, and composition were largely normal in these chimeras, we measured NP-reactive antibody-secreting cells (ASCs) and serum antibody in these mice to investigate whether the absence of TFH-derived BLyS affects affinity maturation. Analysis on highly substituted NP conjugates revealed that both types of chimeric mice had comparable serum levels of total NP-specific IgM (Fig. 6 A) and IgG1 (Fig. 6 B), as well as similar numbers of total NP-specific IgG1 ASCs (Fig. 6 E). However, despite antibody and ASC responses of similar magnitude and isotype composition, the BLyS−/− T cell chimeras had significant reductions in both IgG1 high-affinity antibody (Fig. 6 C) and ASCs (Fig. 6 F). Moreover, the ratio of high-affinity to total antibody (Fig. 6 D) as well as ASCs (Fig. 6 G) was also reduced in the BLyS−/− T cell chimera mice (Fig. 6 D).
reduced when T cells were unable to synthesize BLyS. Thus, T<sub>FH</sub>-derived BLyS is dispensable for early antibody responses, for GCs of normal structure and magnitude, and for ASC differentiation but is required for normal affinity maturation.

**Impaired affinity maturation reflects a failure of focused selection among somatically mutated GC B cells**

The failure to achieve normal levels and proportions of high affinity ASCs and serum antibody might reflect either a defect in the initiation or extent of somatic mutation on its own, or a failure to preserve high affinity clones at the expense of those with spurious or detrimental mutations. To investigate these possibilities, we sequenced heavy chains of sorted NP-specific GC B cells from each chimera and performed three analyses to assess the numbers and pattern of mutations, as well as the presence and strength of positive and negative selection in the framework regions (FWRs) and complementarity determining regions (CDRs).

The pattern of mutations was clearly different between control and experimental chimeric mice. The W33L replacement was present in GC B cells from both chimeras, albeit at reduced frequency in BLyS<sup>−/−</sup> T cell chimeras than the control chimeras (Fig. 6 H). However, GC B cells in control chimeras exhibited a restricted pattern of replacement (R) mutations (Fig. 6 H), whereas GC B cells in BLyS<sup>−/−</sup> T cell chimeras displayed a more broadly distributed pattern of R mutations, with a reduced preference for W33L, and many additional R and S mutations (Fig. 6 H). Furthermore, the R to S ratio in the CDR of WT chimera was much higher than in the BLyS<sup>−/−</sup> T chimera (~8 vs. ~3.5, Table 1). Because altered mutation patterns can influence specificity-based selection, we applied the focused binomial test to determine whether selection was occurring comparably among the GC B cells of these mice (Hershberg et al., 2008; Uduman et al., 2011). This test affords greater specificity in detecting selection than previous R/S ratio methods because it more precisely accounts for inherent biases of somatic mutation through comparisons of the number and distribution of R, mutations in areas of interest (CDR or FWR) to the total level of S mutations in the entire sequence. Results from this test revealed that GC B cells from neither the WT nor the BLyS<sup>−/−</sup> T cell chimeras displayed selection in the FWR, whereas both had significant positive selection in the CDR (P < 0.05).

Finally, to quantify selection strength we used BASELINEe, which yields a full probability distribution of estimated selection strength (Uduman et al., 2011; Yaari et al., 2012). This analysis revealed that both chimeras preserved the FWR, thus indicating similar and negligible levels of negative selection against changes in their FWR regions (Fig. 6 I). In contrast, although antigen-specific GC B cells from both chimeras exhibited positive selection within CDRs, the strength of positive selection in the CDRs of WT chimeras was significantly stronger than in BLyS<sup>−/−</sup> T cell chimeras (P < 0.05 and Fig. 6 I). In conjunction with the selective loss of high affinity ASCs and antibody in BLyS<sup>−/−</sup> T cell chimeras, these findings suggest that T<sub>FH</sub>-derived BLyS is required to promote and maintain GC B cells that achieve high affinity.

**DISCUSSION**

In this study, we examined how BLyS and its receptors influence the GC reaction. The results reveal that BLyS is spatially segregated both at the GC–follicle interface and within GCs themselves. BLyS partitioning between the GC and follicle relies on differential BLyS receptor densities in these regions, which is afforded by IL-21–driven TACI down-regulation in stimulated B cells. Within the GC, BLyS is localized in the LZ, reflecting production by T<sub>FH</sub>/T<sub>FR</sub> cells. Furthermore, results from mixed BM chimeras reveal that although T cell–elaborated BLyS is dispensable for GC initiation and the generation of normal T<sub>FH</sub> and GC B cell numbers, it is required for the persistence of high-affinity GC B cells. Based on these findings, we propose a model whereby B–T cell interactions govern BLyS availability within the GC, allowing cells that have acquired high affinity to survive.

