The Rac2 Guanosine Triphosphatase Regulates B Lymphocyte Antigen Receptor Responses and Chemotaxis and Is Required for Establishment of B-1a and Marginal Zone B Lymphocytes

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The Rac2 Guanosine Triphosphatase Regulates B Lymphocyte Antigen Receptor Responses and Chemotaxis and Is Required for Establishment of B-1a and Marginal Zone B Lymphocytes

Ben A. Croker,*‡ David M. Tarlinton,† Leonie A. Cluse,* Alana J. Tuxen,* Amanda Light,† Feng-Chun Yang,§ David A. Williams,§ and Andrew W. Roberts‡‡

We have defined roles for the hemopoietic-specific Rho guanosine triphosphatase, Rac2, in B lymphocyte development and function through examination of rac2−/− mice. Rac2-deficient mice displayed peripheral blood B lymphocytosis and marked reductions in peritoneal cavity B-1a lymphocytes, marginal zone B lymphocytes, and IgM-secreting plasma cells as well as reduced concentrations of serum IgM and IgA. The rac2−/− B lymphocytes exhibited reduced calcium flux following coligation of B cell AgR and CD19 and reduced chemotaxis in chemokine gradients. T cell-independent responses to DNP-dextran were of reduced magnitude, but normal kinetics, in rac2−/− mice, while T-dependent responses to nitrophyrenyl-keyhole limpet hemocyanin were subtly abnormal. Rac2 is therefore an essential element in regulating B lymphocyte functions and maintaining B lymphocyte populations in vivo. The Journal of Immunology, 2002, 168: 3376–3386.

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correct positioning of T and B cells within the lymphoid areas during an immune response is mediated by altering their response to chemokines (21–23). While GC development and function require B lymphocyte chemoattractant (BLC; CXCL13)-dependent organization, the localization of AFC foci produced during the immune response appears to be due to SDF-1α (CXCL12) (21).

It is evident that the integration of signals transmitted from the BCR, costimulatory molecules, and chemokine receptors is pivotal to B cell development and function. While such integration probably occurs at multiple levels, the Rho family of guanine triphosphatases (GTPases), which includes Rho, Rac, Cdc42, and relatives, are attractive candidates as points of confluence for these signals, as they appear to perform analogous roles in other cell types such as fibroblasts (24). Rac proteins in particular have been directly implicated in extracellular signal integration in T lymphocytes (25). Rac2 is a hemopoietic-specific GTPase that is thought to act in concert with its ubiquitously expressed, highly homologous sister protein, Rac1 (26). Unlike other Rho family GTPases, Rac2 is expressed exclusively in myeloid and lymphoid cells, including B lymphocytes (27), suggesting that it is required for unique processes ascribed to these cells. We have recently described crucial roles for Rac2 in the regulation of superoxide production in neutrophils (28) and chemotaxis in neutrophils, mast cells (29), and hemopoietic stem cells (30).

Several studies suggest that Rac proteins are important for B lymphocyte function and for signaling from the BCR and CD19. Analysis of mice deficient in Vav, a guanine exchange factor for Rac and a catalyst of Rac activity, revealed abnormal Ig class switching in response to T-dependent Ags (31) as well as impaired signaling via the BCR and CD19 (31–34). Furthermore, ligation of CD19 in a human B cell line activates Vav and mitogen-activated protein kinase pathways and induces the association of CD19 with CD26 and Vav (35), suggesting that Rac may be important for CD19-mediated signal transduction.

The majority of studies to date have focused on the roles of Rac proteins in T lymphocytes, whereas little, if any, data directly address Rac2 function in B lymphocytes. In this study we begin to define the physiological roles of Rac2 in B lymphocyte development and function using a variety of in vitro and in vivo assays that directly compare B lymphocytes containing or lacking Rac2.

### Materials and Methods

#### Mice

Rac2-deficient mice backcrossed nine generations onto the C57BL/6 strain and wild-type littermate or C57BL/6 mice were used in these experiments. The generation of Rac2-deficient mice has been described previously (28). Sex- and age-matched mice, 5–25 wk of age, were used in these experiments. For reciprocal transplant experiments, four to seven lethally irradiated mice of each genotype were reconstituted with 5 × 10^6 bone marrow cells from donors of each genotype, and lymphoid compartments were analyzed 8 wk postinjection. Reciprocal, labeled lymphocyte transfer experiments were performed as previously described (36) with minor alterations. Groups of three recipients of each genotype were injected i.v. with unfractionated splenocytes (containing equivalent numbers of B220<sup>+</sup> cells for each genotype, ~30 × 10<sup>6</sup> for each independent experiment) from pooled donors of each genotype, after incubating with 0.5 μM CTG (Cell-Tracker Green CMFDA; Molecular Probes, Eugene, OR) according to the manufacturer’s instructions. The frequency of labeled cells in recipient organs was determined 5 h later by flow cytometric analysis of 100,000 leukocytes. For peripheral blood, recovery was calculated from the frequency of labeled cells, the leukocyte count per milliliter, the number of cells injected, and a presumed blood volume of 2 ml. To examine T-dependent B lymphocyte responses, mice were injected i.p. with either 100 μg 4-nitrophenyl-keyhole limpet hemocyanin (NP-KLH) precipitated with alum and boosted at 6 wk with 4 μg NP-KLH diluted in PBS or with 100 μg NP-KLH in the absence of adjuvant. T-independent type II (TI-II) B lymphocyte responses were examined by injecting mice i.p. with 10 μg DNP-dextran diluted in PBS. Analyses of data were performed using one-way ANOVA and, where appropriate, the Student-Newman-Keuls test.

