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Bam32/DAPP1 Promotes B Cell Adhesion and Formation of Polarized Conjugates with T Cells

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B cell Ag receptors function in both signaling activation of Ag-specific cells and in collecting specific Ag for presentation to T lymphocytes. Signaling via PI3K is required for BCR-mediated activation and Ag presentation functions; however, the relevant downstream targets of PI3K in B cells are incompletely defined. In this study, we have investigated the roles of the PI3K effector molecule Bam32/DAPP1 in BCR signaling and BCR-mediated Ag presentation functions. In mouse primary B cells, Bam32 was required for efficient activation of the GTPase Rac1 and downstream signaling to JNK, but not activation of BLNK, phospholipase C γ2, or calcium responses. Consistent with a role of this adaptor in Rac-mediated cytoskeletal rearrangement, Bam32 was required for BCR-induced cell adhesion and spreading responses on ICAM-1 or fibronectin-coated surfaces. The function of Bam32 in promoting Rac activation and adhesion required tyrosine 139, a known site of phosphorylation by Lyn kinase. After BCR crosslinking by Ag, Bam32-deficient B cells are able to carry out the initial steps of Ag endocytosis and processing, but show diminished ability to form Ag-specific conjugates with T cells and polarize F-actin at the B-T interface. As a result, Bam32-deficient B cells were unable to efficiently activate Ag-specific T cells. Together, these results indicate that Bam32 serves to integrate PI3K and Src kinase signaling to promote Rac-dependent B cell adhesive interactions important for Ag presentation function. The Journal of Immunology, 2010, 184: 6961–6969.

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Abbreviations used in this paper: GC, germinal center; HEL, hen egg lysozyme; LAMP1, lysosome-associated membrane protein 1; MHC-II, MHC class II; PI, pleckstrin homology; PLCγ2, phospholipase Cγ2; RT, room temperature; SKAP, Src kinase-associated phosphoprotein; WM, pretreated with wortmannin (100 nM); WT, wild-type.

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activation of integrins, which promotes cell–cell adhesive interactions (14). PI3Ks produce 3-phosphoinositide second messengers at the plasma membrane, which provide docking sites for pleckstrin homology (PH) domain proteins (25, 26). The downstream targets of PI3K controlling B cell Ag presentation function are currently unknown.

Bam32, also known as DAPP1, was identified during a screen for genes highly expressed in human germinal center (GC) cells (27) and through screens for proteins binding to phosphoinositide products of PI3K (28–30). Bam32 binds PI3K lipid products, phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate, in vitro (28) and is recruited to the plasma membrane in a PI3K-dependent manner upon BCR ligation (27). Bam32 phosphorylation by Src family kinases is also PI3K dependent (30, 31). Bam32 has been implicated in BCR signaling processes, including activation of the GTPase Rac1 and MAPKs ERK and JNK (32–34). All B cell subsets develop normally in Bam32-deficient mice, but they show markedly impaired T-independent Ab responses (32, 35). In contrast, Bam32-deficient mice were reported to mount quantitatively normal responses to T-dependent Ag; however, we recently found that Bam32 deficiency leads to premature dissolution of GCs and impaired affinity maturation (36).

In the current study, we have used Bam32-deficient mice to test whether this molecule is functionally important for nonproliferative B cell functions linked to Rac activation, including B cell adhesion, spreading, and BCR-mediated Ag presentation to T cells. We find that Bam32-deficient B cells have impaired adhesion and spreading on integrin ligands and impaired formation of polarized conjugates with Ag-specific T cells. Together, our findings indicate that Bam32 functions to link the PI3K pathway to enhanced B cell adhesion and Ag presentation functions.

**Materials and Methods**

**Mice**

Bam32 knockout mice were a kind gift from Michel C. Nussenzweig (Laboratory of Molecular Immunology, Rockefeller University, New York, NY) and have been described previously (32). For BCR-mediated Ag presentation experiments, the Bam32-deficient mice were crossed to C57BL/6 MHC congenic mice expressing I-A^d (purchased from The Laboratory of Molecular Immunology, Rockefeller University, New York, NY) and have been described previously (32). In contrast, Bam32-deficient mice were reported to mount quantitatively normal responses to T-dependent Ag; however, we recently found that Bam32 deficiency leads to premature dissolution of GCs and impaired affinity maturation (36).

