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The Unique Antigen Receptor Signaling Phenotype of B-1 Cells Is Influenced by Locale but Induced by Antigen

Michael J. Chumley, Joseph M. Dal Porto, and John C. Cambier

Normal animals contain an autoreactive B lymphocyte subset, the B-1 subset, which is controlled by undefined mechanisms to prevent autoimmunity. Using a \( V_{\text{H}}^{11} V_{\text{9}} \) Ig transgenic mouse, with a specificity prototypic of the subset, we have explored conditions responsible for the previously reported Ag hyporesponsiveness of these cells. We report that peritoneal \( V_{\text{H}}^{11} V_{\text{9}} \) B cells exhibit typical B-1 behavior with high basal intracellular free \( \text{Ca}^{2+} \) and negligible receptor-mediated calcium mobilization. However, splenic B cells from this mouse, while phenotypically similar to their peritoneal counterparts, including expression of CD5, mount robust B-2-like responses to Ag as measured by calcium influx and altered tyrosine phosphorylation responses. When these splenic cells are adaptively transferred to the peritoneal cavity and encounter their cognate self-Ag, they acquire a B-1 signaling phenotype. The ensuing hyporesponsiveness is characterized by increases in both basal intracellular calcium and resting tyrosyl phosphorylation levels and is highlighted by a marked abrogation of B cell receptor-mediated calcium mobilization. Thus, we show that self-Ag recognition in specific microenvironments such as the peritoneum, and we would propose other privileged sites, confers a unique form of anergy on activated B cells. This may explain how autoreactive B-1 cells can exist while autoimmunity is avoided. The Journal of Immunology, 2002, 169: 1735–1743.

B-1 cells represent a specialized subset of B cells that are distinct from the majority of recirculating conventional B cells (termed B-2) in an individual. They are distinguished by tissue distribution, cell surface phenotype, B cell receptor (BCR) signal generation, and capacity for self-renewal (1–4). B-1 cells express high levels of IgM and low levels of IgD and B220, lack CD23, and when found in the peritoneum, express the macrophage marker CD11b (Mac-1). Many B-1 cells (termed B-1a) also express CD5, and it has been proposed that this coreceptor may contribute to the unique characteristics of these cells (5–8). CD5\(^+\) B-1 cells have been shown to display an altered responsiveness to BCR ligation compared with their B-2 cohorts. This is marked by a diminished ability for intracellular \( \text{Ca}^{2+} \) mobilization, aberrant proliferation, and increased apoptosis after BCR cross-linking (6, 7, 9, 10). While B-1 cells only comprise a small percentage of all B cells, they are the primary source of the serum Ab in unimmunized mice, known as natural Ab (3, 11, 12). The majority of natural Ab is low affinity and polyreactive, with the ability to recognize autoantigens as well as those from bacterial and parasitic sources. Studies of mice lacking natural Abs have demonstrated their essential role in providing early protection from a variety of pathogens (13–16).

In wild-type mice, B-1 cells develop predominantly during fetal hemopoiesis (12, 17); however, we and others have shown that cells with a B-1 phenotype can be produced from adult precursors in several model transgenic systems (18–22). In these studies, B-1 cell development appeared to correlate with Ag receptor specificity. Recognition of self Ag, either endogenously or transgenically expressed, was shown to be necessary for the generation and expansion of the B-1 cell population (18, 21, 23). The pleural and peripheral compartments of these mice become replete with cells bearing the B-1 phenotype. The tenets of tolerance induction might predict that although these autoreactive cells may escape deletion in the bone marrow, peripheral mechanisms should render these cells anergic and/or short-lived. Indeed, while the surface characteristic of these cells has been well documented, little is known regarding the forces that drive their altered responsiveness to Ag, and less about how the responsiveness of these cells may differ depending on their anatomical location.

Among the known BCR specificities typical of B-1 cells are autoantigens, such as plasma membrane phospholipids, and conserved epitopes present on common pathogens, such as polysaccharide moieties. One highly represented example is the specificity encoded by germline \( V_{\text{H}}^{11} \) and \( V_{\text{9}} \) Ig genes. This rearrangement is expressed by 5–15% of the peritoneal B-1 cells in normal mice and reacts with phosphatidylycholine (PtC), a normal component of the cell membrane (24). We recently reported that B cells constrained by a \( V_{\text{H}}^{11} V_{\text{9}} \) Ig transgene generated CD5\(^+\), PtC-specific B cells in both the spleen and peritoneum (22). Interestingly, splenic B cells from \( V_{\text{H}}^{11} V_{\text{9}} \) mice exhibit low basal intracellular free \( \text{Ca}^{2+} \) ([\( \text{Ca}^{2+} \)]) levels, and mobilize \( \text{Ca}^{2+} \) following BCR aggregation, suggesting that they are functionally equivalent to nonautoreactive, conventional B-2 cells. However, we also found that transgenic B cells isolated from the peritoneum of these mice display an elevated basal [\( \text{Ca}^{2+} \)] and a greatly reduced ability to mobilize \( \text{Ca}^{2+} \) following BCR aggregation, typical of B-1 cells. Thus, a dichotomy exists in the signaling phenotype of B cells with identical receptor specificity by virtue of their microenvironments.

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3 M.J.C. and J.M.D.P. contributed equally to this study.

