Murine B1 B Cells Require IL-5 for Optimal T Cell-Dependent Activation

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Murine B1 B Cells Require IL-5 for Optimal T Cell-Dependent Activation

Loren D. Erickson, Teresa M. Foy, and Thomas J. Waldschmidt

T cell-dependent (TD) activation of B cells requires both cell surface and cytokine signals delivered by Ag-specific Th cells (reviewed in Ref. 1). The process of TD activation is initiated after recognition of MHC class II:peptide on the surface of B cells by Th cells. Engagement of this complex by the TCR induces expression of CD40 ligand (CD40L), a homotrimer that binds CD40 on the B cell, and invokes a series of events critical for progression into the activation sequence. Further stimulation of Th cells by the activated B cells leads to production of cytokines, the final set of signals necessary for B cell proliferation and differentiation. To date, evidence suggests that the CD40-CD40L interaction is central to the process of TD B cell activation, and is required not only for cell expansion and differentiation, but for germinal center and memory cell formation as well (reviewed in Ref. 2). Although substantial progress has been made in understanding the role of CD40 in B cell activation, most reports examining T-B interaction in the mouse have used conventional splenic B cells. The question thus remains as to whether all mature B cell subsets respond to Th cells in a manner equivalent to that understood for conventional B cells.

The B cell compartment in the mouse is heterogeneous and can be separated into distinct subsets using a number of parameters. B cell subsets can be distinguished by stage of maturation, surface phenotype, anatomical localization, and state of activation (reviewed in Ref. 3). Our laboratory has previously defined four mature murine B cell subsets based upon expression of the intermediate affinity IgE FcR, designated CD23 (4–7). In the spleen, CD23 is present on conventional or follicular B cells, whereas marginal zone B cells are low to negative for this receptor. Similarly, conventional B2 B cells in the peritoneum are CD23+, whereas the B1 B cell population (both B1a and B1b) is CD23–.

Using T cell-independent (TI) stimuli, a number of investigators have compared the functional attributes of purified splenic marginal zone and follicular B cells. When stimulating cells with either soluble anti-IgM (5, 8, 9) or dextran-conjugated anti-Ig (8), follicular B cells respond by proliferating, whereas marginal zone B cells respond poorly or not at all. The lack of response in the marginal zone subset reflects their rapid entry into the apoptotic pathway following surface Ig cross-linking (9). Both populations proliferate strongly in response to LPS (5, 8, 9). Additional studies comparing the ability of marginal zone and follicular B cells to differentiate and isotype switch found both populations fully capable of producing IgM and switching to downstream isotypes when cultured with either LPS plus cytokines (5, 8–10) or dextran-conjugated anti-Ig plus cytokines (8).

A number of groups have also examined the functional characteristics of purified peritoneal B1 and B2 B cells when treated with TI stimuli. B2 B cells, phenotypic counterparts of the follicular B cell subset, proliferate in response to anti-IgM and LPS (5, 11–13). Similar to marginal zone B cells, B1 B cells respond vigorously to LPS, but are unresponsive when cultured with anti-IgM (5, 11–13). The inability to proliferate upon surface Ig cross-linking may be due to their altered cytoplasmic status. Detailed studies by Rothstein and coworkers (11, 14–16) have demonstrated B1 B cells to exhibit constitutively elevated levels of protein kinase C and nuclear activated STAT3, and to have decreased activation of phospholipase C-γ2 and NF-κB after anti-IgM treatment. When assessing the ability of B1 and B2 B cells to differentiate and isotype switch upon culture with LPS plus cytokines, B2 B cells...
were found to produce high levels of IgM, IgG, and IgE, while B1 B cells secreted only high levels of IgM (5, 10, 17). The latter subset produced low levels of IgG and little or no IgE (5, 10, 17).

Given the various response patterns to TI stimuli, the question arises as to whether conventional (follicular and B2), marginal zone, and B1 B cells also exhibit differences when undergoing a TD challenge. A limited number of studies have examined the capacity of purified B cell subsets to respond to either Th cells or CD40 agonists, with mixed results. Studies by Snapper et al. (8) found marginal zone and follicular B cells to proliferate and differentiate equivalently when cultured with an activated Th2 clone. In assessing the capacity of splenic B cell subsets to proliferate when cultured with an anti-CD40 mAb, Kearney and colleagues (9) reported marginal zone B cells to exhibit a greater response compared with follicular B cells. A number of groups investigating the ability of purified B1 and B2 B cells to expand when treated with either anti-CD40 mAb or activated Th2 cells found modest or no differences between the two subsets (11–13, 18). However, in exploring the differentiative potential of B1 and B2 B cells, Tarlinton et al. (17) found a marked difference in the ability of these populations to produce IgM after stimulation with CD40L-transfected fibroblasts plus cytokines. At present, therefore, a clear understanding as to the ability of the various B cell subsets to respond to TD activation is lacking. This study thus assessed the activation potential of all four CD23-defined mature B cell subsets when treated with either soluble CD40L in the presence of cytokines or activated Th2 clones. In addition to proliferation, the capacity of the various subsets to differentiate and isotype switch was measured. The results demonstrate marginal zone, follicular, and peri-toneal B2 B cells to respond in an equivalent manner to TD-directed activation, and to require a similar set of signals to undergo proliferation, differentiation, and isotype switching. In contrast, B1 B cells exhibit different activation requirements when stimulated with CD40L or Th2 cells. Although capable of expanding and differentiating under TD conditions, B1 B cells require IL-5 for optimal responses.

