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Modulation of Antigen Presentation and B Cell Receptor Signaling in B Cells of Beige Mice

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Binding of Ag by B cells leads to signal transduction downstream of the BCR and to delivery of the internalized Ag–BCR complex to lysosomes where the Ag is processed and presented on MHC class II molecules. T cells that recognize the peptide–MHC complexes provide cognate help to B cells in the form of costimulatory signals and cytokines. Recruitment of T cell help shapes the Ab response by facilitating isotype switching and somatic hypermutation, and promoting the generation of memory cells and long-lived plasma cells. We have used the beige (Bg) mouse, which is deficient in endosome biogenesis, to evaluate the effect of potentially altered Ag presentation in shaping the humoral response. We show that movement of the endocytosed Ag–BCR complex to lysosomes is delayed in Bg B cells and leads to relatively poorer stimulation of Ag-specific T cells. Nevertheless, this does not affect Bg B cell activation or proliferation when competing with wild-type B cells for limiting T cell help in vitro. Interestingly, Bg B cells show more prolonged phosphorylation of signaling intermediates after BCR ligation and proliferate better to low levels of BCR cross-linking. Primary Ab responses are similar in both strains, but memory responses and plasma cell frequencies in bone marrow are higher in Bg mice. Further, Bg B cells mount a higher primary Ab response when competing with wild-type cells in vivo. Thus, the intensity and duration of BCR signaling may play a more important part in shaping B cell responses than early Ag presentation for T cell help. The Journal of Immunology, 2012, 188: 000–000.

B cells share features of the adaptive and innate arms of the immune system. Like T cells, they possess clonally diverse receptors that are generated by random recombination events in the bone marrow (BM) and lend specificity to Ag recognition, and like macrophages and dendritic cells, they endocytose, process, and present Ag on MHC class II (MHC-II) molecules to T cells (1). In addition, they possess pattern recognition receptors like Tlr4, Tlr7 and Tlr9 and signaling through the Tlrs can independently activate MAPKs and NF-kB, and lead to cellular activation and proliferation (2, 3). Signaling through the BCR is initiated at the plasma membrane after its translocation into lipid rafts (4), and the major signaling pathways include phospholipase C (PLC), PI3K, and the Ras and the Rho family of GTPases (5, 6).

The exact mechanisms by which the Ag–BCR complex is internalized remain incompletely characterized. However, colocalization of the BCR with clathrin after phosphorylation of the latter by the protein tyrosine kinase src (7) and association of the adaptor molecule B42 with the BCR and components of the endocytic machinery (8) appear to be crucial. The endocytosed Ag–BCR complex is transported to a multivesicular lysosomal-associated membrane protein 1 (LAMP-1)–positive MHC-II–rich compartment (MIC) where the Ag undergoes proteolytic cleavage and the resultant peptides are loaded onto MHC-II molecules for transport to the cell membrane (9–11). The MICs are also known to fuse with autophagosomes (12) to which other signaling molecules like Tlr9 are recruited (13).

Signaling through the BCR appears to continue after internalization into endosomes and trafficking to autophagosome-like compartments (13). Rapid transport of the endocytosed Ag to lysosomes for degradation and presentation on MHC-II will favor rapid recruitment of T cell help that is required for optimal activation and survival of B cells, and the induction of isotype switching and somatic hypermutation (14). However, it will also curtail direct signaling downstream of the BCR. We have tried to analyze the relative roles of these two events in determining the final outcome of B cell activation by using the beige (Bg) mouse, which is deficient in endosome biogenesis. We show that BCR downmodulation after receptor cross-linking is similar in Bg and wild-type (WT) B cells, but that transport of the endocytosed cargo is delayed in Bg B cells, leading to less efficient activation of Ag-specific T cells. However, Bg B cells undergo equivalent activation and proliferation when competing with WT B cells for limiting T cell help in vitro, suggesting that very small amounts of T cell help may suffice for cognate interactions. Both strains of mice show equivalent primary responses to immunization with a T-dependent Ag, but Bg mice show higher frequencies of memory B cells, as well as a higher frequency of plasma cells in the BM. Bg B cells proliferate to lower levels of receptor cross-linking and also show more prolonged phosphorylation of signaling intermediates, suggesting that the strength and/or duration of signaling from the BCR may play an important role in determining the final outcome of B cell stimulation in vivo.
Materials and Methods