The striking anatomical sequestration of BLyS reveals a mechanism for restricting BLyS availability without physical barriers. Based on several observations, we favor the notion that TACI down-regulation on its own is responsible for partitioning BLyS between the FO and GC regions. First, in normal mice, the BLyS binding capacities of FO and GC B cells directly correspond to TACI expression (Fig. 2). Second, TACI-deficient mice exhibit uniformly low levels of bound BLyS on their FO and GC B cells, display corresponding increases in free serum BLyS (Fig. 2), and fail to generate strongly demarcated BLyS-rich and BLyS-poor FO and GC regions after immunization. This relationship between BLyS retention and TACI expression effectively sequesters systemic BLyS in the follicles, and enables the creation of BLyS-poor regions in the GC and follicle.
microenvironments where locally generated BLyS can be leveraged as a selecting agent. This model predicts that an inability to create BLyS-poor microenvironments through this mechanism would thwart competitive selection, yield protracted GC kinetics, and impair the generation of high affinity ASCs. Indeed, despite enlarged and persistent GCs in TACI-deficient mice, the BM ASC pool is reduced (Yan et al., 2001b; Seshasayee et al., 2003; Tsuji et al., 2011; not depicted). Finally, our findings also suggest that TACI, although not required for FO B cell survival, is critical for retaining BLyS on FO B cell surfaces. This may reflect efficient binding of higher order BLyS multimers to TACI, as well as increased avidity afforded by two ligand-binding domains in the TACI trimer compared with only a single, partial binding domain in BR3 (von Bülow and Bram, 1997; Thompson et al., 2001; Bossen et al., 2008).

Because T<sub>FR</sub> cells do not produce IL-21 and are not thought to interact directly with GC B cells (Linterman et al., 2011; Sage et al., 2013), we propose that T<sub>FH</sub> cells orchestrate conditions that foster local BLyS availability in the GC through a bilateral mechanism. First, IL-21 mediates TACI down-regulation on GC B cells, so sustained IL-21 signals will not only maintain GCs but also limit the intrinsic BLyS binding ability of GC B cells. Although this scenario awaits in vivo confirmation, it is consistent with the inability of IL21R-deficient mice to maintain GCs, and suggests that the failure to enforce TACI down-regulation and other aspects of GC B cell character might underlie aspects of this phenotype.

Second, T cells are the predominant source of BLyS in GCs because T<sub>FH</sub> and T<sub>FR</sub>, but not GC B cells, are enriched for BLyS transcripts and co-localize with BLyS protein. This notion is further supported by our observation that the disparity in BLyS distribution within GCs is minimized in mixed BM chimeras where T cells cannot produce BLyS. The signals that regulate BLyS production by T FO lineage cells might involve co-stimulatory signals that regulate other effector functions, such as ICOS or PD-1/PD-L1 (Bauquet et al., 2009; Good-Jacobson et al., 2010). The nature of BLyS expressed by T FO cells is not yet clear. Given that the only known membrane isoform, delta BAFF, is not expressed in the T cell lineage (Gavin et al., 2005), local secretion of BLyS by T cells is a likely explanation of its restricted presence in the LZ.

Combined with prior findings, our results reveal distinct roles for systemic versus locally produced BLyS during the GC reaction, enabling GC initiation and maintenance versus affinity maturation, respectively. The severely reduced naive B cell numbers that accompany systemic BLyS deficiency probably underlie the premature GC termination and reduced class-switched antibody observed previously, because neither of these defects was observed in chimeras where only the T cell lineage lacks BLyS. This interpretation is consistent with the notion that incipient and ongoing GCs can be replenished from the naive pool (Schwickert et al., 2007), as well as recent studies showing that B cell antigen presentation is required for full T<sub>FH</sub> differentiation (Goenka et al., 2011; Kerfoot et al., 2011), both of which predict impaired GC kinetics in B lymphopenic environments.

