Cutting Edge: Germinal Centers Formed in the Absence of B Cell-Activating Factor Belonging to the TNF Family Exhibit Impaired Maturation and Function


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Cutting Edge: Germinal Centers Formed in the Absence of B Cell-Activating Factor Belonging to the TNF Family Exhibit Impaired Maturation and Function

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Germinal centers (GCs) form in B cell follicles and require specific signals for development and maintenance. B cell-activating factor belonging to the TNF family (BAFF) is a fundamental B cell survival factor and therefore may influence GC reactions and subsequent Ab responses. To test this possibility, the effect of BAFF neutralization in immunized mice was assessed. Using B cell maturation Ag-Fc, we demonstrate that BAFF blockade does not inhibit GC formation or somatic hypermutation. However, GCs in B cell maturation Ag-Fc-treated mice dissipated more rapidly than those of control mice and did not form a mature follicular dendritic cell reticulum. Examination of immunized BAFF-null mice validated the BAFF-independent nature of GC formation. Furthermore, Ab responses, including high-affinity responses, were attenuated. This is the first evidence that BAFF is required for maintenance, but not initiation, of the GC reaction, and it further hints that somatic hypermutation within the GC and selection of Ag-specific high-affinity Ab could be uncoupled. The Journal of Immunology, 2003, 171: 547–551.

Generation of humoral immune responses to T-dependent Ags requires cognate T cell-B cell interaction within the periarteriolar lymphoid sheath, resulting in activation of Ag-specific B cells. Thereafter, activated B cells proliferate and migrate to the splenic follicle where they initiate the germinal center (GC)5 reaction. The GC structure is the site wherein B cells continue to undergo rapid proliferation, followed by mutation of Ig V region genes and finally selection of those B cells exhibiting high-affinity Ag receptors into the plasma cell and memory B cell compartments (1, 2). This mutation and selection process requires continuous exposure to Ag. An abundant source of Ag can be achieved by the capture of Ag via immune and complement receptors (3), thus providing a microenvironment whereby constant stimulation of GC B cells is achieved.

Signals mediated by TNF family ligands CD154 (4) and lymphotoxin (LT)αβ (5) have been shown to be critical for initiation and maintenance of the GC reaction and subsequent Ab responses. B cell-activating factor belonging to the TNF family (BAFF) (6, 7) (also known as B lymphocyte stimulator (8); TNF- and ApoL-related leukocyte-expressed ligand 1 (9); TNF homolog that activates apoptosis, NF-xB, and c-Jun N-terminal kinase (10); and zTNF4 (11)), a recently identified member of the TNF ligand family, is a fundamental B cell survival factor and therefore may also be essential for successful GC development and function. A critical role for BAFF in B cell biology was clearly demonstrated by several laboratories. First, investigators showed that in vitro BAFF augments proliferation and Ig production mediated by cross-linking the B cell receptor (6), and functions as a B cell survival factor (12). Second, overexpression of BAFF in BAFF transgenic mice resulted in elevated numbers of peripheral B cells and circulating Ig, and an autoimmune phenotype (11, 13–15). In addition, in vivo administration of soluble BAFF enhanced humoral immune responses (8). Third, we demonstrated previously that administration of the BAFF decoy receptor-Fc fusion protein, B cell maturation Ag (BCMA)-Fc, to block endogenous BAFF activity resulted in a significant reduction of peripheral B cells (16). Validation of the critical role for BAFF in B cell survival/development was achieved by generating mice that lack a functional BAFF gene (17, 18). These mice exhibit a severe loss of peripheral B cells. Little is known about the role of BAFF in GC formation, generation of the B cell memory compartment, and plasma cell development. A single study using the BAFF receptor decoy, transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI)-Fc, reported a complete...
absence of GCs in treated mice (19). However, this same report also noted the presence of high-affinity anti-(4-hydroxy-3-nitrophenyl)acetyl (NP) Ab, albeit at low levels. This suggests an early GC response because high-affinity Ab are derived from GCs. Thus, the role of BAFF in the GC reaction requires further clarification. To this end, we used BCMA-Fc as a BAFF inhibitor and BAFF-null mice to investigate the impact of a BAFF-deficient environment on GC formation and function. In this study, we demonstrate that GC formation is BAFF independent, but that the integrity of the GC reaction is impaired over time in a BAFF-deficient environment. We also find that a mature follicular dendritic cell (FDC) network fails to form in a BAFF-deficient environment. Finally, despite a reduced ability to develop high-affinity cell in BCMA-Fc-treated mice, analysis of V region gene somatic hypermutation within the GC indicates that this function remains intact.

