Multiple Checkpoint Breach of B Cell Tolerance in Rasgrp1-Deficient Mice

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Multiple Checkpoint Breach of B Cell Tolerance in Rasgrp1-Deficient Mice

Amber Bartlett,* Janet E. Buhmann,† James Stone,‡ Bing Lim,§ and Robert A. Barrington*  

Lymphocytic fate decisions are regulated in large part through composite signals generated through Ag receptor complexes. In the case of autoimmune disease, mutations affecting these composite signals via pathways downstream of Ag receptors can alter normal lymphocyte selection, leading to altered survival and activation of self-reactive cells.

Guinea exchange factors RasGRP1 and RasGRP3 predominate in lymphocytes and regulate Ras activation, a central pathway determinant of Ag receptor signal strength (1). Rasgrp1-deficient (Rasgrp1<sup>−/−</sup>) mice develop a lymphoproliferative disorder and exhibit high autoantibody titers (2). Interestingly, ho-normally, the frequency of receptor-edited 564Igi B cells within these germinal centers were significantly increased in Rasgrp1-deficient mice relative to control mice. Taken together, these studies suggest that autoreactive B cells lacking Rasgrp1 break central and peripheral tolerance through both T cell–independent and –dependent mechanisms. The Journal of Immunology, 2013, 191: 000–000.

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To address whether RasGRP1 is important for B cell tolerance, Rasgrp1<sup>+/−</sup> mice were crossed with autoimmune-prone 564IgI mice. The 564IgI strain, described by Berland et al. (15), harbors a knockin BCR transgene originating from a hybridoma isolated from an autoimmune SWR × NZB mouse (16). Self-reactive 564IgI B cells can be identified by an anti-idiotypic Ab. 564IgI B cells exhibit an anergic phenotype and are excluded from follicles, but aged mice develop immune complex deposition in kidneys and measurable titers of anti-nuclear Abs (15). By comparison, we report that Rasgrp1<sup>+/−</sup> 564IgI B cells accumulate at the transitional and mature stages of development and exhibit higher levels of IgM. Developing and transitional 564IgI B cells lacking Rasgrp1 had reduced levels of Bim, suggesting that clonal deletion of these self-reactive B cells was impaired. Whereas 564IgI B cells infrequently localized to germinal centers in Rasgrp1-deficient mice, spontaneous germinal centers developed in 8–10-wk-old Rasgrp1<sup>+/−</sup> mice and contained numerous 564IgI B cells. Further, 564IgI germinal center B cells from Rasgrp1<sup>−/−</sup> mice had increased levels of CD86 compared with 564IgI B cells from control mice. In sum, these studies indicate that autoreactive B cells lacking Rasgrp1 break tolerance early during development and further suggest that both T cell–independent (immature bone marrow and transitional B cells) and –dependent (germinal center B cells) mechanisms are involved in breaking B cell tolerance.

Materials and Methods

Mice

Mice were housed at the University of South Alabama in an American Association for the Accreditation of Laboratory Animal Care–accredited specific pathogen-free facility. Rasgrp1<sup>−/−</sup> (17) and littermate control Rasgrp1 heterozygous mice were maintained on a C57BL/6 background (10 generations backcrossed). Rasgrp1 strains were crossed with 564IgI H and L chain–targeted transgenic mice, also maintained on a C57BL/6 background (15). Importantly, no phenotypic differences were measured between Rasgrp1 heterozygous and wild-type mice (data not shown). Maintenance of breeding colonies and all procedures involving mice were performed according to protocols approved by the University of South Alabama Institutional Animal Care and Use Committee.

