Normal Induction but Attenuated Progression of Germinal Center Responses in BAFF and BAFF-R Signaling–Deficient Mice

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Abstract
The factors regulating germinal center (GC) B cell fate are poorly understood. Recent studies have defined a crucial role for the B cell–activating factor belonging to TNF family (BAFF; also called BLyS) in promoting primary B cell survival and development. A role for this cytokine in antigen–driven B cell responses has been suggested but current data in this regard are limited. A BAFF receptor expressed by B cells (BAFF-R/Br3) is defective in A/WySnJ mice which exhibit a phenotype similar to BAFF–deficient (BAFF−/−) animals. Here, we show that although GC responses can be efficiently induced in both A/WySnJ and BAFF−/− mice, these responses are not sustained. In BAFF−/− mice, this response is rapidly attenuated and accompanied by perturbed follicular dendritic cell development and immune complex trapping. In contrast, analysis of the A/WySnJ GC response revealed a B cell autonomous proliferative defect associated with reduced or undetectable Ki67 nuclear proliferation antigen expression by GC B cells at all stages of the response. These data demonstrate a multifaceted role for the BAFF pathway in regulating GC progression.

Key words: B cell development • germinal center response • B cell memory • immunodeficiency • FDC reticulum

Introduction
T cell–dependent (TD) antigens induce germinal centers (GC) in the follicles of secondary lymphoid organs (1). In GCs, B cells undergo rapid proliferation and Ig V region somatic hypermutation (1–3). B cell mutants with high affinity for antigen are positively selected into the Ab-forming cell or memory compartments (4–6). B cell mutants with low affinity for antigen and apparently B cells with autoreactivity are negatively selected (7, 8). Despite these insights into the pivotal function of GCs in TD B cell responses and peripheral tolerance, the factors that regulate GC B cell survival and selection are still poorly understood.

The cytokine B cell–activating factor belonging to TNF family (BAFF; also called TALL-1, THANK, BLyS, and zTNF4) is a member of the TNF ligand family (9–13). BAFF is predominantly expressed by myeloid cells, including monocytes and DCs (9–12). Of the three known receptors, BCMA (13, 14), TACI (15, 16), and BAFF receptor (BAFF–R) (BR3) (17, 18) expressed on B cells that can bind this cytokine, BAFF–R appears to be specific for BAFF since it does not bind the related cytokine APRIL (17, 18). Studies on BAFF-deficient (BAFF−/−) mice have defined a crucial role for BAFF in primary B cell survival and development (19, 20). Such mice display a loss of transitional type 2 (T2), follicular (B2), and marginal zone B cells. However, BM B lymphopoiesis, transitional type (T1) B cells, B1 cells, and other hematopoietic cell lineages appear unaffected.

Analysis of BAFF transgenic mice revealed expanded B cell numbers in all peripheral B cell compartments (21). BAFF was also shown to augment B cell proliferation induced

Abbreviations used in this paper: BAFF, B cell–activating factor belonging to TNF family; BAFF–R, BAFF receptor; BrdU, bromodeoxyuridine; Bcmd, B cell maturation defect; Bcl-6, B cell lymphoma-6; FDC, follicular DC; GC, germinal center; LT, lymphotoxin; TUNEL, terminal deoxynucleotidyl transferase nick end labeling; HRP, horseradish peroxidase; IC, immune complex; PI, propidium iodine; PNA, peanut agglutinin; T1, transitional type 1; T2, transitional type 2; TD, T cell–dependent.
by anti-IgM antibody in vitro (9, 10). In addition, BAFF promotes increased survival of mature resting B cells in vitro (21, 22). These data indicate a role for BAFF beyond the T1-T2 transition.

The phenotype of A/WySnJ mice closely resembles that of BAFF−/− mice in exhibiting an ~10-fold loss of splenic B cells, including T2, B2, and marginal zone B cells (23–25). As in BAFF−/− mice, BM B lymphopoiesis, T1 transitional B cells, B1 cells, and other hematopoietic lineages appear unaffected (25). The B cell maturation defect (Bond) locus (26) was shown to contribute to this peripheral B cell defect (23, 24). The BAFF-R gene was localized to the Bond locus (17, 18). Expression of a defective BAFF-R, resulting from the mutation of the cytoplasmic domain was reported in A/WySnJ mice (15, 17). These data strongly indicate that the mutated BAFF-R is encoded by the Bond locus.