**Calcium assay**

Following isolation, purification, and equilibration at 37°C for 1 to 2 h in serum-containing medium, mouse B cells were washed and suspended at 10^6/ml in prewarmed serum-free medium. Cells were cultured in Opti-MEM medium (Invitrogen, Carlsbad, CA) containing 10% FCS, 10 μM 2-ME, antibiotics, and the indicated stimulatory Abs and/or cytokines.

**Abs and reagents**

The anti-Rac1 Ab (105-389) was from Upstate Biotechnology (Lake Placid, NY). Anti-JNK, anti–phospho-JNK, and HRP-labeled anti-rabbit IgG Ab were purchased from Cell Signaling Technology (Beverly, MA). Chemiluminescence detection reagents ECL or ECL Advance were purchased from GE Healthcare (Piscataway, NJ). FITC-labeled anti–MHC-II (clone D3.1.3.7) (30) was generously provided by Dr. John Cambier (National Jewish Medical and Research Center, Denver, CO). Anti–LFA-1 was purchased from eBioscience (San Diego, CA). Purified anti-mouse CD40 was from BD PharMingen (San Diego, CA). LPS was from Sigma-Aldrich (St. Louis, MO), and recombinant mouse IL-4 was from PeproTech (Rocky Hill, NJ). CFSE was purchased from Sigma-Aldrich, and CellTrace Far Red was purchased from Molecular Probes.

**Rac1 activation assay**

Rac1 activation was determined using specific pulldown with recombinant p21-activated kinase 1-p21 binding domain, according to the manufacturer's protocol (Upstate Biotechnology). A20 cells (10^6) were washed twice and suspended at 10^6/ml in prewarmed serum-free medium. Cells were cultured in Opti-MEM medium (Invitrogen, Carlsbad, CA) containing 10% FCS, 10 μM 2-ME, antibiotics, and the indicated stimulatory Abs and/or cytokines.

**Calcium assay**

Following isolation, purification, and equilibration at 37°C for 1 to 2 h in serum-containing medium, mouse B cells were washed and suspended at 10^6/ml in prewarmed serum-free medium. Cells were loaded with calcium-sensitive probes fluo-4-AM (7.0 μg/ml) and fura Red-AM (13.0 μg/ml) in a 37°C water bath for 30 min. Excess probes were removed by washing once and suspending cells in RT-equilibrated HBSS. Cells were then analyzed on a FACS Canto II (BD Biosciences), with baseline fluorescence acquired for 30 s, followed by addition of F(ab')2 anti-IgM (20 μg/ml) and then continued acquisition for 5 min.

**Adhesion assay**

Adhesion assays were carried out in 96-well ELISA plates (Costar #3369, Costar, Cambridge, MA) coated with overnight (5 μg/ml) fibronectin (Calbiochem, San Diego, CA) or rICAM (R&D Systems, Minneapolis, MN) diluted in carbonate coating buffer. Coated plates were washed once and suspending cells in RT-equilibrated HBSS. Cells were then analyzed on a FACS Canto II (BD Biosciences), with baseline fluorescence acquired for 30 s, followed by addition of F(ab')2 anti-IgM (20 μg/ml) and then continued acquisition for 5 min.

**Spreading assay**

Spreading assays were carried out in 96-well ELISA plates coated overnight with 5 μg/ml anti–LFA-1 (eBioscience) or 10 μg/ml anti–MHC-II (clone D3.1.3.7) (37) Abs diluted in carbonate coating buffer plus 2% BSA. Plates were washed once with PBS and blocked for 30 min at RT with HBSS plus 2% BSA. Cells were resuspended at 4 × 10^5/ml in prewarmed blocking medium, and 100 μl cells were mixed with the indicated stimuli in the coated plates. Plates were incubated for 1 h at 37°C in a humidified CO2 incubator. At the end of incubation, plates were gently inverted to remove nonadherent cells and medium. Wells were filled with PBS, and plates were covered with sealing tape, inverted, and centrifuged at 100 × g for 3 min. At the end of centrifugation, PBS was removed, and adherent cells were fixed with 1% glutaraldehyde solution for 10 min at RT and then stained for 20 min with 0.1% crystal violet solution. After complete removal of crystal violet solution, stained cells were dissolved in 1% SDS. Crystal violet absorbance was read at 570 nm using an ELISA plate reader (Molecular Devices, Sunnyvale, CA).