Materials and Methods

Mice

Female BALB/c, C3H/HeN, C57BL/6, and DBA/2 mice, 8–10 wk old, were purchased from Harlan Sprague-Dawley (Indianapolis, IN). CD40L-deficient (129 × C57BL/6) × C57BL/6 mice were provided by Dr. Jacques Peschon (Immunex, Seattle, WA). All mice were maintained in the specific pathogen-free animal facility at The University of Iowa (Iowa City, IA).

Flow cytometric analysis

The following mAbs were used for flow cytometric analysis and sort purification of B cell subsets: 6B2, a rat IgG anti-mouse B220; B23A4, a rat IgG anti-mouse CD23; and 2.4G2, a rat IgG anti-mouse CD16/32. The mAbs were semipurified by ammonium sulfate precipitation from serum-free HB101 (Irvine Scientific, Santa Ana, CA) hybridoma culture supernatants. mAbs were conjugated with FITC and PE using standard protocols. Texas Red-conjugated goat Ab specific for IgM (μ-chain) was purchased from Southern Biotechnology Associates (Birmingham, AL), and PE-conjugated rat anti-mouse CD5 mAb (53-7.3) was obtained from PharMingen (San Diego, CA). Biotin-conjugated IC10 (19), a rat IgG anti-mouse CD40 mAb, was provided by DNAx (Palo Alto, CA). Chromatographically purified rat and goat IgG (Jackson ImmunoResearch, West Grove, PA) were used as isotype controls. FITC-Ultra-avidin was purchased from Leinco Technologies (St. Louis, MO). For flow-cytometric analysis, 5 × 10^5 cells were suspended in staining buffer consisting of 5% fetal bovine serum, 25 μg/ml 2.4G2 and 10 μl of rat serum were added to each of the cell suspensions. After 20 min, cells were washed and incubated with FITC-avidin. Subsequent to final washing, cells were analyzed on a Becton Dickinson (San Jose, CA) FACS 440 flow cytometer equipped with a primary argon laser, and a rhodamine 6G CR599 dye head laser (Coherent, Palo Alto, CA) pumped by a secondary argon ion laser. Residual dead cells and cell aggregates were excluded by low angle and orthogonal light scatter. FACS data were collected and analyzed using a DECA VAXstation 3200 computer equipped with DEK software (supplied by Wayne Moore, Stanford University, Stanford, CA). Final graphic output was performed with Canvas (Deneba Software, South Miami, FL) software.

IgM and IgG1 ELISA

The following reagents were used for isotype-specific ELISA tests: Rabbit Ab specific for mouse μ-chain (Jackson ImmunoResearch) and b7-6, a monoclonal anti-murine IgM, were used as capture and detection Abs, respectively, for the IgM ELISA; goat Ab specific for mouse IgG1 (Southern Biotechnology Associates) served as both capture and detection Abs for the IgG1 ELISA. IgG1 and IgM from 6-day culture supernatants was quantitated based on a standard curve. All ELISA used a biotin-conjugated detection Ab, followed by alkaline phosphatase streptavidin (Zymed, San Francisco, CA) and subsequent development with phosphate substrate (Sigma). Absorbance was read at a dual wavelength of 405 and 540 nm using a Bio-Tek Instruments EL309 plate reader (Winooski, VT).

B cell preparation and sort purification

Peritoneal cells were obtained by injecting cold sterile BSS into the peritoneal cavity of BALB/c mice, followed by rigorous massaging of the abdomen and sterile extraction of the cell suspension. Spleen cell suspensions isolated from C3H/HeN mice were T depleted using anti-Thy-1.2 Ab (HO13.4) and complement (Pel Freeze, Rogers, AR). Both cell suspensions were spun through Fico-Lite-LM (Atlanta Biologicals, Norcross, GA), the interface collected, and washed with sterile BSS. For sterile sorting, cell suspensions were resuspended in staining buffer consisting of 5% newborn calf serum in BSS at a concentration of 20 × 10^6 cells/ml. Cells were incubated with FITC anti-B220 and PE anti-CD23 for 20 min at 4°C. The 2.4G2 and rat serum were added to the cells as described above. Cells were then washed, resuspended in sterile staining buffer, and sorted on a Coulter (Hialeah, FL) EPICS 753 flow cytometer. Postsort analysis revealed greater than 95% purity in each of the sorted populations.

