REPORT DOCUMENTATION PAGE

(2)

1b. RESTRICTIVE MARKINGS	
2a. SECURITY CLASSIFICATION:	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution is unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S): NMRI 93-21	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: Naval Medical Research Institute	6b. OFFICE SYMBOL (If applicable)
7a. NAME OF MONITORING ORGANIZATION: Naval Medical Command	
6c. ADDRESS (City, State, and ZIP Code): 8901 Wisconsin Avenue, Bethesda, MD 20889-5055	7b. ADDRESS (City, State, and ZIP Code): Department of the Navy, Washington, DC 20372-5120
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: Naval Medical Research & Development Command	8b. OFFICE SYMBOL (If applicable)
9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER	
8c. ADDRESS (City, State, and ZIP Code): 8901 Wisconsin Avenue, Bethesda, MD 20889-5044	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
62233N	MM33C30	005-1051	DN249507

11. TITLE (Include Security Classification)
Comparative in vitro analysis of proliferation, Ig secretion, and Ig class switching by murine marginal zone and follicular B cells

12. PERSONAL AUTHOR(S) Snapper CM, Yamada H, Smoot D, Sneed R, Lees A, Mond JJ

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
journal article	FROM _____ TO _____	1993	9

16. SUPPLEMENTARY NOTATION
Reprinted from: The Journal of Immunology 1993 April 1; Vol. 150 No.7 pp. 2737-2745

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	B cell; antibody production; lymphocyte transformation

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT: ☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	21. ABSTRACT SECURITY CLASSIFICATION: Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL: Phyllis Blum, Librarian	22b. TELEPHONE (Include Area Code): (301) 295-2188 / 22c. OFFICE SYMBOL: MRL/NMRI

DD FORM 1473, 84 MAR — 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE: UNCLASSIFIED

0022-1767/93/1507-2737$02.00/0
The Journal of Immunology

Vol 150, 2737-2745, No. 7, April 1, 1993
Printed in U.S.A.

Comparative in Vitro Analysis of Proliferation, Ig Secretion, and Ig Class Switching by Murine Marginal Zone and Follicular B Cells[1]

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Abstract. We have previously demonstrated that activation of murine B cells by dextran-conjugated anti-IgD antibodies may serve as a polyclonal, in vitro model system for studying immune responses to T cell-independent type 2 (TI-2) Ag, as exemplified by the bacterial polysaccharides. Because in vivo Ig responses to TI-2 Ag are mediated primarily by B cells resident in the splenic marginal zone, we wished to determine whether this reflected an intrinsic difference in the responsiveness of marginal zone B cells (MZB) compared with follicular B cells (FB) to this class of Ag. In this report we demonstrate that highly purified MZB, isolated by electronic cell sorting, exhibit a lower proliferative response in vitro in response to unconjugated anti-Ig antibody as well as to dextran- or Sepharose-conjugated anti-IgM or anti-IgD antibodies, whereas they proliferate equal to or better than FB when stimulated by other B cell mitogens including LPS, *Salmonella typhimurium* mitogen, or an anti-CD3-activated CD4$^+$ Th2 cell clone. Despite the different proliferative responses of MZB and FB induced by anti-Ig, Ag receptor cross-linkage stimulates comparable increases in intracellular free calcium concentrations in both of these B cell populations. Furthermore, MZB secrete Ig and undergo Ig isotype switching to a comparable degree, relative to FB, in response to both T cell-dependent and T cell-independent stimuli. This suggests that the compartmentalization of TI-2 responses to the splenic marginal zone rather than the follicular zone reflects something other than the intrinsic responsiveness of the B cells from these two sites. *Journal of Immunology*, 1993, 150: 2737.

The marginal zone is a specialized anatomic site in the spleen that contains a B cell subpopulation responsible for generating antibody responses to TI-2[3] Ag (1–3). By contrast, B cells resident in the splenic follicle (FB) secrete antibody in response to TD Ag (4). TI-2 Ag, as exemplified by the polysaccharides, are typically found in abundance in bacterial cell walls, and unlike TD Ag, such as soluble proteins, elicit Ig isotype production that is skewed toward IgM and IgG3 (5). Furthermore, TI Ag, in contrast to TD Ag, are generally ineffective at stimulating a secondary or memory-type immune response (6–8).

The TI-2 Ag-responsive MZB is a mature, noncycling

Received for publication July 15, 1992. Accepted for publication January 6, 1993.

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[1] This work was supported, in part, by Uniformed Services University of the Health Sciences Research Protocols RO74CN and R083BQ, The Burroughs Wellcome Fund Developing Investigator Award, Immunopharmacology of Allergic Diseases (C. M. S.), National Institutes of Health Grants AI24273, and AI27465, and Naval Medical Research and Development Command N0007591WR00018. Opinions and assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Department of Defense or the Uniformed Services University of the Health Sciences.

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[3] Abbreviations used in this paper: TI-2, T cell-independent type 2; TI, T cell independent; MZB, marginal zone B cell; FB, follicular B cell; FcεRII, low affinity Fc receptor for IgE; STM, *Salmonella typhimurium* mitogen; αδ-dex, dextran-conjugated anti-IgD antibody; αμ-dex, dextran-conjugated anti-IgM antibody; TI-1, T cell-independent type 1 PE, phycoerythrin; mIg, membrane Ig; TD, T cell dependent; SN, supernatant; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; PKC, protein kinase C.

and noncirculating cell (9, 10) that expresses high levels of mIgM and low or absent mIgD (11, 12). By contrast the FB is a recirculating cell that expresses intermediate levels of mIgM and high levels of mIgD (11, 12). Evidence suggests that these two B cell subpopulations may represent distinct lineages (2). Recently, Waldschmidt et al. demonstrated that MZB and FB can be clearly separated on the basis of their differential expression of the intermediate affinity receptor for IgE (FcεRII) (13). Thus, MZB fail to express detectable FcεRII whereas FB are FcεRII$^+$.

Because the frequency of primary Ag-specific B cells, even to potent TI Ag, is very low, a detailed analysis of the variables influencing their responsiveness would be difficult if not impossible. We therefore developed an in vitro polyclonal model system for studying B cell activation in response to TI-2 Ag (14, 15). Anti-IgD or anti-IgM antibodies were conjugated to a high molecular dextran in order to simulate the repeating epitope nature of polysaccharides. We recently demonstrated that small, high density B cells activated by αδ-dex proliferated, but failed to secrete Ig unless a B cell maturation factor, such as IL-5, was also added to culture (15). By contrast, αδ-dex stimulated large, low density B cells to secrete Ig in the absence of exogenous cytokines. This differential responsiveness of small and large B cells to induction with αδ-dex was similar to that observed for in vitro hapten-specific antibody responses to conjugates of hapten-Ficoll, a prototypic TI-2 Ag. We further demonstrated that αδ-dex activated B cells could undergo Ig class switching to most, although not all, Ig isotypes, in the presence of appropriate switch and differentiation factors (16, 17).