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were captured. Individual cells were scored as spread/not spread by an independent observer without knowledge of the experimental groups. Scoring was scored as cells having an elongated or irregular shape with membrane processes and a length of >1.5 times the width.  

**BCR-mediated Ag endocytosis, degradation, and colocalization with late endosomes**

To measure BCR internalization, purified B cells were incubated with biotin rabbit anti-mouse IgM for the indicated times. Then cells were fixed with 2% paraformaldehyde in PBS, and biotinylated Ab staining on the cell surface were detected with PE-streptavidin staining as previously described (24). For the Ag degradation assay, B cells (×10⁶) were incubated with 10 μg/ml Ag (rabbit anti-mouse IgM) for the indicated times, followed by cell fixation and permeablization/blocking with 0.1% saponin in PBS containing 2% FCS. Cells were then washed and incubated with Alexa 488 donkey anti-rabbit Ab (Jackson ImmunoResearch Laboratories) for 30 min at 4°C. The mean fluorescence intensity of Alexa 488 staining was quantified on an FACS Calibur flow cytometer (BD Biosciences) and used to calculate the amount of intact immunoreactive Ag relative to the initial amount bound (zero time point). Internalized rabbit anti-mouse IgM and LAMP1 were stained and visualized by confocal microscopy as previously described (24).

**B cell/T cell conjugate assays and IL-2 production**

Purified splenic B cells from C57BL6-IA d or Bam32-deficient-IA d congenic mice (8 × 10⁶/ml) were treated with 0.01% DMSO (control) or wortmannin for 30 min at 37°C followed by extensive washing and then were labeled with CFSE. 2R.50 T cells (specific for rabbit Ig presented by I-A d) (38, 39) were labeled with 2 μM Cell Trace Far Red DDAO-succinimidyl ester dye (Invitrogen-Molecular Probes) for 15 min at RT followed by washing. CFSE-labeled B cells (×10⁶) were mixed with Ag (rabbit anti-mouse IgM Ab; 40 µg/ml) and Far Red-labeled 2R.50 T cells (2.5 × 10⁵). Cell coccultures were maintained in sterile FACS tubes and incubated at 37°C for the indicated times. The tubes were gently shaken to disrupt the pellet immediately before running on the flow cytometer. For confocal microscopy analysis, the B/T conjugates were generated using rhodamine-labeled rabbit anti-mouse IgM Ag and were adhered to slides by centrifugation and stained with Alexa 488-phalloidin as described (24). Assessment of IL-2 production by 2R.50 cells was performed as previously described (24).

**Results**

**Bam32 is required for efficient BCR-induced activation of Rac1 and JNK, but not PLCγ2, BLNK or calcium responses**

We previously found evidence in human and chicken B cell lines that Bam32 can regulate activity of the GTPase Rac1 and downstream signaling to JNK (33). In this study, we examined primary B cells isolated from wild-type (WT) or Bam32-deficient mice stimulated by BCR crosslinking. When levels of Rac1 activation were assessed by specific pulldown assay, Bam32-deficient B cells showed significant reductions in BCR-induced accumulation of Rac1-GTP (Fig. 1A). BCR-induced activation of JNK was also impaired (Fig. 1A), consistent with a previous study on Bam32-deficient B cells (32). In contrast, we found no evidence for reduction in BCR-induced phosphorylation of BLNK or PLCγ2 (Fig. 1B) or reduced calcium responses (Fig. 1C) in Bam32-deficient B cells. In fact, slightly elevated PLCγ2 phosphorylation and calcium mobilization responses were observed in Bam32-deficient B cells, consistent with our findings in Bam32-deficient mast cells (S. Hou, P. Liu, S. Pauls, and A. Marshall, submitted for publication). These results confirmed that Bam32 is selectively required for efficient activation of both Rac1 and its downstream target JNK.