Despite their B lymphopenia, BAFF−/− and A/WySnJ mice do contain splenic B2-like cells based on local and cell surface phenotype (19, 24), indicating that the T1-T2 block is not absolute. Moreover, A/WySnJ and mice in which BAFF function is inhibited can mount high affinity class switched serum antibody responses to TD antigens, albeit at levels lower than normal mice (15, 18, 27). Therefore, we speculated that a GC response could be induced in these mice, allowing assessment of the role the BAFF–BAFF-R pathway might play in this response. Our results suggest that GCs can be efficiently formed in these mice. However, in both A/WySnJ and BAFF−/− mice, GC responses are not sustained. Therefore, our data demonstrate a novel role for the BAFF pathway in the regulation of antigen-driven B cell differentiation.

Materials and Methods

Mice and Immunizations. A/J, A/WySnJ, μMT (on a C57BL/6j background), (A/J × C57BL/6j) F1 (AB6Fl/J), and C57BL/6 mice were purchased from The Jackson Laboratory and were maintained in a pathogen-free facility, BAFF−/− mice were generated (19) and maintained in a pathogen-free facility, and all animal protocols were approved by the Institutional Animal Care and Use Committee. All mice (9–12 wk) were immunized i.p. with SRBC (Lampire Biological Labs) as described (28, 29).

Antibodies. The following antibodies and other reagents were used: FITC–GL7; PE–anti–TCR-β; PE–anti–FcyRII/FcγRIII (clone 2.4G2); PE and PerCP–anti–B220 (clone RA3–6B2); FITC–anti–IgD (clone 11–26c.2a); APC–anti–mouse IgM (clone II/41); biotin–anti–CD35 (clone 8C12); rat IgM anti–mouse GL7; rat IgG antibody to mouse follicular DCs (FDC-M1); streptavidin–CyChrome (BD Biosciences); rabbit polyclonal antibody to Bcl-6/N-3 (Santa Cruz Biotechnology, Inc.); alkaline-phosphatase and biotin–(Fab′)2 mouse anti–rat IgG; HRP donkey anti–mouse IgM and biotin donkey anti–rabbit IgG (Jackson ImmunoResearch Laboratories); HRP–peanut-agglutinin (PNA) (Sigma–Aldrich); biotin–LS136 (anti–AlmAb); HRP–anti–CD4 (clone GK1.5; made in house); anti–Ki67 (clone TEC–3) and streptavidin–alkaline phosphatase (Dako); biotin–anti–B220 (clone RA3–6B2), biotin–anti–IgD (clone 11–26; Southern Biotechnology Associates, Inc.), and streptavidin–PE (Molecular Probes).

Immunohistology. Spleen cryostat sections (5–6 μm) were prepared as previously described (30). Immunohistology was performed using either visible dye staining as described (30), except the Abs were detected using the Vector Blue Alkaline–phosphate Substrate kit III and the Vector NovaRed kit for peroxidase (Vector Laboratories) or immunofluorescent staining as described (29). The stained sections were analyzed using light or fluorescence microscopy (Leitz Diaplan; Axiosplan Universal Microscope; Carl Zeiss MicroImaging, Inc.), and digital images were captured using either an Eastman Kodak Co. camera or an axiocom using the Openlab software.

Cell Cycle Analysis by Flow Cytometry. Cell suspensions prepared from spleens on day 8 post SRBC immunization were RBC depleted using ACT buffer and stained with GL7–FITC, anti–B220–PE and biotin–anti–IgD followed by streptavidin–CyChrome. B220+ IgD– GL7+ GC and B220+ IgD+ GL7+ B cells were separated on a Coulter Epics Elite flow cytometric cell sorter, and propidium iodine (PI) staining was performed on the sorted cells as described (31). PI staining was analyzed using a Coulter Epics XL/MCL analyzer. The data were analyzed using FlowJo software (Treestar).