B cell activation

Enriched or sort-purified populations were washed with sterile BSS and resuspended in medium consisting of RPMI 1640 (Life Technologies, Grand Island, NY) with 10% FCS (HyClone Laboratories), penicillin, streptomycin, L-glutamine, and 2-ME. B cells were cultured at 4 × 10^4 per well in 96-well plates at a 200 μl final volume. The following reagents alone or in combination were added for activation: LPS (Difco, Detroit, MI) at 40 μg/ml; murine rIL-4 (provided by Dr. Charles Maliszewski, Immunex) at 100 U/ml or 1000 U/ml for proliferation and Ig secretion, respectively; murine rIL-5 at 10 ng/ml (R&D Systems, Minneapolis, MN); and soluble rCD40L trimer (provided by Dr. William Fanslow, Immunex) at 200 ng/ml. TRFK 5 (20), a rat IgG anti-mouse IL-5, and MR1, a hamster IgG anti-mouse CD40L (provided by Dr. Randolph Noelle, Dartmouth Medical Center, Lebanon, NH), were used at a concentration of 50 μg/ml. B cell proliferation was measured after 3 days by pulsing with [3H]thymidine during the last 4 h of culture. To quantitate levels of Ig secretion, culture supernatants were collected after 6 days of incubation and tested by isotype-specific ELISA. For activation of B cell subsets with intact Th cells, B cells were seeded at a density of 4 × 10^4 per well in 96-well plates precoated with 145-2C11, a hamster IgG anti-mouse CD3, at 20 μg/ml. T cells were resuspended at 20 × 10^6 per ml in sterile PBS and incubated with mitomycin C (Sigma) at 50 μg/ml for 20 min at 37°C to prevent DNA synthesis. T cells were washed three times with sterile BSS and cocultured with B cells at 4 × 10^5 cells/ml per well (1:1 ratio). Preliminary titration experiments demonstrated 4 × 10^5 B cells and T cells to be optimal for B cell activation.
Th clones

CDC35, an I-A<sup>q</sup>-restricted Th2 clone specific for rabbit γ globulin (21), served as a prototypic Th2 cell line. KO-1, KO-12, KO-18, and WT-25 are H-2<sup>d</sup>-alloreactive Th2 clones generated in our laboratory. KO-1, KO-12, and KO-18 are clones derived from a CD40L-deficient ((129 × C57BL/6) × C57BL/6)F<sub>1</sub>, mouse, and WT-25 was derived from a wild-type C57BL/6 mouse. Briefly, CD40L-deficient or wild-type splenocytes were bulk cultured with irradiated DBA/2 (H-2<sup>d</sup>) splenocytes in the presence of IL-4 and IL-10 (Peprotech, Rocky Hill, NJ). Following 1 wk of culture, the responding cells were plated at limiting dilution and restimulated with DBA/2 splenocytes, cytokines, and 10 μg/ml of the agonistic anti-CD40 mAb IC10. The latter reagent was added to induce appropriate costimulatory activity in the feeder cells. Clonal wells containing CD4 <sup>+</sup> Th cells were expanded, and cytokine secretion profiles were assessed. Upon activation with anti-CD3, KO-1, KO-12, KO-18, and WT-25 secrete >245, 10, 10, and >127 ng/ml of IL-4, respectively, and 23, 12, 7, and 19 ng/ml of IL-10, respectively. Neither IL-2 nor IFN-γ secretion was detected from any of the clones. All activated clones express CD25, whereas only the wild-type clone displays CD40L (data not shown). Clones were maintained by periodic stimulation with irradiated H-2<sup>d</sup> splenocytes and IL-2. T cell clones used in all experiments were harvested 2 wk after their last stimulation. At this time point, T cells were in a resting phase, as measured by morphologic, cytokine, and activation marker analysis (data not shown).

