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*J Immunol* 2003; 170:73-83; doi: 10.4049/jimmunol.170.1.73
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CD19 Function in Early and Late B Cell Development: I. Maintenance of Follicular and Marginal Zone B Cells Requires CD19-Dependent Survival Signals

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Loss of membrane-bound Ig results in the rapid onset of apoptosis in recirculating B cells. This observation implies that a competent B cell receptor (BCR) is not only required for Ag-dependent differentiation, but also for continued survival in the peripheral immune system. Expression of the B cell coreceptor, CD19, is likewise essential for key B cell differentiative events including the formation of B-1, germinal center, and marginal zone (MZ) B cells. In this study, we report that CD19 also exerts a role before Ag encounter by promoting the survival of naive recirculating B cells. This aspect of CD19 signaling was first suggested by the analysis of mixed bone marrow chimeras, wherein CD19+/− B cells fail to effectively compete with wild-type B cells to reconstitute the peripheral B cell compartment. Consistent with this observation, Bromodeoxyuridine- and CFSE-labeling studies reveal a shorter in vivo life span for CD19+/− B cells vs their wild-type counterparts. Moreover, we find that CD19 is necessary for propagation of BCR-induced survival signals and thus may contribute to homeostatic mechanisms of tonic signaling. To determine whether provision of a constitutive survival signal could compensate for the loss of CD19 in vivo, Bcl-2-transgenic mice were bred onto the CD19+/− background. Here, we observe an increase in follicular B cell numbers and selective recovery of the MZ B cell compartment. Together these findings suggest that maintenance of the follicular and MZ B cell compartments require CD19-dependent survival signals.


Throughout B cell development, decisions are made to live, proliferate, differentiate, or die. These cell fate decisions are tightly regulated and collectively determine the constitution of the mature B cell pool that is capable of responding to Ag. Central to such regulation is the continual monitoring of B cell receptor (BCR) expression and signaling competence. This checkpoint regulation first occurs in cycling pre-B cells which require a functional pre-BCR to progress to the small resting pre-B cell stage and the onset of Ig L chain gene rearrangement. Later events of positive and negative selection occur at the immature IgM-only stage, leading to the formation of transitional B cells that up-regulate IgD expression upon egress to the periphery (1, 2). Importantly, however, recirculating B cells need to maintain a signaling-competent receptor for continued survival (3). This tonic signal is apparently necessary to maintain a peripheral pool of Ag-specific B cells that can be recruited into a productive immune response.

Although the BCR is the focal receptor for B cell differentiation, it also integrates signals provided by the coreceptor molecules (e.g., CD19, CD21, CD22, CD72, and FcyRIIB). By modulating Ag recognition and signal transduction through the BCR, the B cell coreceptors provide information as to the nature of the Ag as well as the context in which the Ag is encountered. Of particular note in this capacity is the B cell-restricted cell surface protein CD19. CD19 is a 95-kDa transmembrane protein bearing two extracellular Ig domains and an extensive cytoplasmic tail (4, 5). Onset of CD19 expression occurs at the earliest stages of B cell development before the expression of the pre-BCR. On mature B cells, CD19 is found in a complex with the complement receptor (CD21), the tetraspan membrane protein TAPA-1 CD81 (CD81), and Leu13 (6, 7). Moreover, CD19 associates with members of the BCR complex and is rapidly phosphorylated following BCR crosslinking (8–10). CD19 coligation with surface Ig (sIg) greatly augments B cell activation (11), presumably through the enhanced recruitment or activation of associated kinases and adaptor molecules. Thus, CD19 behaves as an essential downstream element of BCR signaling and is thought to functionally link CD21 with the BCR following corecognition of C3d-bearing Ags (12).

The importance of CD19 in the development and function of B cells was demonstrated through the targeted deletion of the CD19 gene (13, 14). Mice rendered CD19 deficient present multiple B cell defects, including decreased B cell numbers in the periphery as well as a severe reduction in B-1 cells. CD19+/− mice are also functionally impaired in responding to T cell-dependent Ags as evidenced by a sharp reduction in primary and secondary Ab responses and a lack of germinal center (GC) formation. Recently, it has been shown that CD19 is essential for the generation of marginal zone (MZ) B cells (15, 16). In this study, we provide strong evidence that modulation of cell survival is a key property of CD19 function in vivo and accounts for some of the phenotypes observed in CD19+/− mice.

Materials and Methods

Mice

CD19+/− mice on BALB/c (Ighb) (The Jackson Laboratory, Bar Harbor, ME) background (10-generation backcross) were maintained under pathogen-free conditions and handled in accordance with the guidelines set forth...
by the Animal Subjects Program at the University of California, San Diego. BALB/c (IgH)-congenic mice were used as wild-type controls and BALB/c (IgH) wild-type mice were used as recipients for adoptive transfer experiments. B-cell mice (Eμ-bcl-2-22) were purchased from The Jackson Laboratory.