#### Phenotypic characterization of B lymphocyte populations by flow cytometry

B lymphocyte populations were characterized by flow cytometry using FITC-, PE-, or biotin-conjugated mAbs specific for CD45R (B220), IgM, IgD, CD19, CD21, CD23, CD24, and CD5 (provided by Dr. A. Strasser, Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia). Biotinylated Abs were revealed with streptavidin-PE (BD PharMingen, San Diego, CA).

#### In vitro lymphocyte chemotaxis and F-actin assays

The recombinant mouse chemokines SDF-1α (CXCL12) and BLC (CXCL13; R&D Systems, Minneapolis, MN) were used for migration and actin assays. Migration assays were performed as previously described (37). In brief, 5 × 10<sup>6</sup> cells in 100 μl medium were added to the upper chamber of the 5-μm pore size Transwell insert (Costar, Corning, NY). Various concentrations of the chemoattractant were added to the appropriate well. The Transwells were incubated at 37°C in 5% CO<sub>2</sub> for 4 h. Migration of B lymphocytes was determined by flow cytometry using anti-B220 Abs. Duplicate assays were performed for each sample. Actin polymerization was quantified as described previously (37) with minor modifications. Splenocytes (5 × 10<sup>6</sup>) were incubated at 37°C for 5 min before addition of chemoattractant in varying concentrations for 15 s, then fixed, stained (80 nM FITC-phalloidin (Sigma-Aldrich, St. Louis, MO), 0.1 μg/ml α-(1-4)-lysophosphatidylcholine (Sigma-Aldrich), 3.6% formaldehyde, and PBS) and analyzed by flow cytometry. Forty thousand events were acquired, and lymphocytes were identified by forward and side scatter characteristics. Increases in F-actin fluorescence of lymphocyte populations relative to control samples were used to determine percentage increases in filamentous actin (F-Actin).

#### Immunohistochemistry

Spleens were snap-frozen in OCT compound (Sakura Finetek, Torrance, CA), and were stored at ~70°C. Cryostat sections were prepared and stained exactly as described previously (38). Sections were stained with the following reagents in the combinations indicated in the relevant figures: anti-IgG1-HRP (Southern Biotechnology Associates, Birmingham, AL), biotinylated peanut agglutinin, MOMA-1-specific for MZ metabolic macrophages, provided by Dr. I. Caminschi, Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia, F4/80 (Serotec, Oxford, U.K.), RA3-6B2 (anti-B220), and biotinylated KT3 (anti-CD3). Anti-B220 was detected with Abs specific for rat κ chain directly coupled to HRP (BD Pharmingen). Biotinylated primary reagents were detected using streptavidin-alkaline phosphatase (Southern Biotechnology Associates). Alkaline phosphatase was visualized using the Fast Blue Black vector (Laboratories, Burlingame, CA) after endogenous phosphatases were blocked with 2 mM levamisole (Sigma-Aldrich). HRP was visualized using the 3-amino-9-ethylcarbazole substrate kit (Vector Laboratories).

#### ELISA and ELISPOT

Ab titers and the frequency of AFC were determined exactly as previously described (39). Individual Ig isotypes were revealed using specific goat anti-mouse Abs conjugated to HRP (Southern Biotechnology Associates), except IgA, which was detected using biotinylated goat anti-mouse IgA and streptavidin-HRP (Southern Biotechnology Associates). IgG AFC represent a pool of cells secreting IgG1, IgG2a, and IgG2b. For detection of Ag-specific Abs and AFC following immunization, plates were coated with 20 μg/ml NP<sub>6</sub>B and NP<sub>20</sub>BSA diluted in PBS. Bound IgG1 was detected with isotype-specific goat anti-mouse IgG1 directly coupled to HRP. Secondary Abs were detected using the reaction of peroxidase with ABTS (Sigma-Aldrich).

