The Pleckstrin Homology Domain Adaptor Protein Bam32/DAPP1 Is Required for Germinal Center Progression

Ting-ting Zhang, Monther Al-Alwan and Aaron J. Marshall

J Immunol 2010; 184:164-172; Prepublished online 30 November 2009;
doi: 10.4049/jimmunol.0902505
http://www.jimmunol.org/content/184/1/164
The Pleckstrin Homology Domain Adaptor Protein Bam32/DAPP1 Is Required for Germinal Center Progression

Ting-ting Zhang,*† Monther Al-Alwan,*† and Aaron J. Marshall*†,‡

Ab affinity maturation within germinal centers (GCs) requires weeks to complete. Several signaling pathways in B cells have been shown to be required for initiation of the GC response; however, the signaling checkpoints controlling progression and eventual dissolution of the GC reaction are poorly understood. The adaptor protein Bam32/DAPP1 was originally isolated from human GCs and functions downstream of phosphoinositide 3-kinase enzymes, which are known to have critical roles in B cell activation and GC responses. In this study we identify a unique role of Bam32/DAPP1 in promoting GC progression. Bam32-deficient mice show normal GC initiation, but premature GC dissolution after immunization with protein Ag in alum or low doses of sheep red blood cells. Adoptive transfer studies confirmed that Bam32-deficient B cells have an intrinsic impairment in the ability to mount sustained GC responses. Bam32 deficiency was also associated with impaired Ab affinity maturation. Proliferation of Bam32-deficient GC B cells was not compromised; however, these cells show impaired switch to IgG1 and increased apoptosis in situ. GCs formed by Bam32-deficient B cells contain fewer T cells, indicating that Bam32 is required for B cell–dependent T cell accumulation within established GCs. Exogenous CD40 ligand restored GC B cell numbers and switch to IgG1, indicating that Bam32-deficient B cells are competent to respond to CD40 stimulation when ligand is available. These data demonstrate that Bam32 is not required for GC initiation, but rather functions in a late checkpoint of GC progression associated with T cell recruitment and GC B cell survival. The Journal of Immunology, 2010, 184: 164–172.

During B cell responses to T-dependent Ags, key B cell activation and differentiation events occur within germinal centers (GCs) (1–4). The GC response is normally initiated when B cells activated by encounter with Ag and cognate T cell help migrate to the B cell follicles and begin proliferating rapidly in association with the follicular dendritic cell (FDC) network to give rise to a GC. GC B cells proliferate rapidly and undergo mutation and breakage of the chromosomal DNA in the Ig locus while under intensive positive and negative selective pressure (5). GC B cells are highly prone to apoptosis, but can be rescued from apoptosis in culture by stimulation through either CD40 or the BCR (6). Maintenance of GC responses, as well as generation of memory B cells, depends on signaling via CD40L and other T cell–derived signals (7–11). Under some unusual circumstances, GC responses can be initiated in the absence of T cell help; however, these GCs are short-lived and abort prematurely without giving rise to affinity maturation (12, 13), underlining the critical importance of T cell–derived signals in sustaining the GC response through the critical selection period. Our understanding of intracellular signaling pathways that control selective survival of high affinity GC B cells is limited.

The B cell adaptor molecule of 32 kDa (Bam32), also known as DAPP1, was identified during a screen for genes highly expressed in human GC cells (14) and through screens for proteins binding to phosphoinositide products of phosphoinositide 3-kinase (PI3K) (15–17). PI3K enzymes are strongly activated by BCR ligation (18, 19), and the importance of PI3K signaling in B cell development and activation is well established (20, 21). Bam32 binds PI3K lipid products, PI(3,4)P2 and PI(3,4,5)P3, in vitro (15) and is recruited to the plasma membrane in a PI3K-dependent manner upon BCR ligation (14). Bam32 phosphorylation by Src family kinases is also PI3K-dependent (17, 22). Bam32 has been implicated in BCR signaling processes, including activation of the GTPase Rac1, and MAPKs ERK and JNK (23–25). Bam32-deficient B cells develop normally, but show markedly impaired BCR-triggered proliferation in vitro; however, proliferation could be fully recovered with addition of anti-CD40 and/or IL-4 stimuli (23, 26). PI3K-deficient mouse models, as well as CD19 mutants lacking the ability to activate PI3K, show defective GC responses (20, 27, 28). In contrast, Bam32-deficient mice were reported to generate normal size GCs after immunization with SRBCs (23, 26).