Little is known regarding the parameters that determine the differential response patterns of MZB and FB to immunization with distinct classes of Ag in vivo. Thus, it is not clear whether these differences primarily reflect intrinsic properties of these two B cell subpopulations or whether the unique microenvironments in which they localize and/or selective interactions with other cell types account for their distinct responsiveness to antigenic stimulation. To address this issue we obtained highly purified populations of MZB and FB from the spleens of unimmunized mice by utilizing an electronic cell sorter to isolate mIgM^{bright}FcεRII$^-$ and mIgM^{intermediate}FcεRII$^+$ B cells, respectively. These sorted B cell subpopulations were then tested for their relative ability to proliferate, secrete Ig, and undergo Ig isotype switching in response to TI-1 (bacterial LPS), TI-2 (αδ-dex and αμ-dex), and TD modes of activation.

Materials and Methods

Mice

Female DBA/2, BALB/c, and C3H mice were obtained from the National Institutes of Health Small Animals Division (Bethesda, MD) and were used at 8 to 12 wk of age. The experiments were conducted according to the principles set forth in the *Guide for the Care and Use of Laboratory Animals*, Institute of Animal Resources, National Research Council, Department of Health, Education, and Welfare Publication 78-23, National Institutes of Health, Bethesda, MD.

Culture medium

RPMI 1640 (Biofluids, Rockville, MD) supplemented with 10% FCS (GIBCO Laboratories, Grand Island, NY), L-glutamine (2 mM), 2-ME (0.05 mM), penicillin (50 μg/ml), and streptomycin (50 μg/ml) were used for culturing cells.

Reagents

αδ-dex and αμ-dex were prepared by conjugation of Hδ^a/1 (monoclonal mouse IgG2b (b allotype), anti-mouse IgD (a allotype) (18), and B7.6 (monoclonal rat IgG1 anti-mouse IgM) (19) to a high m.w. dextran (2×10^6 m.w.) as previously described (14). Approximately 6 Hδ^a/1 and B7.6 antibodies were conjugated to each dextran molecule. FF1-4D5 (mouse IgG2a (b allotype) anti-mouse IgD (a allotype) (20) was purified from ascites. CnBr-activated Sepharose was purchased from Pharmacia (Piscataway, NJ). Two milligrams of purified FF1 antibody were added/milliliter of packed, swollen Sepharose beads according to instructions included by the manufacturer. LPS W, extracted from Escherichia coli 0111:B4, was obtained from Difco Laboratories (Detroit, MI). *Salmonella typhimurium* mitogen (STM) was obtained from Ribi Immunochemical Research (Hamilton, MT) and was used at 50 μg/ml. The following mAb were purified from ascites: B3B4 (rat IgG2a anti-FcεRII) (21), MKD6 (mouse IgG2a anti-Ia^d) (22), and 2.4G2 (rat IgG2b anti-FcγRII) (23). Selected antibodies were conjugated to FITC (Calbiochem-Behring, San Diego, CA), *N*-hydroxysuccinimidobiotin (Sigma, St. Louis, MO), and Texas Red (Research Organics, Cleveland, OH) by standard protocols. PE-labeled affinity-purified goat anti-mouse IgM antibody was purchased from Southern Biotechnology Associates (Birmingham, AL). Avidin-PE (Phycoprobe) and avidin-allophycocyanin were obtained from Biomeda (Foster City, CA). Purified murine rIL-4 was produced in *E. coli* and was a gift from Dr. Alan D. Levine (Monsanto Company, St. Louis, MO). Murine rIL-5 was produced in the baculovirus system and was a gift from Dr. Gregory Harriman, (National Institutes of Health, Bethesda, MD). Murine rIFN-γ, prepared from Chinese hamster ovary cells, was a gift of Genentech (South San Francisco, CA). Percoll was obtained from Pharmacia.

Preparation and culture of B cells

Enriched populations of B cells were obtained from spleen cells from which T cells were eliminated by treatment with

monoclonal rat IgM anti-Thy-1(H013-4), rat IgG2b anti-CD4 (GK1.5), and rat IgG2b anti-CD8 (2.43), followed by mouse anti-rat Igκ (MAR 18.5) and C by the method of Leibson et al. (24). Small B cells were obtained by the modified (25) discontinuous Percoll gradient centrifugation procedure of DeFranco et al. (26). Cells that formed a band between 60 and 70% Percoll and had a density of 1.080 to 1.086 g/ml were used in all experiments. Functional assays were carried out in 96-well, flat bottom Costar plates (Costar, Cambridge, MA). Cultured cells were incubated at 37°C in a humidified atmosphere containing 6% CO_2. Unless otherwise indicated, cells were cultured at a density of 1.25×10^5 cells/ml. All experiments are representative of at least two to three similar studies.

Cytofluorometric analysis and cell sorting

B cells were stained for 30 min with various combinations of sterile filtered, fluorescence-labeled immunoreagents (final concentration of 10 μg/ml in the presence of a fivefold excess of anti-FcγRII mAb to prevent cytophilic antibody binding) at 10^7 cells/ml in cold clear HBSS containing 3% FCS. Cells were then washed and resuspended in staining buffer at 10^7 cells/ml in preparation for fluorescence analysis and/or cell sorting. Cell sorting generally was carried out over a 5-h period. Sorted cells were cultured immediately after this sorting period and were >95% viable at this time. Control studies in which stained, but nonsorted, cells were used were shown to behave similarly to nonstained, nonsorted cells upon activation with αδ-dex or LPS (data not shown). For analysis, 15,000 cells were collected using logarithmic amplification on a Becton Dickinson FACStar Plus (Mountain View, CA). Only viable cells were analyzed, and any residual macrophages were eliminated from analysis on the basis of characteristic forward and side scatter profiles. Cell sorting was similarly carried out on a FACStar Plus, as well as on a Coulter Epics Elite (Miami, FL), and sorted cells were immediately reanalyzed to confirm their staining profile. Only sorting purities of >95% were considered acceptable for subsequent study.

