IgM<sub>high</sub>CD21<sub>high</sub> Lymphocytes Enriched in the Splenic Marginal Zone Generate Effector Cells More Rapidly Than the Bulk of Follicular B Cells

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Ag encounter will recruit Ag-specific cells from the pool of mature B lymphocytes in the spleen and activate them to perform effector functions: generation of Ab-forming cells (plasma cells) and presentation of Ag to T cells. We have compared the ability of mature follicular and marginal zone cells to develop into effector B cells. The generation of marginal zone B cells and their localization in the marginal sinus area are T cell and CD40 ligand independent, suggesting that they do not represent a postgerminal center population. Compared with mature recirculating follicular B cells, they express several characteristics of previous antigenic experience, including higher levels of B7.1 (CD80) and B7.2 (CD86) when freshly isolated and following in vitro stimulation. After a brief 6- to 8-h in vitro stimulation with LPS or anti-CD40 Abs, marginal zone B cells become potent APCs. In addition, their ability to proliferate and differentiate into plasma cells in response to low doses of T-independent polyclonal stimuli (LPS) is far greater than that of follicular B cells. These findings indicate a functional heterogeneity within splenic mature B lymphocytes, with marginal zone B cells having the capacity to generate effector cells in early stages of the immune response against particulate Ags scavenged efficiently in this special anatomical site. The Journal of Immunology, 1999, 162: 7198–7207.

The stages of B lymphopoiesis in adult bone marrow have been defined by the use of a large panel of cell surface and genetic markers, and as a result, many of the developmental events resulting in the production of newly formed B cells are well characterized (1–3). In contrast, the cellular and molecular events involved in the transition of a newly formed, immature B cell into a long-lived, mature B lymphocyte populating peripheral lymphoid organs are less well understood. Mature B lymphocytes are able to respond to antigenic challenge, and there is an abundance of experimental evidence related to the cellular and molecular interactions of the immune response to T-dependent Ags, leading to germinal center formation and subsequent production of long term memory cells (4–7).

In rodents and humans two categories of mature, long-lived B cells, termed memory and naïve, have been defined that either have or have not encountered the nominal Ag under study (8–11). Phenotypic and immunohistological analyses subdivided mature splenic B cells into follicular (FO) (3) comprising 80–90% and marginal and immunophenotype with the extranodal disease of mucosa-associated lymphoid tissue generically referred as the postfollicular MZ B cells (29). Taken together with recent results from lymphotoxin gene-targeted mice in which both mucosa-associated lymphoid tissue and the MZ of the spleen are lacking, these observations further suggest a link between mature long-lived B lymphocytes in these distinct anatomical sites (5, 30).
Here we report that compared with their nontransgenic littermates, certain Ig heavy chain transgenic (TG) mice have proportionally larger MZ compartments that may constitute up to 50% of splenic B cells, and that the development of this compartment is indeed independent of T cells. We have used these TG mice as a source of large quantities of purified MZ B cells to facilitate the analysis of their functional potential in early stages of in vitro activation. We show that MZ B cells respond better than FO B cells with respect to early in vitro activation events, resulting in up-regulation of T cell costimulatory molecules and a concomitant ability to function as APCs. We also show that MZ B cells proliferate much faster in response to very low doses of polyclonal mitogens and terminally differentiate into plasma cells within hours of activation. Collectively, these findings suggest that MZ B cells have unique signaling and subsequent differentiative potentials that might permit them to react faster than the majority of splenic B cells (FO) in the earliest stages of an in vivo immune response. These functional differences apply to both Ag presentation and the production of short-lived IgM Ab-forming cells that are involved in the early defense against blood-borne pathogens.

Materials and Methods

Animals

Eight- to twelve-week-old C57BL/6 and BALB/c mice were purchased from Charles River Laboratories (Raleigh, NC) or were bred in our mouse facility. All other mice were used at 8–16 wk of age: 81X-C57BL/6 (31), 81X-JHko (32), MD2 (gift from Dr. C. C. Goodnow, Australian National University, Canberra, Australia), MD4 and TCR T (TCR double knockout, C57BL/6 TcrbMmory Fcrpm1/Momt; purchased from The Jackson Laboratory, Bar Harbor, ME), and 81X-TCR T-C57BL/6 (generated by crossing 81X-C57BL/6 with TCR T-C57BL/6 and screening for the TCR double knockout by PCR) were bred and housed in a pathogen-free facility in accordance with institutional policies for animal care and usage.