Microdissection of GCs, PCR, Cloning, and Sequencing. Spleen sections were stained with PNA and LS136 (anti–λ1, mAb); PNA+ λ1+ and PNA+ λ2+ GC B cells were microdissected and processed as described (32). VK1 and V4186.2 (J588 family) genes were amplified as described (33, 34). Vκ genes were amplified using a degenerate universal 5′ primer (35). The kappa 3′ primer (AATCTCTGACCTGTGGACAGT) hybridize in a region of Jκ3 in the intronic sequence. PCR product purification, cloning, sequencing, and somatic mutation analyses were done as described (34).

Terminal Deoxynucleotidyl Transferase Nick End Labeling Assay. Spleen sections were first stained with GL7 as described (30). A terminal deoxynucleotidyl transferase nick end labeling (TUNEL) assay was then performed on the same spleen sections using an ApopTag in situ apoptosis detection kit (Intergen). Alkaline–phosphatase and HRP-labeled antibodies were visualized as described above. GCs were then categorized into small, medium, and large sizes as described (36). Apoptotic activity was assessed by counting the number of TUNEL-positive nuclei in small and medium GCs.

In Situ Bromodeoxyuridine Proliferation Assay. An in situ bromodeoxyuridine (BrdU) proliferation assay was performed using a kit (Roche). Mice were immunized with SRBC as above. At different time points (day 6, 9, and 14) postimmunization, BrdU was administered i.p. 1 h before sacrificing and freezing spleens. One of two adjacent spleen sections (5–6 μm) at each time point were stained with anti–IgD and PNA, and alkaline–phosphatase (IgD) and HRP-labeled (PNA) antibodies were revealed as described above. BrdU uptake was detected on the other section, after the first two staining procedures had been completed as described (37), using mouse anti–BrdU followed by alkaline phosphatase conjugated anti–mouse Ig.

In Vitro Stimulation and Intracellular Staining of B Cells for Ki67. Small dense splenic B cells were isolated and stimulated with antirabbit CD40 (FGK45, a gift from Dr. Anton Rolink, Department of Immunology, University of Basel, Basel, Switzerland) and recombinant murine IL-4 (Pepro Tech) as previously described (36). Resting (0 h) and stimulated (48 h) B cells were cytospun (450 rpm, 10 min) onto charged slides and stained for Ki67 as described above. Fixing and permeabilization were done using a kit from Caltag Laboratories.

Passive Immune Complex Deposition Assay. Mice were immunized with SRBC as above. On day 8 postimmunization, the mice were injected with either rabbit anti–HRP serum or normal rabbit serum followed by HRP 1 d later as described (30). 1 d after that,
the mice were killed, and spleens were frozen and sectioned. HRP activity was visualized via fluorescent staining with goat anti-HRP (Sigma-Aldrich) followed by biotin–donkey anti–goat (Jackson ImmunoResearch Laboratories) and SA-488 (Molecular Probes).

Mixed BM Chimeras. Mixed BM chimeras were created through adoptive transfer of a 1:1 mixture of BM from A/J and μMT (C57BL/6–Igh-6m1Cgn) or A/WySnJ and μMT mice into (A/J × C57BL/6) F1 mice, designated AB6F1. AB6F1 mice were given 1,000 rad whole body irradiation before the injection in the lateral tail vein of ~6 × 10⁶ RBC and T-depleted BM cells. Chimeric mice were allowed to reconstitute for 35 d before immunization with SRBC as above. Mice were killed at different time points (day 6, 9, and 14) postimmunization, and spleens were frozen as described above. Immunohistology was performed on spleen sections of these mice, also as described above.

Results

A/WySnJ and BAFF−/− Mice Have Reduced but Similar Numbers of IgM(low), IgD(high) B Cells Located in Splenic Follicular Areas. Previous studies have suggested that the block in mature B cell development may be more severe in BAFF−/− mice than in A/WySnJ mice (18–20). Therefore, we compared B cell phenotype and locale in the spleens of these mice. Immunohistology analysis revealed that although the follicular areas in both strains are clearly smaller than in control mice, they appear reduced to similar extents (Fig. 1 A). B cells of both strains in these areas stain strongly with anti-IgD and weakly with anti-IgM. Quantitative flow cytometric analysis showed that both strains have reduced but similar numbers of sIgM(low) and sIgD(high) splenic B cells (Fig. 1 B).