4 Address correspondence and reprint requests to Dr. John C. Cambier, Department of Immunology, National Jewish Medical and Research Center, 1400 Jackson Street, Denver, CO 80206. E-mail address: cambierj@njc.org

5 Abbreviations used in this paper: BCR, B cell receptor; 7-AAD, 7-amino actinomycin D; [\( \text{Ca}^{2+} \)], intracellular free \( \text{Ca}^{2+} \); HEL, hen egg lysozyme; NTg, nontransgenic; PEs, peritoneal exudate; PtC, phosphatidylycholine; TNP, trinitrophenol.
Deficits in peritoneal B-1 cell BCR signaling, notably calcium influx, have been reported previously. While aggregation of the BCR on these cells results in tyrosyl phosphorylation of many BCR proximal proteins, including phospholipase C-\(\gamma\), the activation of this enzyme, measured by phosphatidylinositol 4,5-P\(_2\) hydrolysis, is markedly diminished compared with splenic B cells (25). And, unlike splenic B cells, translocation of the transcription factor NF-\(kappa\)B to the nucleus does not occur following BCR aggregation on peritoneal B-1 cells (9). Interestingly, similar changes in BCR signal transduction have been reported in anergic B cells from the hen egg lysozyme (HEL)/anti-HEL model in which the peripheral B cells are constantly exposed to their cognate soluble Ag (26). However, in the HEL model, the BCR exhibits a greatly diminished ability to transduce signals leading to tyrosyl phosphorylation of virtually all receptor proximal signaling intermediaries (27) and calcium mobilization (28). Recently, another model of B cell tolerance has been developed in which an unknown autotigent, with much lower affinity than the HEL/anti-HEL model, is capable of inducing a similar anergic state (29). Here again, peripheral B cells display elevated basal [Ca\(^{2+}\)], and BCR signaling defects similar to the HEL/anti-HEL model. Taken together, these data suggest that CD5+ B-1 cells may display a unique hyporesponsive phenotype that is distinct from anergy. Additionally, the hyporesponsiveness of B-1 cells may not be a simple consequence of Ag exposure, as the site of exposure, such as the peritoneum and other pleural cavities, appears to play a significant contributing role.

In this study, we describe the phenotypic and functional differences between \(V\text{\_11V\_9}\) receptor-bearing splenic and peritoneal B cells. Although both subsets would be classed as B-1 cells by cell surface phenotype, only the peritoneal B cells are hyporesponsive to BCR ligation. We further show that the splenic population becomes hyporesponsive upon movement to the peritoneal milieu and that this transition is a consequence of cognate signaling through the BCR.

Materials and Methods

**Mice**

\(V\text{\_11V\_9}\) transgenic mice, originally obtained from R. R. Hardy (Fox Chase Cancer Center, Philadelphia, PA) on the CB17 background, were backcrossed at least six times on the B10.D2Sn/N background. The 3-83 \(\mu\)g transgenic mice were maintained on the autoantigen-free, nondeleting (H-2K\(^b\)) background of B10.D2Sn/N (The Jackson Laboratory, Bar Harbor, ME). Nontransgenic (NTg) control mice were comprised of \(V\text{\_11V\_9}\) littermates negative for both H and L chain transgenes. Mice were housed and bred at the National Jewish Medical and Research Center, Biological Research facility and used at 8–12 wk of age.

**Cell isolation and tissue culture**

Splenic and peritoneal B cells were prepared as previously described (22). Briefly, purified splenic B cells were obtained by Percoll gradient separation after RBC and T cell lysis; peritoneal B cells were purified from peritoneal lavage by removal of adherent accessory cells and magnetic bead depletion of T cells. Where noted, splenic and peritoneal B cells were treated with CFESE (Molecular Probes, Eugene, OR), as described elsewhere (30). Labeled cells were washed twice with IMDM (Life Technologies, Gaithersburg, MD) and placed in culture at concentrations ranging from 5 \times 10^5 to 2 \times 10^6 cells/ml in complete IMDM at 37°C, or in cell-free peritoneal exudate (PEx). Cell-free PEx was obtained by lavage of several NTg mice with 4 ml each of IMDM (Life Technologies). The combined washes were then centrifuged at 3000 RPM for 10 min; the supernatant was 0.2 \(\mu\)m filtered, and supplemented with FCS. Where noted, PtC-containing liposomes were added to the cultures.

**Phenotypic analysis**

Cells were resuspended in PBS containing 1% BSA and 0.1% sodium azide and incubated with an optimal amount of biotinylated or directly fluoresceinated Ab. mAbs directed against the following mouse cell surface mol-ecules were used: B220 (RA3-6B2; American Type Culture Collection (ATCC), Manassas, VA), CD23 (B3B4; BD Pharmingen, San Diego, CA), CD19 (1D3; BD Pharmingen), CD5 (53-7.313; BD Pharmingen), IgM* (RS-1; ATCC), CD69 (H1.2F3; BD Pharmingen), CD80 (16-10A1; BD Pharmingen), CD86 (GL-1; BD Pharmingen). Fluorescent reagents were either purchased or labeled in-house using a standard protocol and fluoresceinminated tetraacetic acid or 4-hydroxyxysuccinimimid-biotin (Sigma-Aldrich, St. Louis, MO). Cells were incubated for 30 min at 4°C and washed twice in PBS/BSA/azide. Biotinylated Abs were visualized with streptavidin coupled to TriColor, PE, or allophycocyanin (Caltag Laboratories, Burlingame, CA) for analysis on a FACSCalibur (BD Biosciences, San Jose, CA). Forward and side scatter gates were adjusted to include only nucleated cells, and dead cells were excluded based on 7-amin actinomycin D (7-AAD) incorporation (Via-Probe; BD Pharmingen). Postexperiment analysis was performed using CellQuest Pro software (BD Pharmingen).

