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BRIEF COMMUNICATION

Early Growth Response-1 Plays a Non-redundant Role in the Differentiation of B Cells into Plasma Cells

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Early growth response (Egr)-1 is a Cys_2-His_2-type zinc-finger transcription factor. It has been shown to induce survival and proliferation of immature and mature B cells, respectively, but its role in the differentiation of B cells into plasma cells remains unclear. To examine the effects of Egr-1 deficiency on the activation of B cells, naive B cells from *Egr1*$^{-/-}$ mice and their wild-type (WT) littermates were activated to proliferate and differentiate, and then assayed by FACS. Proportions of cells undergoing proliferation and apoptosis did not differ between *Egr1*$^{-/-}$ and WT mice. However, *Egr1*$^{-/-}$ B cells gave rise to fewer plasma cells than WT B cells. Consistently, *Egr1*$^{-/-}$ mice produced significantly lower titer of antigen-specific IgG than their WT littermates upon immunization. Our results demonstrate that Egr-1 participates in the differentiation program of B cells into plasma cells, while it is dispensable for the proliferation and survival of mature B cells.

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Keywords: Egr1, B cells, Plasma cells, Differentiation, Antibody

INTRODUCTION

Ag challenge can activate B cells to proliferate and differentiate into plasma cells. Plasma cells are a unique population of specialized cells that can produce Abs through T-dependent (TD) and T-independent (TI) pathways (1). Abs elicit a variety of effects, such as complement activation and opsonization. Therefore, plasma cells act as key players during the humoral immune response. However, the development program of plasma cell populations is not clearly understood.

The early growth response (Egr) protein family comprises Cys_2-His_2-type zinc finger transcription factors, which share 90% homology in their DNA binding region (2). Egr-1, which is also known as NGFI-A, Zif268, and Krox24, is expressed ubiquitously by many cell types and binds to a consensus DNA motif (GCGGTGGGCG) in many genes, including c-Myc, cyclins D2 and G2, and p19 (3-5). Other Egr-1 target genes identified in B cells include genes encoding TNF, IL-2, CD44, and ICAM-1, which are important for growth and functions of B cells (6).

Many studies have shown that Egr-1 participates in B cell maturation as a positive regulator. Egr-1 is crucial for B lymphopoiesis, especially in the development of pre-B cells and marginal zone B cells (7,8). Induction of Egr-1 expression following engagement with the BCR is asso-

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Abbreviations: Egr, Early growth response; NP-KLH, 4-hydroxy-3-nitrophenylacetyl-keyhole limpet hemocyanin; TD, T-dependent; TI, T-independent; WT, wild-type

ciated with the growth and proliferation of mature B cells, suggesting that Egr-1 plays a role in their activation (2,9). However, it has also been observed that a deficiency in Egr-1 failed to inhibit BCR crosslinking-induced proliferation of mature B cells, due to the compensatory functions of Egr-2 and -3 (8). Egr-1 can exert anti- and pro-apoptotic roles, depending on the cell type. Its anti-apoptotic role is important for the survival of immature B cells during BCR-induced growth inhibition (10,11), while its pro-apoptotic role contributes to the elimination of bortezomib-resistant multiple myeloma cells (12). Although transgenic mice expressing a dominant negative form of Egr-1 develop lower numbers of plasma cells than normal mice (8), it remains unclear whether this effect stems from the role of Egr-1 as none of the other Egr family members were expressed in these transgenic mice.

We investigated the functions of Egr-1 in B cells, and what cellular events in B cells are affected when Egr-1 is deficient. We used mice in which *Egr1* was knocked out ($Egr1^{-/-}$), and found that Egr-1 is involved in the differentiation of naive B cells into plasma cells. However, Egr-1 did not appear to be involved in the proliferation and apoptosis of B cells. Our results highlight the non-redundant role of Egr-1, which acts as a positive regulator of humoral immunity.