Preparation of T cell-derived SN and induction of T cell-mediated B cell activation

The murine $CD4^+$ T cell clone, D10.G4.1 (D10), (conalbumin-specific, $I\alpha^k$-restricted) was considered a Th2 clone based on its secretion, after stimulation, of IL-4 and IL-5, but not IL-2 or IFN-γ, and was maintained as described (27). Cytokine-rich SN from D10 T cells was obtained as follows: resting D10 T cells (1×10^5/ml) were stimulated by adding irradiated (3000 r) spleen cells (5×10^5/ml) from C3H mice ($I\alpha^k$-bearing) acting as antigen-presenting cells in the presence of 100 μg/ml of conalbumin (Sigma). Cellfree SN was harvested 24 h later, aliquoted and stored at −80°C until used. This T cell SN had, on average, 95 U/ml of IL-5 and 365 U/ml of IL-4. For Ag-independent, MHC-unrestricted, T cell-mediated stimulation of B cells (28, 29), 96-well culture plates were first coated with anti-CD3 antibody (2C11) (30) by incubation of 50 μg/ml of 2C11 in PBS at room temperature for 4 h. Plates were subsequently washed three times with PBS. Resting D10 T cells (3×10^4 cells/ml) were then cocultured with either sort-purified MZB or FB cells (1×10^5 cells/ml) in the anti-CD3-coated microtiter wells for induction of B cell proliferation or Ig isotype production. D10 T cells were first irradiated with 3000 r to prevent them from proliferating, without inhibiting their ability to become activated or secrete cytokines.

Measurement of DNA synthesis

DNA synthesis was determined by [^{3}H]TdR uptake (2 μCi/well; 6.7 Ci/nmol; 1 mCi = 37 GBq; ICN, Irvine, CA) over a 16-h period. Cells were harvested onto glass filter paper and [^{3}H]TdR incorporation was determined by liquid scintillation spectrometry.

Quantitation of secreted Ig isotypes

Ig isotype concentrations were measured by ELISA, with Immulon 2, 96-well, flat bottomed ELISA plates (Dynatech Laboratories, Alexandria, VA), which has been described by us in detail elsewhere (31). Briefly, a fluorescent product was generated from cleavage of 4-methylumbilliferyl phosphate (Sigma) by specifically-bound alkaline phosphatase-conjugated antibodies. Fluorescence was measured on a 3 M FluoroFAST 96 fluorometer (Becton Dickinson, Mountain View, CA) and fluorescence units were converted to Ig concentrations by extrapolation from standard curves determined in each assay by using purified myeloma proteins of known concentrations. Each assay system showed no significant cross-reactivity or interference from the presence of other isotypes (IgM, IgD, IgG3, IgG1, IgG2b, IgG2a, IgE, and IgA) found in the culture supernatants.

Determination of intracellular Ca^{2+} concentrations

Our procedure for the measurement of $[Ca^{2+}]_i$ in single cells has been described in detail elsewhere (32). Briefly, cells were loaded with 1.5 μM indo-1 in HBSS containing 1% FCS. The cells were warmed to 37°C and analyzed at 200 cells/s on a dual laser flow cytometer (Ortho Cytofluorograph, Westwood, MA) after addition of appropriate mAb. Data were analyzed using commercially available software (Phoenix Flow Systems, San Diego, CA). The technique is capable of detecting a calcium response in as few as 0.3% of the cells analyzed.

Results

Isolation of marginal zone and follicular splenic B cells by electronic cell sorting

MZB and FB can be distinguished on the basis of their characteristic cell-surface phenotypes (13). MZB

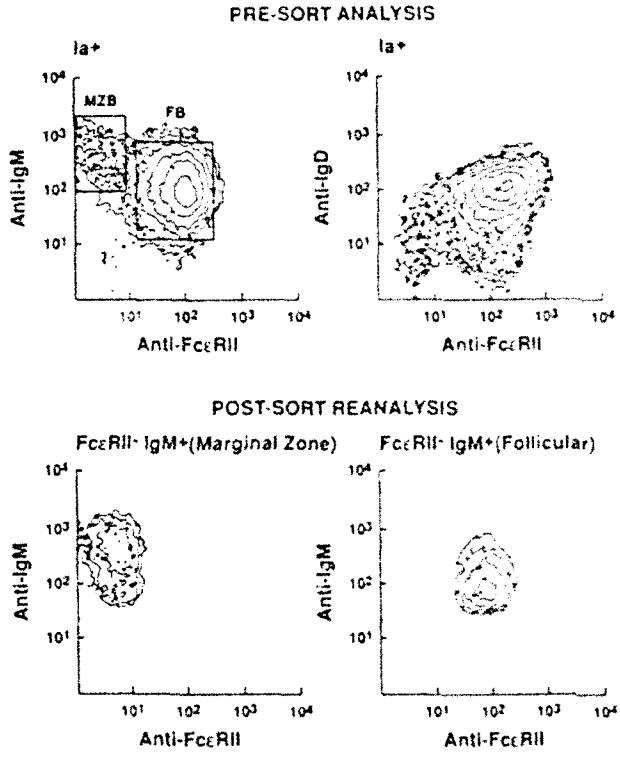

FIGURE 1. Cell surface phenotypes and sorting of MZB and FB. B cells were stained with FITC-anti-FcεRII (B3B4), PE-polyclonal goat anti-IgM, and TR-anti-Ia^d (MKD6). Macrophages were eliminated by forward and side scatter gating. Only Ia^{d+} cells (B cells) are displayed. *Upper panels*: pre-sort analysis-MZB are $mIgM^{bright}Fc\epsilon RII^-$, FB are $mIgM^{intermediate}$ $Fc\epsilon RII^+$(boxed populations). MZB ($Fc\epsilon RII^-$) are also $mIgD^{dull/-}$ and FB ($Fc\epsilon RII^+$) are $mIgD^{bright}$. mIgM staining of MZB was ~4-fold higher than that for FB. Cells within the indicated boxes were isolated by sorting. Sorted cells were analyzed immediately after sorting (*lower panel*) and only purities >95% were acceptable for further study.

are $mIgM^{bright}mIgD^{dull/-}Fc\epsilon RII^-$ whereas FB are $mIgM^{intermediate}mIgD^{bright}$ $Fc\epsilon RII^+$ (Fig. 1). MZB comprised ~4% of the splenic B cells from either DBA/2 or BALB/c mice. For all experiments reported herein highly purified (≥95%) MZB and FB were obtained by staining cells with FITC-anti-FcεRII + PE-anti-IgM and then isolating them by electronic cell sorting.