Cell culture
Splenic B cells were purified by depletion of adherent cells bound to plastic and lysis of T cells using anti-CD4, anti-CD8, and anti-Thy1.2-specific Abs and rabbit complement (Cedarlane Laboratories, Westbury, NY). Cells were cultured at 1 × 10⁶ cells/ml in RPMI 1640 with 10% FCS, penicillin/streptomycin, t-glutamine, nonessential amino acids, sodium pyruvate (Cellgro; Mediatech, Herndon, VA) and 2-ME (Life Technologies, Rockville, MD). Cells were stimulated with anti-IgM F(ab’²), or anti-IgG F(ab’²), (Jackson ImmunoResearch, West Grove, PA), and apoptosis was measured by flow cytometry using propidium iodide (PI). Apoptotic cells were identified by a sub-G₀-G₁ peak after PI staining in 1 mM Tris, 0.1% Triton X-100, 0.1% sodium citrate, 0.1 mM EDTA, and 50 µg/ml PI (pH 8).

Flow cytometry
After harvesting organs, single-cell suspensions were prepared and RBCs were lysed with ACK buffer (0.15 M NH₄Cl, 1 mM KCl, 0.1 mM Na₂ EDTA, pH 7.4). Cells (1 × 10⁶) were stained for 15 min on ice with diluted Ab, washed with PBS containing 1% FCS, and incubated with streptavidin-conjugated fluorochromes when necessary. Abs against the following surface markers were obtained from BD Pharmingen (San Diego, CA): CD4 (heat stable Ag, HSA)-FITC, CD24/HSA-biotin (bio), IgD-FITC, IgM-PE, IgM-bio, CD19-PE, B220-allophycocyanin, B220-PE, CD5-bio, GL7-FITC, bromodeoxyuridine (BrdU)-FITC, and streptavidin-alkaline phosphatase. Anti-IgD bio was purchased from Southern Biotechnology Associates (Birmingham, AL) and anti-B220 TriC was obtained from Caltag Laboratories (Burlingame, CA).

Adoptive transfer
Recipient mice (BALB/c/IgH⁻) were lethally irradiated (1000 rad) and administered antibiotics (neomycin, 1 mg/ml, and polymixin B, 0.1 mg/ml; Life Technologies) in the drinking water postreconstitution. Bone marrow cells from wild-type and CD19⁻/⁻ mice were depleted of B cells using MiniMACS columns (Miltenyi Biotec, Auburn, CA) and anti-B220-conjugated magnetic beads. B cell-depleted bone marrow cells (1 × 10⁶) were injected into the lateral tail vein of irradiated recipients. To generate bone marrow chimera, B cell-depleted bone marrow from age-matched wild-type and CD19⁻/⁻ mice were mixed before injection. Chimeric mice were analyzed 8 wk postirradiation.

Spleenic/lymph node B cell transfer
Wild-type and CD19⁻/⁻ B cells from age-matched mice were purified, admixed at a 1:1 ratio, labeled with 5 µM CFSE (Molecular Probes, Eugene, OR) for 10 min, washed, and injected into the tail vein of wild-type BALB/c mice. Organs were harvested at 5, 18, or 35 days posttransfer and analyzed by flow cytometry.

Auto-reconstitution
Wild-type BALB/c, CD19⁻/⁻ mice, Bcl-2-transgenic, and CD19⁻/⁻/Bcl-2-transgenic mice were sublethally (500 rad) irradiated and allowed to reconstitute for 12–14 days before flow cytometric analysis of bone marrow and spleen cell populations.

BrdU treatment
Age-matched wild-type BALB/c and CD19⁻/⁻ mice were administered BrdU in their drinking water (1 mg/ml, changed twice weekly) for up to 12 wk. Mice were sacrificed following 6, 9, or 12 wk of labeling and analyzed by flow cytometry for the presence of BrdU-positive B cells. Student’s t test was used to determine the level of significance for differences between sample means of wild-type and CD19⁻/⁻ mice at each time point.

Immunohistology
Spleens from CD19⁻/⁻, Bcl-2⁻/⁻, and CD19⁻/⁻/Bcl-2⁻/⁻ mice were frozen in OCT compound above liquid nitrogen and stored at −90°C. Ten-micrometer spleen sections were frozen in OCT compound above liquid nitrogen and stored at −90°C. Ten-micrometer spleen sections were cut on a cryostat and fixed with 4% formaldehyde for 15 min. Slides were then washed with PBS (pH 7.4) and blocked with 10% normal goat serum in PBS for 30 min at room temperature. Slides were incubated with first Ab overnight. Secondary reagents included anti-rat Ig FITC or streptavidin-Cy3 (Zymed Laboratories, San Francisco, CA). The MOMA-1 Ab was purchased from Bachem (King of Prussia, PA) and peanut agglutinin (PNA)-FITC from Vector Laboratories (Burlingame, CA).