GC Kinetics in A/WySnJ and BAFF−/− Mice. The induction of robust GC responses in mice immunized with SRBCs has been shown previously (28, 29). A/WySnJ and A/J control and BAFF−/− and C57BL/6 mice were immunized with SRBC. Spleen sections obtained at multiple time points were stained with PNA or GL7. Defects in the GC response can be manifested by changes in GC size, frequency, and kinetics (34, 36, 38). Therefore, GCs were counted and categorized as small, medium, and large at all time points as described (36). The total number of GCs at day 6 of the response in all four groups was comparable...
were counted per 10
day 6, only small GCs were prevalent at day 9, and GCs
obtained at each time point, each at least 40–50 sections apart, were subjected
(Fig. 3 A, right). This staining was far less extensive, less in-
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points where GCs were observed revealed characteristic
GC substructure. A/J and A/WySnJ GCs from all time
response at day 6, and total GC numbers decreased rapidly
large GCs at any time point, small GCs dominated the re-
regression differed. In A/WySnJ spleens, there were very few
were nearly absent at day 12 (Fig. 2 B).

Figure 2. Kinetics of germinal center (GC) induction. (A) A/J (■) and
A/WySnj (□) mice and (B) BAFF−/− (○) and C57BL/6 mice (●,
BAFF+/+) were immunized with SRBC. Two spleen sections per mouse
obtained at each time point, each at least 40–50 sections apart, were subjected
to immunohistological analysis. Small, medium, and large PNA+ GCs
were counted per 10× field. The data represent at least three mice from
each group at each time point.

(Fig. 2). However, the kinetics and magnitude of GC pro-
gression differed. In A/WySnj spleens, there were very few
large GCs at any time point, small GCs dominated the re-
sponse at day 6, and total GC numbers decreased rapidly
after day 12 (Fig. 2 A). In contrast, the number and size of
GCs at day 6 appeared normal in BAFF−/− spleens, but
there was a dramatic decrease in total GC numbers after
day 6, only small GCs were prevalent at day 9, and GCs
were nearly absent at day 12 (Fig. 2 B).

GC Substructure in A/WySnj and BAFF−/− Mice. Spleen
sections from the SRBC response were stained with
GL7 in combination with a variety of antibodies to define
GC substructure. A/J and A/WySnj GCs from all time
points where GCs were observed revealed characteristic
“sectoring” due to up-regulated FDC-M1 and FcyRIIB
(2.4G2) levels on the FDC reticulum present in a large re-

gion of each GC and low to undetectable levels of expres-
sion of these markers on GC B cells where the FDC net-
work was absent (29). Representative images from day 12
are shown in Fig. 3 A, left panels. In contrast, day 6 BAFF−/−
GCs revealed abnormal staining with FDC-M1 and 2.4G2
(Fig. 3 A, right). This staining was far less extensive, less in-
tense, and more punctate than what is characteristic of nor-
mal GCs. However, the primary FDC reticulum appeared
normal in both BAFF−/− and A/WySnj GCs as indicated by
intensity and distribution of anti-CD35 staining (Fig. 3 B).

T cell migration to the GC was investigated by staining
spleen sections with GL7 and anti–TCR-β. T cell num-
bers and locale in the GCs of A/WySnj (day 12) and
BAFF−/− (day 6) mice were comparable to controls (Fig. 3
C). We also examined the expression of the transcription
factor Bcl-6, which has been shown to be highly and spec-
cifically expressed in GC B cells (39, 40). Bcl-6−/− mice
have impaired GC formation (41). Fig. 3 D shows that Bcl-6
expression in A/WySnj (day 12) and BAFF−/− (day 6) GCs
was comparable to controls. Analysis of T cell locale and
number and Bcl-6 expression at other time points gave re-
results similar to those illustrated in Fig. 3, C and D.