MATERIALS AND METHODS

Mice and immunization

$Egr1^{+/-}$ mice on a C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained in a pathogen-free barrier facility at Hanyang University (Seoul, South Korea). $Egr1^{+/-}$ mice were incross to generate $Egr1^{-/-}$ mice. When $Egr1^{-/-}$ mice and their wild-type (WT) littermates were 6～12-weeks old, they were injected i.p. with a mixture of 4-hydroxy-3-nitrophenylacetyl-keyhole limpet hemocyanin (NP-KLH; 100 μg/mouse; Biosearch Technologies) and alum (Thermo Scientific). Our study was approved by the Institutional Animal Care and Use Committee (HY-IACUC-13-053).

RT-PCR assays

Erythrocyte-depleted spleen cell fractions from WT and $Egr1^{-/-}$ mice were stimulated with 20 ng/ml PMA and 1 μM ionomycin (Sigma-Aldrich) for either 1 or 3 h. Total RNA was isolated using Trizol reagent (Life Technologies). Semi-quantitative and quantitative RT-PCR assays were conducted as described previously (13). The mRNA expression levels of each gene were normalized to that for the gene encoding β2-microglobulin (*B2m*). Differences between samples were normalized using the cycle threshold method (ΔC_t). We used specific oligonucleotide primers targeting *Egr1* (5'-AAC CGG CCC AGC AAG ACA CC-3' and 5'-TGG CAA ACT TCC TCC CAC AAA T-3'), *Egr2* (5'-CTT CAG CCG AAG TGA CCA CC-3' and 5'-GCT CTT CCG TTC CTT CTG CC-3'), *Egr3* (5'-CAA CGA CAT GGG CTC CAT TC-3' and 5'-GGG CAG GCT GCC GAA TCC CG-3'), and *B2m* (5'-CAG TGT GAG CCA GGA TAT AG-3' and 5'-TGA CCG GCT TGT ATG CTA TC-3').

Abs and FACS

Splenic single-cell suspensions from WT and $Egr1^{-/-}$ mice were stained and analyzed using a $FACS_{Canto\ II}$ (BD Biosciences) as described previously (14). The following mAbs and reagents were purchased from BD Biosciences or eBioscience: anti-GL7-FITC, anti-CD4-PE, anti-B220-PerCP, anti-CD11C-allophycocyanin, anti-CD138-allophycocyanin, 7-AAD, and Annexin V-FITC.

B cell cultures

Naive $GL7^-CD138^-CD11c^-B220^+$ B cells from the spleens of WT and $Egr1^{-/-}$ mice were sorted using a $FACS_{Aria\ III}$ (BD Biosciences) and stained with 3 μM CFSE (Molecular Probes). Cells at 1×10^6/ml were cultured in the presence of 20 μg/ml LPS (Sigma-Aldrich) and 10 ng/ml IL-4 (Peprotech), or 5 μg/ml goat anti-mouse IgM (Jackson Immune Research) and 10 ng/ml IL-4. After culturing for 4 days, cells were subjected to flow cytometry.

ELISA

Sera were collected from pre- and post-immunized mice and assayed by ELISA to determine levels of NP-specific Abs. In brief, sera were diluted 1:100,000 in PBS (WelGene) and dispensed into 96-well immunosorbent plates (Nunc) pre-coated with 10 μg/ml NP_8 conjugated to BSA (Biosearch Technologies). A serum sample containing the highest titer of anti-NP_8 IgG was serially diluted and used as a standard. Plates were incubated with an anti-mouse IgG conjugated to biotin (Sigma-Aldrich), followed by streptavidin conjugated to HRP (BD Biosciences).

RESULTS AND DISCUSSION

Egr-1 expression was selectively abolished in *Egr1*$^{-/-}$ B cells

We examined whether depletion of *Egr1* induces compensatory expression of other *Egr* genes. *Egr1* mRNA were detected in unstimulated WT B cells, and were more abundant 1 h later in response to stimulation with PMA and ionomycin. We failed to detect *Egr1* mRNA in *Egr1*$^{-/-}$ B cells, regardless of whether cells were stimulated (Fig. 1). We also found that *Egr2* and *Egr3* mRNA were expressed at similar levels in WT and *Egr1*$^{-/-}$ B cells. Our findings show that deficient *Egr1* expression did not induce compensatory expression of *Egr2* and *Egr3*. Thus, our *Egr1*$^{-/-}$ knockout model appears to be appropriate for investigating the role(s) of Egr-1.