Flow cytometric analysis and Abs

Single-cell suspensions of bone marrow and splenic mononuclear cells (MNCs) were isolated by density-gradient centrifugation using Lymphocyte M (Cedarlane Laboratories, Burlington, NC). To detect 564IgI B cells, MNCs were stained with anti-PNA biotinylated anti-idiotypic Ab (clone B6-256; BioXCell, West Lebanon, NH), followed by streptavidin-allophycocyanin conjugate. Abs used for flow cytometric analyses of B cell subsets in bone marrow and spleen included the following (BD Biosciences, San Jose, CA and eBioscience, San Diego, CA): CD19 (1D3), CD21/CD35 (7G6), CD23 (B3B4), CD40 (M1/69), CD38 (90), CD45R (RA3-6B2), CD86 (GL1), CD93 (AA4.1), IgM (DS.1), IgM<sub>α</sub> (AF6-78), IgM (Nov-41, R6-60.2), IgD (AMS9.1), GL-7, and peanut lectin (agglutinin) (PNA; Vector Laboratories, Burlingame, CA). Levels of Bim were examined by intracellular staining using polyclonal rabbit anti-Bim or isotype control rabbit IgG (Cell Signaling Technology, Beverly, MA).

Cells were analyzed by FACSCanto II and sorted using multilaser FACSaria II-SORP (BD Biosciences) housed in the University of South Alabama College of Medicine Flow Cytometry Core Laboratory. Data were analyzed with FlowJo software (Tree Star, Ashland, OR).

Immunofluorescent analysis of splenic sections

Five-micrometer cryosections of OTC (Tissue-Tek, Torrance, CA) preserved spleens were prepared as described previously (18). After hydration with PBS, sections were incubated with anti-CD16/CD32 (2.4.G2; BioXCell) before incubation to resolve 564IgI B cells using biotinylated anti-idiotypic Ab followed by streptavidin-PE, then counterstained with anti-mouse CD45R-allophycocyanin and PNA-FITC. Images were acquired by using a Nikon A1R confocal microscope (University of South Alabama microscope Core Facility), equipped with Nikon elements software (Nikon Instruments, Melville, NY).

Gene expression

RNA was prepared from FACS-sorted B cells using the RNeasy mini kit (Qiagen, Valencia, CA). cDNA was prepared using iScript (Bio-Rad, Hercules, CA) and normalized by PCR for HPRT and β<sub>2</sub>-microglobulin expression. Bim expression was determined by real-time PCR for SYBR Green incorporation using a CFX96 (Bio-Rad) and the following primers (forward/reverse): HPRT (5′-CTGGTGAAGAGGCACCTCCTGAGT- ACTCTATGATCAGGGCGA-3′), β<sub>2</sub>-microglobulin (5′-CCCGGCC- TCACATTGAAATCCGGGATGTCATTCACTGCTCAGGGTAAT-3′). Statistical analysis

All statistical comparisons reported used the standard two-tailed t test assuming unequal variance.

Results

To understand where and how autoreactive B cells lacking Rasgrp1 (Rasgrp1<sup>−/−</sup>) break tolerance, Rasgrp1<sup>−/−</sup> mice were bred to mice containing a knock-in BCR transgene (564IgI) (15) with polyreactive specificity for ssDNA (16, 21), ssRNA (16, 21), and nucleosomes (22). 564IgI B cells in this model are anergic, though they eventually break tolerance through a TLR7-dependent mechanism that leads to elevated serum levels of 564IgI-derided IgG2a Ab (15). 564IgI-derived Ig expresses a H-chain allotype marker (IGM<sup>+</sup>) and thus can be distinguished from endogenous IgM (IgM<sup>+</sup>). The development and fate of the auto-reactive "a" allotype 564IgI B cells was followed using an anti-idiotypic Abs in combination with antiallootypic reagents. Mice heterozygous for each of the 564IgI H and IgL chain transgenes were used to allow nonautoreactive B cells to be produced to provide competition necessary to visualize selection.