Results
CD19 is required for efficient entry into and maintenance of the peripheral B cell pool
Early in life the composition of the B cell compartment is dynamic, whereas the majority of recirculating B cells in adult animals are relatively long-lived and not rapidly replenished by newly formed cells from the bone marrow (17). We now show that CD19 contributes to the maintenance of the peripheral B cell pool. This notion was initially suggested by the finding that B cell numbers were reduced in adult CD19⁻/⁻ mice (13). This deficiency is more striking early in ontogeny where we observe a 3- to 4-fold reduction in B cells from neonatal spleens (Fig. 1a). At this stage of ontogeny, B cells bearing the B-1 cell phenotype (IgMₜ₉₉, CD5⁺(B1a subtype), CD23⁻) are well represented in wild-type mice, but reduced in CD19⁻/⁻ mice. Interestingly, however, the reduction in B cell numbers we observed in neonatal CD19⁻/⁻ mice is not restricted to B cells bearing the B-1 cell phenotype, but also applies to conventional (B-2, CD23⁺) cells and thus indicates a general impairment in B cell lymphopoiesis.

In adult animals, the B-1 cell population remains depressed while the conventional B cell compartment recovers to some degree. B cells in the adult spleen represent a mixed population of mature and immature cells possessing different rates of turnover since relatively few immature B cells will enter the pool of mature recirculating B cells. As previously noted (18, 19), B cells from CD19⁻/⁻ mice have higher levels of surface IgM expression (Fig. 1b), suggesting that there is an increase in the relative number of IgM⁰/IgDₜ₉₉ (transitional-2) B cells in CD19⁻/⁻ mice. To determine whether CD19⁻/⁻ mice exhibit a developmental block in peripheral B cell maturation, expression of additional mature B cell surface markers was determined (Fig. 1b). A similar number of CD19⁻/⁻ splenic B cells expressed the surface marker 493, which has been associated with immature and transitional B cells (20, 21). In addition, CD19⁻/⁻ and wild-type B cells expressed similar levels of L-selectin, MHCI, and HSA. These data suggest that B cells from CD19⁻/⁻ mice undergo normal maturation, but may express higher levels of slgM as an outcome of selection. Because compensatory effects may obscure the severity of the CD19 defect, we chose to directly evaluate B cell formation in chimeric mice bearing mixed populations of CD45R/B220-depleted bone marrow cells from adult CD19⁻/⁻ and wild-type mice. Animals were lethally irradiated and reconstituted with bone marrow from BALB/c or BALB/c-congenic CD19⁻/⁻ mice or combinations thereof. The B cell compartment of recipient mice was analyzed 8 wk postreconstitution to guarantee recovery of the peripheral immune system. Fig. 1c shows representative flow cytometric data of cells from the bone marrow, spleen, lymph node, and peritoneal cavity of mice reconstituted with 50:50, 80:20, or 20:80 ratios of CD19⁺/⁺ (transitional-2) B cells in CD19⁻/⁻ mice. To determine whether CD19⁻/⁻ mice exhibit a developmental block in peripheral B cell maturation, expression of additional mature B cell surface markers was determined (Fig. 1b). A similar number of CD19⁻/⁻ splenic B cells expressed the surface marker 493, which has been associated with immature and transitional B cells (20, 21). In addition, CD19⁻/⁻ and wild-type B cells expressed similar levels of L-selectin, MHCI, and HSA. These data suggest that B cells from CD19⁻/⁻ mice undergo normal maturation, but may express higher levels of slgM as an outcome of selection. Because compensatory effects may obscure the severity of the CD19 defect, we chose to directly evaluate B cell formation in chimeric mice bearing mixed populations of CD45R/B220-depleted bone marrow cells from adult CD19⁻/⁻ and wild-type mice. Animals were lethally irradiated and reconstituted with bone marrow from BALB/c or BALB/c-congenic CD19⁻/⁻ mice or combinations thereof. The B cell compartment of recipient mice was analyzed 8 wk postreconstitution to guarantee recovery of the peripheral immune system. Fig. 1c shows representative flow cytometric data of cells from the bone marrow, spleen, lymph node, and peritoneal cavity of mice reconstituted with 50:50, 80:20, or 20:80 ratios of CD19⁺/⁺ and wild-type bone marrow cells. In all organs, we observe a greater presence of CD19-positive B cells in excess of the 50:50 ratio at which they were injected. This trend is first noted at the immature slgM-positive stage in the bone marrow and continues in the periphery where the majority of IgD-positive mature B cells are also CD19 positive. Thus, these data illustrate a clear advantage for CD19-expressing B cells in the formation and maintenance of peripheral B cell subsets.