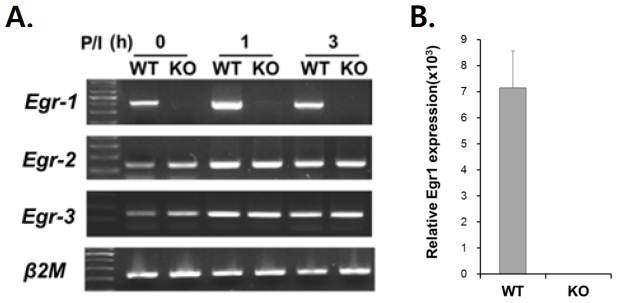

Figure 1. A selective lack of Egr-1 expression in *Egr1*$^{-/-}$ cells. Splenocytes from WT and *Egr1*$^{-/-}$ mice were treated with PMA and ionomycin (P/I) for 1 or 3 h, and assayed by semi-quantitative (A) and quantitative RT-PCR (B). Egr1 mRNA expression was normalized to that for β2m. Data are representative of three independent experiments.

Normal development of naive and germinal center B cells in *Egr1*$^{-/-}$ mice

Given that a deficiency in Egr-1 affects normal development of B-lineage cells (7,8), we assessed whether *Egr1*$^{-/-}$ mice have an abnormal composition of spleen cells. We observed similar proportions of B220^{+} and CD4^{+} cells among WT and *Egr1*$^{-/-}$ mice (Fig. 2). The proportions of B220^{+}GL7^{-} naive B cells and B220^{+}GL7^{+} germinal center B cells within the whole B cell population were similar between WT and *Egr1*$^{-/-}$ mice. Therefore, Egr-1 activity does not appear to be necessary for the development of naive B cells, CD4^{+} T cells, or germinal center B cells.

Differentiation of *Egr1*$^{-/-}$ B cells to plasma cells is defective, while proliferation and apoptosis of *Egr1*$^{-/-}$ B cells is unaffected

We sought to determine whether a deficiency in Egr-1 affects the proliferation and apoptosis of *Egr1*$^{-/-}$ B cells,

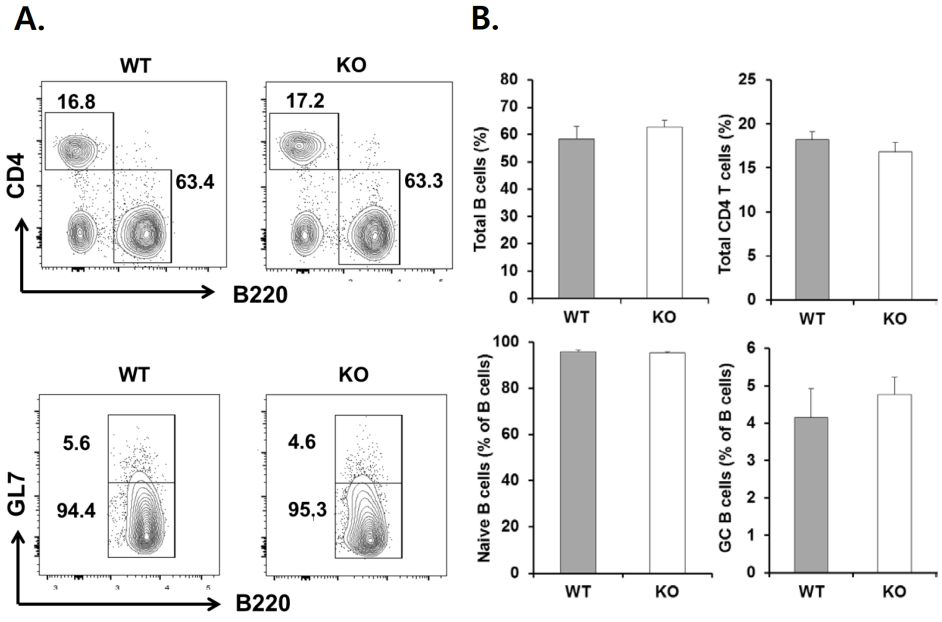

Figure 2. Analysis of naive B, germinal center B, and CD4^{+} T cell populations in *Egr1*$^{-/-}$ mice. (A) Spleen cells from 12 week-old *Egr1*$^{-/-}$ mice and their WT littermates were stained for the presence of GL7, B220 and CD4, and analyzed by FACS. The dot plots show total B (B220^{+}), CD4^{+} T, naive B (B220^{+}GL7^{lo}), and germinal center (GC) B cell (B220^{+}GL7^{hi}) populations with relevant percentages. Presented values are representative of six independent experiments. (B) The proportions (mean±SEM) of indicated cell compartments.