Flow cytometric analysis and sorting

Fluorescin-conjugated (FITC) anti-CD5, anti-CD23, and anti-CD69 as well as biotinylated anti-CD62L, anti-CD25, anti-MHC class I, anti-MHC class II, anti-CD95, anti-syndecan-1, anti-B7.1, and anti-B7.2 mAbs were purchased from PharMingen (San Diego, CA). The anti-CD21 (7G6) Ab-secreting hybridoma was a gift from Dr. Michael Holers (University of Colorado Health Science Center, Denver, CO) (33, 34). The purified Ab was conjugated to PE by Southern Biotechnology Associates (Birmingham, AL). Two-, three-, and four-color surface stainings were performed as previously described (31). Briefly, 5 × 10⁶ cells depleted of RBC by lysis with an ammonium chloride-containing buffer were incubated with a mixture of fluorescein-, PE-, and biotin-conjugated Abs followed by SA-PECy5 (Southern Biotechnology Associates) or streptavidin-allophycocyanin (PharMingen). Cells were incubated for 15 min at each step and were washed with 2% FCS/PBS between steps. Cells were resuspended in either 1% paraformaldehyde or 2% FCS/PBS with 2 μg/ml of propidium iodide to exclude dead cells. Data from stained cell samples were acquired on a FACSCalibur and CellQuest software (Becton Dickinson, Mountain View, CA) and were analyzed with WinList 2.01 (Verity Software House, La Jolla, CA) and WinMDI 2.0 (Trotter@scripps.edu) software programs.

FO and MZ cells were separated by cell sorting using anti-CD23-FITC and anti-CD21-PE Abs as previously described (12). Briefly, single-cell suspensions were made from five or six mouse spleens. T cells were removed from RBC-depleted spleen cells by treatment with anti-Thy1.2 and anti-CD4 Abs and rabbit complement (Cedarlane Laboratories, Hornby, Canada). Viable cells were recovered by centrifugation over a Lympholyte M gradient (Cedarlane Laboratories) at 900 × g at 4°C and were stained with anti-CD23-FITC and anti-CD21-PE as described above. MZ and FO B cells were sorted based on their differential expression of CD21 and CD23 using a FACSStar Plus (Becton Dickinson). When sorting T cells for the Ag presentation assays, BALB/c lymph nodes were used for the single-cell suspension and were stained with B220-FITC and CD43-PE. To control for presentation assays, BALB/c lymph nodes were used for the single-cell suspension and were stained with B220-FITC and CD43-PE. To control, 96-well plates at 5 × 10⁵ cells/ml in 200 μl of medium containing either LPS 20 μg/ml or anti-CD40 Ab (10 μg/ml). Lymphocyte proliferation assays

Sorted FO and MZ B cells were loaded with CellTracker Green CMFDA (Molecular Probes, Eugene, OR) and rhodamine isothiocyanate goat anti-mouse IgM (20 μg/ml; Southern Biotechnology Associates), polyclonal goat anti-rabbit F(ab’)2 anti-IgM (20 μg/ml), anti-CD40 (PGK45, 100 μg/ml, a gift from Dr. Jan Anderson, Basel Institute Immunology, Basel, Switzerland), and IL-4 (100 U/ml) were added in different combinations to the plated cells. IL-4 was obtained from the supernatant of P3 × 63Ag8.653 cells transfected with the appropriate expression constructs. Cells were incubated for 48 h at 37°C in the presence of 10% CO₂. Four hours after the addition of IL-4, cells were washed, incubated with 1 μg/ml of [³H]thymidine was added to each well. Cells were harvested on Filter MATs (Skatron Instruments, Sterling, VA), and [³H]thymidine incorporation was measured in a scintillation counter (Wallac, Gaithersburg, MD).

Cell culture

Cells were sorted as described above and were plated in 0.2–1 ml at a total density of 5 × 10⁶/ml in flat-well (24, 48, or 96 wells, depending on volume) tissue culture plates (Costar). Cells were cultured separately or in a combination with anti-CD40 (10 μg/ml), LPS (20 μg/ml), anti-IgM (20 μg/ml), and IL-4 (100 U/ml) for 1–4 days. At the end of the incubation, the cells were isolated, washed, and stained with the appropriate Abs to determine changes in the expression of cell surface Ags or were used for cytospin preparations.