Reagents

OVA, LPS, DMSO, propidium iodide, E64d, Bafilomycin A1, NHHCl, Nonidet P-40, o-phenylene diamine, parafinaldehyde, saponin (Sigma, St. Louis, MO), avidin (Merck Biosciences, Darmstadt, Germany), biotin (Thermo Fisher Scientific, Rockford, IL), 4-hydroxy-3-nitrophenoacyl (NP) chicken γ globulin (CGG), NP-OVA, NP-BSA (Biosearch Technologies, Novato, CA), PE (Chromoprobe, Maryland Heights, MO), CFA (Difco Laboratories, Detroit, MI), goat anti-mouse Ig, Ig-HRP, IgM-HRP, IgG-HRP, azide-free goat anti-mouse IgM and IgM F(ab')2 (Southern Biotechnolog, Birmingham, AL), anti-CD3 (eBioscience, San Diego, CA), IL-2 (Roche Applied Sciences, Manheim, Germany), H2O2 (Merck), CFSE, sytox green, sytox red ( Molecular Probes, Eugene, OR), streptavidin PE-FITC (Jackson Immunoresearch Laboratories, West Grove, PA), pure/fluo-sytox green, sytox red (Molecular Probes, Eugene, OR), streptavidin-PE, Cy5 and biotinylated anti-IgG1, IgG2a, IgG2b, IgM, IgM (eBiosciences and BD Biosciences, San Jose, CA), PE-rabbit anti-mouse pERK, Alexa Fluor 488-mouse anti-mouse pp38, rabbit anti-mouse pJNK, ppERK, Alexa Fluor 488-mouse anti-mouse pERK, PE-rabbit anti-mouse pJNK, Alexa Fluor 488-mouse anti-mouse pJNK, rabbit anti-mouse pp38, rabbit anti-mouse pp38, rabbit anti-mouse pERK (Cell Signaling Technology, Danvers, MA), streptavidin Alexa 546, goat anti-rat IgG Alexa 488, and goat anti-rabbit IgG Alexa 488 (Invitrogen, Eugene, OR) were used.

Mice

C57BL/6/6 (B6), C57BL/6/6.SJL, C57BL/6/6-Lyn+/- (bg/bg), B6-C-20, and OT-II mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in the Small Animal Facility of the National Institute of Immunology. A WT mouse strain expressing CD45.1 and secreting Ig of the “a” allotype was generated by crossing C57BL/6.SJL mice (CD45.1, Igha) with B6-C-20 mice (CD45.2, Ighb). F2 mice generated from subsequent crossing of the F1 mice were tested for the CD45.1 allotype by flow cytometry of PBMC and for Ig allotype by ELISA with allotype-specific reagents. The WT strain so generated is referred to as B6.SJL-C-20 (Supplemental Fig. 1). Mice were used for experiments at 6–12 wk of age, and all procedures were in accordance with the guidelines of the Institutional Animal Ethics Committee.

BM chimeras

For generation of mixed BM chimeras for competition experiments, recipient B6.SJL-C-20 mice were irradiated at 8 Gy (Biodron Irradiator 2000; Department of Atomic Energy, Mumbai, India) and reconstituted with 3 × 10^7 BM cells containing a 1:1 mixture of cells from two different WT donor (CD45.1, IgGb and CD45.2, IgGb) mice or from WT (CD45.1, IgGb) and Bg (CD45.2, IgGb) mice. The extent of chimerism was tested in PBMC 2 mo later by assessing CD45.1 and CD45.2 proportions on gated B cells in peripheral blood. Mice that showed ~1:1 chimerism were used for experiments 8 wk after reconstitution, and the data for each mouse were normalized to the chimerism noted. Controls included WT and Bg mice irradiated and reconstituted with homologous BM to assess responses in the absence of competition.

B cell stimulation

Single-cell suspensions of splenic cells obtained by mechanical disruption were depleted of erythrocytes with Gey’s solution, and B cells were isolated on a Ficoll-Hypaque gradient (Cedarlane, Burlington, Ontario, Canada) before further analysis. Proliferation was assessed 48 h after culture initiation by thymidine incorporation or 72 h later by CFSE dilution after gating out dead cells with 1 µg/ml propidium iodide or 1 µM sytox red.