MZB exhibit a lower proliferative rate than FB in response to activation by αδ-dex or αμ-dex

We determined that 10 ng/ml of αδ-dex induced maximal proliferation of T-depleted spleen cells, as assessed by [^{3}H]-TdR incorporation (data not shown). To assess the proliferative rate of MZB and FB we stimulated them with 10 ng/ml of αδ-dex (TI-2-like stimulus) and compared their incorporation of [^{3}H]TdR with that obtained after activation with LPS (TI-1-like stimulus). Incorporation of [^{3}H]-TdR upon culture with medium alone was negligible for both MZB and FB (Table I). MZB showed an 8.1-fold lower uptake of [^{3}H]TdR than FB in response to αδ-dex whereas they incorporated 5.3-fold higher amounts of [^{3}H]TdR than FB in response to LPS. The lower proliferative rate exhibited by MZB relative to FB was not caused by the dose of αδ-dex used because lower uptake of [^{3}H]TdR by MZB (3.4- to 16-fold) was observed upon activation with 1, 10, or 100 ng/ml of αδ-dex (Fig. 2). The addition of an IL-4 + IL-5-containing T cell SN to αδ-dex-activated MZB and FB enhanced [^{3}H]TdR uptake by both populations of cells. However, αδ-dex-activated MZB still exhibited a lower incorporation of [^{3}H]TdR than αδ-dex-stimulated FB (Fig. 2).

Table I
*MZB proliferate poorly in response to αδ-dex**

	[^{3}H]TdR (cpm)	
	MZB	FB
Med	252	581
αδ-Dex	2,350	19,500
LPS	61,600	11,700

* Sorted MZB and FB were cultured in medium alone or stimulated with αδ-dex (10 ng/ml) or LPS (20 μg/ml). [^{3}H]TdR was added after 48 h and [^{3}H]TdR incorporation was measured 16 h later.

The differences in [^{3}H]TdR incorporation between MZB and FB upon induction with αμ-dex (0.1 to 100 ng/ml) were even more marked than that observed after αδ-dex activation (Fig. 3). αμ-dex-activated MZB incorporated 5.7- to 124-fold lower amounts of [^{3}H]TdR than FB depending upon the dose of αμ-dex used. The addition of 1000 U/ml of IL-4 variably enhanced [^{3}H]TdR uptake by MZB (0.0- and 5.1-fold) and FB (3.9- and 4.0-fold) activated by 10 or 100 ng/ml of αμ-dex, but the marked differences between the two populations were still apparent (22- and 7.3-fold) (Fig. 4). Visual inspection of MZB cultures clearly showed an early phase of cellular enlargement by a large proportion of cells in response to αμ-dex, which was followed a day or two later by a widespread loss of cell viability, as determined by trypan blue exclusion (data not shown). Although further analyses are required, this suggests that MZB are competent to enter the cell cycle in response to αμ-dex but fail to progress through S phase, as evidenced by a low level of [^{3}H]TdR uptake. Whether or not αμ-dex-activated B cells undergo apoptosis remains to be determined.

To determine whether this diminished proliferative response to αδ- and αμ-dex reflected a general inability to respond to any mode of mIg-mediated B cell activation, we evaluated their responses to unconjugated and Sepharose-bound anti-IgD or anti-IgM. We further wished to compare the proliferative responses of MZB and FB upon stimulation with a second TI-1 like stimulus, STM, and after activation with an anti-CD3-activated Th2 clone (TD-like stimulus). Incorporation of [^{3}H]TdR by MZB was also lower than FB upon activation with unconjugated anti-IgM

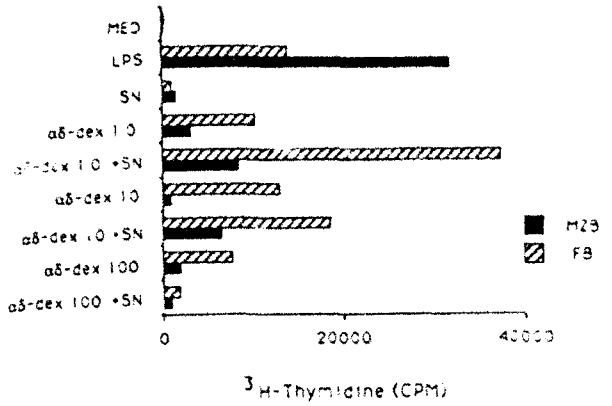

FIGURE 2. MZB and FB proliferative response to varying doses of αδ-dex in the presence or absence of Th2 SN. Sorted MZB and FB were stimulated with αδ-dex (1.0, 10, and 100 ng/ml) in the presence or absence of Th2 (D10) SN (final concentration 25% v/v) or LPS (20 μg/ml). [^{3}H]TdR was added after 48 h and [^{3}H]TdR uptake was measured 16 h later.

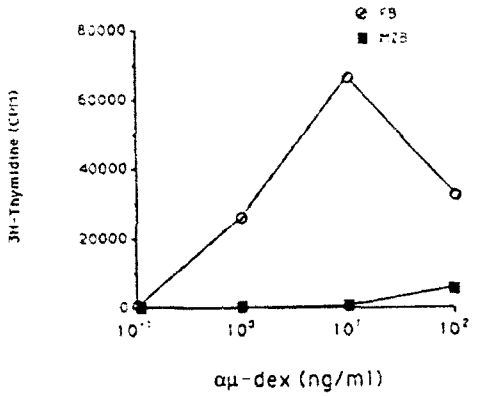

FIGURE 3. MZB and FB proliferative response to varying doses of αμ-dex. Sorted MZB and FB were stimulated with varying doses of αμ-dex as indicated. [^{3}H]TdR was added 48 h after initiation of culture and [^{3}H]TdR uptake was measured 16 h later. [^{3}H]TdR uptake by LPS-activated MZB was 136,000 cpm and LPS-activated FB was 72,300 cpm.

(6.4-fold), unconjugated anti-IgD (2.9-fold), or anti-IgD-Sepharose (8.3-fold) (Table II). By contrast, MZB and FB showed comparable levels of [^{3}H]TdR uptake in response to STM or anti-CD3-activated Th2 cells.