**Bam32 is required for optimal B cell adhesion and spreading responses**

Rac and other Rho GTPases are key players in actin remodeling leading to cell adhesion, polarization, and motility, and, in many systems, their activation is strongly PI3K dependent (40, 41). Recent studies have clearly implicated Rac and Rap GTPases in B cell adhesion and spreading on integrin ligands, such as ICAM.
(14, 42). We thus assessed whether the identified defect in Rac activation may impair the ability of Bam32-deficient B cells to adhere and spread on ICAM-coated plates. BCR crosslinking led to a dose-dependent increase in B cell adhesion to ICAM (Fig. 2A). Bam32-deficient B cells showed reduced BCR-induced adhesion at all stimulation doses (Fig. 2A), suggesting that Bam32 is important for inside-out activation of LFA-1–mediated adhesion. Bam32-deficient B cells were found to express normal cell-surface levels of LFA-1 under all stimulation conditions (data not shown). Reduced adhesion was also observed when plates were coated with the extracellular matrix protein fibronectin, another integrin ligand important for lymphocyte function (Fig. 2B).

Upon encounter with surfaces containing integrin ligands, B cells undergo a transient flattening/spreading response that was recently shown to be dependent on Rac and Rap GTPases (14, 42) and is thought to be important for adhesion, cell–cell interaction, and Ag-gathering behaviors of B cells. It was found that Bam32-deficient B cells are markedly impaired in their ability to undergo cell spreading after plating on anti–LFA-1–coated surfaces (Fig. 3A, 3B), consistent with a functional impairment in GTPase signaling. Interestingly, we also found that B cells undergo a spreading response on plates coated with a stimulatory anti-MHC mAb (37), and Bam32-deficient B cells were also markedly impaired in this spreading response (Fig. 3C). These results identify Bam32 as one of the effector molecules downstream of PI3K that links the BCR signaling to increased B cell adhesion and spreading responses.

**FIGURE 2.** Bam32 is required for efficient BCR-induced B cell adhesion to integrin ligands. Splenic B cells were purified from WT or Bam32-deficient mice, preactivated for 2 d with 1 μg/ml LPS, and then plated on ICAM- (A) or fibronectin-coated (B) wells in the presence of the indicated concentrations of Fab′(a) anti-IgM. After 1 h incubation, nonadherent cells were removed, and plates were fixed and stained with crystal violet. Stained cells were dissolved in SDS buffer, and the OD570 read on an absorbance plate reader. Results shown represent average ± SD of triplicate wells and are representative of three to five independent experiments. Statistical significance was determined by Student t test. *p < 0.05; **p < 0.01 comparing respective WT and Bam32−/− groups.

**Overexpression of WT, but not tyroisin 139-mutant, Bam32 enhances Rac activation, B cell adhesion, and spreading over integrin ligands**

BCR-induced phosphorylation of Bam32 on Tyr-139 is dependent on both PI3K activity and the Src family kinase Lyn (31, 43). To assess the role of Bam32 phosphorylation in B cell adhesion and spreading responses, we overexpressed WT Bam32 or Y139F mutant Bam32 in A20 mouse B lymphoma cells. Transfectants expressing WT Bam32 showed enhanced activation of Rac1 after BCR stimulation (Fig. 4A). This activity is lost in the transfectants expressing Y139F mutant Bam32, indicating that Tyr139 is required for this enhancement. Cells expressing WT Bam32 exhibited increased BCR-induced adhesion to fibronectin compared with parental A20 cells, and this effect was reversed in cells expressing the Tyr139 mutant Bam32 (Fig. 4B). Lastly, cells expressing WT Bam32 showed markedly increased spreading on anti–LFA-1–coated plates, whereas cells expressing Tyr139 Mutant Bam32 showed reduced spreading compared with parental A20 cells (Fig. 4C, 4D). Together with the results from primary B cells, these data strongly implicate Bam32 in integrating signals from PI3K and Lyn to enhance Rac activation and cell adhesiveness.