Autoreactive 564IgI-derived serum Abs

Autoantibody titers (including anti-nuclear, anti-dsDNA, and anti-Sm) are detectable in Rasgrp1<sup>−/−</sup> mice beginning at ∼8 wk of age (2). To assess whether 564IgI B cells exhibit a similar age-dependent break in tolerance in the absence of rasgrp1, serum samples were collected from young (4–6-wk-old) and mature (8–12-wk-old) Rasgrp1<sup>−/−</sup> 564IgI mice. Serum from young Rasgrp1<sup>−/−</sup> mice as well as littermate control mice (Rasgrp1<sup>+/−</sup>) had comparable levels of 564IgI-derided IgM, IgG2a, IgG2b, and IgG3 (Supplemental Fig. 1). Whereas serum 564IgI titers for all isotypes remained similar between young and old littermate control mice, titers in Rasgrp1<sup>−/−</sup> mice increased with age for each of IgM, IgG2a, IgG2b and IgG3. Further, serum 564IgI titers from old Rasgrp1<sup>−/−</sup> mice were significantly higher than compared with those in age-matched littermate control mice (Fig. 1). These data indicate that levels of 564IgI autoreactive Ab are increased in...
mice lacking RasGRP1 ~8 wk of age and suggest that 564Igi B cells break tolerance in the absence of Rasgrp1.

Development of 564Igi B cells

To establish what B cell tolerance checkpoints were breached in Rasgrp1−/− mice, the frequency and number of 564Igi B cells was measured within bone marrows and spleens. No significant differences were observed in the frequency of 564Igi B cells during early development in bone marrows of Rasgrp1−/− and littermate control mice (data not shown). Consistent with previous studies, no apparent differences were observed in the frequency of 564Igi transitional B cells in spleens of young mice with or without RasGRP1 (Table I; 53.3±11.7 versus 46.2±15.0, mean±SE in Rasgrp1−/− versus controls). Though the frequencies were comparable, the number of transitional B cells was increased in Rasgrp1−/− mice compared with littermate controls (Table I; 2.3±0.6×10^6 versus 0.8±0.5×10^6; t test, p=0.003). Similarly, though the frequency of 564Igi B cells in the transitional stage of development was similar between older Rasgrp1−/− and littermate control mice, the number of autoreactive 564Igi B cells within the transitional B cell pool was increased in older Rasgrp1−/− mice (Table I; 3.6±3.1 versus 0.5±0.4×10^6; t test, p=0.04). These data suggest that autoreactive B cells accumulate during B cell development in Rasgrp1−/− mice.

Tolerance in developing B cells

Berland et al. (15) showed that receptor editing occurs in 564Igi B cells and can be measured by the appearance of endogenous IgMα and Igλα B cells. Developing B cells in both the bone marrow and spleen were compared for the frequency of IgMα+ B cells in young Rasgrp1−/− 564Igi and littermate control mice (Fig. 2). In bone marrow, similar frequencies of developing B220+CD23−, fractions B through D) B cells were observed in Rasgrp1-sufficient and -deficient mice (Fig. 2A and data not shown). As shown in Fig. 2, the frequency of IgMα+ cells among B220+CD23− developing B cells was reduced in Rasgrp1−/−/− 564Igi mice compared with Rasgrp1-sufficient littermate control mice. Similarly, the frequency of IgMα+ B cells among B220+CD93+ transitional B cells was reduced ~2-fold in young Rasgrp1−/−/− 564Igi compared with littermate control mice (Fig. 2B, 2C). In each case, IgMα+ B cells were negative for both anti-idiotypic and for IgMα, suggesting that cells edited away from the autoreactive transgenic BCR. Interestingly, the reduced frequency of IgMα+ B cells in mice lacking RasGRP1 was also evident in B cells still expressing transgene-associated IgMα (Fig. 2B). Similar results were observed when Igλα+ B cells was used as an indicator of receptor editing (data not shown). Taken together, these data suggest that RasGRP1, though not required for receptor editing, is required for tolerance in early B cell development.