Materials and Methods

Receptor-Fc fusion protein

The BCMA-Fc fusion protein contains the extracellular domain of human BCMA and the Fc portion of human IgG (IgG1), as described previously (16).

Mice and treatment protocols

Six- to 7-wk-old female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME); BAFF-null mice and wild-type littermates were generated at Biogen, as described previously (17), and maintained in the Biogen animal facility under barrier conditions. All animal experimental protocols were approved by the Biogen Institutional Animal Care and Use Committee. For in vivo studies, 100 µg of BCMA-Fc, polyclonal IgG (Novartis, Basel, Switzerland); or PBS was administered i.p. two times per week for various lengths of time. Mice were bled via the retro-orbital sinus to capture sera. To evaluate whether BAFF is required to initiate the GC reaction, cohorts of NP-CGG-immunized mice that received BCMA-Fc, hIgG, or PBS (Fig. 1A) were sacrificed on days 7, 14, and 21, and their spleens were harvested for histologic examination of PNA+ GC structures. As shown in Fig. 1, treatment with BCMA-Fc did not block GC formation. The number of GCs in the spleens of BCMA-Fc-treated mice was equivalent to hIgG- and PBS-treated controls on days 7 and 14; however, by day 21, BCMA-Fc-treated mice exhibited a significant reduction in the number of GCs compared with controls (Fig. 1B). The remaining GCs on day 21 were also visibly smaller when compared with PBS- and hIgG-treated controls (Fig. 1C). To confirm that the ability of the immunized mice to mount a GC reaction was intact, we performed an ELISA for anti-NP Abs. Total and high-affinity NP-specific Abs were captured on NP22-BSA and NP3-BSA (Biosearch Technologies)-coated plates, respectively. To elaborate anti-NP specific Abs, mouse anti-rat IgG (Jackson ImmunoResearch, West Grove, PA) and streptavidin-PE (Molecular Probes, Eugene, OR) were added. ELISA results showed that the number of high-affinity NP-specific Ab was reduced in BCMA-Fc-treated mice compared with controls. These results suggest that BCMA-Fc inhibits GC formation in BAFF-null mice. To further confirm this, we performed ELISA on sera from mice treated with BCMA-Fc, hIgG, or PBS (Fig. 1A). The number of high-affinity NP-specific Ab was significantly reduced in BCMA-Fc-treated mice compared with controls. These results suggest that BCMA-Fc inhibits GC formation in BAFF-null mice.
form GCs was not due to insufficient blocking of BAFF, mice were given a more intensive regimen of BCMA-Fc treatment. In these experiments, mice received NP-CGG on day 0 and 100 μg of BCMA-Fc, hIgG, or PBS on days 0 to 7. Spleens of the BCMA-Fc-treated mice harvested on day 8 had obvious PNA⁺ GC structures (data not shown), although when compared with controls, they were fewer in number and smaller in size, similar to the day 21 data shown in Fig. 1.

To confirm that GC formation is truly BAFF independent, we examined spleens of BAFF-null mice at several time points after immunization with NP-KLH. The data show that BAFF-deficient mice are capable of forming PNA⁺ structures (Fig. 2A). Not surprisingly, the GCs in BAFF-null mice are smaller, and they dissipate more rapidly with time when compared with littermate control immunized mice (Fig. 2B).

**BAFF blockade impairs the formation of a mature FDC reticulum within the GC.**

Given that BAFF is a B cell survival factor, an obvious role for BAFF in GC maintenance would be a direct effect on B cell survival. Nevertheless, we considered that BAFF may also have an indirect effect on other cell types within the GC. An important component of the GC reaction is the formation of a mature FDC network, which is necessary for Ag trapping and presentation. To determine the impact of BCMA-Fc treatment on the FDC network, splenic tissue sections from mice that received BCMA-Fc, hIgG, or PBS were stained with anti-FDC-M1 to identify mature FDC. Anti-GL7 was used to identify GCs. The spleens examined were harvested 9 or 12 days after NP-CGG challenge. The FDC reticulum within the GC of BCMA-Fc-treated mice was quite scant (Fig. 3). In general, the mature FDC network (FDC-M1) did not appear as organized or mature as in PBS control or hIgG-treated (data not shown) mice despite the fact that the primary reticulum, identified in the GC by CD35 positivity (Fig. 3), was fully intact. Similarly, the FDC reticulum failed to mature in BAFF-null mice.6