#### Proliferation and I<sub>g</sub> production

In vitro proliferation and Ig production assays used a pool of two spleens for each assay. B lymphocytes were purified from spleen by negative sorting after removal of RBC. T lymphocytes, macrophages, and plasma cells were labeled with Abs specific for Thy1, Mac1, and 281-Syndecan, respectively. B cell representation among the negatively sorted leukocytes was determined by subsequently staining the sorted population with anti-B220-PE and was routinely >95%. Proliferation assays were conducted at a concentration of 10<sup>6</sup> cells/ml in 0.2 ml RPMI 1640/2% FCS/2%ME. B lymphocytes were stimulated with LPS (Difco, Detroit, MI) at 20 and 20 μg/ml, F(ab'<sub>2</sub>)<sub>2</sub> anti-IgM (Jackson Immunoresearch Laboratories, West Chester, PA).
Grove, PA) at 1 and 10 μg/ml, and CD40 ligand (CD40L; 1/200 dilution of a baculovirus-infected cell membrane preparation (40)) alone or supplemented with IL-4 and IL-5. Proliferation on day 2 was measured as [H]thymidine incorporation following a 6-h pulse with 0.5 μCi [H]thymidine. Ig production assays were conducted at a cell concentration of 2.5 × 10^3 cells/ml in 400 μl RPMI 1640/2% FCS/2-ME. B lymphocytes were stimulated with LPS, CD40L, and CD40L supplemented with IL-4 and IL-5 as described above. IgM and IgG1 production on day 5 was determined by ELISA. All assays were performed in triplicate. Control wells contained no stimulus.

**Calcium signaling**

Calcium flux experiments were performed as described previously (41). In brief, indo-1/AM (Molecular Probes)-loaded splenocytes were stained with either anti-B220-FTTC or CD21-FTTC and CD23-PE and then labeled with biotinylated anti-CD19 and/or anti-mouse κ chain Abs at the concentrations indicated in the figure. For calcium flux induction, biotinylated Abs were cross-linked with 20 μg/ml avidin. Changes in intracellular calcium concentrations were monitored for 4–6 min by calculating the ratio of the fluorescence emissions at 425 and 530 nm of B220+ cells or follicular B cells (CD21^hi/CD23^hi). Previous experiments have shown that staining with anti-B220 does not influence the outcome of BCR cross-linking (41).

**Results**

**Perturbed B lymphocyte development in Rac2-deficient mice**

Before defining the role of Rac2 in B lymphocyte differentiation, we first established the basic parameters of the immune system in Rac2-deficient C57BL/6 mice. The weight and total cellularity of bone marrow, spleen, lymph nodes, and peritoneal cavity were determined the number of cells present in each of several identifiable cell subpopulations. Immature (B220<sup>hi</sup> HSA<sup>hi</sup>) and mature (B220<sup>lo</sup> HSA<sup>lo</sup>) B lymphocyte cell numbers were reduced by 10 and 40%, respectively, compared with controls, while immature plus MZ B lymphocytes (IgD<sup>lo</sup>IgM<sup>+</sup>) and follicular (IgD<sup>hi</sup>IgM<sup>+</sup>) B lymphocytes were reduced by 50 and 20%, respectively (Table I and Fig. 1A). Together these data indicate that the B cell deficiency in the spleens of rac2<sup>−/−</sup> mice is primarily due to an insufficiency of mature and/or MZ B cells. This issue was clarified by flow cytometric analysis of spleen examining the distribution of CD23- and CD21-expressing cells (Fig. 1B). This showed a near absence of the MZ B cell population (CD23<sup>hi</sup>CD21<sup>hi</sup>), a finding confirmed by immunohistochemistry (Fig. 1C). The distribution of MOMA-1-positive metallophilic MZ macrophages was normal in rac2<sup>−/−</sup> mice (Fig. 1C), suggesting that the absence of MZ B cells in rac2<sup>−/−</sup> mice is not due to a structural defect that prevents cells from accumulating in this area. The localization of T and B lymphocyte populations in spleen, as determined by immunohistochemistry, was normal in rac2<sup>−/−</sup> mice (data not shown). To determine whether the absence of MZ B cells was cell-intrinsic, reciprocal bone marrow transplantation experiments into irradiated recipients were performed. The percentage and number (Fig. 1B) of MZ B cells observed in wild-type and rac2<sup>−/−</sup> recipients 8 wk post-transplant simply reflected the genotype of the donor, with marked deficiency of MZ B cells being recapitulated in all recipients of rac2<sup>−/−</sup> cells. The absence of MZ B cells in recipients of rac2<sup>−/−</sup> cells was also confirmed by immunohistochemistry (data not shown).

