Early Growth Response Genes Regulate B Cell Development, Proliferation, and Immune Response

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Early Growth Response Genes Regulate B Cell Development, Proliferation, and Immune Response

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Egr-1 (early growth response gene-1) is an immediate early gene encoding a zinc finger motif-containing transcription factor. Upon cross-linking of BCR, mature B cells undergo proliferation with an increase in Egr-1 message. Immature B lymphoma cells that express Egr-1 message and protein constitutively are growth inhibited when Egr-1 is down-regulated by negative signals from BCR or by antisense oligonucleotides. To test the hypothesis that Egr-1 is important for B cell development, we examined B cells from primary and secondary lymphoid organs in Egr-1-/-/- mice. Marginal zone B cell development was arrested in these mice, whereas the B cells in all other compartments were increased. To test the hypothesis that Egr-1 function may be partially compensated by other Egr family members, we developed transgenic mice expressing a dominant negative form of Egr-1, which lacks the trans activation domain but retains the DNA-binding domain, in a B cell-specific manner. There was a decrease in B lymphopoiesis in the bone marrow accompanied by a reduction in splenic immature and mature B cells as well as marginal zone B cells in the transgenic mice. Moreover, transgenic mice respond poorly to BCR cross-linking in vitro and T-independent and T-dependent Ags in vivo. The Journal of Immunology, 2008, 181: 4590–4602.
FIGURE 1. Altered B cell subsets in the bone marrow and spleen of Egr-1$^{-/-}$ mice. Flow cytometric analysis of bone marrow cells obtained from Egr-1$^{-/-}$ and littermate controls stained with CyChrome anti-B220 and PE anti-IgM. B220$^{+}$IgM$^{-}$ cells represent pre-pro B, pro-B, and pre-B cells. B220$^{+}$IgM$^{+}$ cells represent immature B, transitional, and recirculating mature B cells (A). Bone marrow cells were stained with PE-Cy anti-B220,

B

Gated on B220$^{+}$ CD43$^{+}$ lymphocytes

C

Gated on B220$^{+}$ CD43$^{+}$ lymphocytes

D

Gated on B220$^{+}$ CD43$^{+}$ lymphocytes

E

Gated on CD23$^{-}$ lymphocytes

F

Gated on CD23$^{+}$ lymphocytes

Wild type $^{+/-}$

Egr1$^{-/-}$

Wild type $^{+/-}$

Egr1$^{-/-}$

Wild type $^{+/-}$

Egr1$^{-/-}$

Wild type $^{+/-}$

Egr1$^{-/-}$

B-2 B cells

C57BL/6

B-1 B cells

Egr1$^{-/-}$

B-2 B cells

Egr1$^{-/-}$

B-1 B cells

Wild type $^{+/-}$

Egr1$^{-/-}$

Wild type $^{+/-}$

Egr1$^{-/-}$
Schnell et al demonstrated that Egr-1 is required for survival of mature thymocytes and newly emigrated thymocytes (13). All four family members are induced upon TCR ligation. Overexpression of Egr-2 and Egr-3 is associated with an increase in the E3 ubiquitin ligase Cbl-b and inhibition of T cell activation. Also, T cells from Egr-3−/− mice display lower Cbl-b and are resistant to in vivo peptide-induced tolerance (14). These data support the idea that Egr-2 and Egr-3 are involved in promoting TCR-induced negative signaling. The role of Egr-1 in macrophage differentiation has also been studied in detail. Using a variety of differentiation-inducible myeloid cell lines, Krishnaraju et al. (15–17) showed that the ectopic Egr-1 expression in normal hemopoietic progenitors stimulates development along the macrophage lineage at the expense of development along the granulocyte or erythroid lineages, regardless of the cytokine used. These observations are in contrast to the phenotype observed in Egr-1−/− mice where differentiation along the macrophage lineage remains normal (18).

In B lymphocytes, cross-linking of the Ag receptor results in egr-1 expression through activation of the p21WAF1/CIP1/MAPK pathway (1, 19). Detailed analysis of the Egr-1 promoter showed that the two most distal serum response elements mediate Egr-1 gene induction (20). Egr-1-binding sites are found within the promoter regions of the genes that encode ICAM-1 and CD44, two cell adhesion molecules important for cell trafficking (20–22). ICAM-1 and CD44 are up-regulated in B cells upon receptor cross-linking. Although the Egr-1 promoter has been studied extensively, its role in B cell development and function is not well understood. The role of Egr-1 in pre-B cell development has been demonstrated previously, and Egr-1 was shown to be important for BP-1 expression in the bone marrow pre-B cells (23). Differential expression of Egr-1 in anergic and naive B cells has also been studied previously with differing conclusions, with an increase in Egr-1 in anergic B cells in one of the studies and no such increase in another (24, 25). It was shown that the Egr-1 gene is methylated and not induced upon BCR ligation of an immature B cell line WEHI-231 (26). This suggests that inactivation of the Egr-1 gene might be important for tolerance induction in B cells. In our laboratory, we find that when mature B cells are cross-linked, they undergo proliferation with an increase in Egr-1 expression whereas neonatal B cells, which are immature, become unresponsive with only a modest increase in Egr-1 (19). This differential expression pattern of Egr-1 during different stages of B cell development may be related to induction of tolerance vs clonal expansion in B cells. Accordingly, microarray analysis of naive and tolerant B cells showed a difference in Egr-1 expression subsequent to BCR cross-linking, followed by a rapid decrease in Egr-1 (27). Moreover, BKS-2 B lymphoma cells, which have an immature B cell phenotype and express Egr-1 constitutively, undergo growth arrest when treated with antisense oligos specific for Egr-1 (28). Microarray studies demonstrate a 100-fold decrease in Egr-1 mRNA when surface BCR is deleted in immature B cells derived from the bone marrow of Cre-LoxP conditional transgenic mice in which Cre activation leads to H chain deletion (29). These data show that Egr-1 is important for B cell survival and that Egr-1 induction downstream of BCR might be important for B cell development. Despite these studies, the in vivo role of Egr-1 during B cell development and functional responses remains to be elucidated.