In contrast to the transfer of fetal liver cells, transfer of bone marrow cells does not lead to efficient reconstitution of B-1 cells (22). In our bone marrow reconstitution experiments, CD19-positive IgMₜ₉₉/IgDₜ₉₉ and IgDlowIgMₜ₉₉ cells are present in the peritoneal cavity (Fig. 1b and data not shown). This latter population bears the phenotype of immature or transitional B cells.
FIGURE 1. Defects in B cell lymphopoiesis and homeostasis in CD19−/− mice. 
a. B cell populations in the spleen of representative 3-day-old CD19−/− and CD19−/− littermates stained for IgM, CD23, and CD5. 
b. Expression surface markers associated with B cell maturation in adult wild-type and CD19−/− animals. 
c. Flow cytometric analysis of bone marrow, spleen, lymph node, and peritoneal cavity of chimeric mice 8 wk after irradiation. Columns are labeled with the relative composition of bone marrow cells injected. Plots of CD45R/B220-positive cells showing relative percentages of CD19-positive and CD19-negative B cells.
FIGURE 2. Shorter life span of CD19−/− B cells. a, Flow cytometric analysis of bone marrow and spleen cells from mice fed BrdU for 9 wk. Rectangles in dot plots show gated populations for histograms. BrdU-positive cells are indicated. b, Bone marrow, spleen, and lymph node cells were stained as in a. Graphs represent the percentage of BrdU-positive cells in the B220hi/HSAlo gate. Wild-type (□) and CD19−/− (●) mice were fed BrdU continuously for 6, 9, or 12 wk (three mice per time point). Statistical significance is indicated by displayed $p$ values. c, A 50:50 mixture of wild-type and CD19−/− splenic or lymph node B cells was labeled with CFSE and injected i.v. Bar graph (three mice per group) shows percentage of CD19-positive CFSE-labeled cells recovered from various organs 5 or 18 days after injection. BM, bone marrow; Spl, spleen; LN, lymph node; PEC, peritoneal cavity; PBL, peripheral blood leukocytes. d, Flow cytometric analysis of cells harvested from spleen 18 days postinjection and showing largely uniform staining for CFSE.
FIGURE 3. Reduced BCR-mediated survival in the absence of CD19. a, Time course of B cell survival in the absence of BCR stimulation and the presence or absence of Bcl-2 overexpression. Apoptotic cells were identified by merocyanine 540 (MC540) staining and gating on MC540<sup>high</sup> cells. Data are means of triplicate cultures. b, Survival of wild-type, Bcl-2-transgenic (Bcl-2), CD19<sup>−/−</sup>, and CD19<sup>−/−</sup>/Bcl-2 B cells after 36 h of stimulation with indicated concentrations of anti-IgM. Apoptotic cells were identified by PI staining and flow cytometric gating on the sub-G<sub>0</sub>-G<sub>1</sub> population (M1) vs cycling cells (M2). Arrow in lower profiles indicates 1 μg/ml concentration selected for displayed histograms. WT, wild type.
(IgM<sup>high</sup>, CD23<sup>neg</sup>, IgD<sup>low</sup>) that is also shared by B-1b cells; however, it is unlikely to represent B-1 cells as it is uniformly CD5 negative (data not shown). CD19<sup>−/−</sup> IgD<sup>neg</sup> B cells were greatly underrepresented in the peritoneal cavity of all recipient mice (Fig. 1c). The severity of this defect may indicate an inability of newly generated CD19<sup>−/−</sup> B cells to enter or survive in the peritoneal cavity.

**CD19<sup>−/−</sup> B cells exhibit increased turnover and reduced survival**

The inability of CD19<sup>−/−</sup> B cells to effectively compete with wild-type B cells suggests a role for CD19 in promoting cell survival. To address this issue, we used long-term BrdU-labeling procedures and flow cytometric analysis. Mice were administered BrdU continuously in their drinking water for 6, 9, or 12 wk. Thus, all unlabeled peripheral B cells were generated before administering BrdU. A representative labeling profile is shown in Fig. 2a where, following 9 wk of labeling, all developing B cells in the bone marrow and transitional B cells in the spleen are labeled, but a fraction of recirculating and follicular B cells remain unlabeled. Comparing wild-type and CD19<sup>−/−</sup> mice after 6 or 9 wk of labeling, we detected a significantly higher percentage of labeled mature B cells (B220<sup>high</sup>, HSA<sup>low</sup>) in the bone marrow, spleen, and lymph nodes of CD19<sup>−/−</sup> mice (Fig. 2b). At 12 wk of BrdU labeling, the percentage of labeled cells in CD19<sup>−/−</sup> and wild-type mice appeared to normalize; which may reflect a countereffect of reduced B cell generation in CD19<sup>−/−</sup> mice. Overall, the differential labeling kinetics indicated that there was a relatively smaller fraction of CD19<sup>−/−</sup> B cells whose survival spanned the labeling period. Using 50% labeling as a relative index of B cell longevity, it was evident that CD19<sup>−/−</sup> B cells turn over more rapidly than their wild-type counterparts (5.5 vs 8 wk).