Adoptive transfer

Mice were immunized i.p. with 10 µg NP-CGG on alum (Superfos Biosector A/S, Frederikssund, Denmark); 2 wk later, splenic B cells were transferred i.v. into syngeneic mice pretreated with 100 µg OVA in CFA 2–3 mo earlier and irradiated (6 Gy) 18 h earlier. Recipients were challenged with 100 µg NP-OVA on alum 20 h later, and NP-specific Ab assessed in sera collected 3 d later by ELISA.

Limiting dilution analysis

Ag-specific B cell limiting dilution analyses (LDAs) were carried out by polyclonal stimulation of B cells followed by Ag-specific ELISA as described previously (15). In brief, unfractionated lymphocytes or purified B cells from draining lymph nodes (DLNs) of mice immunized with NP-OVA were cultured in 96-well flat-bottom plates (Falcon, Franklin Lakes, NJ) from 10^7/well to 100/well (1 plate/cell input). A total of 10^5 thymocytes from normal mice was added as a source of filler cells to all wells. The cultures were stimulated with 10 µg/ml LPS for 7 d (with 12 wells in each plate left as unstimulated controls) and culture supernatants assayed for anti-NP Ab on ELISA plates coated with NP-BSA. Wells that showed an absorbance more than three times that of unstimulated controls in each plate were considered positive for Ab. Estimates of total Ig served as a normalizing control for LDA sensitivity.

ELISA and ELISPOT assays

Ninety-six–well flexiplates (Falcon) or activated multiscreen filter plates (Millipore, Molsheim, France, for ELISPOT) were coated with PE (10 µg/ml), NP-BSA (10 µg/ml), or goat anti-mouse Ig (2–10 µg/ml). The plates were blocked with 1% defatted milk/PBS (or media containing 10% FBS for ELISPOT), loaded with culture supernatant/sera (or cells for ELI-Spot), and bound Ig detected with secondary reagents coupled to HRP in appropriate buffers. Absorbances were read at 490 nm. For detection of high- and low-affinity Abs, plates were coated with NP6-BSA or NP23-BSA, respectively.

Flow cytometry

Cells were incubated with appropriate staining reagents in buffer containing 0.1% sodium azide (Sigma) and 0.5% BSA or 1% FBS for 45 min on ice. Samples were run on a BD-LSR or FACS Aria (BD) flow cytometer. For BCR biotinylated assay, a mixture of biotinylated anti-IgM and incubated at 37°C for different times. Cells were then washed with streptavidin-PE, and residual BCR on the cell surface was assessed. For phosphoflow assays, B cells from WT and Bg mice were stimulated with anti-IgM F(ab')2 for various periods of time (5 min to 6 h), fixed with Cytofix, permeabilized with Phosflow perm Buffer II (both from BD Bioscience), and stained for pERK, pp38, or pJNK (all Abs from Cell Signaling Technology, Danvers, MA). To establish specificity of phosphoflow assays, we pretreated positive control samples with U0126 (Promega, Madison, WI), SB203580, or JNK1 (both from Calbiochem, Merck, India) for pERK, pp38, and pJNK, respectively, for 30 min, and then stimulated (as indicated in Supplemental Fig. 4) in the continued presence of inhibitor. For Ca flux assay, splenocytes were stained with 0.5 µM fluo-AM and 1.5 µM fura red (both from Invitrogen) in the presence of 0.2% pluronic acid (Sigma) and 1.0 mM HSS containing 5% FBS. Cells were stimulated with 10 µg/ml anti-IgM F(ab')2 in the presence of 1 mM CaCl2, and induction of Ca flux was scored by flow cytometry over time. Ionomycin (1 µg/ml; Sigma) was added at 420 s. Flow cytometric data were analyzed using FlowJo software (Tree Star, San Carlos, CA).

Preparation of biotinylated OVA

OVA was dialyzed against biotin labeling buffer (0.1 M NaHCO3, 0.1 M NaCl, pH 7.4), incubated with biotin (10 µg/ml) in phosphate buffer (1 mg/ml protein) dissolved in anhydrous DMSO, and unbound biotin removed by dialysis (0.1 M Tris HCl, 0.1% NaN3, 0.2 M NaCl, pH 7.4).