In this study, we found that Bam32-deficiency lead to normal GC initiation and proliferation, but premature dissolution of GC responses, associated with reduced T cell recruitment into GCs, decreased cell survival and isotype switch, and less efficient affinity maturation. The requirement for Bam32 in GC progression could be restored by treatment with agonistic anti-CD40 Ab. Together, our findings provide evidence that the PI3K effector molecule Bam32 is not required for GC initiation or GC B cell proliferation, but has a unique function in promoting GC progression through a critical selection checkpoint.
Materials and Methods

Mice

Bam32 knockout (KO) mice were a kind gift from Michel C. Nussenzweig (Laboratory of Molecular Immunology, Rockefeller University, New York, NY) and have been described previously (23). δMT mice (Igh-6tm1Cgn) were purchased from The Jackson Laboratory (Bar Harbor, ME), and CD45.1 mice were obtained from Taconic Farms (004007; SJL-Ptprc<sup>a</sup>; Germantown, NY). Age and sex-matched C57BL/6 mice were purchased from Charles River Laboratories (Quebec, Canada). All animals were housed at the Central Animal Care Facility (University of Manitoba, Winnipeg, MB) in compliance with the guidelines established by the Canadian Council on Animal Care.

B cell isolation, stimulation and functional assays

For in vitro functional assays, B cells purified from the spleens of naive mice using anti-CD43 Microbeads (Miltenyi Biotech, Auburn, CA), according to the MiniMacs protocol (Miltenyi Biotech). For proliferation assay, B cells (2 × 10<sup>5</sup> in 200 μl of complete medium) were stimulated in round-bottom 96-well culture plates with goat-anti mouse IgM (Jackson ImmunoResearch Laboratories, West Grove, PA) or purified anti-mouse CD40 Abs (BD Pharmingen, San Diego, CA), alone or together with recombinant mouse IL-4 (PeproTech, Rocky Hill, NJ), for 72 h. In the last 18 h of incubation, cultures were pulsed with 1 μCi of tritiated thymidine. For apoptosis, isotype switch, and plasma cell differentiation assays (29), B cells (1 × 10<sup>6</sup>/ml) were stimulated in 24-well culture plates (2 ml/well) for 3 d with indicated stimuli. At the end of culturing, cells were cultivated and stained with FITC-anti-GL7 (BD Bioscience, San Jose) and DAPI (Sigma-Aldrich, St. Louis, MO) to detect apoptotic cells, or with Abs to IgG1 and CD138 (BD Bioscience) to detect switched cells and plasmablasts by flow cytometry.

Immunization, adoptive transfer, and treatments

Six- to 10-wk-old mice were immunized by i.p. injection of 10 μg OVA protein (Sigma-Aldrich) adsorbed onto 2.0 mg Al(OH)<sub>3</sub> (alum) adjuvant (Imject Alum; Pierce Chemical, Rockford, IL) and splenic GC responses were analyzed at the indicated time points. For adoptive transfer experiments, splenic B cells purified from either wild-type (WT) or Bam32-deficient mice were transferred by i.v. injection (1 to 2 × 10<sup>7</sup>/recipient) into the tail vein of B cell-deficient mice (δMT). For competitive adoptive transfer experiments, splenic B cells from either WT C57BL/6 or Bam32-deficient mice (CD45.2) were mixed with CD45.1 B cells at a 1:1 ratio before adoptive transfer (2 to 4 × 10<sup>7</sup> per recipient) to δMT mice. Six days after transfer, recipient mice were injected i.p. with 10 μg OVA/alum, and GC responses were investigated 12 d after immunization. For in vivo cell proliferation experiments, BrdU (Sigma-Aldrich) in PBS (2.5 mg/mouse) was administered by i.p. injection 5 h before the mice were sacrificed. In some experiments, at days 5 and 10 after immunization, the mice were treated with 50 μg agonistic anti-CD40 (FGK45; Alexis Biochemicals, San Diego, CA) or Rat IgG2a isotype control (eBioscience, San Diego, CA) by i.p. injection.