Results

Proliferative response of B cell subsets stimulated with rCD40L

In an effort to study TD activation of B cells in a polyclonal manner, soluble rCD40L trimer in the absence or presence of cytokines, and anti-CD3-activated Th2 clones were used. This study assessed the response of CD23-defined murine splenic and peritoneal B cell subsets (3) to these CD40-directed stimuli. CD23 positive (follicular) and negative (marginal zone) C3H/Hen splenic B cells, and CD23 positive (B2) and negative (B1) BALB/c peritoneal cavity B cells were sort purified after staining with anti-B220 and anti-CD23 mAb. C3H/Hen mice were chosen as a source of splenocytes, as this strain has a high frequency of marginal zone B cells (4, 7). Although the B220<sup>−</sup>CD23<sup>−</sup> splenic subset contains immature as well as marginal zone B cells, the former is only a minor constituent of this population (7). BALB/c mice were used for isolating peritoneal B cell subsets because of the relatively high proportion of B1 B cells (10). Fig. 1 shows representative pre- and postsort contour plots, and indicates the gating strategies used for sorting B220<sup>−</sup>CD23<sup>−</sup> and B220<sup>−</sup>CD23<sup>+</sup> B cell subsets. The first series of experiments examined the proliferative response of sort-purified B cell subsets when stimulated with rCD40L trimer. Sort-purified B cells were cultured for 72 h with rCD40L in the absence or presence of IL-4. B cell proliferation was measured by [3H]thymidine incorporation during the last 4 h of culture. Fig. 2A demonstrates splenic follicular and marginal zone B cells to proliferate when stimulated with soluble rCD40L. Peritoneal B2 B cells (Fig. 2B) likewise proliferate in response to rCD40L. In contrast, B1 B cells weakly respond to rCD40L, showing levels of proliferation ~20–50% of that observed with B2 B cells. The poor ability of B1 B cells to proliferate when stimulated with rCD40L is even more apparent when IL-4 is present. Previous reports have demonstrated an enhancing role of IL-4 in CD40-directed murine B cell stimulation (9, 19, 22–24). This is again observed in the present experiments, in which the combination of rCD40L and IL-4 markedly enhances the levels of proliferation in follicular and marginal zone B cell subsets (Fig. 2A) as well as peritoneal B2 B cells (Fig. 2B). Although addition of IL-4 to B1 B cells stimulated with rCD40L enhances proliferation, the presence of both stimuli consistently results in a response that is still ~20% of that observed with B2 B cells. Thus, when stimulated with rCD40L, splenic follicular and marginal zone as well as peritoneal B2 B cells respond vigorously, while B1 B cells demonstrate a modest response. This indicates that B1 B cells have a suboptimal mitogenic response to CD40 engagement, a deficit not overcome by the addition of IL-4. It is important to note that the altered response of B1 B cells is not due to the Abs used for sort purification, as cells enriched by negative selection respond similarly (data not shown). Also, preliminary experiments determined all B cell subsets to register their greatest response at 72 h, indicating that differential kinetics cannot account for the findings (data not shown).

Proliferative response of B cell subsets cocultured with activated Th2 cells

When stimulated with rCD40L and IL-4, splenic follicular and marginal zone B cells, as well as peritoneal B2 B cells vigorously proliferate, while B1 B cells demonstrate a suboptimal response. Because the use of rCD40L and IL-4 mimics Th2 conditions, we further tested the capacity of the four B cell subsets to proliferate when stimulated with anti-CD3-activated Th2 cells. Therefore, sort-purified B cells were cocultured with mitomycin C-treated CDC35 Th2 cells in the presence of solid-phase anti-CD3 for 72 h. B cell proliferation was assessed via [3H]thymidine incorporation during the last 4 h of culture. Results shown in Fig. 2B demonstrate that similar to stimulation with rCD40L plus IL-4, both splenic B cell subsets (C) and peritoneal B2 B cells (D) strongly proliferate in response to activated CDC35 cells. Interestingly, peritoneal B1 B cells exhibit proliferation equivalent to that observed with B2 B cells (Fig. 2D). This finding is unexpected given the results with rCD40L, and suggests that intact Th2 cells may provide an additional signal required for optimal B1 B cell stimulation. Control cultures set up in the absence of plate-bound anti-CD3 showed the requirement for T cell activation to observe B cell proliferation.
Expression of CD40 on peritoneal B cell subsets

Whereas Th2 cells induce strong proliferation in B1 B cells, rCD40L and IL-4 support only modest expansion. Although the difference in results may rest with an additional signal supplied by the Th cells, it is also possible that B1 B cells express lower levels of CD40, making it more difficult for soluble rCD40L, as opposed to cell surface CD40L, to cluster a sufficient number of CD40 molecules. This possibility was ruled out by simply documenting CD40 expression levels using three-color flow cytometry. Freshly obtained peritoneal B cells were stained with anti-CD40, anti-IgM, and anti-CD5 Abs, followed by analysis. Fig. 3 clearly shows B1a B cells (IgM<sup>high</sup>, CD5<sup>+</sup>; population A), B1b B cells (IgM<sup>high</sup>, CD5<sup>+</sup>; population B), and B2 B cells (IgM<sup>low</sup>, CD5<sup>-</sup>; population C) to exhibit uniform positive staining. Splenic B cells show similar levels of CD40 expression (data not shown). This suggests that an additional factor supplied by Th2 cells accounts for their ability to optimally stimulate B1 B cells.