To independently confirm the BrdU-labeling experiments and to directly determine whether CD19 promoted the survival of peripheral B cells, 50:50 mixtures of wild-type and CD19<sup>−/−</sup> splenic B cells were labeled with CFSE and cotransferred into nonirradiated wild-type recipients. Labeled cells were enumerated from the bone marrow, blood, and peripheral lymphoid organs at 5 or 18 days posttransfer (Fig. 2c). In this study, we observe a dramatic loss of CD19<sup>−/−</sup> B cells in the spleen and blood by day 18 posttransfer and very few CD19<sup>−/−</sup> B cells in the peritoneal cavity. This effect is due to reduced survival rather than reduced proliferation of B cells.

**FIGURE 4.** B cell generation in CD19<sup>−/−</sup> mice overexpressing Bcl-2. 

*a.* Flow cytometric analysis of B cell subpopulations from bone marrow, spleen, and peritoneal cavity (PeC) cells of CD19<sup>−/−</sup>, Bcl-2-transgenic (Bcl-2), CD19<sup>−/−</sup>, and CD19<sup>−/−</sup>/Bcl-2 mice. Transitional B cells in bone marrow and spleen and B-1a cells in the PeC are rectangular. 

*b.* Flow cytometric profiles of spleen and bone marrow cells from autoreconstituted mice 2 or 14 days after sublethal irradiation. Rectangles show more mature B cell populations bearing high levels of IgD and low levels of HSA.
CD19<sup>−/−</sup> B cells, since the vast majority of transferred cells retain uniform high-intensity CFSE labeling regardless of CD19 expression (Fig. 2f). The lymph node and bone marrow showed less of a disparity in recovery of donor B cells at the selected time points. This distinction could reflect the heterogenous nature of the transferred splenic B cells from wild-type animals, which is also distinct from CD19<sup>−/−</sup> animals insofar as they lack MZ B cells. Since the lymph node contains a more homogeneous population of mature B cells, we repeated the cotransfer experiment using lymph node B cells and extended the analysis to 35 days (Fig. 2c). Here, we observed a dramatic reduction of CD19<sup>−/−</sup> B cells in the spleen, bone marrow, and lymph nodes of recipient mice. Thus, these results are consistent with the BrdU-labeling experiments and together suggest that CD19-dependent survival is an intrinsic property of mature recirculating B cells.

**BCR-induced cell survival is CD19 dependent**

To understand the basis of CD19-mediated B cell survival, we assessed the in vitro growth properties of purified splenic B cells from CD19<sup>−/−</sup> and wild-type mice. Unlike the in vivo situation, where recirculating B cells are exposed to a milieu of cognate and soluble ligands that act in part to promote longevity, B cells are short-lived in culture unless supplied with growth factors or mitogens. CD19<sup>−/−</sup> and wild-type B cells display similar survival properties in the absence of exogenous stimuli that protect cells from apoptosis (Fig. 3a). Because B cell survival in vivo is dependent upon continued expression of the BCR (3), we investigated whether such a signal was in turn dependent upon down-regulation of B cell survival by ectopic overexpression of Bcl-2 was sufficient to rescue the developmental and functional defects associated with CD19 deficiency, or whether these defects were intrinsic to the lack of CD19-induced signaling events distinct from survival. We show that B cell-specific expression of a Bcl-2 transgene leads to increased formation of slgM<sup>high</sup> cells in the bone marrow, spleen, peritoneal cavity, and lymph node from 8- to 10-wk-old CD19<sup>−/−</sup>, Bcl-2, CD19<sup>−/−</sup>/Bcl-2, and CD19<sup>−/−</sup>/Bcl-2 mice obtained by analysis of flow cytometric data. Graphs represent at least five mice each.