FIGURE 1. Bam32 deficiency leads to premature dissolution of GCs. A, WT C57BL/6 (WT) and Bam32-deficient (Bam32<sup>−/−</sup>) mice were immunized with T-dependent Ag OVA/alum. After 7, 10, or 14 d, splenocytes were isolated and stained with FITC-anti-GL7, PE-anti-Fas, and APC-anti-B220 to identify GC B cells. Representative GL7/Fas plots are shown (gated on B220+ lymphocytes). The right panel shows the percentage of GC B cells at different time points expressed as the mean ± SD of at least four mice per point. B, GCs in spleen cryosections were visualized using confocal microscopy. Sections were stained with FITC-anti-GL7 (green), PE-anti-CD4 (red), and Biotin-anti-IgD with secondary Ab Avidin-Alexa647 (blue). The GCs in Bam32-deficient mice have visibly deteriorated by day 10 and are virtually absent at day 14. C, Purified B cells (1.5 to 2.0 × 10<sup>7</sup>) from either WT or Bam32-deficient mice were transferred to B cell-deficient μMT mice. At day 12 after OVA immunization, GC B cells in μMT mice were identified using flow cytometry. Representative flow cytometric plots gated on CD19+ lymphocytes (left) and the percentages of GC B cells for individual mice (right) are shown.
Affinity maturation assay and serum IgM

To measure Ab affinity maturation, WT and Bam32−/− mice were immunized i.p. with 2 μg/ml nitrophenyl-OVA (NP-OVA; Biosearch Technologies) absorbed to 2 mg/ml alum. Sera were collected at days 7, 14, 21 after primary immunization by tail bleeding. Mice were boosted 28 d later with the same dose NP-OVA. Ten days after the boosting, mice were sacrificed and blood was collected by cardiac puncture. NP-specific IgG1 and IgG2a Ab of high and low affinity were detected by ELISA with NP3-BSA and NP20-BSA–coated plates as described earlier (30, 31). The quantities of NP-specific IgG1 and IgG2a in each experiment were expressed in relative units compared with a standard hyperimmune serum, and a ratio of NP-binding over NP20-binding Abs was calculated for determination of total and OVA-specific IgM. ELISA plates (MaxiSorp; Nunc, Naperville, IL; Roskilde, Denmark) were coated overnight at 4°C with goat anti-mouse IgM or 20 μg/ml OVA, respectively, and bound serial diluted serum Abs were detected with biotinylated rat anti-mouse IgM (BD Biosciences).

Flow cytometric analyses

Splenocyte single cell suspensions were prepared and surface stained with combinations of Abs to the following: GL7 (BD Biosciences), Fas (Jo2), B220 (clone RA3-6B2), CD138 (28-1-2), IgG1 (A85-1; BD Biosciences), peanut agglutinin (PNA; Vector Laboratories, Burlingame, CA), CD19 (6D5), goat anti-mouse IgM (SouthernBiotech, Birmingham, AL), CD45.1 (A20; eBioscience), CD45.2 (104; eBioscience). Biotin conjugates were visualized with streptavidin-PerCP-Cy5.5 (BD Biosciences) or streptavidin-din-PE-Alexa647 (Molecular Probes, Eugene, OR). After surface staining, cells were either directly analyzed by flow cytometry, or further stained with the following steps.