Anergic B cells exhibit a significant reduction in membrane BCR levels, resulting from increased endocytosis and decreased transport of new BCRs to the cell surface (23, 24). To assess whether 564Igi B cells in Rasgrp1−/− and control mice had this anergic phenotype, the levels of surface IgMα were determined (Fig. 3A, 3B). Flow cytometric analysis revealed that transitional 564Igi B cells from Rasgrp1−/− mice displayed significantly more IgMα and further bound more anti-Id Ab, compared with transitional 564Igi B cells from control mice. As an internal control, the levels of IgMα in edited 564Igi B cells were compared and found to be similar in both Rasgrp1−/− and control mice. These data are consistent with a role for RasGRP1 in the regulation of BCR levels on the plasma membrane of autoreactive B cells.

Clonal deletion of developing autoreactive B cells is thought to occur in a Bim-dependent manner (25). To address whether Bim was differentially expressed in autoreactive Rasgrp1−/− B cells, 564Igi developing bone marrow B cells (B220+CD23−) and splenic transitional B cells (B220+CD93−) were sorted from young Rasgrp1−/− and littermate control mice. As shown in Fig. 3C, real-time PCR revealed that both developing bone marrow and transitional 564Igi B cells from Rasgrp1−/− mice had reduced levels of Bim mRNA compared with 564Igi B cells from control mice. To determine whether differences at the mRNA level were evident at the protein level, intracellular staining for Bim was performed (Fig. 3D, 3E). A significant reduction in levels of Bim was observed in immature 564Igi B cells from the bone marrow and from spleens of Rasgrp1−/− mice relative to those levels observed in littermate control mice. Together, these data are consistent with enhanced survival of autoreactive B cells in Rasgrp1−/− due to reduced Bim-dependent apoptosis.

Table I. The frequency and number of self-reactive 564Igi+ B cells during development in young and mature Rasgrp1−/− mice

<table>
<thead>
<tr>
<th>Frequency (564Igi B Cells)</th>
<th>Number of 564Igi B Cells (×10^6 cells)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>4–6-wk-old mice</td>
<td></td>
</tr>
<tr>
<td>Rasgrp1−/− (n = 10)</td>
<td>20.0±10.0</td>
</tr>
<tr>
<td>Rasgrp1−/− (n = 9)</td>
<td>29.9±11.1</td>
</tr>
<tr>
<td>8–12-wk-old mice</td>
<td></td>
</tr>
<tr>
<td>Rasgrp1−/− (n = 9)</td>
<td>14.8±10.3</td>
</tr>
<tr>
<td>Rasgrp1−/− (n = 18)</td>
<td>26.8±12.6</td>
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</tbody>
</table>

Frequency of cells within each population was determined by flow cytometry; numbers of cells were determined using total splenocyte counts and frequency of the population as determined by flow cytometry.

Statistical differences comparing age-matched Rasgrp1−/− versus Rasgrp1−/− controls: *p<0.04, **p<0.003.
Signals provided through TLR7 and TLR9 elicit breaks in B cell tolerance by driving expansion of immature/transitional B cells (26–29). Further, B cells with impaired BCR signaling are reported to have enhanced responsiveness to TLR signals. To test whether RasGRP1 affected responses of 564Igi B cells to TLR signals, proliferative responses of splenic B cells from Rasgrp1^{−/−} and control mice were measured (Fig. 4). 564Igi B cells from control mice exhibited marked proliferation in response to TLR7 agonist loxoribine alone and in combination with BCR cross linking using anti-idiotype Ab. As expected from earlier reports (29), transitional B cells proliferated most extensively compared with mature B cells (data not shown). 564Igi B cells from Rasgrp1^{−/−} mice showed similar responses compared with 564Igi B cells from control mice when stimulated with either TLR7 or TLR9 agonists alone and in combination with BCR cross linking. Notably, though the frequency of 564Igi B cells proliferating to TLR and BCR stimuli were comparable, more 564Igi B cells proliferated in response to TLR7 agonist loxoribine, either alone or in combination with anti-idiotypic Ab. Similar results were obtained using TLR9 agonist ODN alone and in combination with BCR cross linking (data not shown). Therefore, autoreactive B cells lacking Rasgrp1^{−/−} responses similarly when stimulated throughTLRs.