Immune Complex Deposition in BAFF−/− and A/WySnj
GCs. These data showed that although GC substructure
is apparently normal in BAFF-R−/− defective A/WySnj
mice, development of a mature FDC reticulum does not take
place in the GCs of BAFF−/− mice. Although the pre-
cise role of FDCs in the GC response remains controver-
sial, their ability to trap and retain native antigen in the
form of immune complexes (ICs) is thought to be impor-
tant for stimulation of GC B cell proliferation and differen-
tiation (42–45).

Passive splenic IC deposition assays were performed on
BAFF−/− and A/WySnj mice undergoing immune re-
sponses to SRBC. These did not reveal any major differ-
ces in IC amounts or locale in A/WySnj and A/J mouse
GCs (Fig. 4, right). In contrast, little IC deposition could be
detected on FDCs in the GCs of BAFF−/− mice (Fig. 4,
left). Thus, the attenuation of the GC response in these
mice may result from a lack of sufficient antigen trapping in
GCs, secondary to pleiotropic perturbations in FDC de-
velopment (see Discussion). In contrast, clues to the mecha-
nism(s) responsible for attenuation of the GC response in
A/WySnj mice were not provided by these studies, neces-
sitating further analysis of this response.

Apoptosis in GCs of A/WySnj Mice. To investigate
whether the decline of GC numbers in A/WySnj mice was
correlated with increased intra-GC apoptosis, TUNEL as-
says were done on spleen sections. Due to the low fre-
cency of large GCs in A/WySnj mice, small and medium
GCs were considered for this analysis. Although the aver-
age number of apoptotic nuclei per GC at all time points
was slightly higher in A/WySnj mice compared with A/J
control mice (Fig. 5), the differences were not statistically
significant (Student’s t test at 90% confidence).

Proliferation in the Very Early Stages of the GC B Cell Re-
sponse in A/WySnj Mice Appears Normal. GC B cell pro-
liferation in A/WySnj mice was evaluated using an in situ
BrdU incorporation assay. As shown in Fig. 6 A, BrdU-
positive cells (red-orange, bottom panels, day 9 and 14)
reflect proliferating B cells in a PNA+ IgD− GC (red-
orange, top panels) revealed in the adjacent section. GC
cell BrdU staining frequency and intensity on day 9 were
comparable in A/J and A/WySnJ mice. In contrast, very few intensely staining BrdU^+^ cells were seen in day 14 A/WySnJ GCs. Since levels of DNA damage are elevated in GC B cells (46), much of the BrdU incorporation could have resulted from DNA synthesis due to repair and not chromosomal...
replication. For this reason, GC B cell proliferative status was investigated by PI cell cycle analysis on GC B cells obtained by FACS® on day 8 post-SRBC immunization (Fig. 6 B). The percentage of B220+/H11001 IgD+/H11002 GL7+/H11001 B cells (defined as GC B cells, bottom panels) that were in S/G2-M phases in A/WySnJ mice was comparable to that in A/J mice. Together, the BrdU and PI data suggest that GC B cells in A/WySnJ mice are proliferating normally during the early but not the intermediate stages of this response. Due to the diminution of GC number and size in A/WySnJ mice, it was technically impossible to perform the PI analysis at later time points.

Reduced Ki67 Expression in A/WySnJ GCs. To further evaluate the proliferative status of B cells in A/WySnJ and BAFF+/H11002 GCs we examined the expression of Ki67 (a nuclear proliferation antigen). As illustrated in Fig. 7 A, we categorized GCs as (+/+), most GC B cells positive for Ki67 staining, (+/−), 10–60% of GC B cells positive for Ki67 staining, and (−/−), 0–9% of GC B cells positive for Ki67 staining.

Analysis of BAFF−/− mice revealed that GL7+ GC cells were uniformly Ki67+/+ at all time points, even in the small GCs prevalent at day 9 and the rare GCs present at day 12 (Fig. 7 B). In striking contrast, on day 6 58% of A/WySnJ GCs had reduced or undetectable (−/−) levels of Ki67 expression. On day 9, >70% of GCs in these mice was moderately positive (+/−). However, on day 12 >90% of A/WySnJ GCs had dramatically reduced or undetectable (−/−) levels of Ki67 expression. A/J mice exhibited bright and extensive (Fig. 7, B and C, +/+ ) Ki67 nuclear staining in activated B cells scattered in white and red pulp areas as early as day 3 (not depicted) and in all GCs at all other time points.

