



A role for Bcl6 in sequential class switch recombination to IgE in B cells stimulated with IL-4 and IL-21

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Abstract

IgE plays an important role in the pathogenesis of allergic diseases and high-affinity IgE memory B cells are differentiated from IgG1 B cells developed in germinal centers. Bcl6, a sequence specific transcriptional repressor, is highly expressed in germinal center B cells and suppresses expression of C ϵ germline transcript. However, a role for Bcl6 in inhibition of the sequential class switching from IgG1 to IgE in germinal center B cells is not known. When splenic B cells from Bcl6-deficient (Bcl6-KO) and Bcl6-transgenic (Bcl6-TG) mice were stimulated with anti-IgM Ab and anti-CD40 Ab plus IL-4, IgG1⁺IgE⁺ B cells were detected in Bcl6-KO B cell culture but not in Bcl6-TG B cell culture. C γ 1 and C ϵ germline transcript in Bcl6-KO B cells were induced earlier than those in wild-type (Bcl6-WT) B cells after stimulation. When activated B cells were simultaneously stimulated with IL-21, expression of C γ 1 germline transcript in Bcl6-WT and Bcl6-KO B cells was enhanced by IL-21 stimulation, indicating that IL-21 is an enhancer of C γ 1 expression induced by IL-4. The amount of C γ 1 germline transcript in the Bcl6-KO B cells was more than that in the Bcl6-WT B cells. Conversely, IL-21 stimulation suppressed C ϵ expression in the Bcl6-WT B cells. However, the suppression was not observed in the Bcl6-KO B cells, suggesting that the IL-21-mediated suppression of C ϵ expression is due to Bcl6. Thus, Bcl6 controls the C γ 1 and C ϵ expression and stabilizes class switching to IgG1 in activated B cells simultaneously stimulated with IL-4 and IL-21.

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1. Introduction

IgE are major contributors to pathology in allergic diseases (Oettgen and Geha, 2001). Despite the importance of the IgE response, little is known about the low frequency of switching to IgE, a physiological role of sequential class switching from IgM via IgG1 to IgE (Mandler et al., 1993; Cameron et al., 2003), and the location of switching to IgE. During primary T cell-

dependent responses, two main phases of B cell activation have been described (MacLennan et al., 1997). Early activation and class switch recombination (CSR) occur often in extrafollicular foci, producing short-lived plasma cells that secrete low-affinity antibodies and generate no memory. A second phase occurs in germinal centers (GCs), where CSR, somatic hypermutation and affinity maturation take place. GC reactions generate long-lived plasma cells and memory B cells with high-affinity mutated Igs (Przylepa et al., 1998). Although CSR to IgE occurs during the GC phase of a T cell-dependent response, IgE⁺ B cells are hardly detected in GCs (Erazo et al., 2007). Recently it has been reported that high-affinity IgE⁺ memory B cells differentiate from IgG1⁺ B cells developed in GCs with an help from T follicular helper (Tfh) cells by the sequential CSR from IgG1 to IgE (Erazo et al., 2007).

In CSR, switch (S) regions located in the 5' side to C region genes are transcribed (germline transcript). These transcripts

Abbreviations: Bcl6-KO, Bcl6-deficient; Bcl6-TG, Bcl6-transgenic; CSR, class switch recombination; GC, germinal center; Blimp1, B-lymphocyte-induced maturation protein 1; Synd, syndecan-1; Tfh, T follicular helper; CBA, cytometry beads array; SD, standard deviation.

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are essential for cleavage of the S regions (Jayata and Alt, 2004). B cells switch to IgE under the influence of IL-4 and CD40 costimulation through a multi-step process involving C ϵ germline transcription and CSR. This CSR to IgE is hardly detected in GC B cells (Erazo et al., 2007). In GCs, Tfh cells (Chtanova et al., 2004) are critical for germinal center formation of activated B cells (Akiba et al., 2005) and predominantly produce IL-21 (Wurster et al., 2002; Chtanova et al., 2004). IL-21 suppresses C ϵ expression in IL-4-activated B cells (Suto et al., 2002) and induces apoptosis to IgE⁺ B cells (Harada et al., 2006). IL-21 also drives differentiation of activated B cells into plasma cells through up-regulation of B-lymphocyte-induced maturation protein 1 (*Blimp1*) mRNA (Ozaki et al., 2004). Thus, few IgE⁺ B cells in GCs may be due to IL-21-induced inhibition of the sequential CSR, IL-21-mediated apoptosis of IgE⁺ B cells and/or IL-21-induced differentiation of IgE⁺ B cells into plasma cells.