Ag presentation

For pinocytic delivery of Ag to cells, B cells were pulsed with 10 mg/ml OVA in serum-free media for 1 h. To target OVA to the BCR, we treated cells sequentially with 10 µg/ml biotinylated anti-IgM, 1mg/ml avidin, and 8 mg/ml OVA-biotin on ice in PBS containing 1% FBS. Ag-pulsed B cells were then plated at titrating cell densities with 10^3.8 T cells (a CD4–restricted T cell hybridoma that recognizes OVA-derived peptides on H-2A and has a LacZ gene under control of a minimal IL-2 promoter) (16). T cell activation was assessed by measuring ß-gal activity colorimetrically with chlorophenol red-ß-galactopurynoside (Boehringer Mannheim) reagent (0.15 M chlorophenol red-ß-galactopurynoside in PBS containing 100 mM 2-ME, 0.125% Nonidet P-40, and 9 mM MgCl2). For Ag presentation assays in the presence of pharmacological inhibitors, OVA was targeted to the BCR and processing allowed in the presence of inhibitor before culturing them with T cells.

In vitro competition assay

OVA was targeted to the BCR of purified B cells from B6.SJL (CD45.1) and Bg (CD45.2) mice, and plated either alone or as a 1:1 mixture with activated
T cells from OT-II transgenic mice (T cells were stimulated with 100 ng/ml anti-CD3 for 48 h and rested with 2 μg/ml IL-2 for 48 h) in varying B cell/T cell ratios. B cell activation after cognate interaction with T cells was assessed by CD69 upregulation (at 12 h) and CFSE dilution (at 48 h) of coculture on gated CD45.1+ and CD45.2+ B cells.

Western blots

Cell pellets were lysed in cold buffer containing 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 25 mM KCl, 5 mM MgCl2, 0.1% Triton X-100, 0.02% Na3VO4, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM PMSF, and 1 mM sodium orthovanadate (Boehringer Mannheim), and the lysate boiled in SDS loading dye. Samples were vortexed to reduce viscosity and centrifuged to remove debris. Extracts from 1–1.5 × 106 cells were resolved by SDS-PAGE and blots treated with rabbit mAbs against phospho-Erk1/2, JNK1/2, p38, or PLCγ2 (Cell Signaling Technology) followed by peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology). Blots were stripped and reprobed for rabbit mAbs against total Erk1/2, p38, and PLCγ2 (Cell Signaling Technologies, Danvers, MA). Bands were detected with ECL (Amersham Pharmacia Biotech, Little Chalfont, U.K.). Western blots were carried out with titrating amounts of cell lysates from positive control cell lines to ensure that detection was in the linear range.

Microscopy

Unstimulated B cells or B cells treated with 10 μg/ml LPS for 48 h were treated with biotinylated goat anti-mouse IgM on ice, incubated for various time periods, fixed with 1% paraformaldehyde, permeabilized with 0.03% saponin, blocked with 1% FBS/PBS, and stained with rat anti-mouse LAMP-1. Secondary Abs were Alexa Fluor-streptavidin and donkey anti-rat IgG (Molecular Probes). For image acquisition, an Olympus 1×-81 inverted laser scanning confocal system was used. Pinhole size was kept at 1.0 μJ, which corresponds to an axial resolution of 0.186 μm with a 100×/1.4 NA objective. Eight-bit images were collected using Fluoview FV10-SW software (Olympus, Hamburg, Germany) with a step-size between 0.1 and 0.5 μm, and a digital zoom of 2. Colocalization was carried out slice by slice for each cell, with z step size of 0.42 μm, and 30 cells were analyzed for each time point. Images were processed using Adobe Photoshop software.

Results

Delayed transport of internalized BCR to lysosomal compartments in Bg B cells

Preliminary experiments established that the Bg mouse strain that carries the lyst mutation that leads to defective endosome biogenesis has no maturational defect in the B cell compartment, as assessed by IgM/IgD staining of B cells in the spleen, by their expression of CD69 upregulation (at 12 h) and CFSE dilution (at 48 h) of coculture on gated CD45.1+ and CD45.2+ B cells. Western blots

The microscopy data indicated that a significant fraction of endocytosed Ag–BCR complexes is retained in early endosomal compartments and therefore inaccessible to lysosomal processing. We reasoned that if this was indeed true, Ag presentation by WT and Bg B cells was equivalent if resting B cells were used (Fig. 2D). Thus, Bg B cells are relatively poor at presenting BCR-targeted Ag to T cells, and unlike WT B cells, they are unable to enhance their Ag presentation capability upon activation.