Selection of autoreactive germinal center B cells

To determine whether later tolerance checkpoints were compromised in Rasgrp1^{−/−} 564Igi mice, B cell development in 8–12-wk-old mice also was assessed. Along with significant splenomegaly and lymphadenopathy, mature Rasgrp1^{−/−} mice had significantly higher frequency and number of splenocytes and lymph node cells (data not shown). The frequency and number of 564Igi B cells in spleens was likewise increased in Rasgrp1^{−/−} mice compared with age-matched littermate controls (Table I; 26.8 ± 12.5 versus...
Interestingly, a relatively high percentage of 564Igi B cells lacking RasGRP1 stained positive with PNA (and CD95, not shown), consistent with the phenotype of germinal center B cells (Fig. 5A). Similar results were observed in lymph nodes (Supplemental Fig. 2). To validate the increased frequency of 564Igi germinal center B cells, histological analysis of spleen sections was performed. Although PNA-staining clusters were infrequent in littermate control mice, Rasgrp1<sup>−/−</sup> mice had both an increase in PNA-staining germinal centers as well as an increase in 564Igi B cells within germinal centers (Fig. 5B, 5C). These data indicate that germinal centers in Rasgrp1<sup>−/−</sup> contain self-reactive B cells and further are consistent with germinal centers being the source for self-reactive Ab production in Rasgrp1<sup>−/−</sup> mice.

Germinal center B cells undergoing Ag selection in the light zone recruit T cell help prior to differentiating into memory and Ab-secreting cells. To examine further whether the absence of Rasgrp1<sup>−/−</sup> affects germinal center maturation and selection, the frequency and number of 564Igi B cells expressing T cell co-stimulatory CD86 was determined (Fig. 6). In littermate control

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**FIGURE 4.** Proliferative responses of Rasgrp1<sup>−/−</sup> B cells to TLR stimulation. CFSE-loaded splenic B cells from littermate control (top panels) and Rasgrp1<sup>−/−</sup> (bottom panels) mice were cultured in the presence of TLR7 agonist loxoribine with and without BCR cross linking using anti-idiotypic (Id) Ab as indicated. The loss of CFSE fluorescence intensity was measured in 564Igi B cells (Id<sup>+</sup>). No statistically different proliferative response was apparent between control and Rasgrp1<sup>−/−</sup> B cells. Data are representative from one of three independent experiments.
mice, of the few 564Igi germinal center B cells, only a small percentage were positive for CD86. In contrast, a significantly higher percentage of 564Igi germinal center B cells from Rasgrp12/2 mice stained positive for CD86 (Fig. 6A). In addition, the level of CD86 on 564Igi germinal center B cells was elevated in Rasgrp12/2 mice relative to control mice (Fig. 6B). These data are consistent with at least a portion of the autoantibody response being T cell dependent and provide further evidence that spontaneous germinal centers in Rasgrp12/2 mice promote self-reactive B cell maturation that leads to the production of self-reactive Ab production.

Discussion
Early evidence demonstrated that RasGRP1 is important for T cell development (4, 6, 17), but that it had little impact on B cell development (2). Despite this, Rasgrp11/− mice displayed high levels of serum autoantibodies (2), suggesting that it may be necessary to examine self-reactive B cells directly to understand whether RasGRP1 contributes to B cell development. In this study, we demonstrate that 564Igi B cells lacking RasGRP1 break tolerance at multiple checkpoints during development. During bone marrow development and at the transitional stage, self-reactive B cells lacking RasGRP1 accumulate by a mechanism appearing to involve BCR-mediated levels of Bim. Mature self-reactive B cells further escape tolerance by entering germinal centers that form without active immunization and differentiate to produce autoantibody.