To evaluate whether A/WySnJ B cells have a generic defect in ability to express high levels of the Ki67 antigen, we stimulated purified splenic B cells from A/J and A/WySnJ mice with anti-CD40 and IL-4 in vitro and assayed expression of Ki67. Fig. 7 D illustrates that the majority of B cells from both strains became strongly Ki67 positive after 48 h of stimulation. The slight reduction in Ki67-positive cells observed in A/WySnJ cultures was not significant and may have resulted from higher levels of non–B cell contamination due to the B lymphopenia of these mice.

The GC Defect in the A/WySnJ Mice Is B Cell Autonomous. To determine whether the attenuated GC response of A/WySnJ mice was due to a B cell defect, we generated mixed BM chimeras of A/J (Bcmd) and A/WySnJ (Bcmd) mice with anti-CD40 and IL-4 in vitro and assessed expression of Ki67. Fig. 7 D illustrates that the majority of B cells from both strains became strongly Ki67 positive after 48 h of stimulation. The slight reduction in Ki67-positive cells observed in A/WySnJ cultures was not significant and may have resulted from higher levels of non–B cell contamination due to the B lymphopenia of these mice.

**Figure 4.** Immune complex trapping in A/WySnJ and BAFF−/− GCs. A/WySnJ, A/J, BAFF+/+ mice, and C57BL/6 mice were immunized with SRBC and subsequently injected with HRP and anti-HRP to evaluate IC trapping as described in Materials and Methods. The figure illustrates representative splenic areas in adjacent sections stained either with anti-CD35 to reveal the follicular reticulum or with an anti-HRP antibody. Original magnification of images was 10X. Arrows indicate areas of IC deposition on the follicular reticulum.
in Fig. 8 B, FDC “maturation” and “sectoring” in these chimeric mice on day 9 post-SRBC immunization were comparable to that observed in A/J and A/WySnJ mice, respectively (Fig. 3 A). Ki67 expression, or lack thereof, (Fig. 8 C) and T cell numbers and locale (Fig. 8 D) in GCs of chimeric mice were also comparable to those in their nonchimeric counterparts. The kinetics of the GC response was also comparable (not depicted) to those in A/J and A/WySnJ mice (Fig. 2). These data indicate that the defect in the A/WySnJ GC response is B cell autonomous and not caused by perturbed microenvironments or accessory cells.

Precursors of the GC B Cell Response in A/WySnJ Mice Appear Not To Be B1 Cells. The development of B1 cells is normal in both A/WySnJ and BAFF−/− mice (19, 25). Therefore, we investigated whether the precursors of GC B cells in A/WySnJ mice could be B1 cells. These cells have been shown to express VH genes with either no or only short stretches of N sequence additions at V-D-J junctions (47, 48). We analyzed the N-sequence diversity at V-D-J junctions (Table I) of the VDJ rearrangements of VH genes recovered by microdissection and PCR amplification of A/WySnJ SRBC-induced GCs. The N regions were comparable in length (7–18 nucleotides) and nucleotide composition (55% G+C) to those in A/J GCs (5–20 nucleotides and 60% G+C).

Somatic Hypermutation in A/WySnJ GCs. Since somatic hypermutation generates the V region substrates for antibody affinity maturation, a process that takes place in GCs (1–7), perturbations in hypermutation in A/WySnJ GCs might contribute to GC diminution. In normal mice, the hypermutation process is activated after GC formation, and a high frequency of V region mutation becomes evident during the intermediate and latter stages of the response (49, 50). A/J and A/WySnJ mice were immunized with SRBCs, and PNA splenic GC B cells were microdissected for genomic DNA amplifications and sequencing of VH and VL genes. The mutation frequencies in day 9 (intermediate) and day 16 (late) A/WySnJ GCs were comparable to those in A/J mice (Table II). Moreover, the chemical nature and distribution of mutations in V region genes obtained from A/WySnJ GCs were also comparable to those from A/J mice (unpublished data).