Effect of lysosomal inhibitors and cycloheximide on Ag presentation

The microscopy data indicated that a significant fraction of endocytosed Ag–BCR complexes is retained in early endosomal compartments and therefore inaccessible to lysosomal processing. We reasoned that if this was indeed true, Ag presentation by WT cells should be more sensitive to inhibition by pharmacological inhibitors of lysosomal processing. To examine this, we targeted OVA to the BCR of purified WT or Bg B cells and used them as APCs to stimulate the T cell hybridoma 13.8, with T cell activation being read out as IL-2 promoter activity (16). As shown in Fig. 2, resting Bg B cells presented Ag less well than WT B cells at early time points but were able to stimulate T cells equivalently by 24 h (Fig. 2A). Significantly, when B cells preactivated with LPS for 24 h were used as APCs, WT B cells were superior to Bg cells even at 24 h (Fig. 2B). When B cells were given soluble OVA for pinocytic uptake, presentation by WT and Bg B cells was equivalent if resting B cells were used (Fig. 2C). However, WT cells were superior if activated B cells were used (Fig. 2D). Thus, Bg B cells are relatively poor at presenting BCR-targeted Ag to T cells, and unlike WT B cells, they are unable to enhance their Ag presentation capability upon activation.
WT cells in the absence of inhibitor. As shown in Fig. 3A–C, Ag presentation by WT cells is inhibited by all three inhibitors at all three doses used. Presentation by Bg B cells, in contrast, is significantly inhibited only at the higher concentrations used, indicating that presentation by Bg B cells is relatively far less sensitive to lysosomal inhibitors. No significant difference was seen in the presentation of soluble OVA by the two cell types in the presence of inhibitors (data not shown).

Antigenic cargo in early endosomes can theoretically associate with recycling, as well as with newly synthesized MHC-II molecules (17). To determine whether the Ag presentation by Bg B cells occurred largely on recycling MHC molecules, we added the protein synthesis inhibitor cycloheximide (CHX) to restrict presentation to recycling MHC-II molecules. We found CHX almost completely abrogated presentation of BCR-targeted OVA by B cells from both strains of mice, indicating that newly synthesized MHC-II molecules were necessary for presentation of Ag after BCR-mediated endocytosis. Presentation of soluble OVA, however, was only partially reduced, indicating that Ag taken up pinocytotically may be presented on recycling, as well as newly synthesized MHC-II (Fig. 3D).

Unimpaired ability of Bg B cells to access limiting T cell help in vitro

The relatively poor Ag-presenting capability of Bg B cells raised the possibility that they may be able to access T cell help less well than WT B cells in vivo. To examine this, we first set up in vitro competition assays where a mixture of WT (CD45.1) and Bg (CD45.2) B cells presented OVA to varying numbers of OT-II transgenic T cells, and B cell activation and proliferation were read out at 12 and 48 h, respectively. As B cell/T cell ratios in culture decreased from 3:1 to 3:0.01, the intensity of CD69 staining and the proportion of cells that had upregulated CD69 decreased, but the staining profiles were similar in WT and Bg B cells whether present alone or in competition with each other (Fig. 4). Further, when CFSE-labeled B cells presenting OVA were cultured with OT-II cells, the overall CFSE dilution decreased with decreasing T cell help, but the proliferation was equivalent in WT and Bg B cells cultured alone or in competition (Fig. 5).

