MATERIALS AND METHODS
Materials and Methods

Animals

6-8 weeks old BALB/C mice were obtained from animal house facility at our institute.

Materials

All fluorescent labeled antibodies used in flow cytometric analysis, the allotype-specific (anti mouse IgMa and IgG1a) and anti-B220 antibodies were purchased from Pharmingen (San Diego, CA). Heavy chain specific horseradish peroxidase (HRPO) labeled goat anti-mouse secondary antibodies were obtained from Sigma Chemical Co. (St. Louis, MO), HRPO labeled anti-rabbit IgG, anti-rat IgG, and antibodies used in westerns (antibodies against CREB, NF-κB) were purchased from Santacruz Biotech Inc. (SantaCruz, CA). EGTA, PMA, db-cAMP, Staurosporine, Forskolin, Genistein, Nystatin, and Methyl-β-cyclo dextrin were obtained from Sigma Chemical Co. (St. Louis, MO), whereas H89, H-9, Calphostin C, TMB-8, Ionomycin and antibodies against phospho-CREB were from Calbiochem (San Diego, CA). Biotinylated PNA, streptavidin-alkaline phosphatase (AP) and streptavidin-HRPO conjugates were purchased from Vector laboratories (Burlingame, CA). All the tissue culture media was purchased from Gibco.

Peptide Synthesis

Peptide CT3 (sequence: DIEKKIAKMEKASSVFNVNS) was synthesized by the solid phase method (Merrifield, 1986; Stewart and Young, 1984) on a Millipore9050 automated peptide synthesizer (Millipore, Bedford, MA) using the F-moc chemistry. Crude peptides were purified to at least 95% purity by reverse phase HPLC on a C-18 column (15m, Pak, 19x300mm; Waters, Milford, MA) using an aqueous gradient of 0 to 70% acetonitrile in 0.1% TFA. Identities of all peptides were ascertained by amino acid analysis.
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Generation of Ascites

6-8 weeks old irradiated BALB/c mice were primed with 500 μl IFA/pristane, i.p., 3-4 days prior to the injection of cells. The anti-IgD hybridoma cells, JA 12.5, were washed and suspended in 300ul plain RPMI and injected into mice intra-peritoneously. Ascites was collected by inserting an 18-gauge needle in the peritoneum cavity.

Purification of Antibodies

Antibodies were precipitated from ascites using 60% ammonium sulphate, dissolved in PBS (pH 7.2) and dialyzed against PBS 0/N. The antibodies were then purified by affinity chromatography over anti-rat-IgG coupled to the cyanogen bromide activated sepharose beads. Antibodies were incubated with the affinity matrix overnight at 4°C on the rotating shaker. After this, the flow through was removed and the column was washed with 10 column volumes of PBS, (pH 7.2) followed by 10 column volumes of PB containing 1M NaCl, and finally again with 5 column volumes of PBS, pH 7.2. Bound antibodies were then eluted in glycine HCl buffer (pH 2.7), and then immediately neutralized with 1M Tris buffer (pH 8.0) (Harlow and Lane, 1988). The neutralized eluent was subjected to buffer exchange into 0.5 M Tris buffer containing 2mM EDTA (pH 8.0) and concentrated using centricon filters, cutoff 30 KDa (Amicon). Purity of the antibody fraction was ascertained by SDS-PAGE.

F(ab)₂ fragments of anti-IgD antibodies were generated by pepsin digestion. For this, purified anti-IgD antibody was dialyzed extensively against 100mM sodium citrate (pH 3.5). 5 μg of papain was added for each mg of antibody and incubated at 37°C in water bath for 10 hrs. Reaction was stopped by adding 1/10 volume of 3.0 M Tris (pH 8.8). F(ab)₂ fragments were purified over a Sephadex G-200 column and analyzed by SDS-PAGE (Harlow and Lane, 1988).
Purification of resting B lymphocytes:

For the enrichment of B cells, splenocytes from 6-8 weeks old BALB/C mice were first depleted of RBCs by lysis with RBC lysis buffer (8.3 g/l ammonium chloride in 0.01 M Tris-Cl pH 7.5). Cells were washed thoroughly and resuspended in RPMI containing antibiotics with 20% FCS. Adherent cells were removed by panning on plastic surface tissue culture plates at 37 °C for 1 hour. From the supernatant T cells were removed in two rounds by incubating with a mixture of magnetic beads coated individually with anti-CD90 (Thyl.2), anti-CD4, and anti-CD8 antibodies. Separation was done by MACS column. Subsequent to this, resting B cells were purified by discontinuous percoll density gradient. For this 2.5 ml 70% percoll was taken in 15ml falcon and overlayed with 66%, 60% and finally 50% percoll. 2.5 ml of cell suspension in RPMI-10 was overlayed on the top of percoll gradient and centrifuged in swinging bucket rotor at 2,500 rpm for 30 min at 4 °C. High density cells at 66% - 70% interface were collected, washed in HBSS and cultured in RPMI-10. This fraction contained between 96% to 98 % of slgD⁺ B220⁺ cells as determined by flow cytometry, with CD3⁺ cells accounting for less than 2% of the population.

Stimulation of resting B cells:

Purified resting B cell population was cultured at 2x 10⁶ cells/ml in 10% RPMI containing antibiotics (1x10⁵ units/liter). Cells were stimulated with anti-IgD at a final concentration of 10 μg/ml and cultured for a period of 60 hr. Cells were washed with HBSS at the end of incubation and their viability was ascertained with trypan blue, which remained more than 95%. The same culture conditions were also employed when alternate stimulants such as ionomycin, PMA, db-cAMP, A23187, PDBu and Forskolin were used. When the effects of inhibitors were to be examined the cells were first pre-equilibrated with the appropriate inhibitor at 37°C for 1 hr prior to the addition of the stimulant. The final concentration of the various inhibitors used were as follows: EGTA,
3 mM; TMB-8, 50 μM; genistein, 60 μg/ml; Calphostin C, 0.1 μM; and H89, 100 nM. These values were optimized in preliminary titration experiments, and range from 1-2 times the individual IC₅₀ values. Preliminary experiments also established that the presence of the above inhibitors was most critical during the first hour of stimulation with anti-IgD. Thus, addition of these agents at 1 h or more after the addition of anti-IgD resulted in a marked reduction (by 50% or more) in the extent of inhibition observed.

**FACS staining:**

At the end of culture period cells were washed with FACS wash buffer (PBS with 0.5% BSA and 0.01% azide), and the Fe receptor on the cells were blocked by incubating cells with anti-CD16/CD32 antibody. Cells were then stained with B220-biotin followed by labelling with streptavidin-PE. After washing with FACS buffer, cells were stained with PNA-biotin, or biotin labeled antibodies against IgD, CD24 (J11D), I-A, GL7, CD80 and CD86. Subsequently, cells were washed and incubated with Streptavidin–FITC. All the incubations were carried out at 4 °C for 1hr. All the antibodies were diluted in FACS wash buffer in 1:1000 dilution. Following this cells were washed with wash buffer and fixed in fixing solution (PBS with 0.01% azide and 0.1% paraformaldehyde). Finally two-color analysis of cells was carried out on a FACS Calibur flow cytometer (Becton Dickenson Immunocytometry Systems). Logarithmically amplified fluorescence data were collected on 1 x 10⁴ viable cells as determined by forward scatter intensity and by exclusion of propidium iodide-stained cells.

**Adoptive Transfer:**

Resting B cells from BALB/C Igh₄ mice were either left untreated or treated with different doses of anti-IgD for 48 hrs. Wherever required cells were preincubated with inhibitors for 1 hr at 37°C. Following this, cells were loaded with 30 μg/ml synthetic peptide CT3 and cultured for 12 more hrs. Loading of cells with peptide CT3 did not result in further phenotypic alterations over that observed with anti-IgD stimulation alone. Cells were washed and transferred intravenously (2 x 10⁶ cells/mouse).
into CT3-primed (primed four days earlier at the base of tail with 50 μg/mouse of CT3 emulsified in CFA) BALB/c IgH<sup>b</sup> mice. After ten days of B cells transfer spleens were removed from the recipients and frozen in OTC compound (BDH, UK) for cryo sectioning.