Phenotypic characterization of B lymphocyte populations in lymph node (central and peripheral) revealed a trend similar to that observed in the spleen, with a decrease in the proportion of B lymphocytes noted (wild type, 45 ± 6%; rac2<sup>−/−</sup>, 36 ± 5%; n = 12–13; p < 0.05), although the reduction in absolute number of B220<sup>+</sup> lymph node cells was not statistically significant (Table I). As assessed histologically, Peyer’s patches were of normal architecture and cellularity (data not shown). A second striking deficiency in lymphocyte development was evident within the

<table>
<thead>
<tr>
<th>Tissue</th>
<th>n</th>
<th>Marker</th>
<th>No. of Cells (× 10&lt;sup&gt;6&lt;/sup&gt;)</th>
<th>Wild type</th>
<th>rac2&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<tr>
<td>Peripheral blood</td>
<td>10–11</td>
<td>B220&lt;sup&gt;+&lt;/sup&gt;</td>
<td>3.9 ± 1.4 (× 10&lt;sup&gt;6&lt;/sup&gt;)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.6 ± 1.6&lt;sup&gt;b&lt;/sup&gt; (× 10&lt;sup&gt;6&lt;/sup&gt;)&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Bone marrow</td>
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<td>Total/Femur</td>
<td>28.5 ± 5.2</td>
<td>27.9 ± 7.5</td>
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<td></td>
<td></td>
<td>B220&lt;sup&gt;+&lt;/sup&gt;</td>
<td>6.3 ± 1.7</td>
<td>4.6 ± 1.5&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>IgD&lt;sup&gt;lo&lt;/sup&gt;IgM&lt;sup&gt;+&lt;/sup&gt;</td>
<td>55 ± 5.6</td>
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<td>Lymph nodes</td>
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<td>B220&lt;sup&gt;+&lt;/sup&gt;</td>
<td>19 ± 6</td>
<td>14 ± 5</td>
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<sup>a</sup> B220 is a marker for B lymphocytes. HSA, CD24. Figures represent mean ± SD.

<sup>b</sup> Value of p < 0.05.

<sup>c</sup> Value of p < 0.005.
peritoneal cavity of rac2−/− mice. Cytological analysis showed a decrease in the proportion of lymphocytes in the peritoneal cavity (data not shown). Flow cytometric analysis revealed this decrease to be specific for the CD5+ sIgM+ B-1a lymphocytes, which were decreased in both proportion and total cell number in rac2−/− mice (Table I and Fig. 1D).

Thus, analysis of the B cell subpopulations in both bone marrow and the periphery reveals a critical role for Rac2 in the formation of the recirculating lymphocyte population in the bone marrow and of splenic MZ and peritoneal B1a cells.

**Decreased serum IgM and IgA, but increased serum IgG1 and IgG2b, in Rac2-deficient animals**

To determine whether the aberrant B cell development observed in rac2−/− mice had a functional corollary, we measured the levels of several Ig isotypes in the serum of rac2−/− and control mice (Fig. 1E).
The concentrations of IgG1 and IgG2b were elevated 2-fold in the serum of 8- to 11-wk-old rac2^-/- mice compared with wild-type littermates, whereas serum IgM was reduced by 95% and IgA by 50%. The relative levels of serum IgG2c and IgG3 were similar between wild-type and rac2^-/- mice (data not shown). IgE is normally present at low concentrations in serum, and in this assay was not detected in either wild-type or rac2^-/- serum, indicating a concentration of <80 ng/ml (data not shown).

We next used an ELISPOT assay to investigate whether the changes in serum IgM and IgG could be attributed to alterations in the frequency of AFC in bone marrow and spleen. We found the frequency of IgM AFC to be reduced by 75% in the spleen and by 85% in the bone marrow of rac2^-/- mice compared with controls (Fig. 2B). The reduction in IgM AFC in rac2^-/- mice was also evident in immunohistochemical stains of spleen sections using Abs to IgM (data not shown). Given that the total number of leukocytes in bone marrow and spleen was normal in rac2^-/- mice (Table I), the reduced frequency of IgM AFC in rac2^-/- mice translated into a significant reduction in total number.

Thus, in addition to the B cell developmental defects observed as a consequence of Rac2 ablation, rac2^-/- mice show reduced titers of IgM and IgA together with a reduced frequency of IgM AFC.

**DIFFERENTIATION OF RAC2^-/- B CELLS INTO AFC IS NOT INTRINSICALLY BLOCKED**

To clarify whether the observed in vivo deficiency of IgM AFC resulted from a B cell-intrinsic developmental defect, we next measured the ability of purified spleen B lymphocytes to differentiate into AFC in vitro. B lymphocytes were stimulated with two concentrations of LPS to induce polyclonal activation and IgM production in the absence of BCR-specific activation signals. As shown in Fig. 2C, no differences in IgM production were revealed in response to 2 or 20 μg/ml LPS. We next compared the responses of rac2^-/- and control B cells to a T cell-derived signal, CD40L. Stimulation with CD40L alone produced low quantities of IgM in both wild-type and rac2^-/- lymphocytes. Supplementing CD40L with IL-4 and IL-5 enhanced IgM production ~100-fold in both control and rac2^-/- B lymphocytes, although IgM production by rac2^-/- B lymphocytes remained at 25% of that by control B lymphocytes (p < 0.05). The production of IgG1 in response to stimulation with CD40L/IL-4/IL-5 was indistinguishable between wild-type and rac2^-/- lymphocytes, indicating that rac2^-/- lymphocytes respond normally to IL-4 and IL-5 and are able to class switch to IgG1 in response to a T-dependent stimulus.
Defective Ag receptor-mediated proliferation in Rac2-deficient B lymphocytes