For BrdU staining, 1 to 2 × 106 cells were fixed and permeabilized twice, followed by BrdU exposure using deoxyribonucleoside I (Sigma-Aldrich) to digest genomic DNA at 37°C for 30 min, according to the manufacturers protocol (BD Biosciences). After extensive washing, the cells were stained with FITC anti-BrdU (eBioscience) at room temperature (RT) for 20 min.

To stain active caspases in apoptotic cells, CaspGlow Fluorescin Caspase Staining Kit (Biovision, Mountain View, CA) was used. Cells (1 × 106) were resuspended with 300 μl of RPMI640 containing 10% FCS. FITC-VAD-FMK (1 μl) was added to the culture and then incubated for 30 min at 37°C with 5% CO2. After extensive washing, cells were suspended in FACs buffer and transferred to an FACS tube for sample acquiring.

For Annexin V staining, 1 to 2 × 106 cells were resuspended with 1 ml Annexin V–binding buffer (BD Biosciences); 100 μl was transferred to another FACs tube and then incubated with 5 μl FITC-anti Annexin V (BD Biosciences) and 100 ng DAPI for 15 min at RT, followed by adding 400 μl Annexin V–binding buffer at the end of incubation.

Cells were immediately acquired on an FACS Calibur or FACS CantoII (BD Biosciences), and data were plotted using FlowJo software (TreeStar, Ashland, OR). DAPI staining was detected using violet laser (405 nm) excitation and the PacificBlue detection channel.

Immunofluorescence microscopy

Spleens from immunized mice were harvested, embedded in O.C.T. Compound (Tissue-Tek, Torrence, CA), and snap frozen in liquid nitrogen. Frozen sections (8–10 μm thick) were fixed in cold acetone for 15 min and dried in air for 10 min. The slides were blocked with 5% horse serum for 30 min at RT and then stained overnight at 4°C with biotinylated anti-IgD (SouthernBiotech) or biotinylated-anti-CD35 (BD Biosciences). After washing with PBS, the slides were stained with mixtures of Abs: FITC-labeled anti-GL7, PNA, or FITC-labeled anti-Ki67 (BD Biosciences); PE-anti-CD4, and streptavidin-Alexa647 (Molecular Probes) for 2 h at RT. After extensive washing, the slides were mounted in Prolong Gold anti-fade reagent (Molecular Probes). The sections were then viewed under an inverted confocal microscope (Ultraview LCI; PerkinElmer, Wellesley, MA). The mean fluorescence intensity (MFI) of CD4 staining within GC was determined by analyzing images with ImageJ software (National Institutes of Health, Bethesda, MD) as follows. First, an irregular region was drawn around the GC area as determined by GL7+ IgD-staining. Then, “RGB stack” was selected to display only the CD4 channel (red). The MFI of CD4 within the defined region was then calculated using the “Analyze>Measure” function.

Statistics

Statistical significance was assessed using Student’s t test: *p < 0.05; **p < 0.01; and ***p < 0.001.

Results

Bam32 deficiency leads to premature dissolution of GCs

We compared the kinetics of the GC response in WT or Bam32-deficient mice immunized with protein Ag OVA in alum. B220+GL7+ Fas+ GC cells were present in similar frequencies at day 7 after immunization; however, Bam32-deficient mice showed a decline in GC cells starting at day 10 postimmunization (Fig. 1A). GC B cells are strikingly absent in Bam32-deficient spleens at day 14, a time point when the WT response reached its peak (Fig. 1A). Similar reductions in GC populations at day 14 were observed using PNA staining to identify GC cells (data not shown). Previous studies of Bam32-deficient mice found no obvious deficiency in GC formation after immunization with SRBCs (23, 26). Consistent with these findings, we found that immunization with 10% SRBCs results in similar robust GC responses in both WT and Bam32-deficient mice; however, immunization with 1% SRBC revealed reduced GC responses in Bam32-deficient mice at day 12 postimmunization (Supplemental Fig. 1).