Ig secretion/switching of B cell subsets stimulated with rCD40L

Previous in vitro studies with murine B cells have demonstrated CD40 agonists to induce Ig secretion, and in the presence of IL-4, isotype switching (23–27). Based on these observations, we sought to examine the ability of sort-purified B cell subsets to undergo Ig secretion and switching when stimulated with rCD40L in the absence or presence of IL-4. Sort-purified B cells were cultured for 6 days with the designated stimuli, and culture supernatants subsequently assayed for IgM and IgG1 using isotype-specific ELISA. Table I lists two experiments and summarizes the levels of Ig secretion from the four CD23-defined B cell populations. The results demonstrate that splenic follicular and marginal zone B cells secrete large amounts of IgM when cultured with rCD40L, and significantly higher levels with the addition of IL-4. Although isotype switching to IgG1 is minimal in cultures with rCD40L alone, follicular and marginal zone B cells exhibit a dramatic rise in secreted IgG1 in parallel cultures containing both rCD40L and IL-4. Peritoneal B2 B cells respond similarly to splenic B cells in that they are fully capable of IgM secretion and switching to IgG1 when cultured under the same conditions. Surprisingly, B1 B cells are also capable of producing Ig levels comparable with the other B cell subsets when cultured with rCD40L in the absence or presence of IL-4. Thus, while B1 B cells proliferate suboptimally compared with follicular, marginal zone, and B2 B cells, they are fully capable of differentiating and switching under the conditions used. Also listed in Table I are results using LPS plus IL-4 as positive control. This combination elicits IgM and IgG1 responses that are similar to those using rCD40L and IL-4.

Ig secretion/switching of peritoneal B cell subsets cocultured with activated Th2 cells

Because B1 B cells are fully capable of differentiation and isotype switching in response to rCD40L and IL-4 (Table I), it was anticipated that this subset would likewise secrete IgM and IgG1 when challenged with activated Th2 cells. Sort-purified peritoneal B1 and B2 B cells were cultured with mitomycin C-treated and anti-CD3-activated CDC35 cells for 6 days. Table II depicts two experiments, which demonstrate both peritoneal B cell subsets to be fully capable of secreting IgM production and switching to IgG1. In data not shown, sort-purified follicular and marginal zone B cells also demonstrate excellent IgM and IgG1 secretion when cultured with activated Th2 cells. Thus, as expected, B1 B cells demonstrate a differentiative potential equal to that of the other subsets. In some experiments, LPS and IL-4 were used as positive control.

Effect of anti-CD40L and anti-IL-5 Abs on Th2-mediated activation of B1 B cells

Should Th2 cells be providing an additional stimulus necessary for optimal B1 B cell activation, the question arises as to whether this signal is a cell surface or secreted factor. It is also important to understand whether this additional factor works in conjunction with or is independent of CD40-CD40L interactions. To distinguish between these possibilities, sort-purified B1 B cells were cultured with CDC35 Th2 cells in anti-CD3-coated wells alone, or...
in the presence of neutralizing Abs to CD40L and/or IL-5. If B1 B cells use stimuli for proliferation that are independent of CD40-CD40L interactions, then blockade of CD40L should still allow for significant expansion. The results of such an experiment are shown in Fig. 4A, and indicate that blockade with anti-CD40L mAb only modestly affects the proliferative response of B1 B cells. The same experiment with conventional B cells results in a marked loss of B cell proliferative activity (data not shown). This suggests that B1 B cell expansion can be driven by Th2-derived factors independently of CD40 ligation. Fig. 4A also shows the blocking effect of anti-IL-5 mAb. An Ab to IL-5 was chosen because previous reports demonstrated B1 B cells to constitutively express IL-5R, and to preferentially respond to this cytokine in vitro (28–32). Fig. 4A illustrates that, similar to CD40L blockade, neutralization of IL-5 results in only a modest inhibition of CDC35-driven B1 B cell proliferation. Thus, although IL-5 may support expansion of the B1 subset, it appears not to be a limiting factor in this system. Of interest, the addition of both anti-CD40L and IL-5 mAbs to Th2-driven B1 B cell cultures results in a marked loss of proliferative activity (Fig. 4A). Taken together, these observations suggest that CD40 ligation and IL-5 are required for optimal B1 B cell expansion. However, if either of these two elements is unavailable, B1 B cells are still capable of proliferating in the presence of activated Th2 cells. This further suggests that CD40 engagement and IL-5 can independently induce B1 B cell proliferation in the presence of another stimulus, albeit to a lesser extent. As demonstrated in Fig. 2, rCD40L and IL-4 are capable of inducing modest levels of B1 B cell expansion, suggesting IL-4 to be this third element.