Discussion

Previous findings on the influence of BAFF on B cell behavior led to speculation that this cytokine might play a role in the antigen-driven stages of B cell development (9, 10, 13, 21, 22). In testing this hypothesis, we found that GCs can efficiently form in BAFF−/− and in BAFF-R−/− defective A/WySnJ mice. However, the GC response is not sustained in either strain, and our data strongly suggest that the mechanisms by which GC integrity is compromised differ. Therefore, although BAFF is dispensable for the initiation of the GC response, signaling via this pathway...
Figure 7. Staining of GC B cells for Ki67, a nuclear proliferation marker. Spleen sections from days 6, 9, and 12 post-SRBC immunization were stained with anti-Ki67 (red) and GL7 (green). (A) GCs were categorized into bright (+/+), moderately positive (+/−), and substantially reduced or undetectable (−/−) levels of Ki67 staining. (B) Ki67 expression in A/WySnJ and A/J control and BAFF−/− and C57BL/6 GCs at different time points. (C) Percentage of A/J and A/WySnJ GCs in each category. These data represent 35–50 randomly selected GCs from five mice from each group at each time point. Original magnification of images was 25×. (D) The percentage of Ki67+/− cells in resting (0 h) and in vitro anti-CD40 and IL-4–stimulated (48 h) splenic B cells purified from A/J (hatched bar) and A/WySnJ (white bar) mice.
The data represent one A/J induced by SRBC immunization of BAFF, partially responsible for the failure of this response in the GC response (1, 42, 43), this defect may be at least partially due to... clusters lack the "mature" FDC reticulum characteristic of GCs. Since FDCs have been suggested to play a central role in the development of the primary and secondary GC reticulum, the cells that form the primary and secondary GC reticulum could be distinct (29, 44), with the development of the latter being critically dependent on BAFF. FcγRIIB-deficient mice also display a defect in FDC maturation (29, 45). Our observation that FcγRIIB fails to be induced on FDCs in BAFF−/− mice suggests that BAFF is required for early steps in the FDC maturation pathway.

Given the central role played by B cell–derived lymphotxin (LT) in the development of follicles (51, 52), it should also be considered that the primary defect in GC progression results from insufficient B cell LT production secondary to absence of BAFF stimulation. In an LT-deficient environment, the mature FDC network may fail to develop or be destabilized, precluding the provision of generic B cell growth and survival factors and downstream steps necessary for GC progression, such as IC trapping, antigen presentation to B cells, and B cell costimulation. Indeed, previous studies showed that administration of LTβR-Ig or anti-LTβ to mice with preexisting GCs resulted in dissolution of the FDC network in those GCs (53). Identification of the cellular source of BAFF that regulates GC development is an important goal for future investigation. Since normal lymphocytes are incapable of producing this cytokine (9, 10), a GC myeloid or stromal source of BAFF is implicated.

Although the lack of GC progression in BAFF−/− mice may be due to a GC accessory cell deficiency, this does not appear to be the case in A/WySnJ mice. In these mice, a cell autonomous defect in the capability of GC B cells to receive BAFF-derived signals appears to lead to their inability to sustain the rapid cell cycle progression required to maintain the response. The expression of Ki67 in the GCs of these BAFF−/− mice is most easily explained by a negative effect of Ki67, the perturbed Ki67 expression characteristic of A/WySnJ mice (Fig. 5). This defect is not due to an intrinsic inability of B cells to express Ki67, since primary A/WySnJ B cells uniformly expressed high levels of Ki67 after in vitro stimulation with anti-CD40 and IL-4. This raises the interesting possibility that the BAFF-R regulates expression of intracellular factors essential for the sustenance of normal GC B cell cycle progression, such as Ki67.