In addition to *Blimp1* expression, IL-21 also induces expression of a transcriptional repressor factor, Bcl6, in these populations (Ozaki et al., 2004; Arguni et al., 2006), even though *Blimp1* and Bcl6 are known to repress transcription of the genes encoding each other (Angelin-Duclos et al., 2000; Reljic et al., 2000; Shaffer et al., 2000, 2002; Calame et al., 2003). Bcl6 negatively regulates C ϵ expression through the C ϵ promoter (Harris et al., 1999) and is predominant in GC B cells of human tonsils (Cattoretti et al., 1995; Onizuka et al., 1995) and mouse lymphoid organs (Fukuda et al., 1995). However, a role for Bcl6 in inhibition of the sequential CSR from IgG1 to IgE in GC B cells is not known. To determine whether Bcl6 regulates the sequential CSR, we examined CSR to IgE in activated B cells from Bcl6-deficient (Bcl6-KO) and Bcl6-transgenic (Bcl6-TG) mice by in vitro stimulation of those B cells with anti-IgM Ab and anti-CD40 Ab plus IL-4. Here, we show that IgG1⁺IgE⁺ B cells were detected in Bcl6-KO B cell culture but not in Bcl6-TG B cell culture. C γ 1 and C ϵ germline transcript in Bcl6-KO B cells were induced earlier than those in wild-type (Bcl6-WT) B cells after stimulation. Simultaneous stimulation of activated B cells with IL-21 enhanced C γ 1 expression in both Bcl6-WT and Bcl6-KO B cells, but suppressed C ϵ expression in Bcl6-WT B cells but not in Bcl6-KO B cells. Thus, Bcl6 controls C γ 1 and C ϵ expression and stabilizes CSR to IgG1 in activated B cells simultaneously stimulated with IL-4 and IL-21. Implication of these findings is discussed.

2. Materials and methods

2.1. Mice

C57BL/6 mice were purchased from Japan SLC Co. Ltd. (Hamamatsu, Japan). Bcl6-KO mice (Fukuda et al., 1997) were backcrossed to C57BL/6 mice for more than 10 generation. To construct expression plasmids for transgenic mice, murine Bcl6 cDNAs were subcloned into the *Bam*H1 site of the Lck distal promoter expression cassette (pLck(d)-Bcl6-hGH) (Wildin et al., 1995). Transgenic mice were produced by the method described by Nagy et al. (2003). Briefly, C57BL/6 mice were used to obtain fertilized eggs, and the pLck(d)-Bcl6-hGH plas-

mid linearized by *Spe*I digestion were microinjected into a male pronuclear of fertilized eggs. The injected eggs were returned to the oviducts of pseudo-pregnant mothers of ICR strain. The exogenous DNA in tail DNA from transgenic mice was detected by slot blot analysis. All mice were maintained under specific pathogen free conditions in the animal center of Graduate School of Medicine, Chiba University. The care of all animals used in the present study was in accordance with Chiba University Animal Care guidelines.

2.2. Purification of splenic B cells

Splenic B cells were enriched by depleting non-B lineage cells from spleen cells. In brief, spleen cells were incubated with biotin-anti-mouse CD43 Ab (PharMingen, San Jose, CA). In some experiments, spleen cells were incubated with anti-mouse CD43 Ab and biotin-anti-mouse IgG1 Ab (PharMingen). These cells were subsequently reacted with immunomagnetic beads coated with streptavidin (Milenyi Biotec, Gladbach, Germany). Labeled cells were removed by a MACS system (Milenyi Biotec). The resulting B cell fraction contained >95% of B220⁺ B cells.