Immunofluorescence analysis of tissues sections and cytospins

Spleens embedded in OCT compound (Lab-Tek Products, Napeville, IL) were flash-frozen in liquid nitrogen. Frozen sections were cut, air-dried, fixed in ice-cold acetone, blocked with normal horse serum, and stained with MOMA-1-rat IgG2a κ, a gift from Dr. Georg Kraal, specific for mouse splenic metaphilic macrophages) developed with goat anti-rat IgG-biotin and followed by a third layer of strepavidin-AMCA (Vector Laboratories, Burlington, CA). The sections were washed and then stained with a mixture of FITC rat anti-mouse CD5 (clone 53-7.3, 10 μg/ml; PharMingen) and rhodamine isothiocyanate goat anti-mouse IgM (2 μg/ml; Southern Biotechnology Associates). Cytospins were fixed in alcohol for 30 min at 20°C and stained with rhodamine isothiocyanate goat anti-mouse IgM (2 μg/ml; Southern Biotechnology Associates). Sections and cytospins were washed and mounted in Fluoromount G (Southern Biotechnology Associates) and viewed with a Leica/Leitz DMRB fluorescence microscope equipped with appropriate filter cubes (Chromatechnology, Battleboro, VT). Images were acquired with a C5810 series digital color camera (Hamamatsu Photonic System, Bridgewater, NJ). Images were processed with Adobe PhotoShop and IP LAB Spectrum software (Signal Analytics Software, Vienna, VA).

Ig secretion by mature B cell subsets

MZ and FO B cells were sorted based on their differential expression of CD21 and CD23 as described above and were plated in 96-well flat-bottom plates (Costar) at 1 × 10⁶ cells in 200 μl with 20 μg/ml LPS. Supernatants were harvested at each of three time points (3, 7, or 10 days). Igs secreted into the supernatant were detected by ELISA. Briefly, 96-well plates were coated with purified goat anti-mouse IgM (1 μg/ml; Southern Biotechnology Associates) overnight at 4°C. The plates were washed and blocked with 1% BSA in PBS for 1 h at room temperature. Supernatants were diluted and added to the plates along with titrated purified mouse IgM 5-Chloromethylfluorescein diacetate (CMFDA) labeling of B cells

Sorted FO and MZ B cells were loaded with CellTracker Green CMFDA (Molecular Probes, Eugene, OR) according to the manufacturer’s recommendations. In brief, sorted B cells were washed and resuspended at 10⁶ cells/ml in serum-free RPMI 1640 with 1.25 μM CMFDA and were incubated for 30 min at 37°C. Cells were washed and incubated for another 30 min at 37°C in serum-free RPMI 1640, washed again, and plated in 96-well plates at 5 × 10⁵ cells/ml in 200 μl of medium containing either LPS 20 μg/ml or anti-CD40 Ab (10 μg/ml).
MARGINAL ZONE B CELLS AS EARLY EFFECTOR CELLS

FIGURE 1. Heavy chain TG mice have a large MZ population that develops independently of T cells. Spleen sections and FACS profiles of C57BL/6, 81X-C57BL/6, MD2-C57BL/6, 81X TCR T-C57BL/6 (TCR targeted)-C57BL/6, and TCR T-C57BL/6 are shown. Sections were stained for B cells (anti-IgM, red), T cells (anti-CD5, green), and marginal sinus metallophilic macrophages (anti-MOMA-1, blue; original magnification, ×100). Note the lack of CD5-positive T cells in the TCR T mice. Flow cytometry was performed on spleen suspensions stained with anti-CD23 and anti-CD21 Abs. FACS profiles were obtained by gating on lymphoid cells, and the percentages of FO and MZ cells were calculated from the lymphoid gate. Profiles are representative of at least five adult mice of each genotype.

Results

Generation of CD21highCD23low B cells (MZ) is T cell independent

We have used the differential expression of the CD23 and CD21 surface Ags by MZ and FO B cells to identify these mature subsets in the mouse spleen. MZ B cells express high levels of CD21 and low levels of CD21, while FO B cells express CD21 and moderate levels of CD21 (12, 13, 36). In adult C57BL/6 mice, MZ B cells comprise approximately 5% of the lymphocytes (Fig. 1). However, in heavy chain VH81X TG (81X) mice (37), the MZ is almost 3 times higher proportionally than that in littermate (LM) control mice (81X TG, 15.1 ± 1.3%; LM, 6 ± 0.9%; n = 5). This increased percentage of MZ cells is relative and is due to an overall decrease in the absolute numbers of FO B cells (3.4 ± 0.25 vs 10.1 ± 1.5 × 10^6/spleen, respectively, in TG and LM; n = 5). In contrast, the absolute number of MZ B cells is remarkably conserved between TG and LM (1.85 ± 0.1 and 1.9 ± 0.1 × 10^6/spleen; n = 5). Topographic evidence of smaller follicles and larger MZ is observed in spleen sections stained to display the MZ in heavy chain TG mice, in 81X mice crossed to the TCR TCR T mice. Similar results.