Earlier reports indicated that Rasgrp11/− mice bred onto a C57BL/6 background apparently failed to produce autoantibodies (5). This contrasts with the initial description of the same mice with a hybrid 129:B6 background (2) and raised the possibility that other genetic components were necessary for autoimmunity. A more recent study using mice (B6 background) engineered to produce truncated RasGRP1 lacking the C-terminal tail domain found increased serum autoantibody compared with controls (30), arguing against other genetic contributions to autoantibody levels in mice with defective RasGRP1-mediated signaling. Using a Rasgrp1−/− strain maintained on a C57BL/6 background, we show that autoantibody production is readily detectable. By measuring 564Ig-specific autoantibody, we demonstrate that all IgG subclass–specific autoantibody levels are increased in Rasgrp12/2 mice. Further, levels of 564Ig-specific IgM and IgA (data not shown) also were elevated. Therefore, our data indicate that deficiency in RasGRP1 predisposes to autoimmunity in mice.

TLRs have a demonstrable role in breach of B cell tolerance (26, 31, 32). Genetic duplication of TLRs predispose to lupus-like
A striking consequence of Rasgrpl deficiency is the development of germinal centers without immune manipulation. 564IgI B cells are readily detectable in germinal centers of Rasgrpl−/− mice. Th type 1 cells are considered the key effector population regulating disease in Rasgrpl−/− mice (3). Our data demonstrate that the frequency of CD86-positive self-reactive germinal center B cells is increased in Rasgrpl−/− mice, suggesting that they are better able to recruit T cell help. T follicular helper cells are critical for enhancing B cell survival and differentiation in germinal centers, both through cognate and noncognate interactions (44, 45). The importance of T follicular helper cells has not been established in lymphopenic autoimmune environments, though they are considered important in other autoimmune models (46). It will be important to assess the frequency of T follicular helper cells and whether these T cells also are required for autoantibody production in Rasgrpl−/− mice. That 564IgI is increased for all Ig isotypes measured is consistent with multiple cytokines contributing to autoimmunity, and therefore, we speculate that other T follicular helper cells can contribute to autoimmunity in Rasgrpl−/− mice.

FIGURE 6. 564IgI germinal center (GC) B cells from Rasgrpl−/− mice upregulate CD86. The frequency of CD86 positive 564IgI B cells in germinal centers of Rasgrpl+/− (black bar) and Rasgrpl−/− (gray bar) mice was measured in (A). Representative mean fluorescence intensity (MFI) of CD86 on the surface of 564IgI GC B cells in Rasgrpl+/− (left panel) and Rasgrpl−/− (right panel) mice is shown in (B). Data in (A) are represented as mean ± SE from three independent experiments; n = number of mice in each group. *p < 0.0004.
We currently do not understand the triggers generating germinal centers in Rasgrp1−/− mice. Interestingly, the development of these germinal centers is not apparent until Rasgrp1−/− T cell numbers reach at least normal levels (R.A. Barrington, unpublished observations). Homeostatic expansion as a result of lymphopenia is associated with development of autoimmunity in the context of certain genetic predispositions (47). It is well-documented that homeostatic proliferation of T cells in Rasgrp1−/− mice is dysregulated (4). In both induced and naturally developing lymphopenic environments, expansion of lymphocytes is directed by cytokine storms. The level of a number of proinflammatory cytokines is elevated in Rasgrp1−/− mice (R.A. Barrington, unpublished observations), though whether these cytokines potentiate germinal center development and autoantibody has not been determined.

In summary, our studies reveal the importance of RasGRP1 for B cell tolerance. The accumulation of self-reactive B cells early in B cell development supports a direct role for RasGRP1 in B cell tolerance. Through DAG and calcium-binding domains, it is likely that RasGRP1 modifies the strength of signals generated through the BCR signaling complex. The increased participation of self-reactive B cells in spontaneous germinal centers in Rasgrp1−/− mice, and the increase in Ig class-switched self-reactive serum Ab is consistent with additional T cell–dependent mechanisms of peripheral tolerance. Because both deficiency and polymorphisms in RasGRPs in regulation of lymphocyte proliferation. Annu. Rev. Immunol. 34: 858–861.