**FIGURE 5.** Summary of partial rescue of B cell numbers in CD19<sup>−/−</sup> mice overexpressing Bcl-2. Absolute numbers of B cell subsets from bone marrow, spleen, peritoneal cavity, and lymph node from 8- to 10-wk-old CD19<sup>−/−</sup>, Bcl-2, CD19<sup>−/−</sup>/Bcl-2, and CD19<sup>−/−</sup>/Bcl-2 mice obtained by analysis of flow cytometric data. Graphs represent at least five mice each.
marrow of both wild-type and CD19<sup>-/-</sup> animals (Fig. 4a). In wild-type animals, these immature/transitional B cells are particularly sensitive to negative selection and express low levels of Bcl-2 and Bcl-x<sub>L</sub> and A1 (26, 27). Hence, egress from the marrow to the periphery is normally accompanied by a dramatic loss in B cell numbers (28), which can be attenuated through increased expression of Bcl-2. Curiously, a significant proportion of the slgM<sup>high</sup> cells in the bone marrow of Bcl-2-transgenic mice coexpress high levels of slgD, a population that is thought to be restricted to the spleen (29). To determine whether these cells are newly generated or recirculating B cells, we performed a kinetic analysis of B cell differentiation following sublethal irradiation (Fig. 4b). After 14 days of autoreconstitution, the majority of splenic B cells in CD19<sup>-/-</sup> and CD19<sup>-/-</sup> mice bear the phenotype of transitional B cells (IgM<sup>high</sup>, IgD<sup>low</sup>, HSA<sup>high</sup>; Figs. 4b and 5 and data not shown). However, in the presence of forced Bcl-2 expression, a large population of mature B cells (IgM<sup>low</sup>, HSA<sup>low</sup>) is present in CD19<sup>-/-</sup> mice and, to a much lesser extent, CD19<sup>-/-</sup> mice (Figs. 4b and 5). This population is also present 2 days postirradiation, suggesting that they represent radioresistant cells present before treatment (Figs. 4b and 5). Interestingly, radioresistance appears to require both elevated levels of Bcl-2 and progression to the mature B cell stage, since immature/transitional B cells overexpressing Bcl-2 remain sensitive to this level of irradiation. Altogether, these findings indicate that the reduced numbers of peripheral B cells in CD19<sup>-/-</sup> mice cannot be wholly explained by a failure to upregulate Bcl-2 at the immature-mature B cell transition.

With maturation of the lymphoid compartments, expression of Bcl-2 on the CD19<sup>-/-</sup> background led to a general increase in B cells bearing the phenotype of conventional or B-2 cells (Fig. 4a, summarized in Fig. 5), such that B cell numbers in the spleen of adult CD19<sup>-/-</sup> animals approximate that of heterozygous counterparts. However, cell numbers did not reach the levels seen in Bcl-2-transgenic mice that retain CD19 expression. This partial rescue is also selective for B-2 cells since B-1 cells are not restored in the peritoneal cavity of Bcl-2-transgenic mice on the CD19<sup>-/-</sup> background (Fig. 4a). To determine whether the Bcl-2-mediated increase in B-2 cells is accompanied by a recovery of B cell function in CD19<sup>-/-</sup> mice, immunizations were performed with SRBCs (Fig. 6) and the T cell-dependent Ag, DNP-keyhole limpet hemocyanin (data not shown). If CD19<sup>-/-</sup> B cells are sufficiently activated but do not survive within the GC environment, then we may expect to see a rescue of GC formation on the Bcl-2-transgenic background. Examination of frozen spleen sections 7 days postimmunization revealed a high incidence of well-formed GC in wild-type and Bcl-2-transgenic mice (Fig. 6); however, provision of Bcl-2 was not sufficient to rescue GC B cell differentiation in the absence of CD19. This finding suggests that CD19 is necessary early in the recognition of and/or response to protein Ags leading to the generation of GC B cells. Overall, these findings suggest that ectopic expression of Bcl-2 can compensate for CD19-associated defects in the maintenance of recirculating follicular B cells, but cannot substitute for Ag-induced differentiation signals.

Provision of Bcl-2 rescues MZ B cell formation in CD19<sup>-/-</sup> mice

MZ B cells are key participants in the capture of and response to blood-borne Ags in the spleen (30). This B cell subset appears late in ontogeny and may represent an Ag-selected population primed to respond to further antigenic challenge (15, 31). The size of the MZ B cell population shows some strain variation; however, CD19<sup>-/-</sup> mice on the BALB/c, 129, or mixed C57BL/6/129 background all present a deficiency in MZ B cells (Refs. 15 and 16 and data not shown). To determine whether this deficiency results from impaired survival of cells destined for the MZ B cell compartment, we performed immunohistochemical staining for IgM and IgD in spleens of wild-type, Bcl-2, and Bcl-2/CD19<sup>-/-</sup> mice. Remarkably, overexpression of Bcl-2 on the CD19<sup>-/-</sup> background led to a recovery of the MZ B cell population as evidenced by the formation of an outer ring of IgM<sup>high</sup>IgD<sup>low</sup> cells adjacent to the marginal sinus (Fig. 7a). These findings were corroborated by immunohistochemical staining for metallophilic macrophages and B cells using Abs against MOMA-1 and CD45R/B220, respectively (data not shown), and resolution of the MZ B cell population (CD21<sup>high</sup>, CD1<sup>d</sup>low) by flow cytometry (Fig. 7b). Electronic gating on the CD21<sup>high</sup>CD1<sup>d</sup>population shows that the vast majority of the cells are also CD23 negative, which is consistent with their designation as MZ B cells and distinct from CD23-positive follicular B cells (Fig. 7b). These data suggest that either selection into or maintenance in the MZ B cell compartment is dependent upon a CD19-mediated survival signal. This trait is in common with follicular B cell homeostasis, but distinct from CD19-dependent generation of B-1 and GC B cells.