and whether naive *Egr1*$^{-/-}$ B cells can effectively differentiate into plasma cells. Naive B cells sorted from WT and *Egr1*$^{-/-}$ mice were labeled with CFSE and cultured with LPS plus IL-4 or anti-IgM Ab plus IL-4, and then subjected to flow cytometry. The vast majority of cells proliferated, as determined by the decrease in CFSE intensity (CFSE^{dim} cells), when treated with both types of stimulants (Fig. 3). The proportions of CFSE^{dim} cells were not significantly different between WT and *Egr1*$^{-/-}$ mice, regardless of the stimulants used (88.7±1.2 for LPS/IL-4-treated WT versus 89.5±2.1 for LPS/IL-4-treated KO, p=0.67; 88.2±1.4 for anti-IgM/IL-4-treated WT versus 82.9±4.5 for anti-IgM/IL-4-treated KO, p=0.46). In addition, the proportions of early apoptotic (Annexin V$^+$7-AAD$^-$) and late apoptotic (Annexin V$^+$7-AAD$^+$) cells were equivalent between WT and *Egr1*$^{-/-}$ mice. However, the proportion of CD138$^+$CFSE^{dim} cells among *Egr1*$^{-/-}$ cells was approximately 50% of that seen for WT cells. These results demonstrate that a deficiency in Egr-1 impaired the differentiation of B cells into plasma cells, while proliferation and apoptosis of these cells were unaffected. Thus, Egr-1 appears to play an important role in plasma cell differentiation, and its depletion cannot be adequately compensated for by other Egr family members. Results from previous studies have shown that Egr-1 activity is associated with the proliferation of mature B lymphoma cell lines (2,9). Therefore, it is likely that, rather than not participating in proliferation, the functions of Egr-1 are redundant and compensated for by other Egr family members, as suggested previously (8).

Egr1$^{-/-}$ mice produce less Abs than their WT littermates

To validate the *in vitro* effects of Egr-1 deficiency on plasma cell development, we immunized *Egr1*$^{-/-}$ mice and their WT littermates with NP-KLH and alum. We then determined serum titers of Abs with high affinity for NP at 14 and 21 days post-immunization (dpi). The anti-NP$_8$ IgG was detected at both dpi; its titer was significantly lower in *Egr1*$^{-/-}$ mice at 21, but not 14, dpi than that in WT mice (Fig. 4). Our findings reveal that Egr-1 is involved in the production of Abs, possibly by promoting the devel-