Although Egr-1−/− mice have been generated, no significant effect of Egr-1 deficiency on B cell development has been reported (30). This could be due to the redundancy of Egr-1 function given that this family has three other members with similar transcriptional activation properties (31, 32). Previous studies of Egr-1−/− mice did not examine the effect of Egr-1 deficiency on specific stages of B cell development. Hence, we performed a detailed analysis of B cell development in Egr-1−/− mice. In addition, we generated transgenic mice in which the function of all Egr-1 family members is inhibited by a dominant-negative (DN) Egr-1 construct in a B cell-specific manner. We show that Egr family members are important for B lymphopoiesis and proliferation, and that follicular B cells lacking this transcription factor are defective in proliferative response to Ag receptor stimulation. Egr-1, in particular, is essential for marginal zone (MZ) B cell development. We also demonstrate that Egr family members are critical for immune responses in vivo.

Materials and Methods

Mice

Egr-1−/− mice were generated and backcrossed to C57BL/6 (B6) mice as described earlier and were a gift from Dr. Jeffrey Milbrandt (30). Dominant-negative Egr-1 (ΔEgr-1) construct was generated as described previously (20, 33). A FLAG tag was added to this construct at the C terminus and was then cloned into a plasmid containing the Ig H chain promoter and μ enhancer (obtained from Dr. N. Muthusamy, Ohio State University, Columbus, OH) so that the transgene would be expressed only in B cells (34). DN-Egr-1-transgenic mice were generated by pronuclear microinjection by the University of Kentucky Transgenic Facility (Lexington, KY). We generated four founders in the (C3H × B6)F1 background. One line has been backcrossed for nine generations onto a B6 background.

Reagents and cell lines

Abs to Egr-1 (C-19), c-Myc, and cyclin D2 were obtained from Santa Cruz Biotechnologies, and Abs to Egr-2 were purchased from Covance Research Products. Anti-β-actin mAb was obtained from Sigma-Aldrich. The immature B lymphoma cell line BKS-2 was isolated and maintained in vivo as a splenic tumor in our laboratory (35). Female CBA/N (Xid) mice were obtained from The Jackson Laboratory. Mice were housed in microisolator cages in our American Association for Laboratory Animal Accreditation and Certification-approved rodent facility. BKS-2 B lymphoma cells obtained from the spleens of CBA/N mice were depleted of T cells with a mixture of anti-T cell Abs and complement as described (35). Normal splenic B cells were prepared according to procedures described previously (36). The characteristics of the monoclonal rat anti-mouse μ chain Ab, AK11, were described previously (37, 38).

FITC anti-CD43, PE anti-BP-1, and allophycocyanin anti-HSA for fractions A–C′ (B). B220−CD43+HSA−BP-1− represents fraction A, B220−CD43−HSA−BP-1− represents fraction B, B220−CD43+HSA−BP-1− represents fraction C, and B220−CD43−HSA−BP-1− represents fraction C′. Bone marrow cells were stained for PE-Cy anti-B220, FITC anti-CD43, PE anti-IgM, and allophycocyanin anti-IgD for fractions D–F (C). B220−CD43+IgM−IgD− represents fraction D, B220−CD43−IgM+IgD− represents fraction E, and B220−CD43−IgM−IgD+ represents fraction F. Percentages indicate relative values of each subset compared with other subsets in the same histogram. D, Resolution of immature transitional B cell subsets and the follicular B cells in the spleen of 10-wk-old Egr-1−/− and littermate controls by staining splenocytes with FITC anti-IgM, PE-Cy-anti-B220, allophycocyanin-anti-AA4.1.1, and PE-anti-CD23 and analyzed on a FACSCalibur. B220−AA4+ cells represent immature transitional B, B220−AA4+ cells represent mature follicular B. There were 100,000 events collected; data are representative of the average of six mice in each group. E, Flow cytometric analysis of marginal zone B cells in the spleen of Egr-1−/− and the littermate control by staining with FITC anti-CD21, PE-Cy-anti-B220, allophycocyanin-anti-HSA, and PE-anti-CD23. MZ B cells are HSA−CD21+ gated on CD23+ splenocytes. F, Peritoneal B cell subsets were identified by staining with PE-Cy-anti-B220 and PE-anti-CD23 and analyzed by flow cytometry. Peritoneal B-1 cells represent B220−CD23− cells. Data are representative of three independent experiments.
Cell preparation and staining
Suspensions of BM cells were flushed from tibiae and femurs and spleno-
cytes were prepared through crushing of spleens in HBSS. Cells were
washed and then incubated with optimal dilutions of the indicated Abs in
polyethylene round-bottom tubes in a final volume of 100 µL. After 30
min on ice, cells were washed twice with FACS buffer and, when appropriate,
cells were incubated for 20 min on ice before two final washes with flu-
orochrome-conjugated streptavidin to reveal staining by biotinylated Abs.

Flow cytometric analyses
PE-Cy5 anti-CD45/SR220 (RA3-6B2), PE anti-CD1, FITC anti-CD43,
FITC anti-IgM, PE anti-IgM, PE anti-CD23, FITC anti-CD21, FITC anti-
CD5, biotin-anti-CD24/HSA (30F1), and anti-sIgD Abs were obtained
from eBiosciences. Analyses were conducted on a dual-laser flow
cytometer (FACS Calibur; BD Immunocytometry Systems) or a MoFlo cell
sorter (DakoCytomation). All flow cytometry data were analyzed with
CellQuest software.

Retroviral production and transduction of B lymphoma cells
Egr-2 and Wilms’ tumor Egr-1 (WT-Egr) constructs cloned into the
retroviral vector LZR5pnBMN-linker-internal ribosomal entry site-enhanced
GFP (LZRS) encompassing an internal ribosomal entry site were described
previously (32). Retroviral vectors were transiently transfected into Phoe-
nix packaging cells using the Lipofectamine transfection system (Invitro-
gen) according to the manufacturer’s protocol. Transfection efficiency was
assessed by determining the percentage of Phoenix packaging cells ex-
pressing enhanced GFP by FACS. Virus-containing supernatants were
harvested from transfected Phoenix cells and pretreated with 5 µg/ml po-
lybrene (Sigma-Aldrich). BKS-2 B lymphoma cells were harvested from serum-
free OCTEM, single-cell suspensions were incubated at a concentration of
1 × 10^6 cells/ml/well of a six-well plate for 2 h at 30°C in 2 ml of viral
supernatants, and the plates were spin infected. At the end of the 2-h in-
fec tion period, virus supernatant was discarded, and fresh IF-12 medium
was added to the cells, which were cultured for 48 h in a 5% CO₂ humid-
fied incubator. After 2 days, cells were sorted for GFP using a MoFlo cell
sorter, and sorted cells were plated and proliferation measured 48 h later
as described in the next section.