**Cell proliferation**

Before all analyses of cell proliferation, cells were counted on a hemocytometer using trypsin blue exclusion, and the frequency of B220+ cells was determined by immunofluorescence. For proliferation, 5 \times 10^5 B cells were cultured in triplicate or quadruplicate wells of a 96-well microtiter plate for 24 or 48 h. Culture conditions included the addition of LPS (Sigma-Aldrich), anti-mtigM (B76), PMA (Sigma-Aldrich), and ionomycin (Sigma-Aldrich). Next, 1 \(\mu\)l [\(\text{H}\)]thymidine (DuPont NEN, Boston, MA) was added per well, and samples were incubated for an additional 12–16 h of culture was performed. Cells were harvested onto filter paper, and radioactivity was counted.

**Immunoblotting**

Cells (2 \times 10^6) were treated for 2 min at 37°C with either medium or rabbit anti-mouse IgM mAb F(ab′)\(_2\) (Zymed Laboratories, San Francisco, CA) and lysed in 0.5% CHAPS lysis buffer (150 mM NaCl, 10 mM Tris (pH 7.5), 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 mM PMSF, 10 mM NaF, 0.4 mM EDTA, 1 mM aprotinin, 1 mM \(\alpha\)-amantypsin, and 1 mM leupeptin). Lysates were kept on ice for 15 min, then centrifugation was performed at 14,000 rpm for 10 min at 4°C. Supernatants were mixed with SDS reducing sample buffer and boiled for 5 min. Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes and visualized using specific Abs in conjunction with ECL (DuPont NEN). To detect tyrosine phosphorylated proteins, an anti-tyrosine phosphotyrosine Ab was used (Oncogene, Boston, MA). Polyvinylidene difluoride membrane was then stripped and reprobed for Syk as a loading control. Polyclonal rabbit anti-Syk antisera was prepared in our laboratory.

**PtC liposomes**

Liposomes were prepared by a method similar to that used by Mercolino et al. (24). Briefly, a liposome preparation was made using 25% dis- tearylophosphatidylcholine (Avanti Polar Lipids, Birmingham, AL), 30% cholesterol (Avanti Polar Lipids), 30% sphingomyelin (Avanti Polar Lipids), and 15% disatearylphosphatidylethanolamine in a 90:10 mixture of chloroform/methanol, and the organic solvent was removed by evaporation under nitrogen gas. PBS (pH 7.1) was added to the preparation to a final concentration of 10 \(\mu\)M. The lipids were emulsified by sonication and extruded through a 0.2-\(\mu\)m filter. For 95% PtC liposomes, a 95% disatearylphosphatidylcholine and 5% disatearylphosphatidylethanolamine liposome preparation was extracted as above. All samples were stored under nitrogen gas.

**Analysis of calcium mobilization**

Purified splenic and peritoneal B cells were loaded with Indo-1AM (Molecular Probes) for 45 min at 37°C. Cells were washed twice, resuspended at a concentration of 2 \times 10^6 cells/ml in IMDM and 5% FCS, and stimulated with F(ab′)\(_2\) rabbit anti-mouse Ig (Ramlg; Zymed Laboratories) or PtC liposomes. Mean fluorescence was evaluated using a BD-LSR (BD Biosciences), which allows for 7-parameter flow cytometry including real time analysis of changes in UV fluorescence on gated populations. Data analysis was performed using CellQuest (BD Pharmingen) and FlowTree (TreeStar, San Carlos, CA) software. In separate experiments, splenic B cells were isolated as previously mentioned, labeled with CFSE, and either adoptively transferred into NTg recipients or in vitro culture. Calcium analysis was then performed on isolated, B220+ 7-AAD-, and Indo-1AM-loaded peritoneal ceil from these recipients, or from cultures.

**PtC adsorption**

Anti-PtC Abs produced from the 10E8 hybridoma were obtained from R. R. Hardy (Fox Chase Cancer Center). Originally derived from CDS\(_B\) B
cells that recognize bromelain-treated mouse RBCs, the 10E8 hybridoma secretes $V_{H}11V_{9}$ Abs that cross-react with PEC. Purified 10E8 or isotype-matched control anti-trinitrophenol (TNP) Abs were biotinylated, as previously mentioned. The biotinylated Abs were then prebound to streptavidin-coated magnetic beads (Dynal Biotech, Lake Success, NY) at 100 μg Ab/mg beads. The Ab-coated beads were then washed five times with PBS and added to the cell-free PEX at 5 mg beads/ml. Ag was adsorbed by rotating the mixture for 45 min at 4°C. Beads were then removed by sequential rounds of magnetic separation.

Results

Phenotypic and functional differences between peritoneal and splenic B-1 cells

To begin to address the molecular mechanisms underlying the functional differences between the clonally related $V_{H}11V_{9}$ splenic and peritoneal B-1 cells, we compared their expression of a number of phenotypic markers. As shown in Fig. 1A, the most striking differences included the lower level of B220 and higher level of surface IgM on peritoneal vs splenic $V_{H}11V_{9}$ B cells. These phenotypic differences are generally used to differentiate B-1 from B-2 (B220 high IgM low) cells, the latter represented by NTg splenic B cells (Fig. 1A). Yet the expression levels of other cell surface markers used to differentiate B-1 from B-2 cells, including the presence of CD5 and CD43 and absence of CD23, were similar on splenic and peritoneal $V_{H}11V_{9}$ B cells, suggesting a B-1 classification. And, despite the higher B220 and lower IgM expression levels in the splenic cells, they do not match the levels of B220 and IgM expressed on normal splenic B-2 cells from NTg or 3-83μg Ig transgenic control mice (22).