**Germinal Center Staining:**

6μm thick sections were cut with cryostat and thaw-mounted on glass slides. Sections were air dried and fixed in ice-cold acetone for 15 min, dried at 37°C O/N, and stored at -80°C until use.

When required frozen sections were thawed at and rehydrated in GC buffer (1X PBS filtered) for 20 min. To quench endogenous peroxidase activity 0.1% phenylhydrazine was added on sections and kept at RT for 10 min. Sections were washed twice with GC buffer. For blocking nonspecific sites sections were incubated with 1/1 (v/v) solution of 3% BSA (in PBS) and mouse nonimmune serum for 1 hr at 37°C. After washing, sections were incubated with 20 μg/ml PNA-biotin in HEPES buffer (pH-7.5) for 90 min at 37°C. Sections were washed thrice and followed by incubation with streptavidin-–HRPO (5 μg/ml in PBS) for 45min. All the incubations were carried out in humidified chamber.

For allele specific staining, sections were incubated with IgH<sup>a</sup>- allele (IgG<sup>a</sup> plus IgM<sup>a</sup>) specific antibodies (5μg/ml in PBS) at 37°C O/N. Slides were washed thrice with GC buffer and further incubated with 5μg/ml streptavidin-AP for 45min. Sections were washed thoroughly and developed in a sequential manner for bound conjugates. The HRPO conjugate was first detected using AEC staining kit where a red color was obtained for allele specific cells. After washing, AP conjugate was developed with blue substrate kit for the detection of PNA<sup>+</sup> cells.
Intracellular Calcium measurement:

For monitoring Ca^{2+} 2 x 10^7 cells were labeled with 1\mu M FLUO-3-AM in 10 % RPMI at 37^\circ C for 1 hr. Cells were washed extensively with HBSS and resuspended in RPMI-10. Cells were stimulated with varying doses of either anti-IgD or ionomycin and the resultant effects were monitored by flow cytometry.

cAMP measurement

For the measurement of intracellular cAMP, cells were first equilibrated in serum-free medium for 4h, after which they were plated in 100\mu l aliquots (containing 1 x 10^5 cells) into wells of a 96-well tissue culture plate. Quadruplicate wells were then stimulated with the anti-IgD (or anti I-A) for 1h at 37^\circ C. Cells were lysed in lysis buffer and cAMP levels were measured in lysates using the BIOTRAK EIA kit (Amershams), following the protocol recommended by the manufacturer.

To determine the effects of exogenous addition of db-cAMP on intracellular cAMP levels, cells were first incubated with varying concentrations of db-cAMP for 1 h at 37^\circ C. Cells were then washed extensively in serum free medium, and the intracellular levels determined as described above.

Separation of membrane and cytosolic proteins:

5 x 10^7 resting B cells were either left untreated or treated either with aIgD (10 \mu g/ml) or varying concentrations of PMA and incubated at 37^\circ C for 10 minutes. Cells were washed twice with HBSS, suspended in 100\mu l ice cold Buffer A (0.25M sucrose,10mM HEPES,10mM \beta – mercaptoethanol,1mM PMSF, and 1mM sodium orthovanadate, pH 7.4) collected into Dounce Homogenizer and subsequently lysed with 30 strokes. The cellular homogenates were ultracentrifuged at 100,000 x g for 45 min. the cytosolic supernatant fraction was collected and resultant pellet (membrane fraction) was resuspended in Buffer B (i.e. Buffer A containing 1% Triton X-100) at 4 \degree C and
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ultracentrifuged as before. Detergent solubilized supernatant was collected and stored at 4 °C.