2.3. Cell culture

Purified B cells (5.0×10^5 ml⁻¹) were cultured in RPMI medium (Sigma Chemical Co. St. Louis, MO) supplemented with 10% FCS (Intergen, New York, NY), 50 μ M 2-ME, 10 mM HEPES, 100 μ g/ml of streptomycin (Wako Chemical Co., Osaka, Japan) and 100 U/ml of penicillin G potassium (Banyu Pharmaceutical Co., Tokyo, Japan). For B cell activation, purified B cells were stimulated with F(ab)2 fragments of anti-mouse IgM Ab (1.0×10^{-6} μ g/ml, Alpha Diagnostic Co., San Antonio) and anti-mouse CD40 monoclonal Ab (1.0 μ g/ml, BD PharMingen). These B cells were cultured with rIL-4 (20 U/ml) (Karasuyama and Melchers, 1988) in the presence or absence of IL-21 (30 ng/ml, R&D Systems Inc.) in a humidified atmosphere at 37 °C with 5% CO₂. Live cell numbers were counted under a microscope after staining with trypan blue (Invitrogen Corporation, Auckland, New Zealand).

2.4. Monoclonal Abs, flow cytometric analysis and purification of IgG1⁺ B cells

Single-cell suspensions were prepared from cultured B cells or spleen cells. Abs used were monoclonal FITC-anti-IgG1 (A85-1, PharMingen), phycoerythrin-anti-B220 (RA3-6B2, PharMingen), biotinylated anti-syndecan-1 (Synd, a plasma cell marker) (281-2, PharMingen) and biotinylated anti-IgE Abs (R35-72, PharMingen). Biotinylated Abs was detected by allophycocyanin-conjugated streptavidin (PharMingen). Cells were analyzed on a FACSCalibur (BD, Franklin Lakes, NJ) using CellQuest software (Beckton Dickinson). Dead cells were excluded on the basis of forward- and side-light scatter parameters, and propidium iodide (2 μ g/ml) staining. Cultured B cells were stained with FITC-anti-IgG1 (PharMingen) and biotinylated anti-IgE (PharMingen) and IgG1⁺ and IgG1⁻ B cells

were sorted by a FACSVantage (Becton Dickinson, San Jose, CA).

2.5. RT-PCR analysis

RT-PCR was performed as described elsewhere (Takamori et al., 2004). Total RNAs were extracted from cultured B cells with the Trizol reagent (Life Technologies, Gaithersburg, MD). Total RNAs were reverse-transcribed using SuperscriptTM (Life Technologies) and oligo(dT) (Pharmacia, Piscataway, NJ) in a final volume of 20 μ l, and the cDNAs (1 μ l) were used for PCR. After an initial 5-min incubation at 94 °C, 38 cycles of PCR were carried out using the following conditions: denaturation at 94 °C for 60 s, annealing at 58 °C for 60 s and polymerization at 72 °C for 60 s. PCR products were separated on a 1% agarose gel. PCR primers for the cDNA amplification were the following: C ϵ sterile transcript: 5'-CACAGGGGGCAGAAGATG-3' and 5'-AGGGGTAGAGCTGAGGGTTC-3'; C γ 1 sterile transcript: 5'-CAGATCTTTGAGTCATCCTATCACG-3' and 5'-TACATATGCAAGGCTTA-3'; β -actin: 5'-GTTTGAGACCTTCAACACC-3' and 5'-GTGGC-CATCTCCTGCTCGAAGTC-3'. Quantification of the PCR product in relation to the β -actin signal as 1.0 was performed on a Macintosh computer using the public domain NIH Image program.

2.6. Real-time quantitative RT-PCR

Real-time PCR with the cDNA was conducted using a SYBR Green PCR Master Mix (PE Applied Biosystems) and run on an ABI Prism 7000 Sequence Detection System (PE Applied Biosystems). Primers were as follows: C ϵ sterile transcript: 5'-CACAGGGGGCAGAAGATG-3' and 5'-AGGGGTAGAGCTGAGGGTTC-3'; C ϵ switched transcript: 5'-CTCTGGCCCTGCTTATTGTTG-3' and 5'-AGTTCACAGTGCTCATGTTCAG-3'; C ϵ mature transcript: 5'-TACGACGAGAACGGGTTTGCTTAC-3' and 5'-AGTTCACAGTGCTCATGTTCAG-3'. β -actin: 5'-CCAGCCTTCCTTCTTGGGTAT-3' and 5'-TGGC-ATAGAGGTCTTTACGGATGT-3'. Data were analyzed using Sequence Detector software (PE Applied Biosystems). In addition to duplicate reactions for gene expression in each sample, each real-time PCR plate contained reactions for generating standard curves using serial dilutions of a known quantity of cDNA. The data were reported as the ratio of the calculated amount of candidate RNA in a given sample by the calculated amount of the housekeeping control (β -actin) gene in the same sample.