**Bam32 is not required for BCR-mediated Ag endocytosis and processing**

We previously found that Bam32 can regulate endocytosis of BCR-Ag complexes in human and chicken B cell lines, presumably through its impact on Rac-mediated cytoskeletal alterations (44). However, we found that Bam32-deficient primary B cells exhibited no impairment in BCR internalization (Fig. 5A), consistent with previous results suggesting that BCR endocytosis in mouse primary B cells is PI3K independent (24). We thus explored whether Bam32 may be required for Ag targeting to the Ag processing compartment after initial endocytosis. Our data demonstrated no difference in Ag colocalization with the late endosomal marker LAMP1 was observed (Fig. 5B). We also observed similar levels of Ag colocalization with H2M, a resident of the MIIIC lysosome/late endosome compartment (data not shown). Furthermore, the kinetics of Ag degradation after uptake were found to be similar in WT and Bam32-deficient B cells (Fig. 5C). However, slower kinetics of Ag degradation were observed when WT B cells were pretreated with wortmannin, indicating a potential role for PI3Ks in this process, independent of Bam32 (Fig. 5C). Together, these data indicate that Bam32 is not required for BCR endocytosis and trafficking and also suggest that, at least in primary mouse B cells, these events are not sensitive to reduced Rac activation.

**Bam32 promotes BCR-mediated Ag presentation and formation of polarized conjugates with cognate T cells**

We examined whether the function of Bam32 in promoting B cell adhesion and spreading responses may have an impact on formation of stable Ag-specific B/T conjugates. We established a flow cytometric assay to measure Ag-specific conjugate formation between mouse B cells and 2R.50 T cells, which recognize rabbit Ig Ag presented in the context of I-Ad (38, 39). Resting B cells were labeled with CFSE and then pretreated with DMSO vehicle or wortmannin before mixing with Ag and Cell Trace Far Red-labeled 2R.50 T cells. After 6 h incubation, the percentage of B cell-T cell conjugates was determined by flow cytometric assay to measure Ag-specific conjugate formation between mouse B cells and 2R.50 T cells, which recognize rabbit Ig Ag presented in the context of I-Ad (38, 39). Resting B cells were labeled with CFSE and then pretreated with DMSO vehicle or wortmannin before mixing with Ag and Cell Trace Far Red-labeled 2R.50 T cells. After 6 h incubation, the percentage of B cell-T cell conjugates was <2% in the absence of Ag, but increased 2–4-fold in the presence of Ag (Supplemental Fig. 1A). Bam32-deficient B cells showed ~50% reduction in binding Ag-specific T cells, and this inhibition was still apparent after 24 h of coculture (Fig. 6A, 6B). Wortmannin treatment experiments confirmed that conjugate formation is substantially dependent on...
PI3K activity (Fig. 6B). Impaired conjugate formation did not appear to be due to defective chemotaxis, because Bam32-deficient B cells showed normal chemotaxis ability in transwell chamber assays (Supplemental Fig. 1B). We further examined the conjugates formed by WT or Bam32-deficient B cells using confocal microscopy. It was found that the conjugates formed by Bam32-deficient B cells show markedly reduced polarization of F-actin toward the B-T interface (Fig. 6C). These data demonstrate the role of PI3K and Bam32 in promoting the B cell’s ability to form polarized conjugates with Ag-specific T cells.

Formation of a relatively stable immune synapse is thought to be key in promoting sustained T cell signaling required for full development of Ag-specific T cell responses. Overexpression of WT, but not tyrosine 139-mutant, Bam32 enhances Rac activation, B cell adhesion, and spreading. A, Western blots showing levels of active or total Rac1. The indicated A20 transfectants were assessed for BCR-induced activation of Rac1 (A20 = parental A20 cells; Bam32 WT = A20 overexpressing WT Bam32; Bam32-Y139F = A20 expressing tyrosine 139-mutant Bam32). Cells were resuspended and incubated for 1 h in serum-free medium and then stimulated with 10 μg/ml F(ab)2 rabbit anti-mouse IgG for the indicated times (min). Results are representative of three experiments. B, A20 transfectants were assessed for BCR-induced adhesion to fibronectin poststimulation with increasing doses of anti-BCR (Medium = no stimulus, or 1 or 10 μg/ml anti-BCR). Results shown represent data obtained from three to five independent experiments. Statistical significance was calculated using Student t test comparing parental A20 to Bam32 WT. C, The indicated A20 transfectants were plated on wells coated with 5 μg/ml anti-MHC-II Abs for 1 h. Representative images of cell spreading are shown. D, Percent of cells showing spreading as determined by visual scoring of an independent observer. Results shown represent average cell spreading ± SD obtained from three to four independent images totaling at least 200 cells per group. **p < 0.01 using Fisher’s exact test comparing the indicated transfectant groups to the corresponding parental A20 group.