TG and LM MZ B cells have similar functional properties

The increased percentage of MZ B cells in Ig heavy chain TG mouse models compared with normal mice permits the isolation of greater numbers of these cells and facilitates subsequent functional analyses. However, before using these mice as a source of MZ B cells, we first determined whether their ability to respond differently from FO B cells in vitro (12) was preserved. As shown in Fig. 2A, TG MZ B cells isolated from mice that either have (81X) or do not have T cells (81X TCR T) share functional characteristics similar to those reported previously for MZ B cells from normal BALB/c mice (12) responding more vigorously to CD40 (40,471 ± 4,325 cpm) and to LPS stimulation (228,959 ± 2,540 cpm) compared with FO B cells (7,225 ± 640, 138,416 ± 1,765 cpm, respectively). Similar to normal mice, TG FO B cells (47,359 ± 5,140 cpm) proliferate better in response to BCR cross-linking (Fig. 2A) than do MZ B cells (391 ± 130 cpm), which are very sensitive to BCR ligation and die by apoptosis (12). Due to the fact that a similar increase in the percentage of MZ B cells is observed when 81X TG is crossed onto a JH knockout background (39) (data not shown), cells derived from either 81X-C57BL/6 or 81X-JH T mice were used for functional experiments and gave similar results.

By following the decrease in fluorescence of CMFDA-labeled purified B cells with the number of cell cycles, we observed that MZ cells begin to divide more quickly when stimulated by CD40 ligation or LPS (Fig. 2B). The relative decrease in CMFDA levels also indicated that MZ divide more than FO B cells within the first 48 h of culture with anti-CD40 Abs (1.5-fold) or LPS (2- to 3-fold). By 4 days, the difference is even more distinct for anti-CD40 stimulation, while for LPS the MZ cultures are overgrown,
but surviving MZ and FO cells have gone through similar numbers of cycles.

**MZ B cells are not activated but can be quickly stimulated to up-regulate T cell costimulatory molecules**

Since MZ B cells divide vigorously in response to stimuli, it was of interest to determine whether these cells constitutively express surface Ags that would indicate their readiness to collaborate with T cells and engage in an immune response. MZ cells are not activated per se, since they do not express increased levels of typical activation markers, including CD69, CD25, MHC class I, MHC class II, and CD95, compared with FO B cells (data not shown) (40, 41). However, these cells do express surface Ags characteristic of a memory phenotype indicative of a history of prior Ag encounter. In contrast to FO B cells, MZ B cells express increased levels of CD62L and IgD, but have increased levels of CD44 (data not shown) (41). Moreover, the basal level of the costimulatory molecules B7.1 and B7.2 on MZ B cells is higher than that on FO B cells (Fig. 3A). These data further support the hypothesis that MZ B cells have been previously primed, and together with their ability to rapidly enter the cell cycle, suggest that these B cells are potential candidates to provide T cell costimulation.

Higher constitutive levels of B7.1 and B7.2 on MZ B cells suggested that MZ B cells might further increase the surface expression of these molecules after stimulation. Indeed, there was up-regulated expression of B7.2, but not B7.1, in sorted purified cells activated with anti-µ Abs for 24 h, an increase that was higher for MZ than for FO B cells (Fig. 3B). Stimulation with IL-4, LPS, or anti-CD40 also increased the expression of B7.2 on both FO and MZ B cells, with the MZ B cells always expressing higher levels. In addition, B7.1 expression was amplified on MZ B cells to a greater extent than on FO B cells when stimulated with either LPS or anti-CD40 Abs. The expression of other activation Ags was also analyzed after stimulation with either anti-CD40 or LPS, and similar up-regulation of CD25 and CD69 was observed on both MZ and FO B cells, although levels of CD25 on MZ B cells were slightly higher after LPS stimulation (Fig. 3C).