Proliferation assay
T-depleted B cells from the Egr-1^-/- mice, DN-Egr transgenic mice, and
appropriate littermate controls were incubated at 2 × 10^6 cells/well in
triplicate in 96-well flat-bottom plates in medium consisting of IMDM and
Ham’s F-12 medium supplemented with 10 mM glutamine, 10 mM HEPES, 0.5 mg/ml gentamicin, and 5 × 10^-2 M-2-mercaptoethanol. Stimuli added in-
cluded F(ab’2) goat anti-IgM (μ-chain specific; ICN Pharmaceuticals), LPS (Sigma-Aldrich), or anti-CD40 (clone 1C10). After 44 h, cultures were pulsed with 1 µCi of [3H]thymidine and harvested 4 h later for scintillation counting. BKS-2 cells were cultured in IF-12 medium (1:1 mixture of
IMDM and Ham’s F-12 (+10% FCS; Atlanta Biologicals). To measure proliferations, 2 × 10^7 cells were cultured in 200 µl of medium. The cells were harvested onto filter mats using a cell harvester (Packard). The levels of
radioactivity incorporation were measured with a Matrix 96 beta
radiation counter (Packard). Results are presented as the arithmetic mean
of triplicate cultures ± SE and statistical significance of different treatments
is evaluated by Student’s t test. Percentage of control response was defined
as (cpm in the treated group/cpm in the untreated group) × 100.

Real-time PCR
Total RNA was isolated from B cells with the Tri-reagent (Sigma-Aldrich),
and 2 µg of total RNA was subsequently used to make cDNA using the
Superscript II reverse transcriptase (Invitrogen) according to the manufac-
turer’s protocol. RT-PCR was performed on an ABI Prism 7000 machine
using Taqman-based Egr-1, Egr-2, and Egr-3-specific primers and probe
(Applied Biosystems). The GAPDH-specific primers and probe were used
for loading control (Applied Biosystems).

Western blotting
T-depleted B cells from the Egr-1 knockout, DN Egr-transgenic and li-
ternate mice were rested for 3 h in serum, insulin, progesterone-free IF-12
medium and then were stimulated with anti-IgM (25 µg/ml) or PMA (10
ng/ml) for 1 h. Cells lysates were prepared in 1× SDS sample buffer or 1%
Triton X-100 as described earlier and were subjected to SDS-PAGE and
Western blot analysis (36). Western blots were analyzed by probing the
membrane using various primary Abs (Egr-1 and Egr-2) followed by HRP-
conjugated secondary Abs (Santa Cruz). The blots were developed with
Kodak X-OMAT films which were scanned with a flat-bed scanner (UMAX
Technologies). Alternatively, the blots were scanned on a Kodak Image
Station 2000RT (Eastman Kodak). For reprobing, membranes were
stripped using a solution containing 62.5 mM Tris-HCl, 2% SDS, and 100
mM 2-β-ME at 65°C for 20 min. The relative integrated OD of the protein
bands was estimated using Scion Image software (Scion Corp.) or Kodak
Image Station software.

In vivo immunizations and plaque-forming cell (PFC) assays
Mice (both DN-Egr-transgenic and littermate controls) were immunized
i.p. with 10 µg of trinitrophenyl (TNP)-Ficoll or 10% v/v of SRBC in 0.1
PBS. The number of IgM anti-TNP-secreting cells was determined
5 days after immunization using a glass slide version of the tech-
nique of localized hemolysis in a gel as described (39). Briefly, a 1-ml
packed cell volume of SRBC (Colorado Serum) was coupled with 2,4,6-
trinitrobenzenesulfonic acid (Eastman Kodak) following published proto-
cols. The splenocytes were washed with HBSS and then mixed with 50 µl
of 13.5% (v/v) TNP-coupled SRBC, 200 µl of 2× base Eagle’s medium
(Life Technologies), and 200 µl of 1.6% agarose (FMC Bioproducts) and
poured onto a glass microscope slide (Goldseal). The slides were incubated
for 1 h at 37°C, and the plaques were developed during an additional 1-h
incubation at 37°C with guinea pig complement (Pel-Freeze Biologics). The
plaques representing Ab-forming cells were viewed using a light microscope
(Pel-Freeze Biologics). The plates representing Ab-forming cells were viewed under a low power
microscope. Ag-specific Ab-forming cells were calculated by taking the
number of plaques for the Ag-immunized mice and subtracting the number
of plaques obtained in the vehicle-immunized mice. Results are provided as the
arithmetic mean ± SE, and the statistical significance of different treat-
ments is evaluated by Student’s t test.

ELISA
Serum Ig levels were measured by mouse Ig isotyping kit (BD
Biosciences).