Capacity of CD40L-deficient Th2 clones to activate B1 B cells

To further explore the possibility that B1 B cells can respond to activated Th2 cells in the absence of CD40-CD40L interactions, sort-purified B1 B cells were cultured with CD40L-deficient Th2 clones in anti-CD3-coated wells. CD40L-deficient H-2^d alloreactive Th2 clones (KO-12, KO-18) were generated from CD40L^-/- splenocytes, as described in Materials and Methods. A wild-type clone (WT-25) was similarly derived from a normal C57BL/6 mouse. As shown in Fig. 4B, purified B1 B cells proliferate optimally in the presence of an activated wild-type Th2 clone. These B1 B cells also expand in the presence of activated CD40L-deficient Th2 clones, although to a reduced extent. In all groups (Fig. 4B), optimal amounts of IL-4 were added to normalize for various secretion levels of this cytokine. Consistent with Fig. 4A, elimination of both CD40L and IL-5, by addition of an anti-IL-5 Ab to wells containing KO-12 and B cells, completely abrogates the response. This set of experiments again indicates that both CD40L and IL-5 are required for optimal B1 B cell expansion. When only one of these signals is present, modest B1 B cell proliferation can still occur if another factor, likely to be IL-4, is present. In data not shown, conventional B cells failed to exhibit any proliferative response when cultured with anti-CD3-treated CD40L-deficient Th2 clones.

Direct effects of IL-4, IL-5, and CD40L on B1 and conventional B cell proliferation

The data with both wild-type and CD40L-deficient clones (Fig. 4) suggest B1 B cells to require IL-5 and CD40 ligation for optimal proliferation. Furthermore, should either be lacking, B1 B cells can still expand in the presumed presence of IL-4. To confirm this hypothesis, sort-purified B1 B cells were cultured with combinations of IL-4 and IL-5 either in the absence or presence of rCD40L. Purified splenic B cells were used as a source of conventional B cells for comparison, with results shown in Fig. 5. As predicted, B1 B cells cultured with IL-4 and IL-5 in the absence of rCD40L proliferate to a modest but significant extent. None of the cytokine combinations induce proliferation in conventional B cells in the absence of CD40 ligation. Upon addition of rCD40L, conventional splenic B cells expand maximally in the presence of IL-4 and suboptimally with IL-5. The converse is true with B1 B cells, in which IL-5 induces optimal proliferation, and similar to Fig. 2B, IL-4 provides only modest co-stimulation. The addition of both IL-4 and IL-5 does not enhance the response of either the conventional or B1 B cell groups. It is thus clear that B1 B cells use IL-5 and CD40L for maximal proliferation during Th2-directed stimulation. Moreover, IL-4 and CD40L, or IL-4 and IL-5 are capable of promoting B1 B cell stimulation, although to a lesser extent. Thus, B1 B cells have several options for undergoing expansion, including a CD40-independent means.

Table I. Capacity of rCD40L to drive differentiation in purified splenic and peritoneal B cell subsets

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* CD23^-/- and CD23^+ B cells were sorted purified from spleen or peritoneum as described in Fig. 1. B cells (4 x 10^6) were cultured in medium alone, 200 ng/ml rCD40L with or without 1000 U/ml IL-4, or 40 μg/ml LPS with IL-4. After 6 days of culture, supernatants were harvested and isotype-specific ELISA was performed as described in Materials and Methods.
Because B1 B cells are capable of proliferating in the absence of CD40-CD40L interactions, the question arises as to their ability to differentiate in a CD40-independent manner. Tables I and II already demonstrate B1 B cells to differentiate and switch comparable with follicular, marginal zone, and B2 B cells. Therefore, sort-purified B1 B cells were incubated for 6 days with mitomycin C-treated wild-type (CDC35 or WT-25) or CD40L-deficient (KO-1, KO-12) Th2 clones in anti-CD3-coated wells, and culture supernatants subsequently assayed for total IgM and IgG1 using isotype-specific ELISA. Optimal concentrations of IL-4 were again added to normalize for possible differences in secreted levels of this cytokine. The results in Table III show that B1 B cells do indeed secrete IgM and isotype switch when cultured with activated CD40L-deficient T cells, indicating their ability to differentiate independent of CD40 ligation. In data not shown, conventional B cells fail to differentiate when similarly cultured with CD40L-deficient Th2 clones.

### Table II. Capacity of Th2 cells to drive differentiation in purified peritoneal B cell subsets

<table>
<thead>
<tr>
<th>Population</th>
<th>Stimulus</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Secreted Ab (ng/ml)</td>
<td>Secreted Ab (ng/ml)</td>
<td></td>
</tr>
<tr>
<td>B2 B cell</td>
<td>IgM</td>
<td>IgG1</td>
<td>IgM</td>
</tr>
<tr>
<td>Medium</td>
<td>165</td>
<td>&lt;1</td>
<td>1</td>
</tr>
<tr>
<td>Th2act + IL-4</td>
<td>30,874</td>
<td>1,863</td>
<td>11,031</td>
</tr>
<tr>
<td>LPS + IL-4</td>
<td>37,299</td>
<td>2,368</td>
<td>ND</td>
</tr>
<tr>
<td>B1 B cell</td>
<td>Medium</td>
<td>167</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Th2act + IL-4</td>
<td>31,674</td>
<td>1,482</td>
<td>11,954</td>
</tr>
<tr>
<td>LPS + IL-4</td>
<td>25,636</td>
<td>997</td>
<td>ND</td>
</tr>
</tbody>
</table>

a CD23+ and CD23− B cells were sorted purified from peritoneum as described in Fig. 1. B cells (4 × 10⁶) were cultured in medium alone, 4 × 10⁶ mitomycin C-treated and anti-CD3-activated CDC35 Th2 cells with 1000 U/ml IL-4, or 40 μg/ml LPS with IL-4. After 6 days of culture, supernatants were harvested and isotype-specific ELISA was performed as described in Materials and Methods. Control experiments testing the capacity of nonactivated (resting) CDC35 Th2 cells to induce Ig secretion in splenic B2 B cells demonstrated 150–430 ng/ml of IgM and no IgG1 after 6 days of culture.