Higher frequency of memory cells and long-lived plasma cells in Bg mice

After immunization with NP-OVA, WT and Bg mice showed equivalent primary responses as determined by serum Ab levels and the frequency of plasmablasts in DLNs (Fig. 6A, 6B), and there was no significant difference in the affinity maturation profiles (data not shown). However, secondary responses in irradiated adoptive hosts challenged with soluble Ag after transfer of splenocytes from immunized WT and Bg mice indicated that memory B cell frequencies were higher in primed Bg mice (Fig. 6C). This was also supported by higher frequencies of responding cells seen in LDAs set up with DLNs at various times after immunization. On day 42, for instance (Fig. 6D), the frequencies of responding cells seen in LDAs set up with DLNs at various times after immunization. On day 42, for instance (Fig. 6D), the frequencies of responding cells were 10-fold higher in Bg mice (1/3 × 10^4 in Bg DLN versus 1/3 × 10^5 in WT DLN). We also examined whether generation of long-lived plasma cells was affected in Bg mice. To do this, we immunized mice with 10 μg NP-CGG in CFA, and after the primary response had died down, they were challenged with 5 μg soluble NP-CGG and the frequency of NP-specific plasma cells in spleen and BM estimated 5 and 65 d later by ELISPOT assay with titrating numbers of input cells. Plasma cell frequencies were higher in the spleen of Bg mice at both time points. No differences were observed in the BM on day 5, but the frequencies were significantly higher in Bg BM on day 65 (Fig. 6E).
The two allotype-specific Abs available have different sensitivities, with the anti-Ighb IgG reagents being 2-fold more sensitive (∼2) when Ighb-secreting WT cells are in competition with Igha-secreting WT cells. Very little specific IgG is detectable at days 0 and 7, and this contributes to some noise and to an apparent reversal of the ratio at early time points. Surprisingly, we found that Bg B cells responded better in such competition scenarios, indicating that optimal B cell Ag presentation for rapid recruitment of T cell help in vivo is not a crucial limiting factor in determining the efficacy of B cell priming to T-dependent Ags.

Sustained signaling in Bg B cells after BCR cross-linking

One possible explanation for the higher response of Bg B cells after immunization with T-dependent Ags is that delayed transport of the endocytosed Ag–BCR complex to lysosomes may allow for prolonged signaling downstream of the BCR and more than make up for any effects related to delayed Ag presentation to T cells. We tested this by looking at calcium flux and phosphorylation of signaling intermediates after BCR ligation. We found equivalent calcium flux in the two cell types (Fig. 7A). The signaling was quantified and averaged over five experiments. No statistically significant differences were seen between peak signal (p = 0.14) or sustained signal (p = 0.29 at 150 s and p = 0.46 at 200 s), and this is not surprising because the density of surface BCR is similar in the two strains and events that occur within seconds of BCR ligation are unlikely to be affected in Bg B cells before receptor internalization. However, p38, JNK, and ERK showed either higher phosphorylation or remained phosphorylated for longer periods in Bg B cells by Western blot assays (Fig. 7C–F). Flow cytometric phosphoflow assays confirmed this (Supplemental Fig. 4), and when detection was extended to 6 h after stimulation, they indicated that phosphorylation levels had returned to baseline levels by 4 h in both strains. No differences were seen in the phosphorylation of PLCγ. Bg B cells also proliferated better to lower levels of BCR cross-linking (Fig. 7B). Together, these data indicate that BCR signaling is amplified in Bg B cells and that this can lead to better proliferation in the presence of limited amounts of Ag.

Discussion

Optimal B cell responses to most protein Ags require help from CD4 T cells in the form of costimulatory signals and secreted cytokines. Early B–T interaction in vivo leads to B cell proliferation in extrafollicular foci and is responsible for early Ab production (18–20). A few activated B cells that escape terminal differentiation into plasma cells at this site migrate into the follicle where they initiate germinal center reactions that are characterized by extensive and rapid B cell proliferation, isotype switching, somatic hypermutation, death, and differentiation of survivors into plasma cells and memory cells. These events are shaped by the availability of T cells and follicular dendritic cells in germinal centers, and on the affinity-based selection of mutated BCRs (21–27).

B cells are most efficiently stimulated with cognate T cell help rather than with nonspecific bystander help, and they achieve this by processing the endocytosed Ag into peptides that are then loaded on MHC-II molecules for recognition by specific primed T cells. In this report, we have tried to assess the effect of delayed or suboptimal Ag presentation by B cells and the associated recruitment of cognate T cell help in determining the final outcome of B cell stimulation. To do this, we made use of the Bg mouse, which arose from a spontaneous mutation in the lys gene that encodes a widely
expressed cytosolic protein and whose mutation leads to defective endosome biogenesis (28–30). Various cells from Bg mice and from patients suffering from Chediak–Hegashi syndrome who manifest the effects of the \textit{lyst} mutation in humans (31, 32) show compromised function. These include cells that rely on lysosomes and lysosome-related vesicles such as melanosomes, platelet dense granules, and cytotoxic granules for their function (33–41).