Immunofluorescence staining of spleen sections revealed GC initiation in both WT and Bam32-deficient mice, with normal positioning of GL7+ foci at the interface of T and B zones at day 7 postimmunization (Fig. 1B, left) and no obvious difference in segregation of FDC and non-FDC zones (Supplemental Fig. 2). Consistent with flow cytometric data, smaller GCs were observable by staining of spleen sections from Bam32-deficient mice at day 10 postimmunization; moreover, those GCs appear abnormal, with a less cohesive core of GL7−bright cells, and a significant number of IgD+ cells interspersed within the GC area (Fig. 1B, middle). By day 14, no obvious GCs were detectable in the spleens of Bam32−/− mice, whereas the number and size of WT GCs was increased compared with day 7 (Fig. 1B, right). To determine whether the

![FIGURE 2. Reduced affinity maturation in Bam32-deficient mice. WT or Bam32-deficient mice were immunized with T-dependent Ag NP-OVA and boosted at day 28. At the indicated days, sera were collected for measurement of low and high affinity NP-specific IgG1 (A) and IgG2a (B) Abs. Low-affinity NP-specific Abs were detected using NP20-BSA–coated plates, whereas high-affinity Abs were detected using NP3-BSA-coated plates. The ratio of NP20/NP3-binding, used as index of affinity maturation, is plotted for each mouse.](http://www.jimmunol.org/)

Downloaded from http://www.jimmunol.org/ by guest on April 20, 2017
impairment in GC responses is due to a B cell–intrinsic function of Bam32, purified Bam32-deficient or control B cells were adoptively transferred to B cell–deficient hosts. Host animals receiving Bam32-deficient B cells showed significant reduction in the frequency of GC B cells generated 12 d after immunization (Fig. 1C). Together these results demonstrate that Bam32 deficiency leads to premature decay of the GC response.

Reduction affinity maturation of anti-NP–specific B cells in Bam32-deficient mice

We assessed whether premature termination of GC affects Ab affinity maturation. After immunization with NP-OVA, sera were collected at 1-wk intervals, and levels of Ab binding to OVA, NP3-BSA or NP20-BSA were determined. In WT C57BL/6 mice, high-affinity NP3-binding Abs appeared only at later time points after immunization, resulting in an increasing ratio of NP3-binding/NP20-binding Ab over time, reaching affinity maturation ratios of 0.91 and 0.69 for IgG1 and IgG2a, respectively (Fig. 2). Although progressive Ab affinity maturation was also detectable in Bam32-deficient mice, the ratio of NP3/NP20-binding achieved over the course of the response was significantly reduced, reaching ratios of 0.67 and 0.43 for IgG1 and IgG2a respectively (Fig. 2). These results are consistent with impairment in progressive affinity selection within GC.

Bam32 is not required for GC B cell proliferation

We investigated whether selective defects in B cell proliferation may account for impaired GC progression in Bam32-deficient mice. As previously reported (23, 26), Bam32-deficient B cells show markedly impaired in vitro proliferative responses to BCR stimulation, but proliferation can be rescued by the addition of anti-CD40 and IL-4 (Supplemental Fig. 3A). In fact, dose response experiments revealed significantly increased proliferation to either anti-CD40 or IL4, whereas proliferation in response to LPS was similar to controls (Supplemental Fig. 3B). To assess GC B cell proliferation in vivo, we first examined the expression of the nuclear proliferation Ag Ki67. At day 7 after immunization, both WT and Bam32 KO mice show many Ki67⁺ cells present within GCs surrounded by Ki67⁻ IgD⁺ B cells (Fig. 3A, left). Similar frequencies of Ki67⁺ cells were observed in WT and Bam32 KO mice (Fig. 3A, right). These results are consistent with the absence of selective defects in GC B cell proliferation in Bam32-deficient mice.
duced rate of DNA synthesis among GC B cells. This result suggests that Bam32-deficient B cells do not have a proliferative disadvantage, but appear to have some advantage over WT B cells, such that they became overrepresented in the initial pool of GC B cells. Interestingly, the mice receiving the mixture of Bam32−/− and WT cells showed an overall reduction in the frequency of GC B cells at day 12 compared with mice receiving only WT cells (Fig. 3C, bottom). This result supports the GCs that were initially populated with Bam32-deficient B cells later underwent premature dissolution. Together, the preceding results strongly support the conclusion that Bam32 is not required for GC initiation and GC B cell proliferation.