Table 1. Immature B cell accumulation in the bone marrow and spleen of Egr-1^-/- mice and a decrease in marginal zone but not follicular B cells in the spleen of Egr-1^-/- mice

<table>
<thead>
<tr>
<th>Bone Marrow (×10^6 cells/organ)</th>
<th>Spleen (×10^6 cells/organ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B220+ (pre-pro, pre, immature, transitional, and mature recirculating) B cells</td>
<td>B220+ IgM- (pre-pro, pre, and pre) B cells</td>
</tr>
<tr>
<td>B220+ IgM+ (immature, transitional, and mature recirculating) B cells</td>
<td>B220+ AA4+ transitional B cells</td>
</tr>
<tr>
<td>B220+ AA4+ follicular and marginal zone B cells</td>
<td>CD23- HA5 CD21+ marginal zone B cells</td>
</tr>
</tbody>
</table>

Wild type (+/+): 1.8 ± 0.4 (15 ± 2) 0.16 ± 0.2 (10 ± 3) 1.64 ± 0.6 (5 ± 2) 5.1 ± 0.8 (4 ± 2) 25 ± 5 (28 ± 4) 4 ± 0.3 (16 ± 2) 1.01 (4 ± 1) NS <0.05

Egr-1^-/-: 3.0 ± 0.3 (34 ± 4) 0.72 ± 0.2 (29 ± 2) 2.28 ± 0.3 (9 ± 2) 12.5 ± 0.3 (14 ± 2) 26 ± 3 (30 ± 3) 1 ± 0.1 (4 ± 1) NS <0.05

* Absolute numbers of the indicated B cell populations are obtained by multiplying the frequencies of cells by the number of cells harvested from two tibiae and two femurs or the spleen from each mouse. Various B cell precursor members were estimated by FACS as shown in Fig. 1. Values represent mean ± SE of B cell numbers from six mice each in all cases except for MZ B cells where four mice each of Egr-1^-/- and Egr-1^-/- were used. Numbers in parentheses represent percent of cells with indicated phenotype in each tissue. p values were calculated for absolute numbers with Student’s t test, using data from littermate mice as the control population.
Results

Increase in immature B cells in the bone marrow and spleen of Egr-1−/− mice

The number of B220+ cells in the bone marrow was increased in Egr-1−/− mice (Table I). Analysis of B cell development according to the Hardy protocol showed a significant increase in the number of pre-pro B cells, pro-B cells, pre-B cells (B220+CD43+IgM−) and immature B cells, transitional and mature recirculating B cells (B220+CD43−IgM+) in the bone marrow of mice lacking Egr-1 (Fig. 1A and Table I; Ref. 40). Further subfractionation of pro-B cells using the BP-1 and HSA markers into A (pre-pro-B), B (pro-B), C (pre-BI), and C' (pre-BII) populations revealed a decrease in fraction C' in Egr-1−/− mice compared with wild-type mice (Fig. 1B). Typical FACS profiles are shown in various panels of Fig. 1, and summary data based on several mice are shown in Table I. The reduction in fraction C' is likely due to a decrease in high BP-1 expression, a marker for this fraction, which in turn is likely due to a direct role of Egr-1 in enhancing BP-1 expression (23). Decreased BP-1 expression does not appear to affect B cell maturation given that we observed an increase in fractions D and E, which are thought to be derived from fraction C' (Fig. 1C). There appeared to be a decrease in fraction F (recirculating follicular B cells) in the Egr-1−/− mice. Currently, it is not clear whether this is due to homing and/or retention defect in the bone marrow. A homing defect is consistent with the finding that Egr-1 regulates ICAM-1 and CD44, adhesion molecules involved in trafficking (20, 21). Thus, in the Egr-1−/− mice, there is an overall increase in pre-pro-B (fraction A), pro-B (fraction B),...
pre-BI (fraction C), and immature (fraction E) B cells in the bone marrow, suggesting that Egr-1 regulates B lymphopoiesis at a very early stage possibly the common lymphoid progenitors (CLP). The increased output of immature B cells in the bone marrow was reflected in the spleen of 10-wk-old DN-Egr-transgenic mice and littermate controls by staining splenocytes with FITC anti-IgM, PE-Cy-anti-B220, allophycocyanin-anti-AA4.1, and PE-anti-CD23 and analyzed on a FACS Calibur. B220⁺AA4⁺ cells represent immature transitional B cells; B220⁺AA4⁻ cells represent mature follicular B cells and MZ B cells. Subfractionation of B220⁺AA4⁺ immature B cells based on IgM and CD23 expression reveals IgM⁺CD23⁻ transitional T1 cells and IgM⁺CD23⁺ transitional T2 cells. Subfractionation of B220⁺AA4⁻ mature B cells based on IgM and CD23 expression reveals IgM⁺CD23⁻ MZ B cells and IgM⁺CD23⁺ follicular B cells. There were 100,000 events analyzed; data are representative of the average of eight mice in each group. C, Flow cytometric analysis of MZ B cells in the spleen of transgenic and the littermate controls by staining with FITC anti-CD21, PE-Cy-anti-B220, allophycocyanin-anti-HSA, and PE-anti-CD23. Data are representative of four independent experiments. D, Flow cytometric analysis of peritoneal B cells in the peritoneum of transgenic and the littermate control by staining with FITC anti-B220 and PE-anti-CD23. The cells were gated on B220 (hence there are no cells in the upper and bottom left quadrants), and the B220 gated B220⁺CD23⁻ cells represent the B-2 B cells, whereas B220⁺CD23⁺ cells represent the B-1 B cells. Profiles for two individual mice are shown.

FIGURE 3. Genotypic and phenotypic characterization of DN-Egr-1-transgenic mice. A, PCR of tail DNA samples of the transgenic and the littermate controls of four lines (top) and the RT-PCR of the RNA samples obtained from B220⁺ B cells of indicated mice (middle). Western blot analysis of DN-Egr expression based on the expected molecular mass of ~25 kDa of the truncated protein (bottom). B, Resolution of immature transitional B cell subsets and the follicular B cells in the spleen of 10-wk-old DN-Egr-transgenic mice and littermate controls by staining splenocytes with FITC anti-IgM, PE-Cy-anti-B220, allophycocyanin-anti-AA4.1, and PE-anti-CD23 and analyzed on a FACS Calibur. B220⁺AA4⁺ cells represent immature transitional B cells; B220⁺AA4⁻ cells represent mature follicular B cells and MZ B cells. Subfractionation of B220⁺AA4⁺ immature B cells based on IgM and CD23 expression reveals IgM⁺CD23⁻ transitional T1 cells and IgM⁺CD23⁺ transitional T2 cells. Subfractionation of B220⁺AA4⁻ mature B cells based on IgM and CD23 expression reveals IgM⁺CD23⁻ MZ B cells and IgM⁺CD23⁺ follicular B cells. There were 100,000 events analyzed; data are representative of the average of eight mice in each group. C, Flow cytometric analysis of MZ B cells in the spleen of transgenic and the littermate controls by staining with FITC anti-CD21, PE-Cy-anti-B220, allophycocyanin-anti-HSA, and PE-anti-CD23. Data are representative of four independent experiments. D, Flow cytometric analysis of peritoneal B cells in the peritoneum of transgenic and the littermate control by staining with FITC anti-B220 and PE-anti-CD23. The cells were gated on B220 (hence there are no cells in the upper and bottom left quadrants), and the B220 gated B220⁺CD23⁻ cells represent the B-2 B cells, whereas B220⁺CD23⁺ cells represent the B-1 B cells. Profiles for two individual mice are shown.
spleen of Egr-1−/− mice (Fig. 1E and Table I). Alternatively, we looked at the MZ B cell population based on CD21 and CD23 staining and found that CD21+CD23− MZ B cells were reduced in the Egr-1−/− mice compared with the wild-type mice. There was a significant decrease in B-1 cells in the peritoneum of the knockout mice based on their B220+CD23− phenotype (Fig. 1F).