The lower proliferative responses of MZB relative to FB upon Ag-receptor cross-linkage is not associated with differences in stimulation of increased intracellular Ca^{2+} concentrations

To assess the functional status of the Ag receptor on MZB and FB we measured the uptake of Ca^{2+} into the cell $[Ca^{2+}]_i$ immediately upon stimulation with varying concentrations (0.3 to 10 μg/ml) of unconjugated anti-IgM. Both the mean $[Ca^{2+}]_i$ and the percentage of responding cells observed upon activation with anti-IgM were roughly comparable for MZB and FB (Fig. 5). This indicated that

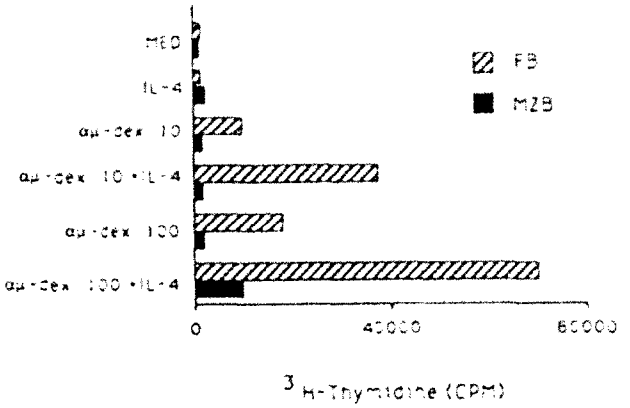

FIGURE 4. MZB and FB proliferative response to αμ-dex ± IL-4. Sorted MZB and FB were stimulated with 10 or 100 ng/ml of αμ-dex in the presence or absence of 1000 U/ml of IL-4. [^{3}H]TdR was added 48 h after initiation of culture and [^{3}H]TdR uptake was measured 16 h later.

Table II
MZB proliferate poorly in response to unconjugated and Sepharose-bound anti-Ig[a]

	[^{3}H]TdR cpm	
	MZB	FB
GαIgM	13,000	88,110
GαIgD	27,500	80,500
FF1-Seph	3,390	28,000
STM	196,000	146,800
αCD3-T cell	25,339	20,583

[a] Sorted MZB and FB (5×10^5/ml) were cultured in the presence of 50 μg/ml of polyclonal goat anti-mouse IgM or IgD (GαIgM or GαIgD). MZB and FB (1.25×10^5/ml) were further stimulated with 1% v/v Sepharose anti-IgD (FF1-Seph), STM (20 μg/ml), or anti-CD3-activated D10 T cells (0.4×10^5/ml). [^{3}H]TdR was added after 48 h and [^{3}H]TdR uptake was measured 16 h later.

the differences in anti-Ig-induced proliferation must reflect differences in the activational pathway distal to this point.

MZB and FB secrete Ig and undergo Ig class switching to a comparable degree in response to TI- and TD-like stimuli

Our finding that MZB show impaired mIg-dependent B cell proliferation was in apparent contrast to the reports demonstrating that the MZB are the predominant responding cells in a TI-2 antibody response (1–3). To evaluate the in vitro ability of these cells to secrete Ig and undergo Ig class switching in response to mIg cross-linking we stimulated MZB and FB with αδ-dex + IL-5 in the presence or absence of IL-4. MZB secreted 1.4- to 2.7-fold greater amounts of IgM than FB although viable cell yields of MZB were 1.3- to 2.0-fold lower than that for FB (Table III). MZB and FB secreted comparable amounts of IgG1 upon addition of IL-4 to αδ-dex + IL-5-activated cells (MZB = 2750 ng/ml of IgG1 (day 5 viable cells = 2.0×10^5/ml); FB = 3000 ng/ml of IgG1 (day 5 viable cells = 3.0×10^5/ml)). MZB viable cell yields (0.60×10^5/ml) on day 4 were 7.2-fold lower than FB (4.3×10^5/ml) in response

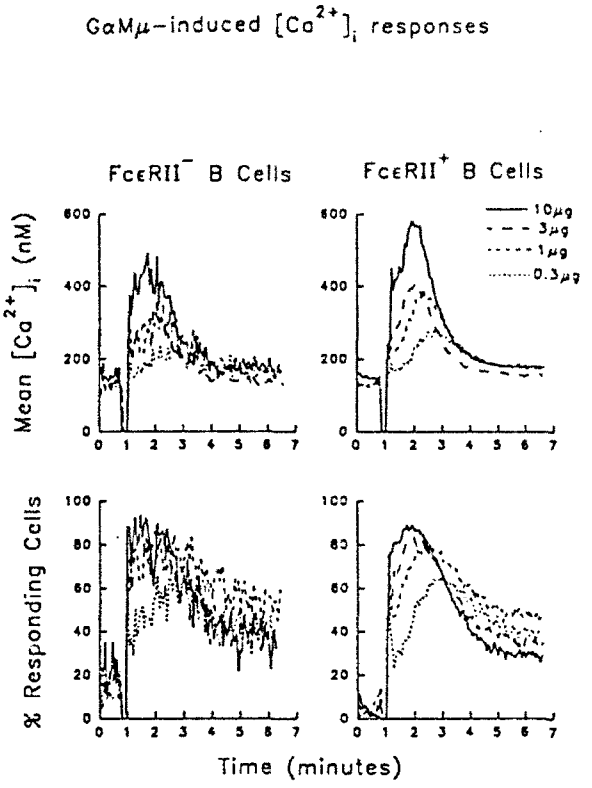

FIGURE 5. Calcium response of MZB and FB to anti-IgM. Sorted MZB (FcεRII⁻) and FB (FcεRII⁺) were loaded with indo-1 and stimulated with polyclonal goat anti-mouse IgM (0.3 to 10 μg/ml).

Table III
Time course of IgM secretion by MZB and FB in response to αδ-dex + IL-5[a]

	IgM Secretion (ng/ml)				Viable Cells (x10³/ml)			
	Day 2	Day 3	Day 4	Day 5	Day 2	Day 3	Day 4	Day 5
MZB	34	160	575	800	1.3	2.1	3.0	0.5
FB	25	60	220	450	2.2	3.5	4.0	1.0

[a] Sorted MZB and FB were cultured with αδ-dex (3 ng/ml) + IL-5 (150 U/ml). Culture SN was removed from separate wells on the days indicated for determination of IgM concentrations by ELISA and viable cell numbers by trypan blue exclusion.

to αμ-dex alone, confirming that MZB and FB were adequately sorted. In additional experiments it was observed that neither MZB nor FB secreted detectable Ig when cultured in medium or IL-5 alone (data not shown).

We further evaluated the ability of MZB and FB to secrete Ig and undergo Ig isotype switching in response to a TI-1 (LPS) and TD-type stimulus. LPS stimulated comparable amounts of IgM and IgG3 (33) by MZB and FB after 5 days in culture, although MZB secreted 22- and 4.1-fold more IgM than FB after 2 and 3 days of LPS stimulation, respectively (Table IV). The IgM response of MZB 2 and 3 days after LPS stimulation represented only 1.2 and 6.7% of the response observed after 5 days of LPS activation. The kinetics of the LPS-induced IgG3 response were comparable for MZB and FB.