The elevated level of CD80 expression on $V_{H}11V_{9}$ splenic and peritoneal B cells (Fig. 1A) suggests that both these cell populations have previously encountered Ag (29). This is not surprising given a model for positive selection in the development of B-1 cells; however, the somewhat higher CD80 expression on the $V_{H}11V_{9}$ peritoneal population could represent a more recent or continuous exposure within the peritoneal cavity. In support of this hypothesis, several other hallmarks of Ag recognition were evident in these peritoneal B cells, including an elevated basal [Ca$^{2+}$], and reduced ability to further mobilize Ca$^{2+}$ following BCR ligation (Fig. 1, B and C, respectively), as well as an elevated basal level of cellular protein tyrosine phosphorylation. This phenotypic transition is a feature of most, if not all peritoneal $V_{H}11V_{9}$ B cells (Fig. 1A and C). These data suggest that the peritoneal cavity may harbor factors that drive low-level activation and BCR desensitization in the $V_{H}11V_{9}$ B cells. Interestingly, unlike the receptor desensitization seen in the HEL/anti-HEL anergy model (27, 28), the disruption in BCR signaling in the $V_{H}11V_{9}$ peritoneal B cells is downstream from the most proximal events, as BCR aggregation induces similar and substantial tyrosine phosphorylation of cellular substrates in both splenic and peritoneal $V_{H}11V_{9}$ cells (Fig. 3D and unpublished data).

Consistent with published results (31), peritoneal B cells from wild-type mice were less responsive than splenic B cells to mitogenic stimuli (Table I), with the exception of PMA. Presumably, the elevation in basal [Ca$^{2+}$], allows these cells to respond to the phorbol ester in the absence of a Ca$^{2+}$ ionophore. Importantly, $V_{H}11V_{9}$ as well as NTg peritoneal B cells were less responsive than their splenic counterparts, supporting the possibility that residence in the peritoneal cavity results in B cell hyporesponsiveness. These results suggest that two signaling defects exist in peritoneal B-1 cells: a defect in an intermediary signaling event between the BCR and Ca$^{2+}$ mobilization, and a defect that affects mitogenic signaling via multiple receptors, e.g., LPS.

$V_{H}11V_{9}$ splenic B cells can survive in the peritoneal cavity following adoptive transfer

The differences in phenotypic and functional responses seen between splenic and peritoneal $V_{H}11V_{9}$ B cells prompted our hypothesis that the peritoneum offers a unique microenvironment for $V_{H}11V_{9}$ B cells, leading to the altered phenotype of these resident B cells. To address this hypothesis, we transferred $V_{H}11V_{9}$ splenic B cells into the peritoneal cavity and assessed their phenotype at various time points. However, because the survival of splenic $V_{H}11V_{9}$ B cells in the peritoneum had not been established, we performed i.p. or i.v. adoptive transfers of CFSE-labeled splenic $V_{H}11V_{9}$ and 3-83μg B cells into age- and allotype-matched NTg littermates and assessed their presence in the spleen and peritoneum of recipients 24 and 72 h later. Fig. 2 shows that $V_{H}11V_{9}$, but not 3-83, splenic B cells can reside in the peritoneal cavity for at least 72 h when transferred i.p. In fact, even when transferred i.v., splenic $V_{H}11V_{9}$ B cells have the capacity to migrate to and populate the peritoneum. As expected, both 3-83 and $V_{H}11V_{9}$ splenic B cells can be found in small numbers in the spleen following i.v. transfer. The inability of splenic 3-83 μg B cells to populate the peritoneum in numbers comparable with $V_{H}11V_{9}$ B cells following i.p. transfer was also anticipated and may reflect cell death or migration to sites other than the spleen (Fig. 2). These data demonstrate the ability of splenic $V_{H}11V_{9}$ B cells to survive in either the splenic or peritoneal microenvironment.
Locale-dependent conversion from splenic to peritoneal B cell phenotype

To address the mechanisms underlying the apparent locale-specific phenotype of V\textsubscript{H}11V\textsubscript{9} peritoneal cells, we analyzed the phenotype of V\textsubscript{H}11V\textsubscript{9} splenic B cells transferred into the peritoneal cavity of NT\textsubscript{g} recipient mice. We have previously determined that splenic V\textsubscript{H}11V\textsubscript{9} B cells not only survive long-term in vitro (22), but can also survive long-term in the peritoneum when transferred i.p. into NT\textsubscript{g} recipient mice (Fig. 2). Importantly, after 72 h, the i.p. transferred splenic V\textsubscript{H}11V\textsubscript{9} B cells (Fig. 3B) exhibited elevated basal [Ca\textsuperscript{2+}]\textsubscript{i} and poor Ca\textsuperscript{2+} mobilization responses to BCR aggregation similar to resident peritoneal cells (Fig. 3B). Furthermore, these transferred splenic B cells acquired other characteristics of peritoneal B-1 cells, such as elevated surface IgM (Fig. 3C) and elevated basal whole cell tyrosine phosphorylation (Fig. 3D, lane 5 vs lane 3). Once again, the site of disruption of the BCR signaling cascade must lie downstream from the initial tyrosine phosphorylation events as the i.p. transferred B cells showed substantial tyrosine phosphorylation following BCR ligation (Fig. 3D, lane 6). It is noteworthy, however, that the pattern of proteins phosphorylated is partially distinct from splenic V\textsubscript{H}11V\textsubscript{9} B cells.