We also determined the kinetics of B7.1 and B7.2 expression on sort-purified FO and MZ B cells cultured with anti-CD40 (10 µg/ml), anti-IgM (20 µg/ml), or LPS (20 µg/ml) or alone. At intervals, cells were isolated and stained to determine the relative mean fluorescence intensity (MFI) of each Ag expressed on FO cells. One representative experiment of 10 is shown.

**FIGURE 2.** TG MZ B cells display functional characteristics similar to those of MZ B cells from normal mice. A, Proliferation assays of TG MZ and FO B cells from 81X TG and 81X TCR knockout mice with anti-CD40 (10 µg/ml), LPS (20 µg/ml), and anti-IgM (20 µg/ml). B, MZ cells rapidly enter into cell cycle after LPS and CD40 stimulation. MZ and FO B cells from 81X TG mice were loaded with CMFDA (see Materials and Methods) and stimulated in culture with LPS or anti-CD40. The relative decrease in CMFDA levels indicates that MZ divide more than FO B cells within the first 48 h of culture with anti-CD40 Abs (1.5-fold) or LPS (2- to 3-fold). By 4 days, the difference is more distinct for anti-CD40 stimulation, while for LPS the MZ cultures are overgrown, but surviving MZ and FO cells have gone through similar numbers of cycles. A representative experiment of three is shown.

**FIGURE 3.** MZ B cells express higher levels of T cell cosignaling molecules that are up-regulated to higher levels after in vitro stimulation for 24 h. A, Splenocytes from 81X TG mice were stained for CD21 and CD23, and basal levels of B7.1 or B7.2 are shown for MZ and FO populations. B and C, Sorted FO and MZ B cells from 81X TG mice were cultured with anti-IgM (20 µg/ml), IL-4 (100 U/ml), LPS (20 µg/ml), and anti-CD40 (10 µg/ml) for 24 h and stained for B7.1 and B7.2 (B) or for CD25 and CD69 (C) expression. The vertical lines indicate the MFI of each Ag expressed on FO cells. One representative experiment of 10 is shown.
significantly increased on FO, but not MZ, cells over that observed following addition of the individual stimuli (Fig. 4B). By contrast, B7.1 expression was further up-regulated by the combined stimuli on MZ cells. The dramatic increase in B7.1 on FO cells after LPS plus anti-CD40 treatment is of interest, since both agents showed a minimal effect when added alone. All these data predict a complex picture in which the B cell subsets’ ability to provide T cell costimuli will depend on the nature, timing, dose, and combination of signals that the B cell received.

Monoclonal and polyclonal MZ and FO B cells have similar functional characteristics

All previous experiments used a polyclonal population of B cells from normal or Ig heavy chain TG mice. Since it was possible that the results reported in the above experiments were influenced by certain B cell clones in the mixture, we determined whether a monoclonal population of B cells with either FO (CD21int CD23 high) or MZ (CD21 high CD23 low) phenotype from the anti-HEL TG mice MD4 had similar characteristics (44, 45). As shown in Fig. 4C, B7.1 and B7.2 expression is higher on MZ than on FO B cells 24 h after incubation with LPS. However, the differences between MZ and FO B7 up-regulation were slightly smaller than those observed with a polyclonal B cell population and faded by 72 h, indicating that individual clones might differ in their ability to provide B7 cosignals (Figs. 3B and 4C). Also, similar to TG mice in which B cells contain only a common heavy chain, MD4 MZ (14,754 ± 2,760 cpm) proliferate better than FO B cells (386 ± 190 cpm) when stimulated with anti-CD40 Abs, while FO (12,380 ± 870 cpm) out-proliferate MZ B cells (1,622 ± 280 cpm) when treated with anti-IgM Abs. The ability to proliferate in response to LPS still follows the same pattern, with MZ cells cycling better than FO B cells, although the differences between these two subsets are smaller than those seen with B cells from normal or heavy chain TG mice. These data show that independently generated B cell clones with identical receptors are heterogeneous with respect to different stimuli (anti-IgM, anti-CD40) depending on their FO or MZ phenotypes. This functional heterogeneity indicates that unknown stochastic selective events influence the fate of a B cell depending on whether it is present in a certain microenvironment and not another at a particular time point in its life span.