2.7. Cytometry beads array (CBA)

Splenic B cells (1.0×10^5 well⁻¹) were cultured for 7 days at 37 °C in 5% CO₂. Culture supernatants were collected on day 7 after stimulation. Amounts of IgM, IgE and IgG1 Abs in the supernatants were measured by CBA. The mouse immunoglobulin isotyping CBA kit (BD Bioscience-Pharmingen) contains seven bead populations with distinct fluorescence intensities. The beads were coated with cap-

ture antibodies specific to seven mouse heavy-chain isotypes and mixed with phycoerythrin-conjugated detection antibodies. These capture beads were incubated with recombinant standards or test samples to form sandwich complexes. The capture beads were analyzed according to the manufacturer's instructions. The results were generated in graphic and tabular format using CBA analysis software (BD Bioscience-Pharmingen).

3. Results

3.1. Effect of Bcl6 on differentiation of IgG1⁺IgE⁺ B cells from naive B cells stimulated with anti-IgM and anti-CD40 plus IL-4

Splenic naive B cells from Bcl6-TG, Bcl6-WT and Bcl6-KO mice were stimulated with anti-IgM and anti-CD40 plus IL-4. Development of IgG1⁺ and IgE⁺ B cells in the culture was analyzed on a FACS. As shown in Fig. 1A, percentages of IgG1⁺ B cells were inversely correlated with the amount of Bcl6 in B cells until day 5 of culture. IgE⁺ B cells were detected in Bcl6-KO culture but not in Bcl6-WT and Bcl6-TG culture on day 3 of culture. IgE⁺ B cells developed in Bcl6-WT culture on day 4 of culture. Percentages of IgE⁺ B cells in Bcl6-WT culture became similar to those in Bcl6-KO culture on day 4 of culture. However, the percentages in Bcl6-TG culture were still less than those in Bcl6-WT and Bcl6-KO culture until day 5 of culture. Surprisingly, IgG1⁺IgE⁺ (double positive) B cells were clearly identified in Bcl6-KO culture and Bcl6-WT culture on day 3 and day 4 of culture, respectively. However, these double positive B cells were hardly detected in Bcl6-TG culture until day 5 of culture.

Kinetic curves of total, IgG1⁺, IgE⁺, IgG1⁺IgE⁺ and Synd⁺ B cell numbers clearly indicate the negative effect of Bcl6 on proliferation and CSR until day 4 of culture (Fig. 1B). As Bcl6 negatively regulates expression of Blimp1 which is an essential factor for plasma cell differentiation, Synd⁺ B cell numbers in Bcl6-KO culture were more than those in Bcl6-WT and Bcl6-TG culture. Although IgG1⁺IgE⁺ B cell numbers in Bcl6-WT culture increased to the similar level to those in Bcl6-KO culture on day 7 of culture, those were very small in Bcl6-TG culture until day 7 of culture. These results suggest that Bcl6 protects sequential CSR from IgG1 to IgE.

We examined amounts of sterile, switched and mature C ϵ transcript in those B cells from Bcl6-WT and Bcl6-KO culture by real-time RT-PCR. Although the amount of sterile C ϵ transcript in Bcl6-KO B cells was similar to that in Bcl6-WT B cells until day 4 of culture, the amount of switched C ϵ transcript in the Bcl6-KO B cells was more than that in the Bcl6-WT B cells after day 3 of culture (Fig. 1C). The amount of mature C ϵ transcript increased in both Bcl6-WT and Bcl6-KO B cells on day 4 of culture.

In order to confirm sequential CSR from IgG1⁺ B cells via IgG1⁺IgE⁺ B cells to IgE⁺ B cells, IgG1⁺ and IgG1⁻ B cells in Bcl6-WT and Bcl6-KO culture 60 h after stimulation were isolated by a FACSVantage. These IgG1⁺ and IgG1⁻ B cells were further cultured with IL-4 for 2 days. Fig. 2A