**BAFF blockade inhibits T-dependent Ab responses, including high-affinity responses**

BAFF-null mice have previously been shown to exhibit a significantly impaired ability to mount both T-dependent and independent humoral immune responses (17, 18). To address whether the altered GC kinetics impacts Ab responses, sera from BCMA-Fc-treated mice were analyzed for NP-specific titers. Although BCMA-Fc treatment (Fig. 1A) did not result in a significant decline in the day 7 or day 14 anti-NP response, a statistically significant decline was evident on day 21 (Fig. 4, left panel). BCMA-Fc treatment also resulted in a significant reduction in the secondary anti-NP response when compared with hIgG and PBS controls (Fig. 4, right panel). These data are consistent with previously published reports using BAFF inhibitors (19, 22).

The quality of the Ab response was evaluated by assessing the abundance of high-affinity anti-NP Ab on day 21 of the primary response. For this analysis, sera were pooled from all mice in each group. High-affinity anti-NP Ab was determined using low (NP₃) density of hapten bound to plates. As shown in Table I, BCMA-Fc-treated mice exhibited a significantly smaller amount of high-affinity anti-NP Ab when compared with hIgG- and PBS-treated control mice. These data correlate with an underlying deficiency in GC processes.

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**FIGURE 2.** The GC reaction in BAFF-null mice. Frozen spleen sections derived from three BAFF-null mice were analyzed at different time points after immunization with NP-KLH. A, GCs were stained with PNA (brown) and follicular boundaries were revealed with mAb MOMA-1 which stains metallophilic macrophages (blue). A representative image is shown from day 10 of the anti-NP response. Arrows point to PNA⁺ GCs (×100 magnification). B, Immunofluorescent staining of spleen sections from wild-type (□) and BAFF-null (■) mice 6 and 12 days after immunization with NP-KLH was performed using GL7-FITC to determine the number of GCs per ×10 field. Data represent counts from at least two ×10 fields from three sections. *, p < 0.05.

**FIGURE 3.** The impact of BCMA-Fc treatment on the FDC reticulum. Splenic tissue sections were incubated with anti-FDC-M1 to visualize mature FDC (red, left panel), anti-GL7 to visualize GCs (green, both panels), and anti-CD35 (red, right panel) to visualize the primary reticulum (×200 magnification).
A BAFF-deficient GC environment does not impact the frequency of somatic hypermutation

An important function within the GC is the process of somatic hypermutation and generation of plasma cells secreting high-affinity Ab. Because BCMA-Fc-treated mice exhibited an unstable GC response, we hypothesized that this impairment resulted in a decline in somatic hypermutation, thus creating a diminished capacity for high-affinity Ab production. To address the validity of this hypothesis, adjacent splenic sections from various treatment groups and BAFF-null animals immunized with NP-CGG were stained with NP-CGG-biotin or anti-IgM-PE or anti-IgG-FITC or anti-IgD-FITC and PNA-HRP to identify A+ or NP+ GCs. The λ L chain-bearing Ag-specific GCs were microdissected, and the Vα1 gene was PCR amplified from extracted genomic DNA using specific primers. Amplified fragments were cloned and sequenced. The data were obtained from four to six animals for each individual group, and GCs were microdissected on day 9 or 12 postimmunization. Seven to 17 sequences were analyzed for each group. Analysis of five GCs from control PBS-treated animals yielded an average mutation frequency of 0.58%, which is in agreement with that reported earlier (21). The mutation frequency obtained from five GCs of the hlgG-treated group was 1%. Eight GCs from the BCMA-Fc-treated group yielded a mutation frequency of 0.722%, which was determined not to be statistically different from the PBS control group. BAFF-null animals (six GCs) revealed a slightly lower mutation frequency of 0.31%. Results from this analysis are shown in Table II. Overall, the data indicate that the decline in the NP-specific high-affinity Ab response in a BAFF-deficient environment does not result from loss of somatic hypermutation within the GC.