Ag-dependent conversion from splenic to peritoneal B cell phenotype

We next sought to define the factor(s) within the microenvironment that drives acquisition of the peritoneal B-1 phenotype. We

Table I. Proliferation of V\textsubscript{H}11V\textsubscript{9} Ig transgenic and NT\textsubscript{g} splenic and peritoneal B cells

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>V\textsubscript{H}11V\textsubscript{9} Spleen Mean SEM</th>
<th>NT\textsubscript{g} Spleen Mean SEM</th>
<th>V\textsubscript{H}11V\textsubscript{9} PCs Mean SEM</th>
<th>NT\textsubscript{g} PCs Mean SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>5.36 1.23</td>
<td>4.32 2.30</td>
<td>1.72 0.57</td>
<td>4.02 0.02</td>
</tr>
<tr>
<td>Anti-IgM (10 (\mu)g/ml)</td>
<td>13.18 2.35</td>
<td>37.65 10.40</td>
<td>6.40 2.80</td>
<td>11.20 3.00</td>
</tr>
<tr>
<td>LPS (20 (\mu)g/ml)</td>
<td>278.91 71.84</td>
<td>318.53 63.29</td>
<td>15.62 0.95</td>
<td>81.20 12.60</td>
</tr>
<tr>
<td>PMA (400 nM)</td>
<td>5.75 0.31</td>
<td>17.94 3.63</td>
<td>47.92 2.56</td>
<td>36.90 2.90</td>
</tr>
<tr>
<td>PMA (400 nM) +</td>
<td>48.45 11.09</td>
<td>306.73 61.04</td>
<td>10.09 1.09</td>
<td>12.78 4.49</td>
</tr>
<tr>
<td>Ionomycin (400 nM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Proliferation of purified splenic and peritoneal B cells as measured by \([^{3}\text{H}]\)thymidine incorporation following 48 h of in vitro culture. A total of 5 \times 10^6 B cells was cultured in triplicate wells under the specified conditions, followed by a 12-h culture with 1 \(\mu\)C of \([^{3}\text{H}]\)thymidine/well. Mean and SEM are stated as cpm \(\times 10^{-3}\) from three separate experiments.
another Ig transgenic (3-83). Splenic B cells from NTg littermates or aggregation (Fig. 4B) evocation was a decrease in the number of cells responsive to BCR basal [Ca\(^{2+}\)]. Membrane-bound Ag can be distinguished from host peritoneal cells (A) by CFSE labeling. B, Isolated peritoneal cells (from gates A and B in A) as well as freshly isolated splenic V\(_{H11V9}\) B cells (C) were loaded with indo-1AM, and [Ca\(^{2+}\)] was monitored following stimulation with F(ab\(^{2}\))\_anti-\(\mu\) (1 \(\mu\)g/10\(^6\) cells). C, Surface IgM is up-regulated on peritoneal B cells, and on splenic B cells adoptively transferred to the peritoneum of NTg mice. V\(_{H11V9}\) splenic and peritoneal B cells were first analyzed for surface IgM expression by flow cytometry. Then purified splenic V\(_{H11V9}\) B cells were labeled with CFSE and 15 \(\times\) 10\(^6\) cells were injected either i.p. or i.v. into NTg recipients. After 72 h, CFSE\(^{-}\) splenic B cells (for i.v. transfer) or CFSE\(^{+}\) peritoneal B cells (for i.p. transfer) were isolated, and surface IgM was compared with freshly isolated splenic V\(_{H11V9}\) splenic B cells (filled gray). Mean fluorescent intensities for examined populations are given. Data shown are representative of three duplicate experiments. D, Splenic V\(_{H11V9}\) B cells adoptively transferred into the peritoneal cavity display an elevated basal tyrosine phosphorylation profile similar to peritoneal V\(_{H11V9}\) B cells. A total of 10 \(\times\) 10\(^6\) purified splenic B cells was labeled with CFSE and injected i.p. into V\(_{H11V9}\) Ig transgenic mice. After 72 h, peritoneal cells were sorted for resident V\(_{H11V9}\) B cells (B220\(^{+}\)CFSE\(^{+}\)) and transferred splenic V\(_{H11V9}\) B cells (B220\(^{+}\)CFSE\(^{+}\)) similar to gates A and B above. Freshly isolated splenic V\(_{H11V9}\) B cells provide a control. After stimulation with F(ab\(^{2}\))\_anti-\(\mu\) (1 \(\mu\)g/10\(^6\) cells), stimulated and unstimulated B cell lysates (1.5 \(\times\) 10\(^6\) cell equivalents), were immunoblotted with anti-phosphotyrosine Abs. Membranes were then stripped and rebotted with anti-Syk as a control for equivalent loading.