Discussion

Generation and maintenance of mature lymphocytes is an active process requiring a competent Ag receptor. However, the nature and strength of signal required to effect positive selection and ensure continued survival in the periphery is not well understood. For B cells, the absence of defined selecting ligands further obfuscates the nature of the selection process, although analysis of the changing repertoire of Ag specificities provides strong evidence that such selective pressures exist (28, 32). In this study, we provide evidence that the B cell coreceptor CD19 contributes to B cell survival, which impacts further differentiation of selected B cell subsets.

CD19 acts as a downstream effector for slg and CD21 (CR2) (33). Thus, B cell recognition of internal and foreign Ags alike will result in the recruitment and phosphorylation of CD19, resulting in augmented B cell activation. This augmentation is apparently necessary for B cells to respond to paucivalent protein Ags, but is dispensable for responding to most, but not all, T cell-independent Ags (13, 14, 34–36). To date, the role of CD19 in B cell generation and maintenance has not received significant attention, despite the
observation that CD19−/− mice show a reduction in peripheral B cell numbers (13). Therefore, to assess the contribution of CD19 to the generation and maintenance of mature B cells, we directly compared the ability of CD19−/− and wild-type B lineage cells to differentiate and survive in an irradiated syngeneic host. In this competitive situation, CD19−/− B cells exhibited a clear disadvantage in contributing to the peripheral B cell pool. The inability of CD19−/− B cells to compete effectively with wild-type B cells could be explained in part by a shorter intrinsic life span. This hypothesis is supported by long-term BrdU-labeling studies where we demonstrated a much higher rate of turnover for mature (B220high, HSAlow) CD19−/− B cells than their wild-type counterparts. However, after 12 wk of BrdU administration, the percentage of labeled CD19−/− B cells was similar to that of wild type. This finding suggests that another parameter, such as reduced generation of CD19−/− B cells, may contribute significantly as the labeling period persists and gradually leads to a reduced number of B cells entering the periphery. Therefore, we corroborated these studies with direct labeling and cotransfer of wild-type and CD19−/− B cells. Here, we observe a selective and rapid loss of CD19−/− B cells from all lymphoid tissues. Thus, we document a novel role for CD19 in promoting B cell survival in vivo. Such a role may underlie the therapeutic basis of targeted B cell ablation studies using anti-CD19/genistein conjugate Abs which mediate specific inhibition of CD19-associated tyrosine kinase activity and result in the directed apoptosis of human B cell leukemias and lymphomas (37).

Because some level of BCR-mediated signaling is required for the continued survival of naive and memory B cells in the absence of overt Ag (3, 38), we reasoned that CD19 may be necessary to propagate this survival signal. Support for this idea was provided by the demonstration that CD19−/− B cells are indeed impaired in BCR-mediated rescue from spontaneous apoptosis in vitro. This effect occurs at levels of anti-IgM stimulation that does not induce significant cell cycle progression, and thus should also be considered in the context of reported defects in proliferation (13) that are ameliorated by costimulation with survival factors such as IL-4 (14). In vivo, it would be expected that CD19−/− B cells are hyporesponsive to tonic signaling through the BCR, thus precipitating their rapid loss in the absence of compensatory up-regulation

FIGURE 7. Bcl-2 overexpression rescues MZ B cell formation in CD19−/− mice. a, Frozen spleen sections were stained with anti-IgD FITC and anti-IgM Cy3. The MZ is indicated by arrows. Figures are representative of several follicles from at least three sets of mice. b, Flow cytometry profiles of MZ B cells bearing high levels of CD1d and CD21 (squares). Absolute cell numbers with SD (×10^6) are provided, based upon four groups of mice. The lower histogram shows the distribution of CD23 expression on the CD21high/CD1dhigh population. Displayed plots were gated on IgMhigh/IgDneg spleen cells.
of sIg levels, as observed (18, 19), or perhaps increased receptor affinity for some internal ligands, as implicated by the recent work of Shih et al. (36).