Not much is known about the effect of the \textit{lyst} mutation on B cell function; however, an earlier report has indicated that there is a delay in peptide loading and transport of MHC-II–peptide complexes to the surface of EBV-transformed B cells from Chediak–Hegashi patients (42). We reasoned that the compromised transport of endocytosed Ag–BCR complexes to lysosomes for processing in Bg B cells could lead to less efficient Ag presentation, and hence to their compromised ability to recruit T cell help, with concomitant effects on downstream events that rely on T-B cooperation. By confocal microscopy tracking, we have shown that transport of endocytosed BCR to lysosomal compartments is delayed in resting primary B cells from Bg mice, with a significant proportion of the BCR outside LAMP-1+ve compartments even 2 h after endocytosis.

Further, delivery of the endocytosed BCR to lysosomes in Bg B cells did not improve even when they were preactivated with LPS, with 60% of the BCR remaining outside LAMP-1+ compartments even 12 h after endocytosis (Supplemental Fig. 3). Not surprisingly, the delayed transport of endocytosed cargo results in relatively poor stimulation of Ag-specific T cells by resting

FIGURE 5. CFSE dilution of WT (CD45.1, solid lines) and Bg (CD45.2, dotted lines) B cells stimulated alone (WT/Bg alone) or in competition (WT/Bg in competition) for 24 h with titrating numbers of activated OT-II T cells after targeting of OVA to the BCR. The various B: T cells ratios used are indicated in each panel. Representative of two independent experiments each.

FIGURE 6. B cell responses in WT and Bg mice. (A and B) Primary responses scored as serum Ab levels by ELISA (A, 7–10 mice/group) and frequency of plasma cells in DLNs by ELISPOT assay (B) in WT and Bg mice immunized with 25 \( \mu \text{g} \) NP-CGG/alum or 10 \( \mu \text{g} \) NP-CGG/CFA, respectively. Differences are not significant. (C) Secondary responses in irradiated carrier-primed hosts after adoptive transfer of splenic cells from WT and Bg mice; immunized 2 wk earlier with 10 \( \mu \text{g} \) NP-CGG/alum; \( n = 5 \) per group. (D) LDA of DLN cells from WT and Bg mice 42 d after immunization with 10 \( \mu \text{g} \) NP-CGG/CFA cells pooled from three mice in each group. (E) Frequency of NP-specific plasma cells in spleen and BM of WT and Bg mice 5 or 65 d after a secondary challenge (four mice per group). (F) Ratio of NP-specific Ab made by WT (Ighb) or Bg (Ighb) cells to that made by WT (Ighb) cells in WT/WT and WT/Bg irradiation BM chimeras various times after immunization with 100 \( \mu \text{g} \) NP-OVA/alum (\( n = 9 \) per group). Significance: \( p = 0.001 \) (days 14 and 21). Data are representative of two independent experiments each, except (E), where LDAs were set up on days 7, 14, 21, and 42 from a single priming. Data for other time points are not shown.
B cells and even poorer stimulation by LPS-activated B cells (Fig. 2). We used LPS as a surrogate activator in lieu of potent adjuvants that can cause bystander stimulation of B cells in lymph nodes draining the Ag during immunization or infection. B cell activation and migration to the B-T border in vivo may therefore occur concomitant to Ag uptake or may even precede Ag uptake. Taking the finding that both resting and activated Bg B cells are relatively poor Ag presenters when compared with WT B cells together with the possibility that Ag-specific T cells may be limiting, we predicted that Bg B cells may be at a disadvantage when competing with WT B cells for cognate T cell help. However, when we targeted OVA to the BCR of cells from Bg and WT mice, and put them in competition for T cell help in vitro that was provided by activated CD4 T cells from OT-II mice, cells from the two strains showed equivalent activation and proliferation, with both cell types showing decreasing but equivalent activation and proliferation as T cell numbers decreased (Figs. 4, 5).