Hyperproliferation of B cells from mice that lack Egr-1

Because Egr-1 is rapidly induced upon Ag receptor stimulation, and Egr-1 was shown to promote cell proliferation in other cell types (41, 42), we determined whether Egr-1 is critical for BCR-induced B cell proliferation. B cells from both Egr-1−/− and littermate control mice were stimulated with anti-IgM for 48 h, and proliferation was measured by thymidine incorporation. There was a 2-fold increase in the proliferative response of Egr-1−/− splenic B cells to anti-IgM at three different doses (5, 10, and 25 μg/ml; Fig. 2A). The increased BCR response in Egr-1−/− mice might be due to compensation by other family members like Egr-2 (which was also shown to be up-regulated during BCR cross-linking in normal B cells) and Egr-3 (27, 43). The other possibility is that Egr-1 is a negative regulator of B cell activation and that the response is higher upon deletion. The former hypothesis is supported by our observations that Egr-2 is expressed by the Egr-1−/− B cells and that a lower mobility band appears in anti-IgM- or PMA-activated cells in the Egr-1−/− but not the littermate wild-type mice (Fig. 2B). This slow moving band appeared to be due to phosphorylation, because it became undetectable when Egr-1−/− B cells were stimulated with anti-IgM in the presence of ERK (PD98059) or JNK (SP600125) inhibitor. Unlike the Egr-1 knockouts, Egr-2 is not modified in the littermate controls. In addition, the Egr-2 and Egr-3 genes were also up-regulated in Egr-1−/− B cells and wild-type B cells upon BCR cross-linking (Fig. 2, B–D). To test the compensatory role of Egr-2, we used the BKS-2 B lymphoma model in which lymphoma growth is inhibited by BCR cross-linking with an accompanying decrease in Egr-1 (19, 28). Ectopic expression of Egr-2 using a retroviral vector rescued immature BKS-2 B lymphoma cells from BCR induced growth inhibition (Fig. 2E). On the other hand, the CD40 response remained comparable between the knockout and the littermates, suggesting that Egr-1 may not function downstream of CD40 signaling pathways.

Generation of DN Egr-transgenic mice

To understand further the role of Egr-1 in B cells, we generated transgenic mice expressing a DN form of EGR-1 in a B cell-restricted manner using V_{H} promoter and the Eμ enhancer. The DN-Egr construct contained the DNA-binding domain of Egr-1 but lacked the N-terminal trans activation domain (22). When cotransfected into fibroblasts with an Egr-1-dependent reporter, this mutant was shown to inhibit the transcriptional activity of both endogenous Egr-1 and exogenously expressed wild-type Egr-1 protein (22). In addition, this construct was shown to block the activity of all four Egr family members (32). The founders, lines 50, 36, and 18 were generated in (C3H × B6)F1 mice. The founders and the transgene positive offspring were backcrossed to B6 mice for nine generations. The presence of the transgene in the founders was confirmed by Southern blot and in the offspring of subsequent generations by tail DNA PCR (Fig. 3A, top). Expression of the transgene in splenic B cells was verified using RT-PCR (Fig. 3A, middle) and by Western blot (Fig. 3A, bottom). Line 50 was used for all the studies except when indicated specifically.

Reduced B lymphopoiesis in the bone marrow and reduced mature B cells in the spleen of dominant negative Egr-transgenic mice

We observed a significant decrease in the number of pre-B cells and immature B cells in the bone marrow (B220−CD43−BP-1 IgM− and B220−CD43−IgM−, respectively) of transgenic mice expressing the DN Egr-1 (Table II). Thus, in the DN Egr-transgenic mice, there was an overall decrease in pre-pro-B (fraction A), pre-B (fraction B), pre-BI (fraction C), pre-BII (fraction C′), and immature (fraction E) B cells in the bone marrow suggesting that Egr transcription factors regulate B lymphopoiesis at a very early stage, possibly the CLPs. The decrease in the output of immature B cells in the bone marrow was reflected in the spleen B cells of transgenic mice that showed a 2-fold reduction (Table III). There was a 2-fold decrease in the frequency of cells by the number of cells harvested from the spleen of each mouse. p < 0.05; p values were calculated for absolute numbers with Student’s t test, using data from littermate mice as the control population.

Table II. Decrease in B-lymphopoiesis in DN-Egr mice

<table>
<thead>
<tr>
<th>B220+ Bone Marrow (Pre-pro, Pre-, Immature, Transitional and Mature Recirculating) B Cells (×10⁶ cells/organ)</th>
<th>B220+ AA4+ Transitional B Cells (Spleen) (×10⁶ cells/organ)</th>
<th>B220+ AA4− Follicular and Marginal Zone B Cells (Spleen) (×10⁶ cells/organ)</th>
<th>CD23+ HSA+ CD21+ Marginal Zone B Cells (Spleen) (×10⁶ cells/organ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (+/+)</td>
<td>2.0 ± 0.4 (30 ± 2)</td>
<td>9.1 ± 1.0 (7 ± 2)</td>
<td>26 ± 5 (38 ± 6)</td>
</tr>
<tr>
<td>DN-Egr transgenic</td>
<td>0.9 ± 0.3 (14 ± 4)</td>
<td>4.5 ± 0.6 (3 ± 2)</td>
<td>14 ± 3 (12 ± 5)</td>
</tr>
<tr>
<td>P</td>
<td>0.05</td>
<td>0.05</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

* Mean ± SE of absolute numbers in millions of the indicated populations were calculated by multiplying the frequencies of cells by the number of cells harvested from two tibia and two femurs and spleen from each mouse (n = 8), p values were calculated for absolute numbers with Student’s t test, using data from littermate mice as the control population. Numbers in parentheses represent the percentage of cells in the given tissue.