First cultured V\(_{H11V9}\) splenic B cells in cell-free PExs to determine whether a soluble factor is responsible for the locale-dependent changes in Ag receptor signaling. As shown in Fig. 4A, splenic V\(_{H11V9}\) B cells underwent a time-dependent increase in basal [Ca\(^{2+}\)] following culture in PExs. Concomitant with this elevation was a decrease in the number of cells responsive to BCR aggregation (Fig. 4B). Splenic B cells from NTg littermates or another Ig transgenic (3-83\(\mu\)g) mouse line treated equivalently did not show this change. Interestingly, V\(_{H11V9}\) peritoneal B-1 cells cultured in medium for as little as 24 h demonstrated a significant reduction in basal [Ca\(^{2+}\)]. However, this decrease never reached the resting [Ca\(^{2+}\)] levels observed in ex vivo splenic V\(_{H11V9}\) B cells.

One obvious explanation for these observations is that this transition of V\(_{H11V9}\) B cells is driven by Ag in the PExs. To assess whether Ag can elicit similar changes in vitro, we prepared liposomes containing physiologically relevant levels of PIC (~25%) and assessed their ability to modulate [Ca\(^{2+}\)] in V\(_{H11V9}\) splenic

Table II. Proliferation of V\(_{H11V9}\) splenic-derived B cells 72 h following transfer into the peritoneum.

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>V(_{H11V9}) Spleen</th>
<th>V(_{H11V9}) PCs</th>
<th>V(_{H11V9}) Spleen Transferred i.p.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
</tr>
<tr>
<td>Medium</td>
<td>6.12</td>
<td>2.35</td>
<td>3.25</td>
</tr>
<tr>
<td>Anti-IgM (10 (\mu)g/ml)</td>
<td>19.52</td>
<td>5.32</td>
<td>4.23</td>
</tr>
<tr>
<td>LPS (20 (\mu)g/ml)</td>
<td>255.30</td>
<td>98.27</td>
<td>22.66</td>
</tr>
<tr>
<td>PMA (400 nM)</td>
<td>6.32</td>
<td>1.80</td>
<td>21.02</td>
</tr>
</tbody>
</table>

* Purified splenic V\(_{H11V9}\) B cells were injected i.p. into 3-83 \(\mu\)g Ig transgenic mice, which have previously been shown to have few peritoneal B cells (Chumley et al., Ref. 22). After 72 h, these cells were isolated from the peritoneum, along with fresh splenic and peritoneal V\(_{H11V9}\) B cells, and proliferation assays were performed as stated in Table I. Mean and SEM are stated as cpm \(\times\) 10\(^{-3}\) from three separate experiments.
three independent experiments. T
ured with 25% PtC liposomes, splenic V H 11V/H11006
allow assessment of responding populations. Mean
80%). However, Cell recoveries were equivalent from all PEx cultures (V/H11022
/Puri
ed splenic V H 11V/PtC-containing liposomes (at the arrow) and monitored
acquire the peritoneal B cell [Ca 2+/H9262
speci
city, splenic V H 11V/Fig. 5
illustrates the inability of NTg B cells to respond
B cells. Fig. 5
B cells. Indo-1AM-loaded B cells were stimu-
FIGURE 4. Culture in cell-free PEx causes splenic V H 11V/9 B cells to acquire the peritoneal B cell [Ca2+], phenotype. Purified splenic B cells from V H 11V/9 Tg, 3-83 (anti-H-2Kb) transgenic, and NTg control mice were cultured in cell-free PEx or in medium alone. Peritoneal B-1 cells isolated from V H 11V/9 Tg mice were included for comparison. Cells were removed at 24 and 72 h, loaded with indo-1-AM, and analyzed as previously described. A, The maximum percentage of cells responding (exceeding 2 SDs above the resting mean) to stimulation (1 μg F(ab’)2 anti-μ/106 cells). B, The mean basal [Ca2+], is displayed for both culture conditions. Cell recoveries were equivalent from all PEx cultures (>80%). However, insufficient cell recovery in some media only cultures after 72 h (#) did not allow assessment of responding populations. Mean ± SEM is shown for three independent experiments.
B cells. Fig. 5A illustrates the inability of NTg B cells to respond to PtC liposome stimulation. Interestingly, V H 11V/9 splenic B cells, which responded normally to anti-μ stimulation, displayed only a gradual rise in [Ca2+], following 25% PtC liposome stimulation. Liposomes containing very high levels (95%) of PtC were capable of inducing a Ca2+ mobilization response similar to anti-μ in splenic (Fig. 5A), but not peritoneal V H 11V/9 B cells (data not shown). It is likely that the reduced avidity for liposomes containing a more physiologic proportion of PtC accounts for the differences in splenic V H 11V/9 B cell responses. Following 24 h cultured with 25% PtC liposomes, splenic V H 11V/9, but not NTg B cells exhibited increased basal [Ca2+], and a reduced ability to respond to further BCR ligation, similar to the hyporesponsiveness shown for peritoneal B cells, and splenic B cells cultured in PEx (Fig. 5B).
These data suggest that PtC is essential for the phenotypic transition of V H 11V/9 B cells in the peritoneum. To test this hypothesis, we depleted PEx of PtC by adsorption using magnetic beads coated with the anti-PtC mAb 10E8, encoded by the same
V H 11V/9 pairing. As shown in Fig. 6, V H 11V/9 splenic B cells cultured for as little as 24 h in PEx or PEx mock depleted, using an isotype-matched control Ab to TNP, exhibited an increase in basal [Ca2+], and showed a diminished capacity for BCR-mediated [Ca2+], mobilization. By contrast, cells cultured with PEx precleared with 10E8 Ab did not elevate basal [Ca2+], and retained their ability to mobilize calcium following BCR aggregation. Acquisition of unresponsiveness was complete by 72 h in cultures using PEx or mock-adsorbed PEx, with resting basal [Ca2+], reaching levels similar to that observed in peritoneal B-1 cells. After 72 h, 10E8-adsorbed cultures remained capable of responding to BCR stimulation; however, a slight increase in resting [Ca2+], was observable at this time point (Fig. 6). This marginal increase in resting [Ca2+], of splenic V H 11V/9 B cells may reflect other components of the PEx, but we cannot exclude the possibility that antigenic forms of PtC reappear in prolonged culture because of cell death or normal cell blebbing. Thus, PtC appears necessary to cause the conversion of signaling-competent splenic V H 11V/9 B cells to cells with a peritoneal B-1 signaling phenotype.
Discussion
BCR specificity for autoantigen has emerged as a critical factor in the development, expansion, and function of the B-1 cell population. In this study, we have demonstrated that when they occur in the spleen, B cells from the V H 11V/9 anti-PtC transgenic mice display a surface phenotype commonly associated with B-1 cells, including the expression of the inhibitory coreceptor CD5, yet exhibit B-2-like responses to Ag. However, peritoneal B cells from the same animal, displaying a very similar surface phenotype, exhibit a typical B-1-like unresponsiveness to BCR aggregation. This hyporesponsiveness can be induced in splenic V H 11V/9 B cells by exposure to the microenvironment of the peritoneum, either through adoptive transfer or in vitro culture. After 72 h under such conditions, these cells exhibit an increase in resting [Ca2+], and basal tyrosyl phosphorylation levels and a striking abrogation of [Ca2+], mobilization following BCR aggregation. This transition is predicated on receptor specificity, as NTg and 3-83αβ splenic B cells do not acquire this phenotype when similarly treated, and is strongly influenced by the microenvironment, the latter serving minimally as a source of cognate Ag.