**Bam32-deficient B cells recruit fewer CD4+ T cells into the GC**

In the course of our confocal imaging analyses, we observed that day 10 GCs formed in Bam32-deficient mice seemed to show less staining of CD4+ cells within the PNA+ GC area. Subsequent image analysis confirmed that CD4 staining within Bam32-deficient GCs is significantly reduced (Fig. 4A, 4B). Lack of T cell localization within Bam32-deficient GCs is not due to a global defect in development of follicular helper T cells, because similar numbers of CXCR5+ICOS+ Tfh cells are present in these mice (Supplemental Fig. 4). Adoptive transfer experiments were performed to test whether deficiency of Bam32 in B cells is sufficient to cause defective T cell entry into GCs. Fewer CD4+ T cells were observed within GCs formed in μMT recipients receiving Bam32-deficient B cells, compared with those receiving WT B cells (Fig 4C). Recent data support the model that T cell entry into established GCs is dependent on Ag-specific interactions with GC B cells (32). The present results suggest that signals transmitted via Bam32 are required for B cell–dependent T cell entry or retention in GCs.

**Bam32 deficiency leads to reduced isotype switch within GCs**

To further examine the role of Bam32 in B cell differentiation within GCs, we examined expression of a number of differentiation-associated cell surface markers at day 10 postimmunization. There was no significant difference in expression of MHCII, CD86, or CXCR4 between WT and Bam32 KO GC cells (data not shown); however, Bam32 KO GC cells showed a marked skewing toward IgM+ cells, with the frequency of IgG1-switched GC cells decreased by 3-fold on day 10 (Fig. 5A). Significant increases in total and Ag-binding IgM were observed in serum of immunized Bam32-deficient mice (Fig. 5B). Impaired isotype switch within GCs was further confirmed by immunofluorescence staining of spleen sections (Fig. 5C). In contrast, purified Bam32-deficient B cells were functionally competent in class switch to IgG1 when stimulated in vitro with anti-CD40+IL-4 (Fig. 5D). Together these results demonstrate reduced switching to IgG1 within Bam32-deficient GCs, and suggest that this may be due to lack of access to CD40 ligand and/or other switch factors within GCs, rather than an intrinsic inability of Bam32-deficient B cells to switch. This local reduction of IgG1-switched cells and premature dissolution of GCs in immunized Bam32-deficient mice did not result in significant changes in systemic IgG1 levels (data not shown), consistent with previously published studies (23, 26).

**Bam32 deficiency leads to increased apoptosis within GCs**

Initiation of apoptosis in WT and Bam32 GC cells was investigated by staining with fluorescently labeled VAD-FMK caspase inhibitor to detect active caspases. Strikingly, the frequency of cells staining with CaspGlow reagent is significantly increased in Bam32-deficient GC cells (Fig. 6A). This increase is apparent at days 7 and 10 after immunization and occurs among both IgM+ and IgM− GC cell populations, providing an argument against selective apoptosis of the more differentiated IgM− population. Consistent with
CaspGlow staining results, the frequency of Annexin V+ DAPI cells (early apoptotic cells) among Bam32-deficient GC cells was also significantly increased (Fig. 6B). Bam32-deficient B cells do not seem intrinsically prone to apoptosis because in vitro stimulation of Bam32-deficient B cells with anti-IgM+anti-CD40 generates comparable levels of apoptotic cells (Fig. 6C). Together, these results indicate that signaling via Bam32 is required for development of mature GCs capable of promoting GC B cell survival in situ.