b ND, not done.

### FIGURE 4. Roles of CD40L and IL-5 in Th2-driven B1 B cell proliferation

A total of 4 × 10⁶ sort-purified peritoneal B1 B cells was cultured for 72 h with 4 × 10⁶ mitomycin C-treated and anti-CD3-activated Th2 cells. Where indicated, 50 μg/ml of neutralizing mAbs against CD40L and/or IL-5 was added. In A, B1 B cells were cultured with activated CDC35 Th2 cells. In B, B1 B cells were cultured with activated Th2 clones derived from wild-type (WT-25) and CD40L-deficient (KO-1, KO-12) mice. IL-4 (100 U/ml) was added to all groups in B to normalize for IL-4 production among clones. As a positive control in B, B1 B cells were cultured in the presence of 200 ng/ml rCD40L plus IL-4. Proliferation was measured as described in the legend to Fig. 2. Bars represent means and SDs of quadruplicate wells. Data are representative of two (A) and four (B) independent experiments.

### FIGURE 5. Comparative ability of IL-4 and IL-5 to support proliferation in peritoneal B1 and conventional B cells

A total of 4 × 10⁶ purified B1 cells and T cell-depleted BALB/c spleen cells was cultured 72 h with 100 U/ml IL-4, 10 ng/ml IL-5, or the combination of both in the absence or presence of 200 ng/ml rCD40L. Splenic B cells were used to ensure sufficient numbers of conventional B cells for all groups. Proliferation was measured as described in the legend to Fig. 2. Bars represent means and SDs of quadruplicate wells. Data are representative of three independent experiments.
center reactions, the finding that marginal zone B cells share the same TD activation requirements suggests this latter population to be capable of similar functions. The observation that follicular and marginal zone B cells respond strongly to CD40-dependent stimulation is consistent with the work of Snapper and coworkers (8), who examined differentiation and switching in response to activated Th2 clones, and Oliver et al. (9), who tested the proliferative response to an agonistic anti-CD40 Ab. In the latter study, marginal zone B cells responded quantitatively better than follicular B cells, a result not observed in our experiments (Fig. 2). The reason for the difference is not apparent, although different CD40 agonists and means of enriching for marginal zone B cells were used.

Compared with conventional and marginal zone B cells, B1 B cells proliferate suboptimally when treated with rCD40L or rCD40L and IL-4. The poor response of B1 B cells to CD40 ligation is consistent with the inability of this population to proliferate when cultured with anti-IgM (10–13) or anti-CD38 (13) Abs. The reason for the poor response of B1 B cells is not readily apparent, but may be linked to the altered intracellular status of these cells. Compared with conventional B cells, Rothstein and colleagues (11, 14–16) demonstrated B1 B cells to exhibit constitutively heightened levels of activated protein kinase C and nuclear STAT3, and to have diminished activation of phospholipase C-

### Discussion

The present study represents a first attempt to systematically define the TD activation requirements of all major mature B cell subsets found in the mouse. This is also the first report to assess the functional capacity of murine CD40L−/−CD40+ Th2 cell clones. B cell populations were separated based on CD23 expression, and activated with either rCD40L and cytokines or intact activated Th2 clones. The results demonstrate that sort-purified conventional B cells (follicular or B2 B cells) and splenic marginal zone B cells require both CD40 ligation and IL-4 for optimal expansion, differentiation, and isotype switching. In contrast, sorted peritoneal B1 B cells respond optimally in the same readouts to rCD40L and IL-5, although rCD40L and IL-4 or IL-5 and IL-4 stimulates this population to a lesser extent. Thus, whereas conventional and marginal zone B cells appear to use a uniform set of stimuli to undergo T cell-driven activation, B1 B cells can use a variety of signals provided by Th2 cells, including combinations that do not require CD40-CD40L interactions.