While the reduced frequency of IgM AFC in rac2−/− mice does not appear to result from an intrinsic block in B cell differentiation, it may reflect deficiencies in BCR-mediated signaling. To determine the response of rac2−/− B lymphocytes to BCR cross-linking, purified B cells were stimulated with a series of mitogens, including F(ab′)2 anti-IgM. A significant defect in the proliferation of rac2−/− B lymphocytes was observed in response to cross-linking the BCR with 1 μg/ml anti-IgM (Fig. 2D; p < 0.05). This defect was ameliorated by a higher concentration of anti-IgM (10 μg/ml). No defects were observed in response to stimulation with LPS, CD40L, or CD40L supplemented with IL-4 and IL-5. Thus, Rac2 appears to have a specific role in B cells in setting the threshold of responsiveness to BCR cross-linking.

Maintenance of calcium flux is impaired in Rac2-deficient B lymphocytes in response to BCR or CD19 ligation

An immediate consequence of BCR ligation is the fluxing of intracellular calcium in B cells. The magnitude of this flux is related to the extent of BCR cross-linking and can be influenced by the coligation of accessory molecules such as CD19 (positively) and CD22 (negatively) with the BCR (42). To determine whether Rac2 deficiency influenced calcium flux in response to BCR ligation, we measured intracellular Ca2+ using the calcium-sensitive fluorescent indicator, indo-1/AM. In response to a low level of BCR cross-linking (0.01 μg/ml anti-κ), rac2−/− B cells showed a reduced Ca2+ flux (Fig. 3A, upper left panel). However, just as a high concentration of F(ab′)2 anti-IgM restored normal proliferation in rac2−/− B cells, a 10-fold increase in the concentration of anti-κ restored Ca2+ flux in rac2−/− B cells to that of controls (Fig. 3A, upper right panel). We next determined whether coligation of CD19 with BCR amplified Ca2+ flux in the absence of Rac2. BCR and CD19 were cross-linked with either 0.1 or 0.01 μg/ml anti-κ and anti-CD19. While coligation of CD19 with BCR clearly enhanced the Ca2+ flux in both rac2−/− and wild-type B cells relative to BCR cross-linking alone, the maintenance of elevated levels of Ca2+ was less in rac2−/− B cells (Fig. 3B). This was particularly meaningful when high concentrations of anti-κ (Fig. 3B, right panels) were used, because, when used alone, this reagent induced a response of equal magnitude in both rac2−/− and wild-type B cells (Fig. 3A, upper right panel). Cross-linking CD19 in the absence of BCR ligation engendered a small and late Ca2+ flux in control B cells and no change in rac2−/− B cells, consistent with diminished signaling through CD19 in rac2−/− B cells (Fig. 3A, lower right panel). To exclude the possibility that the observed differences in Ca2+ flux were explained trivially by the absence of MZ B cells, Ca2+ flux was specifically analyzed in follicular B cells, and the differences observed between genotypes were identical with those observed for the total B cell populations (data not shown). Collectively, these results indicate a role for Rac2 either in the signal transduction pathways emanating from the BCR and resulting in Ca2+ mobilization or in determining the efficiency of BCR ligation. They further indicate that Rac2 is required for the full enhancement of signals engendered by the coligation of CD19 with BCR.

Decreased B lymphocyte chemotaxis in response to chemokines

Abnormalities in chemotaxis in response to a variety of chemotaxtrants have been noted in Rac2-deficient neutrophils (28), mast cells (29), and hemopoietic stem cells (30). We therefore assessed the responses of rac2−/− and control lymphocytes to a range of chemokines. Chemotaxis of splenocytes was reduced by 50–75% in response to all concentrations of SDF-1α and BLC tested (Fig. 4, A and B). Migration of unstimulated splenocytes (control) was also markedly reduced, indicating that chemokinesis, or random movement, was also reduced. This latter observation was confirmed by additional studies showing that migration of rac2−/− splenocytes in a zero gradient of chemokine (equal concentrations of chemokine in the upper and lower chambers of a Transwell) was

![Figure 3](http://www.jimmunol.org/)