### FIGURE 3

Bam32 promotes B cell spreading on anti–LFA-1 or anti–MHC-II–coated surfaces. WT or Bam32-deficient splenic B cells were activated overnight with 10 μg/ml anti-BCR alone or together with 1 μg/ml LPS or cultured in medium alone and were then plated on wells coated with 5 μg/ml anti–LFA-1. Cell spreading was microscopically assessed after 1 h. A, Representative images of plated cells showing reduced cell spreading by Bam32-deficient B cells. B, Images were scored for cell spreading by an independent observer blinded to the experimental conditions. C, Cell spreading on plates coated with 10 μg/ml anti-MHC was assayed as in A and B. Results shown represent average cell spreading ± SD obtained from three to five independent images of at least 150 cells per group. **p < 0.01 using Fisher’s exact test comparing WT and Bam32−/− groups.

### FIGURE 4

Overexpression of WT, but not tyrosine 139-mutant, Bam32 enhances Rac activation, B cell adhesion, and spreading. A, Western blots showing levels of active or total Rac1. The indicated A20 transfectants were assessed for BCR-induced activation of Rac1 (A20 = parental A20 cells; Bam32 WT = A20 overexpressing WT Bam32; Bam32-Y139F = A20 expressing tyrosine 139-mutant Bam32). Cells were resuspended and incubated for 1 h in serum-free medium and then stimulated with 10 μg/ml F(ab)2 rabbit anti-mouse IgG for the indicated times (min). Results are representative of three experiments. B, A20 transfectants were assessed for BCR-induced adhesion to fibronectin poststimulation with increasing doses of anti-BCR (Medium = no stimulus, or 1 or 10 μg/ml anti-BCR). Results shown represent data obtained from three to five independent experiments. Statistical significance was calculated using Student t test comparing parental A20 to Bam32 WT. C, The indicated A20 transfectants were plated on wells coated with 5 μg/ml anti–LFA-1 or 10 μg/ml anti–MHC-II Abs for 1 h. Representative images of cell spreading are shown. D, Percent of cells showing spreading as determined by visual scoring of an independent observer. Results shown represent average cell spreading ± SD obtained from three to four independent images totaling at least 200 cells per group. **p < 0.01 using Fisher’s exact test comparing the indicated transfectant groups to the corresponding parental A20 group.
We thus examined levels of IL-2 secreted by 2R.50 cells when presented Ag by WT or Bam32-deficient B cells (Fig. 6D). Similar to our previous findings (24), IL-2 release by 2R.50 cells was detected only when both Ag (rabbit anti-mouse IgM) and B cells were present and is inhibited when B cells are pretreated with wortmannin (data not shown). IL-2 production increased in an Ag dose-dependent manner, but this response was significantly reduced when Bam32-deficient B cells were used as the APC (Fig. 6D). Bam32-deficient B cells expressed MHC-II and CD86 molecules on their cell surface at levels comparable to controls (Supplemental Fig. 1C), indicating that

**FIGURE 5.** Bam32 is not required for Ag internalization, targeting, or degradation. A, WT or Bam32-deficient B cells were stained with biotinylated rabbit anti-mouse IgM on ice, incubated at 37°C for the indicated times, and BCR remaining on the cell surface was detected with PE-streptavidin. Results are presented as mean fluorescence intensity normalized to the nonincubated sample in each experiment (0 time point). Results are representative of three similar experiments. B, Left panel, representative images showing the colocalization between BCR and LAMP1. Right panel, graph showing mean correlation coefficients of BCR and LAMP1 for at least 50 cells per group. C, WT or Bam32-deficient B cells were incubated with Ag (rabbit anti-mouse IgM) prior to fixation at the indicated time points. The level of intact immunoreactive Ag remaining was detected by staining permeabilized cells with donkey anti-rabbit secondary Ab and flow cytometry detection. Bars show mean fluorescence intensity ± SD of three independent experiments. *p < 0.05 in Student *t* test comparing WM-treated versus untreated. WM, pretreated with wortmannin (100 nM).