Table IV
Time course of IgM and IgG3 secretion by MZB and FB in response to LPS[a]

	IgM Secretion (ng/ml)			
	Day 2	Day 3	Day 4	Day 5
MZB	225	1,300	8,125	19,375
FB	10	320	7,500	15,000
	IgG3 Secretion (ng/ml)			
MZB	<2.4	3.6	120	600
FB	<2.4	<2.4	64	240

[a] Sorted MZB and FB were cultured in the presence of LPS. Culture SN was removed from separate wells on the days indicated for determination of IgM and IgG3 concentrations by ELISA.

Addition of IL-4 to LPS-stimulated MZB and FB led to comparable inductions of IgG1 (34) and IgE (35) secretion 6 days after initiation of culture (Table V). Likewise IL-4 inhibited LPS-induced IgM and IgG3 production (36) by MZB and FB to a similar extent (Table V). Similarly, LPS-activated MZB and FB secreted comparable amounts of IgG2a in response to IFN-γ (37) (Table VI).

Because FB have been specifically implicated in TD responses we wished to test whether MZB and FB differed in their Ig isotype responses to activation with an anti-CD3-activated Th2 clone, D10.G4.1 (D10). D10-activated MZB secreted 6.7-fold more IgM and comparable amounts of IgG1 relative to similarly activated FB (Table VII), indicating that both populations could secrete Ig and undergo Ig class switching in response to T cell activation.

Discussion

The requirements for induction of TI and TD responses are different. Thus, responses to TI, but not TD, Ag can be elicited in the absence of T cell help (38, 39) but only poorly in neonatal mice (40) or in adult mice that have been splenectomized (41, 42). Recent investigations have demonstrated that these differences in activation requirements for TI and TD Ag may reflect the fact that different subsets of B cells respond to these Ag. B cells that localize to the splenic marginal zone have been reported to be the predominant population responding to TI-2 Ag (1–3), whereas B cells that localize to the follicular zone are the cells responsive to TD Ag (4). The studies that have been reported could not discriminate whether the differences in responses of these populations reflected intrinsic differences in the responsiveness of the B cells themselves or whether they reflected the activity of different interacting cells in these compartments. We therefore undertook these studies to investigate this point.

The difference in proliferation between MZB and FB upon Ag-receptor cross-linkage was observed using a number of distinct modes of Ag-receptor ligation including dextran-conjugated anti-Ig, Sepharose-linked anti-Ig, and high doses of unconjugated anti-Ig. Thus, this difference

Table V
Ig isotype production by MZB and FB stimulated with LPS or LPS + IL-4[a]

	Ig Secretion (ng/ml)					
	IgM		IgG3		IgG1	
	MZB	FB	MZB	FB	MZB	FB
LPS	58,750	18,750	700	600	190	<12
LPS+IL-4	3,375	500	90	24	3,750	1,200
	IgG2b		IgG2a		IgE	
	MZB	FB	MZB	FB	MZB	FB
LPS	875	215	750	230	<6	<6
LPS+IL-4	40	<24	35	<24	1,440	1,500

[a] Sorted MZB and FB were cultured in the presence of LPS (20 μg/ml) with or without 10,000 U/ml of IL-4. Culture SN was removed after 6 days for determination of Ig isotype concentrations by ELISA.

Table VI
Ig isotype production by MZB and FB stimulated with LPS or LPS + IFN-γ[a]

	Ig Secretion (ng/ml)					
	IgM		*IgG2a*		*IgG2a*	
	MZB	FB	MZB	FB	MZB	FB
LPS	206,000	100,000	775	525	310	410
LPS+IFN-γ	103,125	93,750	215	105	1,250	2,050

[a] Sorted MZB and FB were stimulated with LPS (20 μg/ml) with or without 10 U/ml of IFN-γ. Culture SN were removed 6 days later for measurement of Ig isotype concentrations by ELISA.

Table VII
IgM and IgG production by MZB and FB stimulated with an anti-CD3-activated Th2 clone[a]

	Ig Secretion (ng/ml)			
	IgM		IgG1	
	MZB	FB	MZB	FB
Med	56	<10	<1.2	<1.2
αCD3-Th2	6,000	900	2,400	1,350

[a] Sorted MZB and FB (1.25×10^5/ml) were stimulated by D10 Th2 cells (0.4×10^5/ml) in the presence of plate-bound anti-CD3 antibody. Culture SN was removed 6 days later for measurement of IgM and IgG1 concentrations by ELISA.

did not depend upon whether or not the anti-Ig stimulus was TI-2-like (i.e., αδ-dex or αμ-dex). Similarly, the starting cell density did not appear to influence the differential proliferative response of MZB and FB because cells stimulated with unconjugated anti-Ig were plated at high density whereas cells stimulated with dextran- or Sepharose-anti-Ig were cultured at a relatively low cell density. Although MZB and FB express different levels of mIgM and mIgD, the differences in anti-Ig-induced proliferation could not be accounted for simply on this basis because similar differences in proliferation were seen over a wide range of anti-Ig concentrations. The impaired proliferative response of MZB to anti-IgM antibody does not reflect absent Ig-mediated signal transduction because anti-IgM stimulated comparable elevations in $[Ca^{2+}]_i$ in both MZB and FB. The impaired mIg-mediated proliferative response was most apparent when anti-IgM was used as the stimulus. Although proliferative responses of MZB to anti-IgD were clearly inferior to that of FB cells, they were nonetheless higher than anti-IgM-stimulated responses.

Although the mechanism underlying the poor proliferative response of MZB to Ag-receptor cross-linkage is unknown, the MZB response is reminiscent of that observed upon Ag-receptor cross-linkage of immature B cells. Thus, anti-Ig cross-linkage of either neonatal B cells or WEHI 231, a lymphoma with an immature phenotype, fails to stimulate proliferation, but instead induces apoptosis and/or tolerization (43–46). This is associated with an apparently normal calcium response, as we have observed for MZB, but a failure to activate PKC and the PKC-linked genes egr-1 and c-fos. Cross-linkage of mIgD on WEHI 231, which was induced to express mIgD through IgD gene transfection, did not result in the delivery of a negative signal in contrast to that observed for mIgM (47). Whether this relates to the more profound differences in proliferation between MZB and FB when stimulated by αμ-dex, as opposed to αδ-dex, is of interest. Although MZB, like immature B cells, express an $mIgM^{bright}mIgD^{low/-}Fc\epsilon RII^{-}$ phenotype, the MZB has been shown to be a mature, noncycling cell (10).