Table III. Reduction in absolute number of splenocytes and B cells in the spleen of DN-Egr-transgenic mice

<table>
<thead>
<tr>
<th>Total Splenocytes (×10⁶)</th>
<th>B220+ B Cells (×10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Line 50</td>
<td>Wild type 88 ± 10 10 40 ± 7 10</td>
</tr>
<tr>
<td></td>
<td>Transgenic 56 ± 6 10 21 ± 6 10</td>
</tr>
<tr>
<td>Line 36</td>
<td>Wild type 62 ± 8 12 24 ± 5 12</td>
</tr>
<tr>
<td></td>
<td>Transgenic 37 ± 8 12 13 ± 2 12</td>
</tr>
</tbody>
</table>

* Mean ± SE of absolute numbers of the indicated populations were calculated by multiplying the frequencies of cells by the number of cells harvested from the spleen of each mouse. p < 0.05; p values were calculated for absolute numbers with Student’s t test, using data from littermate mice as the control population.
FIGURE 4. Defective proliferation of B cells from DN-Egr-transgenic mice. A, T-depleted B cells from littermate and the DN-Egr (line 50)-transgenic mice were cultured for 48 h with medium alone or with indicated concentrations of anti-IgM, and proliferation measured as described in Materials and Methods. B, T-depleted B cells from littermate and the DN-Egr-1-transgenic mice of three different founder lines were cultured for 48 h with medium alone or with indicated concentrations of anti-IgM and proliferation measured as described in Materials and Methods. C, T-depleted B cells from littermates and the line 50 DN-Egr-transgenic mice were cultured for 48 h with indicated concentrations of LPS or anti-CD40 and proliferation measured as described in Materials and Methods. D, Sorted follicular B cells (B220^HSA^{low}CD21^{hi}) from littermate and the DN-Egr-1-transgenic mice were cultured for 48 h with medium alone or with indicated concentrations of anti-IgM, and proliferation measured as described in Materials and Methods. The differences between the transgenic and wild-type mice stimulated with anti-IgM (at all doses) were statistically significant (p < 0.05). Data are representative of five
We found a significant decrease (3-fold) in the MZ B cell population in the spleen of transgenic mice compared with wild-type controls (B220<sup>+</sup>CD23<sup>−</sup>HSA<sup>−</sup>CD21<sup>+</sup> fraction; Fig. 3C and Table II). There was a significant decrease in B-1 cells in the peritoneum of the transgenic mice based on their B220<sup>+</sup>CD23<sup>−</sup> phenotype (Fig. 3D).

Defective proliferative response of B cells that lack Egr-1 activity

Because Egr-1<sup>−/−</sup> mice showed an enhancement of B cell proliferation in response to BCR cross-linking, presumably due to compensation by Egr-2 and Egr-3, we asked whether inhibition of all family members affected B cell proliferation. B cells from both transgenic and nontransgenic littermate control mice were stimulated with anti-IgM for 48 h and the proliferation was measured by thymidine incorporation. There was a decrease in the proliferative response of splenic B cells to anti-IgM at all doses (Fig. 4A). This defective response was evident in all the three founder mice (Fig. 4B). Similar results were obtained with a fourth founder line (data not shown). Alternatively, this defect was not observed when B cells were treated with LPS or CD40 ligation, suggesting that Egr family members were downstream of BCR-signaling but not TLR4 or CD40 pathways (Fig. 4C). The reduced proliferative response of splenic B cells from the transgenic mice can be attributed to failure of the follicular B cells to respond to Ag receptor stimulation or due to an increase in the proportion of immature B cells in the periphery. To clarify this issue, we sorted the follicular B cell population (mature B cells) from both transgenic and littermate controls based on their CD21 and HSA expression and measured their proliferative response to BCR cross-linking. The HSA<sup>low</sup>CD21<sup>+</sup> fraction from the transgenic mice proliferated less efficiently than did the littermate at two different doses of anti-IgM (Fig. 4D). Interestingly, cyclin D2 up-regulation was defective at 25 μg/ml but not at 50 μg/ml anti-IgM, suggesting that higher doses of anti-IgM might overcome the defective cyclin D2 up-regulation (Fig. 4E). Moreover, transgenic B cells failed to up-regulate c-Myc in response to two different doses of anti-IgM. Alternatively, CD40 ligation-induced c-Myc up-regulation remained comparable with wild-type, suggesting that Egr is downstream of B cell receptor and is critically dependent on c-Myc to enter the cell cycle (Fig. 4F). Nevertheless, our data provide a mechanistic basis for Egr-1-induced cell proliferation in B cells as evidenced by its role in regulating c-Myc levels and to some extent cyclin D2 levels. These data suggest that Egr family members are critical for BCR-induced proliferation of normal mature B cells. Also, cell adhesion molecule CD44 failed to up-regulate, when independent experiments. E and F. B220 bead-purified B cells were cultured in vitro for 6 h in the presence or absence of indicated stimuli, and lysates were probed for c-Myc and cyclin D2 protein levels by Western blotting. Blots were stripped and probed for β-actin for protein loading. G. T-depleted B cells from littermates and the line 50 DN-Egr transgenics (n = 2) and the littermate mice (n = 2) were cultured for 48 h with or without anti-IgM, and cells were analyzed for CD44 expression by flow cytometry. The average MFI of CD44 expression in anti-IgM-stimulated cells from two mice is 112 ± 4 for DN-Egr B cells and 480 ± 20 for littermate controls. Data are plotted as a histogram comparing untreated and treated groups in littermates and transgenics. The p value for CD44 expression is <0.05 comparing littermate and the DN-Egr transgenics.
previous studies demonstrating CD44 as a target gene of Egr-1 in B cells (20).