Exogenous CD40 ligand can bypass the requirement for Bam32 in GC progression

To investigate whether premature collapse of Bam32 GC responses can be rescued by an exogenous T cell–derived signal, we treated mice with agonistic anti-CD40 Ab, starting at day 5 postimmunization. By day 12, the group of Bam32-deficient animals treated with anti-CD40 Ab showed fully restored GC responses (Fig. 6C). In contrast, animals treated with a control Ab did not show restored GC responses. The overall reduction in GC responses in control Ab-treated mice may be due to FcR-mediated inhibitory effects. Anti-CD40 treatment also restored the frequency of GC cells expressing IgG1 to levels similar to WT mice (Fig. 6D).

Discussion

Collectively, the results reported in this study provide evidence that a signaling process involving Bam32 is essential for GC progression through a critical checkpoint. In contrast, Bam32 is not required for GC initiation or proliferation of GC B cells, consistent with different signaling requirements (or different thresholds) for GC initiation versus progression. Previous studies on Bam32-deficient mice found normal induction of GC responses after immunization with SRBCs (26), and our results using 10% SRBC immunization are consistent with these findings. However, premature dissolution of GCs in Bam32-deficient mice is clearly apparent upon immunization with lower doses of SRBC or OVA protein Ag. The basis for premature collapse of the GC response appears to relate to increased apoptosis among GC B cells, which also correlates with decreased switch to IgG1. These phenotypes suggest that Bam32-deficient GC B cells fail to sufficiently access T cell–derived signals such as CD40L, and our results indicate a failure of B-dependent T cell entry into GCs. The requirement for Bam32 in GC maintenance can be overcome by treatment with agonistic anti-CD40 mAb, indicating that Bam32-deficient B cells
are capable of mounting a sustained response when the required GC progression signals are provided exogenously. We speculate that a similar effect may occur during immunization with high doses of SRBC, known to polyclonally activate lymphocytes through iron-containing compounds such as hemin (33). The relatively subtle signaling defect in Bam32-deficient B cells (allowing GC responses to initiate normally) provides a unique opportunity to study a relatively late GC progression checkpoint.

Interestingly, we found evidence that Bam32-deficient B cells are specifically hyper-responsive to stimulation via CD40 or IL-4. In vitro proliferation in response to these stimuli is significantly increased, and these stimuli dramatically synergize with BCR stimulation to restore proliferation. Previous studies also found that addition of IL-4 and/or anti-CD40 can restore BCR-triggered proliferation of Bam32-deficient B cells (23, 26), and these published data are also consistent with the trend of increased responsiveness of Bam32-deficient B cells to these T-dependent signals. Our data indicate that, after immunization of Bam32-deficient mice with T-dependent Ag, initial B cell proliferation is robust. In addition, in competitive adoptive transfer, Bam32-deficient cells appear to initiate a GC response more efficiently than WT cells. Although the reasons for this hyperresponse to T cell–derived stimuli are not known, it is possible that this may partially obscure the effects of subsequent GC collapse. For example, although IgG1 switching in GCs is clearly decreased by day 10, systemic IgG1 responses (measured by serum titers) are in the normal range (data not shown). Previous studies of Bam32-deficient mice also found no alteration in IgG1 responses after immunization with T-dependent Ag (23, 26). Our interpretation of these collective data are that Bam32-deficiency leads to strong initiation of the T-dependent response and strong production of low affinity IgG1 and IgM, such that the impairment in the systemic Ab response is only discernable at later time points, at the level of affinity maturation.