**FIGURE 6.** Bam32 promotes formation of polarized conjugates with Ag specific T cells. A, B and T cell conjugates were quantified using flow cytometry by coculturing CFSE-labeled B cells and Cell Trace Far Red-labeled 2R.50 T cells with rabbit IgG Ag for 6 h. Representative dot plots show reduced conjugate formation with Bam32-deficient or wortmannin-treated WT B cells. B, Bar graph showing the conjugates formed after 6 or 24 h of coculture expressed as mean percentage compared to WT control ± SD of three independent experiments. Statistical significance was determined by *t* test. C, Left panel, confocal microscope imaging of conjugates formed by WT or Bam32-deficient B cells. B cells were incubated with Cy5-labeled rabbit anti-mouse IgM at 37°C for 60 min before mixing with 2R.50 T cells for another 60 min. Conjugates were fixed, spun onto slides, and stained with Alexa 488-labeled phalloidin to detect F-actin. Right panel, bar graph showing percentage of conjugates exhibiting F-actin polarization, pooled over two experiments analyzing a total of 95 WT and 71 Bam32−/− conjugates. Error bars indicate the SD between the two experiments, and statistical significance was determined using Fisher’s exact test. D, B cells from WT or Bam32-deficient mice were mixed with various doses of Ag and 2R.50 T cells. The level of IL-2 in supernatants was assessed by ELISA as a measure of T cell activation. Results are representative of eight similar experiments. Statistical significance was determined by *t* test. *p < 0.05; **p < 0.01; ***p < 0.001.
reduced T cell activation was not due to failure to upregulate costimulatory molecules. Together, our results indicate that Bam32 functions to promote formation of stable B/T conjugates needed for efficient T cell activation, likely by promoting adhesion to integrin ligands, such as ICAM, expressed on T cells.

Discussion
Formation of stable adhesive interactions between B cells and T cells is an essential step that allows for bidirectional signal transfer (20, 48, 49). Achieving such functional cognate interactions between B and T cells requires Ag-induced alterations in cell migration and adhesiveness to bring cells together and facilitate the relatively low-affinity recognition of MHC-peptide complexes on the B cell surface. We have previously documented the requirement for PI3K p110δ signaling in regulating B-T conjugate formation (24). The present results identify Bam32 as one of the PI3K effector molecules required for this function, acting at the level of BCR-induced activation of B cell adhesion and spreading. These findings also provide the first evidence that Bam32 plays a role in primary B cell functions beyond cell proliferation. Our data in this study confirm in primary B cells that Bam32 is required for BCR-induced Rac activation (33). Rac-mediated cytoskeletal rearrangement is likely important for integrin activation and formation of polarized conjugates with T cells (50, 51). This interpretation is consistent with recent studies showing that PI3K, Rac, and Rap are required to activate B cell adhesion and spreading mediated by the LFA-1 integrin (14, 42). The Rac activator Vav1 was also shown to be required for dendritic cell adhesion (52). A recent study found that p110δ PI3K is required for LFA-1-mediated B cell adhesion and integrin-mediated marginal zone B cell localization in vivo (53). We have also found that p110δ signaling is required for optimal B cell adhesion responses in vitro (data not shown). Given that Bam32 membrane recruitment and phosphorylation at Y139 are absolutely PI3K dependent (27, 30, 31), and Y139 was required for Bam32 to promote adhesion and spreading in the current study, we conclude that Bam32 is functioning downstream of PI3K in its plasma membrane-associated, phosphorylated state. Treatment of Bam32-deficient cells with PI3K inhibitor further reduced their function in adhesion and Ag presentation (data not shown), consistent with the interpretation that other components of the PI3K pathway are also involved. Thus, our working model is that Bam32 functions in concert with other molecules downstream of PI3K to orchestrate Rac activation and cytoskeletal rearrangements required for optimal integrin-mediated cell–cell conjugate formation.