In contrast to systemic BLyS deficiency, local BLyS production is expendable for normal GC initiation, composition, and magnitude but appears critical for the survival of high affinity GC B cell clones generated by SHM. We favor this interpretation for several reasons. First, the reduced generation of high affinity, but not low affinity, ASCs and antibody reflects the absence of high affinity clones in the GC pool. Although BLyS can increase antibody secretion (Schneider et al., 1999), we consider this an unlikely mechanism for the selective decrease in the proportion of high affinity antibody because total NP-specific antibody concentrations were unchanged. Second, the presence of numerous mutations, including the W33L replacement mutation, indicates that SHM occurs; however, these GC B cells had acquired additional replacement mutations that reduced the strength of positive selection but did not abrogate the ability to bind antigen. It remains possible that the modestly reduced GC B cell numbers have an impact on high affinity antibody production in our mixed chimeras. However, the comparable output of class-switched ASCs and antibody, as well as the equivalent number of total R mutations among GC B cells in these mice and controls, argues in favor of altered selection rather than insufficient numbers of appropriately mutated cells. These possibilities are not mutually exclusive, and in either case posit a key role for T<sub>FH</sub> cell–derived BLyS in the emergence or preservation of high affinity GC B cells.

Based on these findings, it is tempting to speculate that GC selection exploits the same relationship between BLyS availability and BCR signal strength that is active during transitional B cell selection, where increasing BCR signal strength imposes a greater need for survival signals via BR3. In the GC context, this would afford the deletion of all B cells experiencing strong BCR signals, such as those associated with the acquisition of self-reactivity. However, high affinity GC B cells that retain antigen specificity and hence antigen-presenting ability will be selectively spared, as they receive concentrated BLyS signals during cognate T<sub>FH</sub> interactions. Indeed, high affinity B cells present more peptide than low-to-intermediate affinity B cells (Schwickert et al., 2011) and likely engage in longer cognate T<sub>FH</sub> cell interactions, thus competing more effectively for BLyS. This model remains consistent with recent findings because some GC B cells maintain BCR signals (Khalil et al., 2012), and damped BCR signaling may be overcome by exceptionally high affinity. Alternatively, high affinity GC B cells may be more prone to death due to increased expression of pro-apoptotic mediators as a consequence of stronger BCR signals. This could also be balanced by the up-regulation of anti-apoptotic molecules by T<sub>FH</sub> cell–derived BLyS. In either case, this general model resolves the seeming conundrum of simultaneous negative selection and affinity maturation within GCs because most B cells experiencing high affinity BCR engagement will be eliminated unless rescued by BLyS, and it predicts that events thwarting this mechanism could foster autoimmunity.
MATERIALS AND METHODS

Mice and immunizations. Mice were maintained and used in accordance with the University of Pennsylvania Animal Care and Use Guidelines. University of Pennsylvania Institutional Animal Care and Use Committee approved all animal experiments. 8–14 wk-old C57BL/6, TCR-β-deficient (B6.129P2-Tcrβ−/−;Jli) (B6.129P2-Tcrβ−/−;Jli) (B6.129P2-Tcrβ−/−;Jli), SJL (B6.129P2-Tcrβ−/−;Jli), OTII transgenic females (B6.129P2-Tcrβ−/−;Jli) (B6.129P2-Tcrβ−/−;Jli) were purchased from The Jackson Laboratory or NCI. TACI-deficient (von Bülow et al., 2001), IL21R-deficient (Ozaki et al., 2002), BLYS-deficient (Schiemann et al., 2001), and FoxP3-eGFP knockin (Lin et al., 2007) mice were previously described. To generate mixed BM chimeras, BM from 8–12 wk-old TCR-β−/−/BLYS−/− or WT mice was depleted of CD3, Gr1+, and B220 cells using MACs magnetic bead depletion (Miltenyi Biotec), and then mixed in a ratio of 25:75 TCR-β−/−/BLYS−/− or 75:25 TCR-β−/−/WT and injected i.v. into lethally irradiated (950 rad) WT mice. The mice were allowed to reconstitute for ≥1 wk. Mice were immunized i.p. with 50 µg NP-CGG or NP-OVA (Biosearch Technologies) with an NP conjugation ratio of 15 or 16 in alum.

BLYS detection. To detect bound BLYS on the surface of B cells, splenocytes incubated on ice were first labeled with rat anti-mouse BLYS antibody and subsequently labeled with PE-conjugated donkey anti-rat Ig (H+L) secondary antibody (Jackson Immunoresearch Laboratories). The cells were washed and blocked with purified rat IgGs and then stained for other cell surface antigens. BLYS concentration in serum samples was determined by ELISA by Human Genome Sciences, Inc. (Scholz et al., 2008).