Our results differ markedly from observations made with mice lacking the PI3K docking site in CD19, which show diminished proliferation in nascent GCs and decreased serum IgG titres, in addition to subsequent GC collapse (28). GC formation was restored in CD19-deficient mice by coinactivating PTEN (thus deregulating PI3K signaling), further suggesting that PI3K-dependent signals are key for GC responses (34). Also, these results are distinct from those observed in mice exhibiting global defects in PI3K signaling. For example, inactivation of the PI3K catalytic subunit p110δ leads to failure to generate GCs because of impaired B cell development, proliferation, and survival (27); however, signaling via PI3K is also important for restraining activation-induced cytidine deaminase expression and class switch recombination (35, 36). Clearly, PI3K-dependent signals are critical for both GC initiation and progression,
and the present results implicate Bam32 as one of the PI3K-dependent effector molecules that comes into play, particularly during GC progression.

Because Bam32 is not required for B cells to respond to T cell–derived signals, we hypothesize that it is required for B cells to obtain access to these signals via Ag-mediated B:T conjugate formation. Our results suggest that Bam32-deficient GC B cells are unable to promote CD4+ T cell accumulation within established GCs, a function recently shown to depend on cognate B:T interactions (32). We have found that PI3K is required for efficient BCR-mediated Ag presentation and formation of stable B:T conjugates (37), consistent with the model that signaling through this pathway promotes B:T cognate interactions within GCs. Our recent results indicate that Bam32 is also required for efficient BCR-mediated Ag presentation, likely via its role in promoting Rac activation and cell adhesion to ICAM (M. Al-Alwan, S. Hou, T. Zhang, K. Makondo, and A.J. Marshall, submitted for publication). Thus, our working model is that Bam32 deficiency leads to impaired B:T cognate interaction within GCs, limiting the amount of T cell–associated survival signals available to GC B cells. Exogenous provision of anti-CD40 restored GC progression, indicating that CD40 signaling in GC B cells can bypass the requirement for Bam32.

Whereas there is abundant evidence that somatic hypermutation and affinity selection occur within GCs, there are a number of documented cases in which affinity maturation can occur relatively efficiently in the face of disrupted GC responses (30, 38). This may be due to defective proliferation and/or disruption of tissue architecture that precludes formation of robust GCs, whereas normal selective survival mechanisms may remain relatively intact. In the case of Bam32 deficiency, the opposite is true: there is no impairment in GC induction and proliferation, yet affinity maturation is less efficient. These findings are consistent with the idea that initial proliferation within GCs may occur relatively independently of affinity for Ag, with the selective threshold for GC cell survival increasing over time with establishment of more stringent selection checkpoints. Our data show that the reduction in Ab affinity in Bam32-deficient mice is most pronounced at later time points. The inability to detect differences in affinity at earlier time points may partly reflect a technical limitation of the assay and/or may be related to the slow turnover rates of the initial wave of low affinity serum Abs.

The differential signaling requirements for GC initiation versus progression and selection are not well understood. Our results indicate that BCR signaling in the absence of Bam32 is sufficient to allow GC initiation and robust GC B cell proliferation, in marked contrast with other BCR signaling deficiencies such as CD19, PI3K, or BLNK deficiency. A similar phenotype of normal GC initiation, but premature GC collapse, was observed in CD45−/− B cell chimera after immunization. In that case, intrinsically poor survival of CD45−/− B cells seemed to account for the failure to maintain GC responses, whereas Ag presentation function was normal (39). In contrast to Bam32-deficiency, BCR triggering of CD45-deficient B cells induced an abnormally high degree of apoptosis even in presence of CD40 ligation (39). Thus, it is likely that GC B cell survival requires collaboration of survival signals directly emanating from the BCR involving CD45 and signals provided via cognate interactions with T cells involving Bam32.

Acknowledgments

We thank Dr. Michel Nussenzweig for Bam32-deficient mice, and Drs. Edward Clark and Robert Rickert for critical reading of the manuscript.

Disclosures

The authors have no financial conflicts of interest.