MZ B cells can function as potent APCs

Since MZ B cells express high basal levels of B7.1 and B7.2, which are rapidly up-regulated within 8–24 h, we next analyzed the capacity of FO and MZ B cells to present Ag and stimulate alloreactive T cells. Purified FO and MZ B cells, when stimulated with either LPS or anti-CD40 Abs for 8 h, increased their levels of B7.1, B7.2, MHC class I, and MHC class II molecules (Fig. 4A and data not shown). Stimulated or unstimulated B cells from 81X heavy chain TG C57BL/6 mice were irradiated and mixed with sort purified BALB/c T cells. As shown in Fig. 5A, purified unstimulated B cells were unable to induce T cell proliferation. In contrast, LPS- or anti-CD40-stimulated MZ, but not FO, B cells induced vigorous T cell proliferation. T cells stimulated with 2.2 ×

FIGURE 4. A, Zero to 48-h kinetics of B7.1 and B7.2 up-regulation on MZ and FO B cells. Sorted MZ and FO cells were stimulated as follows and were stained for B7 expression at the indicated time points: △, control FO; ●, control MZ; □, anti-CD40 FO; ■, anti-CD40 MZ; ○, LPS FO; ●, LPS MZ; ◊, anti-IgM-FO; ▼, anti-IgM-MZ. Results represent one of three similar experiments. B, Combinations of mitogens are even more potent in up-regulating B7 molecules. Sorted FO and MZ B cells from 81X TG mice were stimulated with LPS (thin line), anti-CD40, or IL-4 (normal line) or with a combination of either LPS plus anti-CD40 or LPS plus IL-4 (dotted line), and their B7 expression was assayed at 24 h by flow cytometry. Results represent one of three similar experiments. C, Homogeneous populations of MZ and FO B cells with the same specificity (anti-HEL) have different functional properties. Sorted FO and MZ B cells from MD4 anti-HEL TG mice were cultured with LPS (20 μg/ml) and stained for B7 at 24 and 72 h (left) or were assayed for proliferation with anti-CD40 (10 μg/ml) and anti-IgM (20 μg/ml; middle) or LPS (20 μg/ml; right). Results represent one of three similar experiments.
10^5 LPS-activated MZ B cells proliferated much more (132,486 ± 9,915 cpm) than T cells incubated with LPS-activated FO B cells (31,068 ± 4,390 cpm). Similarly, T cells stimulated with 1.1 × 10^5 MZ B cells that had been treated with anti-CD40 Abs (55,024 ± 5,870 cpm) proliferated better than T cells stimulated with their FO counterparts (3,849 ± 290 cpm). Thus, MZ, but not FO, B cells when activated by LPS or anti-CD40 for a brief time can up-regulate key costimulatory molecules on the cell surface that help to induce allogeneic T cell proliferation. To determine whether combinations of stimuli and a longer activation time will enable FO to present Ags as well as MZ cells, a similar experiment was performed using a mixture of LPS and anti-CD40 to stimulate the sorted B lymphocyte populations for 8 or 24 h. Although at 8 h the LPS-stimulated or LPS- and anti-CD40-stimulated MZ cells presented alloantigen much better than FO cells, by 24 h LPS- and anti-CD40-activated FO B cells induced T cell proliferation comparable to that in similarly treated MZ cells (Fig. 5B). This correlated with the equivalent level of B7 expression achieved on MZ and FO cells after stimulation with LPS and anti-CD40 in combination but not with each stimulus alone (Fig. 4B).

MZ B cells are highly sensitive to LPS stimulation

Although we have clearly shown that MZ B cells express activation molecules that would potentially enable them to participate in B-T cell interactions, we also studied their involvement in T-independent responses, which is another function potentially associated with these B cells. Although MZ B cells overall are better LPS responders than FO B cells, we next investigated this effect at limiting mitogen concentrations. As already shown, MZ B cells proliferated about twice as much as FO B cells (297,799 ± 7,240 vs 154,180 ± 4,650 cpm) when stimulated with 20 μg/ml LPS. Remarkably, a similar degree of MZ B cell proliferation was seen even with a 10-fold lower LPS concentration (297,799 ± 7,240 cpm at 20 μg/ml vs 292,379 ± 34,010 cpm at 2 μg/ml LPS), while FO B cells showed very little response at this dose (Fig. 6A). Even at 0.2 μg/ml LPS stimulation, MZ B cells proliferated (28,053 ± 1,450 cpm), while FO B cells were totally unresponsive. A kinetic study of LPS (20 μg/ml)-induced cell division showed a faster peak response for MZ cells (48 h) that had decreased by 72 h compared with that for FO cells (Fig. 6B).