FIGURE 5. Splenic V H 11V/9 B cells acquire the peritoneal B cell calcium profile when cultured with PtC. A, PtC and anti-μ effects on [Ca2+], in splenic V H 11V/9 vs NTg B cells. Indo-1-AM-loaded B cells were stimulated with either 1 μg/106 cells of F(ab’)2 anti-μ or 25% PtC-containing liposomes (at the arrow) and monitored by flow cytometry. As an additional control for Ag specificity, splenic V H 11V/9 B cells were stimulated with 95% PtC-containing liposomes. B, Twenty-four hours of continuous culture with 25% PtC liposomes causes elevation of basal [Ca2+], in splenic V H 11V/9 B cells. Purified splenic V H 11V/9 and NTg B cells were cultured (5 × 103/ml) with and without 25% PtC liposomes (20 μg/μl) cells), loaded with indo-1-AM, and stimulated with 1 μg anti-μ/106 cells (arrow).
It is surprising that specificity for a self epitope as common as PtC does not affect all B cells uniformly. One possible interpretation of these results is that differentiation to signaling-inept B-1 occurs only in specific sites such as the peritoneum. This seems discordant with the findings of Liou et al. (32), who found that peritoneal B-1 and splenic B-1-like cells from Ig transgenic mice were similarly resistant to experimental tolerance induction. These results suggest that B-1 cells were phenotypically and functionally identical regardless of locale. Importantly, the cells in these experiments were similar, but not identical to V9 11V9 Tg B cells in having a predisposition, by virtue of their Ag specificity, to become B-1. As mentioned previously, subtle differences in the concentration of Ag and/or its availability could explain these discrepancies. In addition, we would contend that the experimental tolerance procedures used in these studies would, in fact, approximate our hyporesponsive conditioning of V9 11V9 splenic B cells in the medium with PtC (Fig. 6). We propose that peritoneal B-1 cells already exhibit a form of tolerance, albeit far from that classically defined as anergy. Perhaps a more appropriate and a more sensitive indicator of unresponsiveness would be entry into cell cycle, i.e., proliferation (Tables I and II).

In classic examples of peripheral tolerance such as the soluble HEL/anti-HEL model (33, 34), self-Ag encounter renders B cells functionally unresponsive to subsequent BCR challenge with notable increases in basal [Ca\textsuperscript{2+}], and significantly diminished receptor-mediated tyrosyl phosphorylation. It is again important to mention in this study that this anergic unresponsiveness is qualitatively distinct from the B-1 hyporesponsiveness we observe, because induced phosphorylation of virtually all substrates is lost. Interestingly, it has been demonstrated that low levels of CD5 expression on anergic cells in the HEL model may prevent their deletion at earlier stages of development and their inappropriate activation in the periphery (35). And, indeed, V9 11V9 splenic B cells display indications of prior Ag exposure, such as expression of CD5 and CD80. Yet, we observed no deficit in the signaling capability of V9 11V9 splenic B cells as compared with conventional splenic B-2 cells. Our data would support the conclusion that the periphery (i.e., secondary lymphoid organs) of the mice does not promote the acquisition of hypo responsiveness in V9 11V9 B cells. This could be due to a paucity of accessible Ag or the lack of other factor(s) vital to this transition.