**Figure 3**. Maintenance of calcium flux in response to ligation of the BCR and CD19 is compromised in rac2−/− mice. Splenocytes from wild-type (dark line) and rac2−/− (gray line) mice were labeled with the calcium-sensitive indicator, indo-1/AM, and calcium flux of B220+ cells was measured as stated (see Materials and Methods). Baseline intracellular calcium ([Ca2+]i) was measured for 20 s before biotinylated Abs to the κ L chain of the BCR (anti-κ), and/or anti-CD19 were cross-linked with avidin, and calcium flux was monitored for 4–7 min. Data are representative of three independent experiments, each from a pool of two spleens. Arrows indicate the addition of avidin.
decreased relative to that in wild-type splenocytes (data not shown). Flow cytometric analysis of lymphocyte populations before and after migration showed that B lymphocyte migration was reduced 65–75% in response to 800 ng/ml SDF-1α and BLC (inset of Fig. 4, A and B), but only 40% when stimulated with 1500 ng/ml BLC. Migration of rac2−/− lymph node B lymphocytes was also significantly reduced in response to CCL21 and macrophage inflammatory protein-3β (data not shown). These abnormalities were not explained by differences in integrin-mediated adhesion or chemokine receptor expression. Adhesion of purified B lymphocytes via αβ1 and αβ4 integrins was similar between genotypes, as measured in static adhesion assays with fibronectin fragments (data not shown). Furthermore, expression of CXCR5 mRNA, the receptor for BLC, was similar in splenocytes of both genotypes. These data suggest that the role of Rac2 in chemokine responsiveness is in the efficiency of the response, rather than in whether there is a response at all.

Lymphocytes from mice deficient in chemokines and their receptors display markedly reduced homing to lymphoid organs (36, 43, 44). To determine whether the deficits in chemokine responses described above could affect B cell homing, reciprocal transfer experiments of CellTracker Green-labeled splenocytes were performed, with the distribution of B220+ cells analyzed 5 h after i.v. injection. Although a significantly higher percentage of transferred rac2−/− B cells was retained in the circulation regardless of the genotype of the recipient, entry into the spleen was indistinguishable between the genotypes (Fig. 4C). The frequencies of labeled cells recovered from peripheral lymph nodes of either genotype were reduced by 30–50% in two independent experiments when rac2−/− donor B cells were injected rather than wild-type B cells (Fig. 4C). These data confirm the B cell-intrinsic nature of the defect and suggest that the reduced chemokine responsiveness of rac2−/− B cells contributes to their altered distribution in vivo.

Reduced actin polymerization in B lymphocytes in response to chemokines

Chemotaxis is dependent on remodeling of the actin cytoskeleton, and this involves polymerization of globular monomers of actin to form F-actin. Actin polymerization in splenocytes following a 15-s stimulation (the optimal time point for peak polymerization) with BLC and SDF-1α was quantified using phalloidin-FITC. A 50–70% reduction in F-actin generation was recorded in rac2−/− splenocytes in response to stimulation with a wide range of concentrations of SDF-1α (Fig. 4D) and BLC (Fig. 4E). Maximal stimulation of lymphocytes with BLC and SDF-1α did not ameliorate the defect in actin polymerization relative to wild-type lymphocytes.
Rac2-deficient mice have perturbed responses to TI-II and T-dependent Ags

The deficiencies in B cell development revealed by our earlier experiments raised the possibility that the ability of these cells to respond to Ag in vivo may be compromised. The absence of MZ B cells and the diminished responsiveness to BCR cross-linking, for example, may impact on the immune response of rac2−/− mice to T cell-independent Ags, while diminished responsiveness to chemokines and CD19 coligation may influence the outcome of T cell-dependent immune challenge. To determine whether this was indeed the case, we challenged rac2−/− and control mice with T cell-independent and T cell-dependent Ags.

Rac2-deficient and control mice were immunized with the hapten-conjugated polysaccharide, DNP-dextran, a TI-II Ag, and the response was followed by measuring serum Ig able to bind the highly cross-reactive Ag, TNP. As expected from earlier findings (Fig. 2A), TNP-specific IgM was markedly reduced at baseline (Fig. 5, upper panel). Following immunization, the production of TNP-specific IgM was reduced 5- to 10-fold at all time points measured, but both the fold rise over baseline and the rate of increase were the same as those observed for wild-type mice. The production of TNP-specific IgG3 was normal at all time points measured, indicating that class switching in response to the Ag was normal (Fig. 5, lower panel).

Humoral immune responses following immunization with the hapten-conjugated, T-dependent Ag, NP-KLH, in conjunction with alum adjuvant were next tested. Immunohistochemical analysis of the spleens of immunized mice showed normal GC formation in rac2−/− mice (Fig. 6A). Interestingly, the foci of IgG1 AFC that form in the outer PALS as a normal part of the immune response were particularly larger in some rac2−/− spleens (Fig. 6A). This apparent overexuberance was confirmed by the finding of significant increases in the numbers of Ag-specific IgG1 AFC in rac2−/− spleens (Fig. 5, upper panel) 1 wk following immunization and increases in Ag-specific serum IgG1 2 wk following immunization (Fig. 6C; p < 0.05). Consistently, 2- and 4-fold elevations in Ag-specific IgM were observed in rac2−/− sera 1 and 2 wk following immunization (p < 0.01). Migration of AFC to the bone marrow during the course of the immune response appears normal in rac2−/− mice. A similar frequency and proportion of total (NP20-binding) and high-affinity (NP2-binding) IgG1 AFC were apparent in bone marrow both early in the primary response and 1 wk after boosting late in the response (Fig. 6B, lower panel). Because bone marrow AFC originate in the GC, this observation implies that Rac2 is not critical for B cell selection and differentiation in the GC or for the subsequent migration of these AFC. Similarly, the production of high-affinity IgG1 was similar between rac2−/− and wild-type mice, indicating that affinity maturation in the GC was occurring normally (data not shown). Immunization with NP-KLH in the absence of adjuvant induced a substantially lower concentration of Ag-specific serum IgM and IgG1 but revealed no differences between wild-type and rac2−/− mice (Fig. 6C).