To address whether this sensitivity to LPS extended to their ability to differentiate into mature Ab-forming cells, sorted FO and MZ B cells were stimulated with either LPS or anti-CD40 and assessed for their expression of syndecan-1, a surface Ag that is up-regulated on plasmablasts (46). In Fig. 6C it can be seen that some MZ B cells begin to express syndecan within 24 h after LPS treatment, while FO B cells are still negative. By day 3 both MZ and FO B cells have subpopulations expressing syndecan, but compared with FO, MZ has a greater number of very bright syndecan cells. Similar to human tonsillar memory B cells (47), sorted MZ and FO B cells treated with anti-CD40 do not express syndecan-1 (data not shown). In addition to syndecan expression, MZ B cells enlarge very rapidly to form blasts within 24 h, and by day 3 mature to plasma cells containing high levels of cytoplasmic Ig compared with FO B cells-derived blasts (Fig. 6D). Furthermore, MZ B cells from both 81X and MD4 TG mice secrete significantly greater amounts of IgM in a shorter period of time than FO B cells after in vitro LPS stimulation (Table I). These data show that MZ

**FIGURE 5.** Activated MZ B cells function as APCs for alloreactive T cells. Sorted FO and MZ B cells from 81X C57Bl/6 mice were treated in vitro for 8 or 24 h with LPS (20 μg/ml), anti-CD40 (10 μg/ml; A and B), or a mixture of the two (B); washed; irradiated; and added in 2-fold dilutions together with sorted BALB/c lymph node T cells. MZ present alloantigen better than FO cells after short stimulation with only one of the stimuli, while FO cells become effective APCs only after treatment with a combination of both agents. One of five similar experiments is shown.
B cells differentiate into mature plasma cells and secrete large quantities of Abs in response to LPS stimulation.

Discussion

The findings reported in this paper establish a clear functional heterogeneity in the compartment of long-lived splenic B lymphocytes. CD21<sup>hi</sup>IgM<sup>hi</sup> B cells enriched in the MZ are not postgerminal center B cells, since they develop independently of T cells (Figs. 1 and 2) (28). They have the ability to perform effector functions after brief periods of in vitro activation and either become potent APCs for T cells through up-regulation and expression of B7 costimulatory molecules (Figs. 3–5) or differentiate into plasma cells in response to very low doses of polyclonal mitogens (Fig. 6). These findings raise interesting questions about the role of these B cells and their functionality in the course of in vivo immune responses.

It is well documented that besides long-lived B cells, the MZ of the spleen contains several other cell types potentially involved in the early stages of immune responses. MZ and metallophilic macrophages are able to scavenge Ags from the blood, and dendritic cells transport them to the T cell zones in the first few hours after antigenic exposure (48–51) (F. Martin and J. F. Kearney, unpublished observations). MZ B cells also migrate from their normal sites very early after in vivo immunization, with some of them traveling to the T cell zone and subsequently to the B cell follicles.

Table I. LPS-stimulated marginal zone B cells secrete large amounts of IgM earlier than follicular transgenic B cells

<table>
<thead>
<tr>
<th>Mice</th>
<th>Days in Culture</th>
<th>FO (µg/ml)</th>
<th>MZ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>81X JH T</td>
<td>3</td>
<td>0.23 ± 0.03</td>
<td>15.19 ± 4.2</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>45.06 ± 15.2</td>
<td>97.19 ± 17.35</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>100.49 ± 34.97</td>
<td>192.62 ± 71.2</td>
</tr>
<tr>
<td>MD4</td>
<td>3</td>
<td>0.033 ± 0.002</td>
<td>3.12 ± 0.38</td>
</tr>
</tbody>
</table>

FIGURE 6. T-independent polyclonal activators are much more potent for MZ than for FO B cells. FO and MZ sorted B cells from 81X TG mice were assayed for proliferation in response to LPS (decreasing doses: 20, 2, and 0.2 µg/ml (A); increasing time periods: 24, 48, and 72 h (B)) and for up-regulation of syndecan-1 after LPS (20 µg/ml) stimulation (C). Note that cells expressing very high levels of syndecan-1 are much more frequent in the MZ than in the FO population on day 3. Increased cell size and accumulation of cytoplasmic IgM are observed after LPS stimulation (20 µg/ml; original magnification, ×250; D). One of five similar experiments is shown.
It is very likely that the CD24high cells contained a mix of precursors involved in primary and secondary antigenic encounters, leading to the hypothesis that there are different B cell precursors for plasma cells, while the CD24low cells are basically follicular B cells (12, 41), while the CD24low cells are basically follicular B lymphocytes. The events of in vitro B cell activation that we describe here are also mirrored in vivo (F. Martin, A. M. Oliver, and J. F. Kearney, manuscript in preparation). The early difference in generating effector cells associated with FO and MZ would not be dependent on germinal center formation, they exhibit even greater similarity to mouse MZ B cells.