Table I. Inhibition of high-affinity anti-NP production

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<th>Treatment*</th>
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<tr>
<td></td>
<td>hlgG</td>
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<tr>
<td>High-affinity Abb</td>
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* Treatment described in Fig. 1A.

b Micrograms per milliliter from pooled sera of five mice per group.

Table II. Mutation analysis of Vα1 gene segments from microdissected NP+ GCs

<table>
<thead>
<tr>
<th>Genotype/Treatment</th>
<th>Total No. of GCs</th>
<th>Average Mutation Frequency (%)</th>
</tr>
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<tbody>
<tr>
<td>PBS</td>
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<td>0.58</td>
</tr>
<tr>
<td>hlgG</td>
<td>5</td>
<td>1.0</td>
</tr>
<tr>
<td>BCMA-Fc</td>
<td>8</td>
<td>0.722</td>
</tr>
<tr>
<td>BAFF-null</td>
<td>6</td>
<td>0.31</td>
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Discussion

In this study, we have demonstrated, contrary to published data, that GC formation is initiated in both genetically and experimentally induced BAFF-deficient environments. The generation of PNA+ GC structures in BAFF-null mice was particularly noteworthy given the paucity of mature B cells. Nevertheless, these data show that B cells in the spleen can be activated and recruited into the GC reaction in the absence of BAFF, or APRIL, because BCMA-Fc would block APRIL activity as well (7). Interestingly, the data also demonstrate that BAFF is required for the maintenance of the GC reaction, because GCs in BCMA-Fc-treated and BAFF-null mice dissipated more rapidly over time when compared with control animals. This may be due to a direct effect on BAFF function within the GC, such as survival of GC B cells, or to an indirect effect on other cellular components, such as FDC.

The disparity between our observation of GC formation in a BAFF-deficient environment and the converse reported previously (19) using TACI-Fc may reflect the distinct experimental systems that were used. Yan et al. (19) dosed with TACI-Fc daily for 14 days following Ag priming with NP-CGG. Spleens were examined for GCs at only a single time point, day 14 postimmunization. Therefore, it is possible that GC formation occurred early in the response with TACI-Fc treatment, but that GCs dissolved by day 14. Alternatively, it is possible that a TACI-specific ligand exists, and the conflicting results between TACI-Fc and BCMA-Fc would support the notion of a yet-to-be-discovered TACI ligand that is critical for GC formation.

The anti-NP response generated in the presence of BCMA-Fc was also examined. The data were consistent with previous reports (19, 22, 23) and showed that, over time, the primary anti-NP response was significantly reduced with BAFF inhibition when compared with control animals (Fig. 4). This attenuation of the Ab response correlated well with the observed decline in GC stability (Fig. 1, B and C). The diminished Ab response with BAFF blockade could be due to many factors, including reduced survival of peripheral B cells, thus limiting the Ag-specific B cell pool, a reduced frequency and/or survival of NP-specific plasma cells, and/or reduced Ag presentation due to impaired maturation of the FDC network. Still to be elucidated are the roles of BAFF in plasma cell survival, and the survival and functional capability of FDCs in a BAFF-deficient environment.

When compared with control mice, the secondary anti-NP response was also significantly reduced in animals that received BCMA-Fc (Fig. 4), which suggests that the Ag-specific memory B cell compartment failed to be generated. Furthermore, when the quality of the anti-NP response was examined, we found that the lack of BAFF decreased the production of high-affinity Ab. Interestingly, however, the frequency of somatic hypermutation in BCMA-Fc-treated mice was not dramatically altered.
compared with control mice, indicating that the basic processes of somatic hypermutation remained intact. Based on these data, we postulate that high-affinity Ab production and somatic hypermutation can be uncoupled. An exhaustive GC mutation analysis of large numbers of GCs is required to conclusively prove this postulate.

How BAFF impacts the mature FDC network is currently unclear. It is possible that lymphocyte-derived LT, known to have a significant role in maintaining splenic FDC (24), is limiting due to a paucity of B cells in BCMA-Fc-treated mice (Ref. 16 and data not shown). The absence of mature FDC in LT-null mice and their inability to form GCs (5) is consistent with the observation of an immature FDC reticulum in BCMA-Fc-treated mice and the instability of GCs formed in a BAFF-deficient environment.

In summary, our observations reported in this study clarify and further define the role of BAFF in the network of TNF family members.

Acknowledgments

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References