In previous studies using murine B cells, investigators demonstrated TD activation to be CD40-CD40L restricted and optimally use IL-4 (reviewed in Refs. 1 and 2). This is perhaps not surprising because most B cell biologists working in the mouse routinely use splenic B cells, of which follicular B cells comprise the majority, and marginal zone B cells a minority (7). Although it is understood that follicular B cells contribute to TD responses and germinal center reactions, the finding that marginal zone B cells share the same TD activation requirements suggests this latter population to be capable of similar functions. The observation that follicular and marginal zone B cells respond strongly to CD40-dependent stimulation is consistent with the work of Snapper and coworkers (8), who examined differentiation and switching in response to activated Th2 clones, and Oliver et al. (9), who tested the proliferative response to an agonistic anti-CD40 Ab. In the latter study, marginal zone B cells responded quantitatively better than follicular B cells, a result not observed in our experiments (Fig. 2). The reason for the difference is not apparent, although different CD40 agonists and means of enriching for marginal zone B cells were used.

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The question also arises as to how B1 B cells are capable of normal differentiation in response to rCD40L + IL-4 when their proliferative response is muted. Based on the work of several investigators, it is apparent that different regions of the CD40 cytoplasmic tail are capable of initiating distinct functions (34, 38–40). In particular, studies by Bishop and coworkers have demonstrated that CD40-induced up-regulation of B7, CD23, and Fas, and homotypic adhesion map to one part of the cytoplasmic tail, NF-κB activation and Ab secretion to a second, and IL-6 secretion to yet a third. It is thus possible that proliferation and differentiation are likewise initiated by distinct regions of the CD40 cytoplasmic tail, and those elements necessary for CD40-induced proliferation are limiting in B1 B cells, whereas those central for differentiation are not. The present results also imply that signaling components necessary for IL-5-driven expansion are readily available in B1 B cells, and can compensate for the modest proliferative signals initiated by CD40 ligation.

The suboptimal proliferative response of B1 B cells to CD40 ligation is consistent with the reports of Bikah et al. (12) and Lund
et al. (13), who found this population to exhibit a lower response to CD40 stimulation compared with splenic B cells. The difference between the B1 and conventional populations was not as great in these reports as observed in the present study, and might be explained by a different agonist (anti-CD40 Ab) and the use of only semipurified splenic B cells. In contrast, Shirai and coworkers (18) reported that B1 and B2 B cells sort purified from the spleens of autoimmune (NZB × NZW)F1 mice exhibited equivalent levels of proliferation when stimulated with an anti-CD40 Ab in the presence of IL-4 and/or IL-5. The differing results may be explained not only by a different agonist, but the intrinsic hyporesponsiveness of autoimmune B cells (41). It is of interest that, similar to normal BALB/c B1 B cells, B1 B cells sort purified from the peritoneal cavity of NZB mice show a poor response to rCD40L alone or in the presence of IL-4, but respond vigorously upon culture with rCD40L and IL-5 (L. D. E. and T. J. W., data not shown). The present data are also in partial agreement with the study of Tarlinton et al. (17), who demonstrated B1 and B2 B cells to produce IgM and switch to IgG1 when stimulated with CD40L-transfected fibroblasts, IL-4, and IL-5. This study further showed that the frequency of IgG1-switched cells was much lower in the B1 population compared with both splenic and peritoneal conventional B cells. This is at odds with the present findings, in which equivalent amounts of IgG1 are produced by B1 and B2 cells whether stimulating with rCD40L and cytokines or intact activated Th2 clones. Reasons for the differing results are unclear, although different readouts were used (total IgG vs precursor frequency) in the two studies. Finally, previous experiments demonstrating the capacity of adoptively transferred B1 B cells to produce IgM and IgG in response to challenge with a TD Ag are consistent with the present observations (42, 43).

The finding that IL-5 is central in Th2-driven B1 B cell activation confirms and extends a number of studies suggesting a unique role for IL-5 in B1 B cell activation. Previous reports have demonstrated the B1 B cell subset to constitute specifically express IL-5R (31, 32), and to exhibit modest levels of proliferation and differentiation when treated with this cytokine (28–32). IL-5 has also been shown to preferentially enhance the proliferative response of B1 B cells treated with LPS or dextran sulfate (28–30). In addition, studies with IL-5 transgenic and knockout mice have demonstrated a key role for this cytokine in the development and maintenance of the B1 B cell population (44–46). Therefore, it appears that the B1 B cell subset is programmed to preferentially use this cytokine not only for its maintenance, but for cognate encounters with Th cells.

In addition to its role in CD40-directed stimulation of B1 B cells, IL-5 is able to stimulate this subset in the absence of CD40-CD40L interactions. When present with IL-4, IL-5 induces proliferation, differentiation, and isotype switching of B1 cells. This result is of particular interest when examining the humoral response to protein Ags in both CD40L- and CD40-deficient mice (47–49), as well as in X-HIM patients (50). Although germinal centers and a significant portion of the isotype-switched response are absent in mice or humans with CD40 or CD40L mutations, Abs, primarily of the IgM class, are still induced. It is thus possible that in the absence of CD40-CD40L interactions, these protein-specific Ab responses arise from Th2 cytokine-stimulated B1 B cells.

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