The ability of B cells to up-regulate both B7.1 and B7.2 costimulatory molecules has been associated with polyclonal mitogens (LPS), various cytokines, anti-CD40, and, recently, anti-CD21 and anti-CD19 cross-linking signals (59–61). In contrast, anti-BCR signals, either through Ag or anti-IgM Abs, preferentially up-regulate B7.2 (42). The differences in these events between MZ and FO B cells is not simply one of kinetics, since FO cells never reach the levels of B7 expressed by MZ B cells (Fig. 4). These data suggest that there are intrinsic differences within these populations regarding B7 expression following activation, and that the resulting activated MZ and FO effector B cells differ in their ability to use these pathways of costimulation. When these B cell subpopulations are stimulated with a mixture of two different agents (Fig. 4B, LPS plus CD40 or IL-4), for some combinations there is a dramatic increase in B7 molecules compared with the use of each stimulus alone. As a result, the levels of B7.2 expression on FO B cells become comparable to those on MZ B cells, which require only one of the stimuli alone to be maximally stimulated. The attainment of similar levels of expression of B7.2 by both populations is reflected by their equivalent ability to present allografts. These findings predict that depending on the type of in vivo Ag stimulation, MZ or both subsets will participate in Ag presentation. These results also suggest that there is a qualitative difference in the regulation of B7.2 and subsequently of the co-stimulatory activity between FO and MZ B cell subsets. Recently, constitutive expression of the B7.2 molecule has been shown to break the inability of tolerant FO B cells to become activated (62). The higher levels of B7 constitutive expression and its rapid up-regulation on MZ B cells raise questions about mechanisms that control these B cells to avoid autoimmunity and suggest that their repertoire selection and activation are stringently controlled in a healthy organism.

In humans, chronic activation is considered one of the potential steps involved in the induction of lymphomas; in particular for MZ lymphomas, molecular studies support the concept that they are derived from a postfollicular, postgerminal center or a memory B cell (29). In NFS.1+ mice, used as a model of lymphomagenesis, >40% of the spontaneous developing lymphomas derive from the MZ and, surprisingly, express low levels of CD5 and B220, a characteristic of the B1 subset of mouse B lymphocytes (63). In normal mice, splenic B1 cells are infrequent (13) and have not been assayed functionally, but the similarities between peritoneal B1 cells and MZ B cells (lack of proliferation to anti-IgM, larger size, ability to rapidly secrete Abs) suggest some kind of link, if not in their developmental origins at least in function. However, the selection and developmental expansion of B1 cells, in addition to being dependent on costimulation, are probably also linked to the specificity of their receptors (64–67). Since B1 cells are depleted in several Ig TG mouse models that have normal or increased MZ populations (MD4, 81X), these B cells might not have receptors with permissive specificities that select them into the B1 compartment (16, 31).

Recently, several groups have reported that the level of expression of CD1 on MZ B cells is high and comparable to that found on dendritic cells, but there are no data yet on the functional significance of this finding (68, 69). NK T cells, which respond to Ags presented by CD1 molecules, are thought to be early participants in an immune response. The evidence we have presented indicates
that MZ B cells show phenotypic and functional characteristics that would indicate that they are also early responders to antigenic challenge. We propose that certain nonprotein components of bacteri cell walls (Gram-negative derived mitigens such as LPS, newly described mitigens from Gram-positive bacteria (70), and glycolipids presented by CD1 (71)) would be targeted, in the first stage of an immune response, to this special subset of MZ B cells and prepare them for the subsequent phases of plasma cell generation and B-T cell interaction.

Based on the strategic localization of MZ B cells in the spleen and the findings described in this paper, we propose that MZ B cells constitute the first cohort of B cells that respond and participate in an immune response, in particular in conditions of limited T cell help, low concentration of TI mitogens, or low numbers of blood-borne particulate Ags reaching the spleen.

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