Immunohistochemistry. Spleens were immersed in O.C.T. (Tissue Tek), flash frozen using 2-methylbutane cooled with liquid nitrogen, and stored at −80°C. 7–8 µm sections were sliced in a cryostat (HM505E; Carl Zeiss), fixed with cold acetone, rehydrated in PBS, and incubated with antibodies in PBS containing 10% goat serum. Sections were stained with anti-BLYS antibody and then with donkey anti-rat antibody conjugated to FITC (described above), and then stained with PNA conjugated to Rhodamine (Vector Laboratories). For IL-21R, we used an antibody and then with donkey anti-rat antibody conjugated to FITC (described above), and then stained with PNA conjugated to Rhodamine (Vector Laboratories) or Alexa Fluor 647 (Invitrogen) and other cell surface antigens. BLyS concentration in serum samples was determined by ELISA by Human Genome Sciences, Inc. (Scholz et al., 2008).

Flow cytometry. Antibodies or reagents reactive to the following antigens were used for flow cytometry: IgD (clone 11–26), CD4 (clone GK1.5), B220 (clone RA3–68B2), BR3 (clone 7H22–E16), CD62L (clone ME–14), TCR-β (clone H57–597), and CD3e (clone 145–2C11; Bioscience); F4/80 (clone BM8), Fas/Cd95 (clone Jo2), CD19 (clone 1D3), Ly-6G/GR1 (clone RB6–8C5), CCR5 (clone R8F52), and CD35 (clone BC12; BD); Ig Lambda (SouthernBiotech); BLYS (clone 121808) and TACI (clone 166010; R&D Systems); PD1 (clone RMP1–30, BioLegend); and PNA–FITC (Vector Laboratories). NP was conjugated to APC in house. Exclusion of DAPI (Invitrogen) was performed with an ABI 7300 (Applied Biosystems). Relative expression was calculated using 18S ribosomal subunit or GAPDH expression as an endogenous control for cells that were FACs sorted or stimulated in vitro, respectively.

SHM analysis. RNA was extracted from FACs-sorted NP+ GC B cells and reverse transcribed using R-TCy (Rohatgi et al., 2008) primers to enrich for IgG transcripts. cDNA was subjected to two rounds of nested PCR using Expand High Fidelity Polymerase (Roche) with 5′ primers for Vh186.2 (Lu et al., 2001) and 3′ primers for all Jh (Rohatgi et al., 2008). PCR cycling conditions were as follows: 95°C for 2.5 min, 52°C for 4 min, and 72°C for 2 min, followed by 40 cycles of 95°C for 1 min, 52°C for 1 min, 72°C for 1.5 min, and a final extension at 72°C for 5 min. Amplified bands of ~350 bp were purified using PureLink gel extraction kit (Invitrogen) and cloned into pCR–TOPO vector using the TOPO TA Cloning vector (Invitrogen). Plasmid was prepared from individual bacterial colonies (GeneElute HP Plasmid Miniprep kit; Sigma–Aldrich) and Vh inserts were sequenced at the Penn DNA Sequencing Facility.

All sequences were aligned to murine germline heavy chain Vh186.2 using IGGT database (Leffran et al., 2005) and segregated into CDR and FWR (FWR1: 1–25; CDR1: 26–33; FWR2: 34–50; CDR2: 51–58; FWR3: 59–95; CDR3: 96–98). To determine if selection was occurring, we used the focused test, which compares the number of R mutations in the area of interest (CDR or FWR) to the total level of mutation in the whole VH sequence (Hershberg et al., 2008; MacDonald et al., 2010; Udumian et al., 2011). The actual level of S mutations and germine sequence are together used to calculate an expected relative level of R mutations. If the observed level of R mutations is significantly greater or lower than expected, this is an indication of positive selection or negative selection, respectively. As we and others have demonstrated, the analysis of mutations from groups of sequences enhances sensitivity of detection without sacrificing accuracy, thus considering the force of selection on the set of sequences rather than individually.

To relate different sequences from the same experiment, which individually could have different numbers of mutations, we measured selection strength. We converted the observed level of mutation and expected level of mutation to the log odds ratio of the two; higher values indicate more positive selection and vice versa. We quantified selection strength using the BASELINE algorithm available on the web. This algorithm extrapolates from the mutation pattern the distribution of potential selection pressure values that could produce it (Udumian et al., 2011; Yari et al., 2012). This method analyzes all mutations collected from the same experimental condition and takes into account mutations that came from the same sequences and/or clones, and whether they occur in CDR or FWR. It creates a probability distribution function of the estimated selection strength from which these
mutations could arise in those positions. Information about the certainty of the estimated values and the consistency of selection pressures across all sequences analyzed is reflected in the width of the distribution: the narrower the distribution, the more precise is the assessment of selection strength (positive or negative) from the pattern of mutations observed. In this fashion, BASELINE is capable of comparing experiments with unequal sequence numbers, where the calculated distribution of selection strength may not be as narrow.

**Statistical analysis.** Data (other than for SHM analyses) were tested for statistical significance at the test.

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