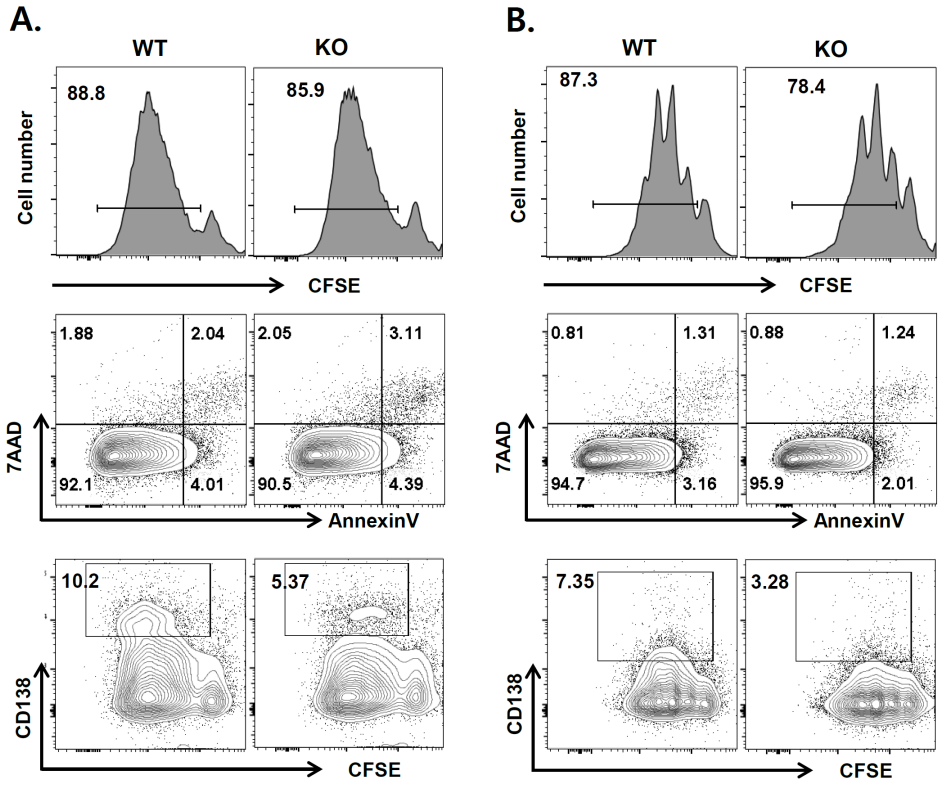

Figure 3. Responses of *Egr1*$^{-/-}$ B cells to polyclonal stimulants. Naive B cells sorted from spleens of WT and *Egr-1*$^{-/-}$ mice were stained with CFSE and stimulated with LPS/IL-4 (A) or anti-IgM/IL-4 (B). After 4 days, cell proliferation, apoptosis and plasma cell development were analyzed by FACS. The data presented are representative of results from more than three independent experiments. Values indicate the percentages of cells within indicated areas.

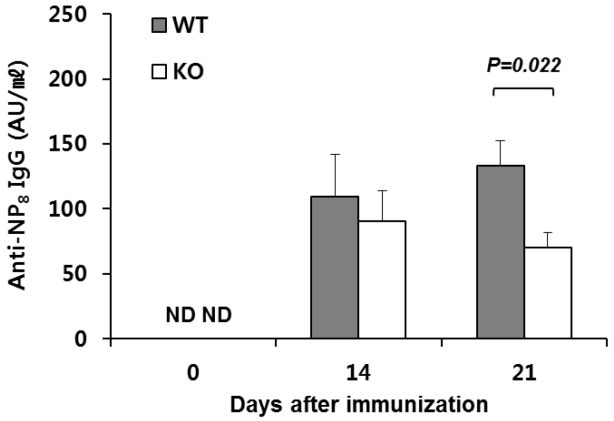

Figure 4. Reduced Ab production in *Egr1*$^{-/-}$ mice. WT and *Egr1*$^{-/-}$ mice were immunized with NP-KLH/alum, and serum was collected at 14 and 21 days after immunization. Titers of NP-specific IgG were detetmined by ELISA. Values represent the mean±SEM arbitrary unit (AU)/ml, pooled from three independent experiments (n=10～12 per group). p-values were determined using unpaired two-tailed Student's t-tests. ND, not detected.

opment of plasma cells through a TD pathway.

Results from several studies have indicated that Egr-1 is possibly associated with plasma cell development. When chronic lymphocytic leukemia B cells were stimulated with phorbol esters, Egr-1 was upregulated, giving rise to cells with a plasmacytic phenotype (15). Egr-1 could bind to the GC-box element of human *PRDM1*, which encodes Blimp-1, a master regulator of plasma cell differentiation (16). In the light of this result, we speculate that the effect of Egr-1 in the differentiation of B cells into plasma cells may be mediated by the induction of Blimp-1 expression. Transgenic mice lacking all Egr family members contain lower numbers of Ab-secreting cells than their WT counterparts (8). However, these studies have provided relatively little information regarding the role of Egr-1 during plasma cell differentiation.

In summary, we found that the proliferation and death of B cells was unaffected by the absence of Egr-1, indicating that Egr-1 is not required for these processes. We also showed that *Egr1*$^{-/-}$ B cells could not efficiently differentiate into plasma cells *in vitro* and *in vivo*, which was a strong indicator of the crucial and non-redundant role of Egr-1 in the commitment of B cells to a plasma cell fate.

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CONFLICTS OF INTEREST

The authors have no financial conflict of interest.

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