**Normal T cell responses in DN-Egr-transgenic mice**

To rule out the possible effects of the transgene in T cell lineage, we analyzed the thymocytes and probed peripheral T cell responses. We did not observe any defect in the thymocyte population (both percentages and the absolute numbers) of the transgenic mice (Table IV). T cell proliferation (Fig. 5A), IL-2 production (Fig. 5B), and CD40L expression in response to anti-CD3 stimulation remained comparable between wild-type and transgenic mice, suggesting that the effects of the transgene are restricted to B cells. Nevertheless, the effect of transgene in cell types other than B cells cannot be completely ruled out.

**Egr family members are critical for immune responses in vivo**

Because Egr-1 was important for BCR-induced B cell proliferation, we next immunized the transgenic mice and tested the in vivo B cell responses. The basal Ig levels of DN-Egr-transgenic mice remained comparable with those of the wild type except for a modest increase in IgG1 (Fig. 6A). There was a significant reduction in Ab formation by the B cells from the transgenic mice compared with littermates in response to both T-independent type 2 Ag TNP-Ficoll (Fig. 6C) and T-dependent Ag SRBC (Fig. 6D) but not T-independent type 1 Ag TNP-LPS (Fig. 6B). Overall, these studies suggest that Egr family members are critical for immune responses in vivo.

**Discussion**

In this study, the importance of Egr-1 in B cell development and proliferation was studied using knockout mice in which Egr-1 gene is deleted and using transgenic mice that express a DN form of Egr-1 in a B cell-restricted manner. Egr-1−/− mice exhibited an increased in B lymphopoiesis in the bone marrow leading to an increase of B cells (mostly immature) in the bone marrow, spleen, and the peritoneum. However, follicular mature B cells were not increased in these knockouts. Egr-1−/− B cells exhibited an increased proliferation response to BCR cross-linking, but responded normally to CD40 ligation. In contrast, B cell lymphopoiesis was reduced in DN-Egr-transgenic mice resulting in a reduction in B cell numbers in the bone marrow and spleen (both immature and mature). In addition, B cells were defective in their proliferative response to BCR cross-linking but not for CD40 ligation or TLR4 stimulation.

This is the first report demonstrating the importance of the transcription factor Egr-1 in B cell development and functional response. The molecular circuitry involving B cell lineage commitment is fairly well characterized, with PU.1 playing a key role in the decision between myeloid and B cell lineage. In this context, the role of the Egr family of immediate early gene transcription factors assumes greater importance given that Egr-1 is downstream of IMDM + Ham’s F-12 (+ 10% FBS) in 48-well plates (Costar) with TNP-LPS (1 and 2 μg/ml) and splenocytes (1 × 10⁶ per culture), for 4 days in 5% CO₂ and at 37°C. The number of IgM anti-TNP-secreting cells was determined using a glass-slide version of the Ab-forming cell assay as described earlier (44). C and D. Mice were immunized with 10 μg of TNP-Ficoll or 10% v/v SRBC i.p. 5 days after immunization; the anti-TNP response for TNP-Ficoll and the anti-SRBC response for SRBC were detected by a PFC assay. *, p < 0.01. This experiment is representative of three mice in each group with duplicate slides for PFC assays for each mouse. Results are representative of three experiments for the TNP-Ficoll response and two experiments for the SRBC response.
of PU.1 (45, 46). Immediate early genes are crucial for cellular responses including the immune cells because they are rapidly induced upon receptor ligation which primes the cells for subsequent late events that regulate cell survival, proliferation, and differentiation (1). The Egr-1 gene is induced by growth factors and cytokines in many cell types (1). The role of Egr-1 in B cell development and proliferation is not known. Egr-1 was shown to be important for BP-1 expression, a marker for pre-B cells in the bone marrow using transgenic mice that express Egr-1 in a B cell-specific manner (23). We and others showed that Egr-1 is rapidly induced in B cells upon BCR ligation.

Cytokine receptors Flt3 and IL-7 and transcription factors PU.1, Ikaros, E2A, Bcl11a, early B cell transcription factor (EBF), and Pax-5 are crucial for the development of B cell lineage precursor cells (46–49). Expression of the receptor tyrosine kinase Flt3 within a subset of multipotent progenitors is one of the earliest events in B cell development. PU.1 and Ikaros are required for expression of Flt3. Flt3 signaling in coordination with PU.1 induces IL-7R. IL-7R signaling induces E2A, which in turn regulates EBF gene in coordination with PU.1. EBF cooperates with E2A and activates the early B lineage gene expression determining the B cell fate. In addition to commitment toward the B cell fate, EBF induces the expression of Pax-5. Pax-5 shut down alternate lineage specifications and promotes commitment to the B cell fate (46–48). In the periphery, Bcl-6, another key transcription factor, is important for the maintenance of germinal center B cells (50).

The phenotype observed in both the knockout and the transgenic mice is contrasting and at the same time highlights the importance of the relative roles of Egr family members in B cell biology. As noted earlier, there are four family members, namely, Egr-1, Egr-2, Egr-3, and Egr-4. The increase in B lymphopoiesis in Egr-1−/− could be due to a negative role of Egr-1 in B cell development or due to a compensatory increase in other family members that further enhance B lymphopoiesis. The later possibility is supported by the finding that B lymphopoiesis is similarly decreased both in the bone marrow and spleen of DN-Egr mice. The fact that Egr-1−/− B cells hyperproliferate and have increased expression of Egr-2 and Egr-3 provides further support to the second model. The possibility of different role for various Egr family members cannot be ruled out.