Discussion

The abnormalities evident in Rac2-deficient mice directly implicate Rac2 in two important processes underpinning B lymphocyte physiology: regulation of signaling thresholds for BCR and CD19 ligation, and regulation of chemokine responses that determine the distribution of B cells.

Rac2 modulates BCR and CD19 signaling thresholds

In vitro, rac2−/− B cells demonstrated both defective proliferation and Ca2+ flux in response to submaximal stimulation of the BCR. Ca2+ flux was also reduced in response to maximal coligation of BCR and CD19 and to CD19 ligation alone. Rather than being absolutely required for BCR or CD19 signaling to occur, Rac2 appears to play a necessary role in grading B cell responses across a range of stimuli intensities and combinations. Consistent with these in vitro observations, Rac2 deficiency in vivo results in abnormal B lymphocyte development in specialized populations of both B1 and B2 lymphocyte lineages. Although the development of precursor and immature B lymphocytes in the bone marrow was normal in rac2−/− mice, mature recirculating B lymphocytes were reduced, as were splenic MZ B cells and peritoneal cavity B1a lymphocytes. For mature recirculating B lymphocyte and B1a lymphocyte populations, recruitment or maintenance of cells is directly dependent upon signals delivered via the BCR and coreceptors such as CD19 (4, 9, 45, 46). In contrast, recent evidence indicates that MZ B cell development is inversely related to the strength of the BCR signaling (13), so the deficiency of MZ B cells in the absence of Rac2 cannot simply be explained by diminished BCR signaling.

In this regard it is pertinent that CD19-deficient mice and Vav-deficient mice share these key phenotypic features of Rac2-deficient mice. The absence of CD19 results in a reduction in MZ B lymphocytes, peritoneal cavity B-1a lymphocytes, and all serum Ig isotypes (11, 14, 17). Vav associates with CD19 (35), and the activity of Vav is augmented by cross-linking of the BCR and CD19 (47). Vav-1-deficient mice lack B-1a cells, and their B cells proliferate poorly in response to cross-linking of the BCR and do not maintain elevated levels of intracellular calcium in response to ligation of the BCR and CD19 (31–33, 48). These abnormalities are more severe when Vav-2 is also absent (49, 50). Collectively, our data and these observations indicate that Rac2 influences BCR and CD19 signaling under physiological conditions and that Vav is probably its major proximal activator. As yet our data do not distinguish between a direct role in signaling cascades emanating from the BCR or CD19 receptors and an indirect role in influencing the assembly of the BCR:coreceptor signaling complex via regulation of actin cytoskeleton remodeling.
**FIGURE 6.** Enhanced responses to T-dependent Ags in rac2<sup>−/−</sup> mice. A. Immunohistochemistry of spleen sections showing the distribution of AFC (brown) adjacent to GC (blue) in wild-type and rac2<sup>−/−</sup> mice on days 7 and 14 of the primary immune response and in unimmunized mice. B. Frequency of NP-specific IgG1-producing plasma cells from spleen (upper panels) and bone marrow (lower panels) at 1 and 2 wk following i.p. immunization with NP-KLH and 1 wk following i.v. boosting with NP-KLH at 6 wk. C. Concentration of NP<sub>20</sub>-specific IgM and IgG1 in wild-type and rac2<sup>−/−</sup> mice at baseline and 1 and 2 wk following i.p. immunization with NP-KLH, as determined by ELISA. Each dot represents one mouse. *, p < 0.05 (rac2<sup>−/−</sup> vs wild type).
Rac2 regulates B lymphocyte migration in response to chemokines

Another prominent consequence of Rac2 deficiency is a disturbed distribution of B lymphocytes in vivo. The 40% increase in B lymphocytes in peripheral blood and the more modest decreases in B lymphocytes in the spleen and lymph nodes suggest that the migration of B lymphocytes from the peripheral blood to secondary lymphoid tissue is diminished. Recirculation of lymphocytes between peripheral blood and secondary lymphoid tissues is modulated by chemoattractant cues (51). The reduced chemotaxis of B lymphocytes in response to a wide range of concentrations of BLC and SDF-1α in vitro, attributable partly to a decrease in actin polymerization, is reflected by the reduced homing of transferred B cells to peripheral lymph nodes. It is likely to explain the B lymphocytosis in peripheral blood and contribute to the minor deficit in B lymphocytes in spleen, lymph node, and bone marrow.