The relative inability of MZB to proliferate in response to mIg cross-linking stimuli is not surprising because it is known that polysaccharide Ag stimulate poor, if any, anamnestic (memory) responses (6–8), which depend heavily on clonal expansion of Ag-specific B cells. Furthermore, in TD responses Ag-mediated selection of high avidity B cells with resultant proliferation and expansion of this population leads, over time after immunization, to increasing avidity of Ag-specific antibodies (4); this response is not seen in response to TI Ag perhaps in part because of the relative inability of these Ag to induce clonal expansion. Thus, the antibody response to TI Ag primarily reflects their ability to rapidly stimulate B cells to secrete Ig in the face of limited B cell proliferation and in the context of limiting ancillary help.

In contrast to TD Ag, TI-2 Ag stimulate the production of IgM and IgG3 (5). This is observed despite the recent observation that TI-2 Ag rapidly localize in the splenic follicle upon their injection (48) and thus presumably are available to interact with FB. The basis for this functional dichotomy is unknown. Our observations that Ig secretion and Ig isotype switching induced by cytokines in combination with either αδ-dex, LPS, or T cell activation were relatively comparable for MZB and FB suggests that the intrinsic properties of MZB and FB do not explain their differential response patterns in vivo. The data in this manuscript demonstrate that differences in immune responses of MZB and FB more likely reflect differences in their his-

tologic milieu, which plays an important role in influencing B cells responses.

Acknowledgments

We thank Hideko Yamaguchi and Michelle Lugo for excellent technical assistance, Dr. Richard Hodes (National Institutes of Health, Bethesda, MD) for rIL-5, Dr. Alan Levine (Monsanto Co., St. Louis, MO) for rIL-4, and Dr. Fred Finkelman (USUHS, Bethesda, MD) for provision of immunoreagents.

References

1. Lane, P. J., L. D. Gray, S. Oldfield, and I. C. M. MacLennan. 1986. Differences in the recruitment of virgin B cells into antibody responses to thymus-dependent and thymus-independent type-2 antigens. *Eur. J. Immunol. 16:1569.*
2. MacLennan, I. C. M., D. Gray, D. S. Kumararatne, and H. Bazin. 1982. The lymphocytes of splenic marginal zones: a distinct B-cell lineage. *Immunol. Today 3:305.*
3. Humphrey, J. H., and D. Grennan. 1981. Tolerogenic or immunogenic activity of hapten-conjugated polysaccharides correlated with cellular localization. *Eur. J. Immunol. 11:212.*
4. Liu, Y.-J., G. D. Johnson, J. Gordon, and I. C. M. MacLennan. 1992. Germinal centres in T-cell-dependent antibody responses. *Immunol. Today 13:17.*
5. Perlmutter, R., M. D. Hansburg, D. E. Briles, R. A. Nicolotti, and J. M. Davie. 1978. Subclass restriction of murine anti-carbohydrate antibodies. *J. Immunol. 121:566.*
6. Baker, P. J., P. W. Stashak, D. E. Amsbaugh, and B. Prescott. 1971. Characterization of the antibody response to type 3 pneumococcal polysaccharide at the cellular level. I. Dose-response studies and the effect of prior immunization on the magnitude of the antibody response. *Immunology 20:469.*
7. Andersson, B., and H. Blomgren. 1971. Evidence for thymus-independent humoral antibody production in mice against polyvinylpyrrolidone and *E. coli* lipopolysaccharide. *Cell. Immunol. 2:411.*
8. Braley-Mullen, H. 1978. Antigen requirements for induction of B-memory cells: studies with dinitrophenyl coupled to T-dependent and T-independent carriers. *J. Exp. Med. 147: 1824.*
9. Kumararatne, D. S., H. Bazin, and I. C. M. MacLennan. 1981. Marginal zones: the major B cell compartment of rat spleens. *Eur. J. Immunol. 11:858.*
10. Kumararatne, D. S., and I. C. M. MacLennan. 1981. Cells of the marginal zone of the spleen are lymphocytes derived from recirculating precursors. *Eur. J. Immunol. 11:865.*
11. Stein, H., A. Bonk, G. Tolksdorf, K. Lennert, H. Rodt, and J. Gerdes. 1980. Immunohistologic analysis of the organization of normal lymphoid tissue and non-Hodgkin's lymphomas. *J. Histochem. Cytochem. 28:746.*
12. Gray, D., I. C. M. MacLennan, H. Bazin, and M. Khan. 1982. Migrant $\mu^+\delta^+$ and static $\mu^+\delta^-$ B lymphocyte subsets. *Eur. J. Immunol. 12:564.*
13. Waldschmidt, T. J., F. G. M. Kroese, L. T. Tygrett, D. H. Conrad, and R. G. Lynch. 1991. The expression of B cell surface receptors. III. The murine low-affinity IgE Fc receptor is not expressed on Ly 1 or "Ly 1-like" B cells. *Int. Immunol. 3:305.*
14. Brunswick, M., F. D. Finkelman, P. F. Highet, J. K. Inman, H. M. Dintzis, and J. J. Mond. 1988. Picogram quantities of anti-Ig antibodies coupled to dextran induce B cell proliferation. *J. Immunol. 140:3364.*
15. Peçanha, L. M. T., C. M. Snapper, F. D. Finkelman, and J. J. Mond. 1991. Dextran-conjugated anti-Ig antibodies as a model for T cell-independent type 2 antigen-mediated stimulation of Ig secretion in vitro. I. Lymphokine dependence. *J. Immunol. 146:833.*
16. Snapper, C. M., L. M. T. Peçanha, A. D. Levine, and J. J. Mond. 1991. IgE class switching is critically dependent upon the nature of the B cell activator, in addition to the presence of IL-4. *J. Immunol. 147:1163.*
17. Snapper, C. M., T. M. McIntyre, R. Mandler, L. M. T. Peçanha, F. D. Finkelman, A. Lees, and J. J. Mond. 1992. Induction of IgG3 secretion by interferon γ: a model for T cell-independent class switching in response to T cell-independent type 2 antigens. *J. Exp. Med. 175:1367.*
18. Zitron, I. M., and B. L. Clevinger. 1980. Regulation of murine B cells through surface immunoglobulin. I. Monoclonal anti-δ antibody that induces allotype-specific proliferation. *J. Exp. Med. 152:1135.*
19. Julius, M. H., C. H. Heusser, and K. U. Hartman. 1984. Induction of resting B cells to DNA synthesis by soluble monoclonal anti-immunoglobulin. *Eur. J. Immunol. 14:753.*
20. Goroff, D. K., A. Stall, J. J. Mond, and F. D. Finkelman. 1986. In vitro and in vivo B lymphocyte activating properties of monoclonal anti-δ antibodies. I. Determinants of B lymphocyte-activating properties. *J. Immunol. 136:2382.*
21. Rao, M., W. T. Lee, and D. H. Conrad. 1987. Characterization of a monoclonal antibody directed against the murine B lymphocyte receptor for IgE. *J. Immunol. 138:1845.*
22. Kappler, J. W., B. Skidmore, J. White, and P. Marrack. 1981. Antigen-inducible, H-2-restricted, interleukin-2-producing T cell hybridomas: lack of independent antigen and H-2 recognition. *J. Exp. Med. 153:1198.*
23. Unkeless, J. 1979. Characterization of a monoclonal antibody directed against mouse macrophage and lymphocyte Fc receptors. *J. Exp. Med. 150:580.*
24. Leibson, H. H., P. Marrack, and J. W. Kappler. 1981. B cell helper factors. I. Requirement for both interleukin 2 and another 40,000 mol. wt. factor. *J. Exp. Med. 154:1681.*
25. Rabin, E. M., J. Ohara, and W. E. Paul. 1985. B-cell stimulatory factor 1 activates resting B cells. *Proc. Natl. Acad. Sci. USA 82:2935.*
26. DeFranco, A. L., F. S. Ravetch, R. Asofsky, and W. E. Paul. 1982. Frequency of B lymphocytes responsive to anti-immunoglobulin. *J. Exp. Med. 155:1523.*
27. Kaye, J., S. Porcelli, J. Tite, B. Jones, and C. A. Janeway, Jr. 1983. Both a monoclonal antibody and anti-sera specific for determinants unique to individual cloned helper T cell lines can substitute for antigen and antigen-presenting cells in the activation of T cells. *J. Exp. Med. 158:836.*
28. Noelle, R., J. J. McCann, L. Marshall, and W. C. Bartlett. 1989. Cognate interactions between helper T cells and B cells. III. Contact-dependent, lymphokine-independent induction of B cell cycle entry by activated helper T cells. *J. Immunol. 143:1807.*
29. Hodgkin, P. D., L. C. Yamashita, B. Seymour, R. L. Coffman, and M. R. Kehry. 1991. Membranes from both Th1 and Th2 T cell clones stimulate B cell proliferation and prepare B cells for lymphokine-induced differentiation to secrete Ig. *J. Immunol. 147:3696.*
30. Leo, O., M. Foo, D. H. Sachs, L. E. Samelson, and J. A.