Measurement of PKC activity:

PKC activation was determined in terms of its translocation from the cytoplasmic to the membrane faction (Chao et al., 1992). PKC activity was assayed in each fraction (25μl) in a reaction mixture containing, in a final volume of 75 μl, 50 mM Tris-HCl, 5mM magnesium nitrate, 1mM calcium chloride, 2μg/ml PMA, 40μg/ml phosphatidyl serine, 0.2mg/ml Histone, 10μCi/ml of [32P]ATP, 50μM cold ATP. The reaction was performed at 25 °C for 15min and was stopped by addition of 1ml ice cold 10% TCA. Proteins were allowed to precipitate at 4 °C for 30 min, and then separated from free [32P]ATP by filtration through glass fiber disks. Precipitated proteins were washed thrice with 20mM tetra sodium pyrophosphate in 5% TCA and then with ethanol. Disks were air-dried and incorporated radioactivity was determined by liquid scintillation spectrometry.

Radiochemical labeling of GPI-linked sIgD (Hooper and Turner, 1992)

For sugar labeling 5 X 10^7 cells were washed with HBSS and resuspended in glucose free RPMI. Cells were preincubated with tunicamycin at a final concentration of 5μg/ml for 1 hr at 37°C. Subsequent to this, 25μCi/ml D-1,6-3H Glucosamine hydrochloride (sp. Activity- 49 Ci/mmol) or D-2-3H Mannose (sp. Activity – 23.90 Ci/mmol) was added to the culture and incubated for 4 hrs at 37 °C.

For fatty acid labelling cells were incubated at 37°C for 10 min. in RPMI supplemented with fatty acid free BSA. 14C-Palmitic acid (850 mCi/mmol) and 9,10-3H-Myristic acid (12.50 Ci/mmol) were added to the culture at the final conc. of 50 μCi/ml and incubated for 4 hrs. Viability was assessed at the end of incubation. Cells were washed extensively and lysed in lysis buffer.
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**Immunoprecipitation:**

Labeled cells from the individual groups were washed and lysed in lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 2.5 μg/ml each of leupeptin, pepstatin and aprotinin, 1 mM sodium orthovanadate and 1% Triton X-100) and kept on ice for 30 min. Lysates were centrifuged at 900 g for 10 min to remove nuclei and cellular debris, and subjected to sucrose density ultracentrifugation. The sIgD from the pooled detergent-soluble and -insoluble fractions from each group was immunoprecipitated by first incubating with biotinylated anti-mouse IgD (10 μg/ml at 4 °C for 1 h in 10 mM Tris containing 1% Triton X-100, 150 mM NaCl, 1 mM each of EDTA and EGTA, and 2.5 μg/ml each of leupeptin, pepstatin and aprotinin, 1 mM PMSF, and 1 mM orthovanadate; pH, 7.5), followed by streptavidin-agarose (at 4 °C for 30 min). Samples were centrifuged, and the pellets washed extensively in the Tris buffer. All immunoprecipitates were then treated with endo-0-glycosidase (1 u/ml for 2 h at 37 °C in PBS) to remove O-linked glycosylation. Further, where necessary, immunoprecipitates from fatty acid-labeled cells were also treated with PI-PLC (1 u/ml at 37 °C for 1 h). Subsequent to this, immunoprecipitated proteins were resolved on a 10% SDS-polyacrylamide gel, and then transferred onto a nitrocellulose membrane. The regions corresponding to the IgH chain (as identified from Western blot analyses) were excised, and processed for the determination of incorporated radioactivity by liquid scintillation counting.