Additional abnormalities in the distribution of subpopulations of B lymphocytes were found in rac2−/− mice. IgM-secreting cells migrate under the direction of chemokine gradients from the peripheral lymphoid tissues to gut-associated lymphoid tissues and to the bone marrow, where they become the major source of serum Ig. Recently, it has been demonstrated that the migration of B-1 lymphocytes from the peritoneal cavity to gut-associated lymphoid tissues and spleen is crucial for their differentiation into plasma cells (16, 52). Indeed, we observed a significant reduction in the frequency of IgM-secreting plasma cells in both spleen and bone marrow of rac2−/− mice. As B1a cells and their plasma cell derivatives are major producers of IgM and IgA (16, 53), it is likely that the marked reduction in serum IgM concentration and the modest reduction in IgA concentration seen in rac2−/− mice reflect both the numerical deficiency in B1a peritoneal cells and the defective migration of these cells to become plasma cells in secondary sites. Importantly, the reduced IgM concentration in the serum of rac2−/− mice was not due to an inability to secrete Ab, as IgM production in response to stimulation with LPS and CD40L was normal. We speculate that the observed deficiency in IgM production in vitro in response to CD40L/IL-4/IL-5 can be attributed to the absence of MZ B lymphocytes in the assays, as these cells produce greater quantities of IgM than follicular B lymphocytes in vitro (12).

Rac2 deficiency perturbs T-independent and T-dependent immune responses

Rac2-deficient mice displayed normal IgG3 responses to the TI-II Ag, DNP-dextran, and normal kinetics of rise in IgM. The consistent reduction in TNF-specific IgM throughout the response is most simply explained by a reduced starting frequency of precursor B cells specific for this Ag. Responsiveness to this class of Ag is primarily a property of MZ B cells, due to both their location in the spleen and unique activation properties (12, 54). The pattern of TI-II response observed is in keeping with the marked reduction in MZ B cells in Rac2-deficient mice and indicates that the residual cells are functionally normal.

The foci of AFC that form in the outer PALS of the spleen during the immune response to T-dependent Ags develop as a consequence of Ag-specific B cell proliferation and migration. B cells first receive T cell help in the inner PALS. They subsequently migrate to the outer PALS, where they proliferate, isotype switch from IgM to IgG, and differentiate into AFC (55). The migration of B cells in the spleen is under the direction of gradients of BLC, SDF-1α, CCL21, and macrophage inflammatory protein-3β (36, 43, 44). Given the significant reductions in lymphocyte chemotaxis in response to several chemokines we observed in vitro, it is surprising that no gross alterations of the architecture of lymphoid tissues or major perturbations in T-dependent immune responses were observed. Subtle abnormalities were evident however: an increase in NP‐specific serum IgM and IgG1 and an increase in the size of extrafollicular AFC foci. It is possible that these are a consequence of prolonged B cell stimulation in the PALS due to defective migration away from Ag-specific T cells along chemotactic gradients of BLC or SDF-1α. No increases in the proliferation of purified B lymphocytes were observed in vitro in response to T-dependent or T-independent stimuli, suggesting that the increase in the size of foci in some Rac2-deficient mice is not due to abnormal proliferation or delayed apoptosis.

The absence of major perturbations in B cell responses to Ag in immunization protocols is in sharp contrast to the profound abnormalities observed in CD19-deficient mice. Similarly, the relatively normal architecture of lymphoid organs (except for the absence of MZ cells) is distinct from the severe consequences for lymphoid tissue organization observed in chemokine- or chemokine receptor-deficient mice (43, 44). These differences indicate that residual signaling downstream of these receptors is sufficient to establish and then maintain correctly functioning lymphoid tissue. We conclude that while Rac2 is a physiological regulator of key cellular responses to the BCR, CD19, and chemokine receptor ligation, redundancy of its activity is apparent. Other GTPases, such as the highly homologous Rac1, are almost certainly involved. Evidence for a compensatory increase in Rac1 activity was demonstrated recently in rac2−/− mice. Reduced homing of transferred B lymphocytes to peripheral lymph nodes is diminished. Recirculation of lymphocytes from peripheral blood to secondary lymphoid tissues is modulated by chemoattractant cues (51). The reduced chemotaxis of B lymphocytes in response to a wide range of concentrations of BLC and SDF-1α in vitro, attributable partly to a decrease in actin polymerization, is reflected by the reduced homing of transferred B cells to peripheral lymph nodes. It is likely to explain the B lymphocytosis in peripheral blood and contribute to the minor deficit in B lymphocytes in spleen, lymph node, and bone marrow.

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References


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