The earlier data suggested the intriguing possibility that although the availability of large or small amounts of T cell help can influence the extent of B cell activation in vivo, relatively small delays in Ag presentation may be unlikely to adversely influence B cell responses. However, the amount of Ag targeted to the BCR in the in vitro competition experiment is not limiting, and the number of activated transgenic CD4 T cells provided even at the lowest dose may surpass the numbers of cognate T cells available in vivo. To overcome these and other limitations of in vitro cultures, we set up competition experiments in vivo with BM chimeras set up with marrow from WT mice (CD45.1, Ighb) and Bg mice (CD45.2, Igha) in irradiated WT mice. After confirming that chimerism was ~50%, we immunized the mice with NP-OVA on alum and tracked the primary Ab response. To our surprise, we found that Bg B cells mounted a higher Ab response in such chimeras (Fig. 6F). Immunization of WT or Bg mice individually, in contrast, led to equivalent primary responses (Fig. 6A, 6B) with no significant differences in affinity maturation (data not shown), but Bg mice showed a higher frequency of plasma cells in their BM and higher recall responses in adoptive transfer experiments (Fig. 6C–E). It is therefore possible that responses in vivo are insensitive either to the magnitude or the kinetics of the Ag presentation delay seen in vitro.

These unexpected results suggested the possibility that delayed delivery of endocytosed Ag–BCR complex to lysosomes in Bg B cells may permit continued signaling from the engaged BCR in early endosomal compartments as has been suggested earlier (13). Indeed, we found that Bg B cells proliferated better to lower levels of BCR cross-linking in vitro and also showed enhanced and more prolonged phosphorylation of signaling intermediates (Fig. 7). The Bg mouse strain has thus allowed us to determine the relative importance of the two events that follow Ag binding by B cells: continued signaling and Ag processing in lysosomes. If the engaged BCR is not delivered to lysosomes for degradation and remains in early endosomes, it appears to remain in a configuration that allows downstream signaling to proceed for a longer period and allows B cells to be stimulated at lower ligand densities.

Together, our data indicate that the strength and/or duration of signaling downstream of the BCR can affect B cell differentiation events, favoring entry of activated cells into the memory and long-lived plasma cell pool. They also indicate that relatively small numbers of T cells can provide adequate help for B cell function, and that short delays in B cell Ag processing and presentation for availing cognate T cell help do not significantly impair B cell activation or proliferation. Thus, nonsynchronous entry of B cells into germinal centers, for instance, is unlikely to affect optimal stimulation of the late-entry cells because of possibly limiting T cell help. A recent report (43) indicates that Ag presentation by B cells could influence their selection into the germinal center reaction, with higher affinity B cells displaying higher densities of cognate peptide-MHC on their surface, monopolizing T cell help at the T-B border. Our data indicate that this limitation may not be constrained by the time taken for effective presentation and/or that at least some compensation may be provided by increased duration of signaling downstream of the engaged BCR as seen in B cells carrying the Bg mutation.
References


Disclosures

The authors have no financial conflicts of interest.
Supplementary figure 1: B6-C20 (CD45.2, Ig\textsuperscript{a}) mice were crossed with B6.SJL (CD45.1, Ig\textsuperscript{b}) mice. The F1 mice were crossed with each other and F2 mice screened for the expression of CD45.1 and Ig\textsuperscript{a}. Brother-sister matings were then carried out for 7 generations before mice were used in experiments.
Supplementary figure 2: B cells from WT (black lines) and Bg (blue lines) mice, gated as shown express equal levels of MHC-II (dotted lines = unstained cells; unbroken lines = stained cells in histogram overlays), show similar profiles in the spleen (IgM vs IgD plots) and proliferate equivalently to LPS in vitro.
Supplementary figure 3: LPS-activated WT and Bg B cells were stimulated with anti-IgM. Co-localization of internalized BCR with and LAMP-1 is poor in Bg B cells even at 12 h.
Supplementary figure 4: Signaling downstream of BCR ligation

A-E: Phosphorylation of kinases analysed by phospho-flow assay. Purified B cells were stimulated with anti-IgM F(ab)² for 30 min or 120 min and phosphorylation of p38 and JNK determined by flow cytometry. Shaded histogram: unstimulated cells.

H-J: Specificity of phospho-flow assays. Splenocytes were stimulated with anti-CD40 (for p-JNK), and PMA+ionomycin (for p-ERK and p-p38) for 30 min to detect phosphorylation. In parallel, cells were pre-treated with 10μM of the indicated inhibitors for 30 min and stimulation carried out in the continued presence of inhibitor. Staining for phosphorylated intermediates was assessed by flow cytometry on gated B cells (p-JNK) or total splenocytes (p-ERK and p-p38). Shaded histogram: conjugate control; black line: unstimulated, blue line: stimulated; red line: stimulated in presence of inhibitor.