**Rafts isolation:**

For the isolation of lipid rafts 1 × 10⁸ cells were washed with ice cold PBS and lysed on ice for 30 min in 1% triton X-100 in TNEV buffer containing protease and phosphatase inhibitors (10 mM Tris-HCl, pH-7.5, 150 mM NaCl, 5 mM EDTA, 2.5 μg/ml each of leupeptin, pepstatin and aprotinin in DMSO and 1 mM sodium orthovanadate). The lysis solution was further homogenized with 20 strokes in Dounce Homogenizer. Lysates were centrifuged at 900 g for 10 min to remove nuclei and cellular debris. 1 ml of cleared supernatant was mixed with 1 ml of 85% sucrose in TNEV and transferred to the
bottom of a Beckman 14 X 89 mm centrifuge tube. The diluted lysates were overlaid with 6 ml of 35% sucrose in TNEV and finally with 3 ml of 5% sucrose in TNEV. The samples were centrifuged in SW41 rotor at 200,000 g for 20 hrs at 4 °C. 1 ml fractions were collected from the top of the gradient. Fractions 4-6 contain detergent insoluble proteins and 10-12 contain detergent soluble protein. Aliquots of each fraction were then resolved by gel electrophoresis and slgD detected with anti-IgD antibodies by a western blot analysis. The efficiency of fractionating procedure was confirmed by monitoring the distribution of GM1-ganglioside, which is predominantly associated with the raft fractions, and CD45R (or B220), which is exclusively present in the detergent soluble fraction.

Disruption of lipid rafts:
Lipid rafts were disrupted by incubating cells with either 10mM MCD for 15 min at 37 °C or with 50 µg/ml nystatin for 10 min at 37 °C.

PI-PLC Treatment of cells:
2 x 10^6 cells were incubated with 1 unit/ml PI-PLC in HBSS for 1 hr at 37 °C. Cells were washed with HBSS and processed.

Preparation of Nuclear and Cytosolic extracts.

Cells were lysed in 200µl lysis buffer containing 10mM HEPES, 10mM KCl, 0.1mM each of EGTA and EDTA containing protease and phosphatase inhibitors and kept on ice. After 20-30 min. 1% NP-40 was added, vortexed and centrifuged at high speed. Supernatant containing cytoplasmic extracts was collected and to the pellet 30µl extraction buffer (20mM HEPES, 0.4 M NaCl, 1mM each of EDTA and EGTA with protease and phosphatase inhibitors) added and kept on ice for 60 min. with intermittent vortexing. Samples were centrifuged at 12,000 rpm for 5 min at 4°C and supernatant containing nuclear extracts were removed. Both cytoplasmic and nuclear extracts were subjected to SDS PAGE and subsequent western blotting for NF-κB, CREB and p-CREB.
**RNA isolation:**

For the isolation of RNA, $5 \times 10^6$ well purified resting B cells were either left untreated or treated with 10μg/ml anti-IgD for 12, 24 or 48 hrs. Cells were washed and lysed in 1 ml of TRIzol reagent (GIBCO BRL) by pipetting, followed by addition of 200 μl chloroform. Contents were mixed by shaking and were centrifuged at 12,000 x g at 4°C for 15 min. Colourless aqueous phase was collected and RNA was precipitated by mixing 0.5 ml of isopropanol. This was followed by a wash with 75% ethanol in DEPC water. Finally RNA pellet was dissolved in DEPC water after drying. The quantity of RNA was measured by taking absorbance at 260 nm.

**RT-PCR.**

cDNA for CD80, CD86, CD24, and MHC II was generated by using QIAGEN one-step RT-PCR kit. Reaction mixtures were prepared as follows-

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNAses free water</td>
<td>variable</td>
</tr>
<tr>
<td>5 x RT-PCR buffer</td>
<td>10.0 μl</td>
</tr>
<tr>
<td>dNTP mix(containing 10mM of each dNTP)</td>
<td>2.0 μl</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>0.6 μM</td>
</tr>
<tr>
<td>Forward primer</td>
<td>0.6 μM</td>
</tr>
<tr>
<td>RT-PCR enzyme mix</td>
<td>2.0 μl</td>
</tr>
<tr>
<td>RNase inhibitor</td>
<td>5 units</td>
</tr>
<tr>
<td>Template RNA</td>
<td>1.0 μg</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>50 μl</strong></td>
</tr>
</tbody>
</table>

Reaction was set on ice and tubes were kept in programmed thermal cycler.
Program used for RT-PCR is as follows-