Since GC B cells in BAFF−/− mice express normal levels of Ki67, the perturbed Ki67 expression characteristic of A/WySnJ GCs is most easily explained by a negative effect on B cell proliferation due to partial function of the BAFF-R, competing signaling by other previously or yet to be characterized receptors for BAFF or both. Regarding the first possibility, recent data suggest that multiple downstream signaling pathways can be induced by BAFF-R engagement (54, 55). The mutated form of the BAFF-R present in A/WySnJ may transmit signals that inhibit rather than promote GC B cell proliferation. Precedence for the idea that single TNF-R family members are capable of both

Figure 8. Reduced Ki67 expression in A/WySnJ GCs is due to a B cell autonomous defect. Spleen sections of A/J + μMT → AB6F1 and A/WySnJ + μMT → AB6F1 chimeric mice from day 9 post-SRBC immunization were stained with (A) anti-B220 (blue) and GK1.5-HRP (red), (B) GL7-FITC (green) and FDC-M1 (red), (C) GL7-FITC (green) and Ki 67 (red), and (D) GL7-FITC (green) and TCRC-β-PE (red). Original magnification of images was 10X in A and 25X in B–D. The data represent one A/J + μMT → AB6F1 and two A/WySnJ + μMT → AB6F1 chimeras.
positive and negative signaling has been established recently for CD40 (56). Regarding the influence of other BAFF receptors in the absence of normal BAFF-R function, since TACI can mediate down-regulation of B cell activity (57, 58) it is tempting to speculate that when BAFF-R function is compromised BAFF induces inhibition of cell cycle progression via TACI.

Yan et al. (15) previously found that in vivo treatment of immunized mice with TACI-Fc abolished the development of splenic GCs, leading to the conclusion that BAFF is required for the GC response. This approach differs fundamentally from our study of mice genetically deficient in BAFF or BAFF-R signaling. TACI-Fc would neutralize not only BAFF, but also APRIL, and perhaps yet to be identified TACI-specific ligands. Further, the degree to which these cytokines are neutralized at their sites of action by such fusion proteins is uncertain. Also, such Ig fusion proteins could alter the activity or survival of cells expressing membrane-bound forms of TNF cytokines. Indeed, we conducted a preliminary examination of the GC response to SRBC in mice treated with BCMA-Ig and found the attenuation of the GC response was moderate in comparison to BAFF/−/− mice (unpublished data).

Certain TI antigens can induce transient GC formation in normal or T cell–deficient mice (59, 60). Several factors

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_V_H/D_J_H_ junctions of rearranged Ig chain J558 family genes PCR amplified, cloned, and sequenced from GCs in SRBC immunized A/WySnJ and A/J mice. PNA+ GC B cells were microdissected using a micromanipulator after the spleen sections were stained with PNA, and then J558 family gene was amplified from the processed PNA+ GC B cells as described previously (34). Shown are three 3′ nucleotides of the V_H gene, the D element (aligned with germline D elements from Ig BLAST), three 5′ nucleotides of J_H and N/P sequences at the V_H/D and D/J_H borders. Short sequence homologies in DJ_H joints are shown in bold and are italicized.
Table II. Somatic Hypermutation Frequency of GC B Cell Ig Light and Heavy Chain Genes in A/J and A/WySnJ GCs

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<tr>
<th>Gene</th>
<th>Total mutation ( ^a )</th>
<th>Total sequence ( ^b )</th>
<th>Frequency (%) ( ^c )</th>
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\(^{a}\)Total number of somatic mutations that was observed in total number of sequences analyzed in a particular gene amplification.

\(^{b}\)Total nucleotide number in sequences that were amplified, sequenced and analyzed for a particular gene.

\(^{c}\)Mutation frequency of a particular Ig light and heavy chain gene in GCs.

\(^{d}\)J558 family heavy chain genes and kappa light chain genes were amplified from SRBC induced A/J GCs.

\(^{e}\)J558 family heavy chain genes, kappa, and \( \lambda 1 \) light chain genes were amplified from SRBC induced A/WySnJ GCs.

and observations suggest that a T cell deficiency does not contribute to the transitory nature of the GC response induced in BAFF\(^{-/-}\) and A/WySnJ mice. First, T cell numbers and locale in the GC are normal in A/WySnJ and BAFF\(^{-/-}\) mice (Fig. 3C). Second, we observed no splenic GC formation in completely T cell–deficient TCR C\(^{H11002}\)/J mice (62).

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