The PI3K signaling pathway is known to intersect with Ras, Rac, and Rap GTPase signaling pathways at many levels. Vav proteins and other guanine nucleotide exchange factors contain phosphoinositol-binding PH domains that can regulate their subcellular localization and GTPase activity (54, 55). Our data implicate Bam32 as another PH domain target of PI3K involved in modulating the balance between GTPase activation and inactivation during B cell activation. Our results regarding Bam32 have similarities with data on Src kinase-associated phosphoprotein (SKAP) and SKAP-HOM adapters, which are also PH domain proteins and were shown to regulate T cell or B cell adhesion, respectively (56–58). Interestingly, these molecules are thought to act by selectively modulating Rap activation (59, 60), whereas we find that Bam32 regulates Rac but not Rap (data not shown). Thus, present results indicate that whereas generation of 3-phosphoinositides by PI3K can directly impact guanine nucleotide exchange factors, PH domain adaptor proteins recognizing these phosphoinositides also play important roles in orchestrating the response. Because both Rac and Rap GTPases are required for B cell adhesion and spreading responses, it seems likely that the nonredundant function of Bam32 and SKAP-HOM in B cell adhesion may reflect their selective impact on these two classes of GTPases.

Although Bam32 was previously found to be required for efficient BCR endocytosis in human and chicken B cell lines (44), we found that endocytosis was not impaired in Bam32-deficient mouse B cells, despite reduced Rac activation. Indeed, we find that complete inhibition of PI3K signaling has little effect on BCR endocytosis in mouse B cells (data not shown). Results from other studies indicate that the requirements for lipid rafts, tyrosine kinases, and actin cytoskeleton in BCR internalization vary depending on the nature of the Ag and B cells (12, 13, 61), and the present data suggest that uptake of soluble Ags by primary B cells may not be highly dependent on signaling via PI3K, Bam32, and Rac.

Consistent with our previous studies on p110δ-deficient B cells (24), we did not find evidence indicating a role of Bam32 in targeting of internalized BCR-Ag into the late lysosomal processing compartments. The present data cannot completely exclude the possibility of some effect of Bam32 deficiency on MHC-II peptide loading or outbound trafficking of MHC-II–peptide complexes, which could influence the number of complexes presented on the cell surface. However, we found no effect of PI3K inhibitors on generation of the hen egg lysozyme (HEL)-I-A<sup>k</sup> (46–61) complex, as detected by flow cytometry using C3H4 mAb (62) on the surface of HyHEL.10 BCR-transfected LK35 cells (63) that were fed HEL (data not shown). This result suggests that PI3K-dependent signaling may not be obligatory in generation of specific peptide-MHC complexes.

Our results clearly indicate that Bam32-deficient B cells have impaired ability to form Ag-specific conjugates with T cells. The conjugates that did form with Bam32-deficient B cells showed less evidence of cytoskeletal polarization based on accumulation of F-actin at the cell-contact interface. Because Bam32-deficient B cells show reduced ability to adhere to ICAM after BCR cross-linking, and LFA-1–ICAM interactions are known to be critical for B cell–T cell interaction (64–66), we conclude that reduced polarized conjugate formation is likely due to defective LFA-ICAM adhesion interactions. We also observed reduced Ag specific T cell activation, as measured by IL-2 production, when Bam32-deficient B cells are used as the APC. The collective data support the conclusion that reduced ability to form stable adhesive interactions with T cells leads to a significant impairment in Ag presentation capacity.

We have recently found evidence supporting a role for Bam32 in T-dependent Ab responses (36). Upon immunization with T-dependent Ag, GCs initiate normally in Bam32-deficient mice, but subsequently collapse, resulting in impaired affinity maturation. This collapse is associated with defects consistent with impaired B-T cognate interactions within the GC (67, 68), including greater GC B cell apoptosis, reduced recruitment of T cells to GC, and reduced isotype switch to IgG1 and hyper-IgM. These results suggest that the defective Ag presentation function of Bam32-deficient B cells markedly impacts their in vivo function.

In summary, our results identify Bam32 as a PI3K effector molecule that regulate BCR-mediated Ag presentation through augmenting Rac activity, cell adhesion, spreading, and formation of stable B/T conjugates.

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Disclosures

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