Reverse Transcription: 30 min 50°C
Initial PCR activation step: 15 min 95°C
3 step cycling: (total 30 cycles)
Denaturation: 1 min 94°C
Annealing: 1 min 47°C
Extension: 1.5 min 72°C
Final extension: 10 min 72°C

Primer sequences-

For CD24 -
Forward primer- 5'- TTACTgCAACCAACCATCTGT-3'
Reverse primer - 5' - TCTCCgCCATTTCTTTCTTTT-3'

For CD86 -
Forward primer- 5'- TCTCAgATgCTgTTTCCgTg-3'
Reverse primer- 5' - ggTTCACTgAAgTTggCgAT-3'

For CD80 -
Forward primer- 5'- CCTTgCCgTTACAACTCTCC-3'
Reverse primer- 5'- CggAAgCAAAAgCAggTAATC-3'

For I-A-
Forward primer- 5'- gTgACCGcGTCCTCGCTCCgC-3'
Reverse primer- 5' - AgACAgTCAACTgAgTCAAAA-3'

PCR product was cloned in pGEMT vector. Cloned product was transformed in JM109 competent cells. Transformed colonies were selected on ampicillin plates and inoculated in Luria broth medium (LB) containing 100 µg/ml of ampicillin. Plasmid DNA was isolated by miniprep (Birnboim and Doly, 1979) and cloned fragments were
excised using EcoRI restriction endonuclease. Excised fragments were resolved on the 1% agarose gel and purified using QIAquick gel extraction kit. Purified DNA fragments were used to generate probe for northern analysis. Later probe was made by random priming method (Amersham).

**Northern blotting:**

30 μg RNA per well was fractionated on formaldehyde agarose gel in MOPS buffer. RNA was transferred overnight onto Hybond-N-nylon membrane (Amersham). Membrane was hybridized with $^{32}$P-labeled full length gene specific probes for CD24, CD80, CD86 and I-A. Blots were exposed to the X-ray film and developed. Blots were then reprobed with $^{32}$P-labeled probe for β-actin as loading control after stripping off the initial probes.

**Generation of membrane IgD$_{H\alpha L}$ (mIgD$_{H\alpha L}$) transfected J558L cells.**

The entire λ$_L$ and the variable IgH segment (including the leader sequence in both cases) was obtained by RT-PCR of the mRNA obtained from the BBE6.12H3 hybridoma cells. In parallel, resting B cells were isolated from BALB/c mouse splenocytes, and the resultant mRNA was taken for generation of the slgD constant region by RT-PCR. The variable IgH and slgD constant regions were then ligated and cloned into the pGEMT vector, and the integrity of the full-length cDNA was verified by nucleotide sequencing. Both the resultant λ$_L$ and slgD cDNAs were independently cloned into the retroviral vector PLNCX-2 (Clontech Laboratories, Palo Alto, CA). These constructs were then separately taken to infect packaging cells (Retropak PT67; Clontech).

Supernatants from these two cultures were pooled in equal amounts and used in two rounds of transfection of J558L cells. Stably transfected cells were selected on the basis of G418 resistance and were expanded before use.
Primer sequences:

\(\lambda_{\mathrm{H}}\) of BBE6.12H3

Forward primer- 5'-CCCCAgCTTATggCCTggATTTCACTTATACTC-3'
Reverse primer- 5'-CCggAATTCCRRACABTCWCASGGRGACARACTCTT-3'

Where R = A, or g
B = C, or g, or T
W = A, or T
S = C, or g

variable Ig\,H segment of BBE6.12H3 (including leader sequence)

Forward primer- 5'-gggAAgCTTATgggATggAgCTGTATC-3'
Reverse primer- 5'-gCgggATCCTgAggAgACTgTgAgTgg-3'

Constant region of sIgD:

Forward primer- 5'-CcCggATCCAAACTTCATCTgTCTTgCAggT-3'
Reverse primer- 5'-CCggAATTCTTACACCTTgATgAAggTgAC-3'