Egr-1, however, has a nonredundant role in the development of MZ B cells, because this population is significantly decreased in both Egr-1−/− and the DN-Egr-transgenic mice. This is consistent with the notion that BCR signaling is required for MZ B cell development (51). Similarly, Egr-1 appears to be uniquely required for the high rate of expression of BP-1, which is decreased in Egr-1−/− mice. Presently, it is not precisely clear at what stage of B cell commitment does Egr-1 have a regulatory role. It is possible that Egr-1 might be affecting the developmental stages as early as the commitment of CLPs toward the B cell lineage. This hypothesis is supported by our preliminary observations that DN-Egr-transgenic mice have a 2-fold increase in B220<sup>+</sup> Sca-1<sup>low</sup> early lineage cells, which are defined as CLPs in the bone marrow (45). Moreover, the model put forth by Singh and coworkers suggests that low levels of PU.1 (transcription factor required for both B and myeloid lineage commitment) in B cells activates Egr-1/2, thus establishing a connection between PU.1 and Egr-1 in lineage stability (52). Alternatively, high PU.1 activates Egr-1 very strongly and promotes macrophage differentiation. These data further support our observations that Egr family members regulate B cell commitment at a very early stage of their development in the bone marrow.

The BCR signal strength model put forward by Pillai et al. proposes that if the BCR of the B cell reacts fairly well (intermediate affinity) to an Ag, then the B cells are induced to differentiate into follicular B cells; but if the BCR of the B cell reacts weakly (weak affinity) to a cognate self Ag, it is prone to receive signals that drive the B cell to become a MZ B cell. The development of B-1 B cells is dependent on strong signals from the BCR (strong affinity) (51). Although there is strong support to this hypothesis, there are some observations that are not fully consistent with this hypothesis. One such observation comes from Rajewsky and coworkers (53) who showed that expression of the EBV LMP2A gene specifically in B cells driven by a weak promoter leads to the generation of both MZ and follicular B cells whereas a strong promoter gives rise to B-1 B cells. Our data suggest that Egr-1 affects the development of both MZ and B-1 B cell development but not the follicular B cells in the Egr-1−/− mice, given that there is a decrease in both MZ and B-1 B cell populations in the spleen and peritoneum, respectively. It is not now clear why a deficiency in Egr-1 affects the development of two B cell subsets (MZ and B-1) but not the development of follicular B cells. One possibility is that the other Egr-1 family members can replace Egr-1 function for follicular B cell development but not for MZ or B-1 B cell development. This hypothesis is supported by the finding that all three subsets are reduced in DN-Egr-transgenic mice (Tables II and III).

We think that Egr-1 has an intrinsic role in B cells, because BCR signal strength affects the development of B cell subsets, as shown by Casola et al. (53) and in several other studies that use BCR signal-deficient mutant mice (54). The fact that the DN-Egr-1 mice that express the DN-Egr protein in a B cell-specific manner exhibit a similar MZ B cell defect further strengthens this argument. One such factor regulated by Egr-1 could be Notch2, which has been shown to be important for MZ B cell development (55). Delta-like 1 (DL1), one of the Notch ligands is also important for MZ B cells and is originally reported to be expressed by B cells (56) but a more recent paper by Moriyama et al. (57) reports that DL1 is expressed by macrophages and dendritic cells but not B cells. It is conceivable that Egr-1 regulates DL1 expression in macrophages or some other stromal cell molecule that affects MZ B cell development. The possibility that Egr-1 may affect stromal cells rather than B cells cannot be completely ruled out at this time.

Egr family members are important not only for B lymphocyte development but also for B cell clonal expansion initiated by B cell triggering. Just as in overall B lymphopoiesis, Egr-1 function can be substituted by other family members in the BCR-induced proliferation responses as shown by their increase in Egr-1−/− B cells, and the ability of ectopically expressed Egr-2 to overcome BCR-induced growth inhibition of immature B cell lymphoma cells. When the functions of all Egr family members are suppressed by DN-Egr, then the follicular B cells are hyporesponsive. The requirement for Egr family members is unique for BCR pathway, because B cell proliferation induced by TLR4 or CD40 signaling is not affected either in Egr-1−/− or in DN-Egr mice. This observation is consistent with the findings that the BCR signaling defect observed in DN-Egr mice is important for in vivo B cell clonal expansion as Ab responses to both TI-2 and TD Ags are decreased.

Upon BCR ligation, there is a significant increase in Egr-1 message as well as protein levels in Egr-1−/− B cells. In contrast to Egr-1, resting B cells express a high basal level of Egr-2 protein which is being modified (most likely phosphorylated) upon BCR ligation in Egr-1−/− but not wild-type B cells. This is the first report of Egr-2 being phosphorylated, a property it shares with Egr-1. This modification is likely to be important because others have shown that phosphorylation vs acetylation potentially modulates the activity of Egr-1 in terms of survival and apoptosis in prostate cancer cells (5). In addition, we find that Egr-2 and Egr-3
message levels are elevated upon BCR cross-linking in Egr-1−/− B cells. Because Egr family members share 90% homology in their DNA-binding region, we propose that in Egr-1−/− mice, Egr-2 and possibly Egr-3 assume a critical compensatory role. Whether such a compensation involves regulation of some of the Egr-1 target genes remains to be explored. At present, target genes that are critically modulated by Egr-2 are not known except that Egr-2 regulates Fas ligand expression in T cells (58, 59). This hypothesis is strengthened by our observations that overexpression of Egr-2 partially overcomes BCR-induced growth inhibition in an immature B cell lymphoma model.

We conclude that Egr family members are positive modulators of B lymphopoiensis, MZ B cell development, BCR-induced B cell proliferation, and T-independent type 2 and T-dependent immune responses (Fig. 6). Our observations demonstrate that Egr-1 is critical for MZ B cell development and its associated TI-2 immune responses. This phenotype is very significant because there are only few instances in which deficiency of a transcription factor leads to a defect in MZ B cell development and TI-2 responses (51). Currently, we are examining some of the target genes that could be potentially modified by Egr-2 in lymphoma cells over-expressing this transcription factor. Moreover, mice deleted for Egr-2 and or Egr-3 could reveal some interesting roles for Egr-2 and Egr-3 in B cell growth response. Further studies on Egr family members will provide new insights into novel players in B lymphocytes in B cell development.

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Disclosures
The authors have no financial conflict of interest.

References

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