While we have provided evidence that a BCR signal is obligatory for the induction of this hyporesponsive phenotype, we do not believe this is a result of receptor desensitization, as these B cells are still capable of eliciting signals in the form of tyrosyl phosphorylation via their BCR. Instead, it is possible that the uniqueness of the microenvironment in which these cells encounter Ag also contributes to this transition. This is at once apparent in the increased longevity and the incremental, yet significant, rise in basal [Ca\textsuperscript{2+}], of even control B cells cultured in cell-free PEx (Fig. 4 and data not shown) and may reflect soluble factors present in this milieu. Several recent studies point to attractive candidate molecules, such as, IL-10, Tall-1 (BAFF), and TGF-β, which are produced by various peritoneal cell populations and have survival and inflammatory effects on B cells (10, 36–39). As hyporesponsiveness is not immediate in our system, such local factors might increase survival and facilitate the acquisition of this phenotype through, for example, de novo synthesis of novel effector molecules. We are currently investigating the individual factors of the peritoneal microenvironment that may support or augment the transition to hyporesponsiveness. Additionally, chemokine receptor expression and proper chemotaxis via these receptors have been shown to be important for the establishment of B-1 peritoneal populations (36, 39).

BCR signaling can influence the response to chemokines and may explain, in part, the propensity for i.p. transferred splenic V9 11V9, but not 3-83μβ, B cells to remain in peritoneal cavity (Fig. 2). Interestingly, i.p. transfer experiments using CFSE-labeled V9 11V9 B cells also showed little indication of cell proliferation despite the obvious presence of PtC in this milieu. It is likely, therefore, that the acquisition of B-1 hyporesponsiveness occurs via a complex pathway in which antigenic signaling through the BCR induces independent phenotypic and functional alterations, possibly based on the strength of BCR signal. Such a model may explain the conversion of bone marrow V9 11V9 cells to a B-1 phenotype in the absence of receptor editing (22), the maintenance of the signaling-competent B-1 phenotype in the spleen, and the full conversion to signaling-inept B-1 cells in the peritoneum.

Based on our results, we suggest that the microenvironment of the peritoneum, and perhaps other pleural cavities, provides a unique milieu conducive to the induction of the hyporesponsiveness observed in our studies. Important elements of this environment include autoantigens, and perhaps cytokines and chemokines not present in the peripheral lymphoid organs. In support of our studies, Qian et al. (20) demonstrated the ability of splenic B cells to differentiate into B-1 cells in vivo. In a BCR transgenic model specific for ribonucleoprotein Sm, transgenic B cells populating the peritoneum expressed a B-1 phenotype, while those in the spleen expressed a B-2 phenotype. Interestingly, it was demonstrated that the strength of BCR signal influenced the ability of anti-Sm B cells to either differentiate into B-1 or remain B-2. The authors concluded that differentiation to B-1 helps maintain tolerance to Sm through the expression of negative regulators of BCR signaling such as CD5. Thus, microenvironment-specific events may determine the likelihood that a given B cell, either adult or fetal derived, enters this pathway. CD5 expression and possibly localization to the peritoneum appear to provide some protection to autoreactive cells otherwise destined for elimination.

A number of findings in this study support the concept that the BCR on CD5+ peritoneal B-1 cells exhibits a qualitatively altered
signal-transducing ability. First, BCR aggregation causes substantial protein tyrosine phosphorylation in peritoneal B cells, but no Ca\(^{2+}\) mobilization, indicating that these cells are not simply anergic. Previous studies (25) and preliminary evidence in our lab (J. M. Dal Porto, manuscript in preparation) indicate that signaling defects may lie in the coupling of the BCR to phospholipase C-\(\gamma\) activation, most likely resulting in the lack of BCR-mediatedinositol 1,4,5-triphosphate production previously reported in peritoneal B cells. Second, we demonstrate that surface IgM levels increase, while B20 levels decrease following localization of V\(_{H}11\)V\(_{9}\) B cells to the peritoneum. Increases in IgM expression can also be induced in vitro by culturing splenic V\(_{H}11\)V\(_{9}\) B cells with PEx, and more specifically IL-10 (data not shown). These alterations in B20 to IgM ratio may shift the equilibrium of Src family kinases toward repression, thereby restricting participation of certain Src family kinases in BCR signaling. Finally, expression of unique effector molecules (Ref. 40 and manuscript in preparation) in peritoneal B-1 cells could cause alterations in the BCR signaling axis. These changes suggest that the normal BCR signaling cascade has been modified as a consequence of exposure to Ag in the peritoneum.

The data presented in this study also suggest an unexpected plasticity in B cells. This plasticity allows cells to maintain particular functional characteristics despite the presence of autoantigen under certain circumstances, while in others they are either deleted or anergized. One advantage of this would be the ability to generate a population that produces biologically important low affinity, polyreactive Abs, while preventing these cells from participating in immune responses that result in class switching and affinity maturation. Stimulation of such a response by endogenous or cross-reactive exogenous Ag could result in the production of pathogenic, high affinity IgG Abs. And, while B-1 cells do not seem to enter germinal centers (11), it is of critical importance that these B-1 cells remain functional and produce natural Abs as a first line of defense against potential pathogens. Boes et al. (13) demonstrated the importance of Abs with PtC specificity by showing that a mutant mouse strain deficient in secreted IgM exhibited increased susceptibility to septic peritonitis induced by cecal ligation and puncture. Most importantly, resistance could be rescued by passive administration of an anti-PtC mAb. Therefore, we propose that the effect of low affinity autoantigen on the PtC-specific V\(_{H}11\)V\(_{9}\) B cells is to establish a signaling set point wherein the BCR transduces qualitatively distinct signals that support survival and Ab production, while not allowing other responses, such as participation in thymus-dependent immune responses. It is appealing to hypothesize that the low number of BCR specificities found within the B-1 population may reflect the availability of Ags at specific sites.

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