Protection from apoptosis is often achieved by the specific induction of pro-survival factors of the Bcl-2 family. Indeed, Roberts and Snow (25) have shown that signaling through CD19 may selectively augment the accumulation of Bcl-2 in resting B cells. Therefore, we determined whether forced expression of Bcl-2 could lead to a rescue of survival-related deficiencies in CD19−/− mice. Normally, Bcl-2 expression is tightly regulated during B cell development with high levels found in pro-B cells and mature B cells, but low levels in pre-B cells and immature B cells (27, 39). This expression pattern is distinct from Bcl-xL and A1, which are up-regulated upon (pre-) BCR engagement or induced during the immature/mature B cell transition, respectively (23, 24). Apart from expression profiles, it is unclear whether these Bcl-2 homologues possess properties distinct from Bcl-2. Mice that overexpress Bcl-2 in the B lineage have an overabundance of B cells and serum Ab, develop autoimmune disease, and have increased GC formation and memory B cell generation (40, 41). We find that provision of Bcl-2 leads to a partial rescue in the number of follicular B cells in CD19−/− mice, suggesting compensation for a survival factor(s) normally induced in the presence of CD19. These findings are consistent with the results of Lam et al. (3), who showed that BCR-dependent survival of mature recirculating B cells can be partially compensated by overexpression of Bcl-2 (3) and, along with our in vitro studies, suggests that B cell persistence in vivo requires CD19 to efficiently propagate tonic survival signals induced by the BCR.

In addition to basal signaling through the BCR complex, successful passage through a given checkpoint in B cell differentiation requires signals through the (pre-) BCR and coreceptor molecules that are of sufficient strength and quality to induce cell cycle progression and differentiation. Recent evidence from gene-targeted and Ig-transgenic mice suggests that MZ B cells also represent an Ag-selected population (42). Selection into the MZ B cell compartment is strictly dependent on Pyk2 and CD19 function (15, 16, 43), whereas Btk appears to play an ancillary role that may be modulated by CD19 signaling (15, 44–46). Although responses of MZ B cells to T cell-independent Ags is facilitated by complement activation and opsonization (43, 47), MZ B cell formation is not impaired in the absence of B cell complement receptors (CD21/CD35) or C3 (43, 45). Thus, the requirement for CD19 in MZ B cell generation is by association with sIg or an unknown ligand. Overexpression of Bcl-2 on the CD19−/− background results in a selective recovery of the MZ B cell subset, suggesting that the precursors to MZ B cells may be present in CD19−/− mice but are not activated sufficiently to up-regulate survival factors such as Bcl-2 and thus fail to further differentiate and colonize the MZ. Alternatively, it has been noted recently that, similar to B-1 cells, MZ B cells are particularly long-lived and less reliant upon the bone marrow for replenishment in the adult animal (48, 49). Thus, this nonrecirculating population may not be sustained in the absence of CD19. Downstream effectors of CD19 signaling that may account for this function include members of the NF-κB family that are known to be required for MZ B cell formation (50–53), perhaps through the activation of target genes such as Bcl-xL and A1, in addition to Bcl-2. Alternatively, we have shown that CD19 promotes activation of the pro-survival kinase Akt (54), suggesting the involvement of a phosphatidylinositol 3-kinase-dependent survival pathway. Characterization of such regulators of MZ B cell survival awaits further investigation.

The data presented here underscore CD19 as an important regulator of cell fate decisions by virtue of promoting cell survival. Nonetheless, it would be inaccurate to classify CD19 simply as a pro-survival molecule. In point of fact, we show that while overexpression of Bcl-2 selectively rescues MZ B cell formation and promotes B cell homeostasis, it does not rescue GC formation or the B-1 B cell population. In the case of B-1 cells, subthreshold signaling through the BCR in the absence of CD19 may preclude commitment to the B-1 cell pathway. This differentiative signal cannot be circumvented simply by increasing cell survival. The CD19-CD21 coreceptor complex also regulates commitment to the GC B cell pathway (33). GC formation is a complement-dependent process requiring cytokines and cognate interactions with CD4 T cells (55). CD19 likely participates in this process by augmenting signals induced by corecognition of Ag by sIg and CD21. In this context, recent work from Pierce and colleagues (56, 57) suggests that CD19/CD21 may be required both for sustained signaling by the BCR and for efficient directed processing of Ags for presentation to T cells. CD19 has also been functionally associated with CD40 (13, 58), which may help explain the absence of GCs in CD19−/− mice. Thus, the role of CD19 in B cell biology is complex and multifaceted, likely depending upon the stage of B cell differentiation and the nature of the Ag encountered, much like the BCR itself.

Acknowledgments

We thank Dr. Michael David and members of the Rickert laboratory for critical reading of this manuscript.

References