Bluestone. 1987. Identification of a monoclonal antibody specific for a murine T3 polypeptide. *Proc. Natl. Acad. Sci. USA 84:1374.*

31. Snapper, C. M., and W. E. Paul. 1987. B cell stimulatory factor-1 (interleukin 4) prepares resting murine B cells to secrete IgG1 upon subsequent stimulation with bacterial lipopolysaccharide. *J. Immunol. 139:10.*
32. June, C. H., and P. S. Rabinovitch. 1991. Measurement of intracellular ions by flow cytometry. In *Current Protocols in Immunology*. Greene Publishing Associates and John Wiley and Sons, New York. *In press.*
33. Kearney, J. F., and A. R. Lawton. 1975. B lymphocyte differentiation induced by lipopolysaccharide. I. Generation of cells synthesizing four major immunoglobulin classes. *J. Immunol. 115:671.*
34. Isakson, P. C., E. Pure, E. S. Vitetta, and P. H. Krammer. 1982. T cell-derived B cell differentiation factor(s): effect on the isotype switch of murine B cells. *J. Exp. Med. 155:734.*
35. Coffman, R. L., and J. Carty. 1986. A T cell activity that enhances polyclonal IgE production and its inhibition by interferon-γ. *J. Immunol. 136:949.*
36. Snapper, C. M., F. D. Finkelman, and W. E. Paul. 1988. Differential regulation of IgG1 and IgE synthesis by interleukin 4. *J. Exp. Med. 167:183.*
37. Snapper, C. M., and W. E. Paul. 1987. Interferon-γ and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. *Science 236:944.*
38. Sharon, R., P. R. B. McMaster, A. M. Kask, J. D. Owens, and W. E. Paul. 1975. DNP-Lys-Ficoll: a T-independent antigen which elicits both IgM and IgG anti-DNP antibody-secreting cells. *J. Immunol. 114:1585.*
39. Barthold, D. R., B. Prescott, P. W. Stashak, D. F. Amsbaugh, and P. J. Baker. 1974. Regulation of the antibody response to type III pneumococcal polysaccharide. III. Role of regulatory T cells in the development of an IgG and IgA antibody response. *J. Immunol. 112:1042.*
40. Mosier, D. E., I. M. Zitron, J. J. Mond, A. Ahmed, I. Scher, and W. E. Paul. 1977. Surface immunoglobulin D as a functional receptor for a subclass of B lymphocytes. *Immunol. Rev. 37:89.*
41. Gray, D., D. Chassoux, I. C. M. MacLennan, and H. Bazin. 1985. Selective depression of thymus-independent anti-DNP antibody responses induced by adult but not neonatal splenectomy. *Clin. Exp. Immunol. 60:78.*
42. Amlot, P. L., D. Grennan, and J. H. Humphrey. 1985. Splenic dependence of the antibody response to thymus-independent (TI-2) antigens. *Eur. J. Immunol. 15:508.*
43. Yellen, A., J. W. Glenn, V. P. Sukhatme, X. Cao, and J. G. Monroe. 1991. Signaling through surface IgM in tolerance-susceptible immature murine B lymphocytes. Developmentally regulated differences in transmembrane signaling in splenic cells from adult and neonatal mice. *J. Immunol. 146:1446.*
44. Benhamou, L. E., P.-A. Cazenave, and P. Sarthou. 1990. Anti-immunoglobulins induce death by apoptosis in WEHI-231 B lymphoma cells. *Eur. J. Immunol. 20:1405.*
45. Sarthou, P., N. Henry-Toulme, and P.-A. Cazenave. 1989. Membrane IgM cross-linking is not coupled to protein kinase C translocation in WEHI-231 B lymphoma cells. *Eur. J. Immunol. 19:1247.*
46. Tisch, R., C. M. Roifman, and N. Hozumi. 1988. Functional differences between immunoglobulins M and D expressed on the surface of an immature B-cell line. *Proc. Natl. Acad. Sci. USA 85:6914.*
47. Ales-Martinez, J. E., G. L. Warner, and D. W. Scott. 1988. Immunoglobulins D and M mediate signals that are qualitatively different in B cells with an immature phenotype. *Proc. Natl. Acad. Sci. USA 85:6919.*
48. Van den Eertwegh, A. J. M., J. D. Laman, M. M. Schellekens, W. J. A. Boersma, and E. Claassen. 1992. Complement-mediated follicular localization of T-independent type-2 antigens: the role of marginal zone macrophages revisited. *Eur. J. Immunol. 22:719.*