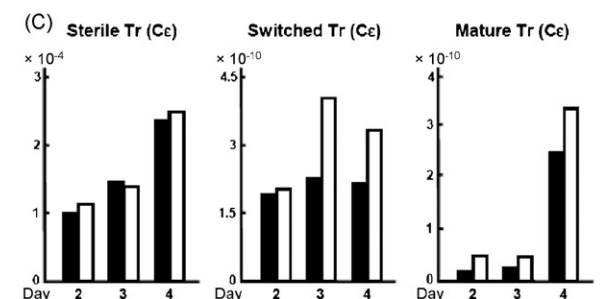
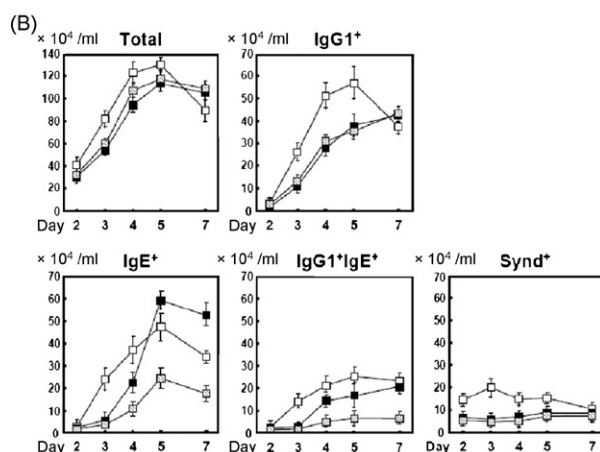
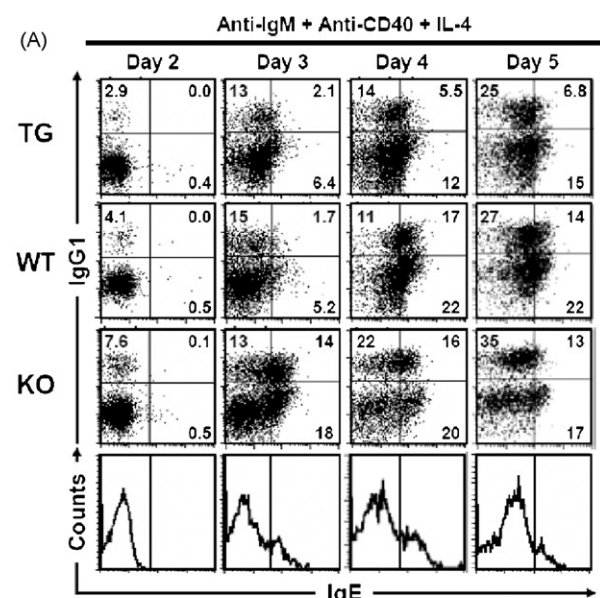


Fig. 1. Effect of Bcl6 on differentiation of IgG1⁺IgE⁺ B cells from naive B cells stimulated with anti-IgM and anti-CD40 plus IL-4. Purified splenic B cells from Bcl6-TG, Bcl6-WT or Bcl6-KO mice were cultured with anti-IgM Ab and anti-CD40 Ab plus IL-4 for 7 days. (A) Expression of IgG1 and IgE on activated B cells was analyzed by a FACS. The number in each corner indicates the percentage of B cells in each square. Histograms were used to determine the threshold of IgE staining. (B) Kinetic curves of total, IgG1⁺, IgE⁺, IgG1⁺IgE⁺ and Synd⁺ B cell numbers in the culture of Bcl6-TG (hatched square), Bcl6-WT (closed square) and Bcl6-KO (open square) B cells. Results represent means and variations (standard deviation, S.D.) from triplicate culture. These results are representative of three independent experiments. (C) Amounts of sterile C ϵ , switched C ϵ and mature C ϵ transcript in Bcl6-KO (open) and Bcl6-WT (closed) B cells were analyzed by real-time RT-PCR.

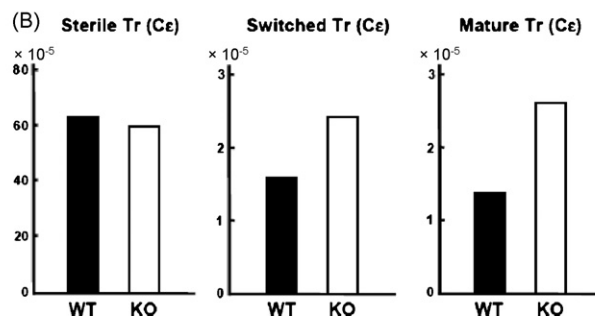
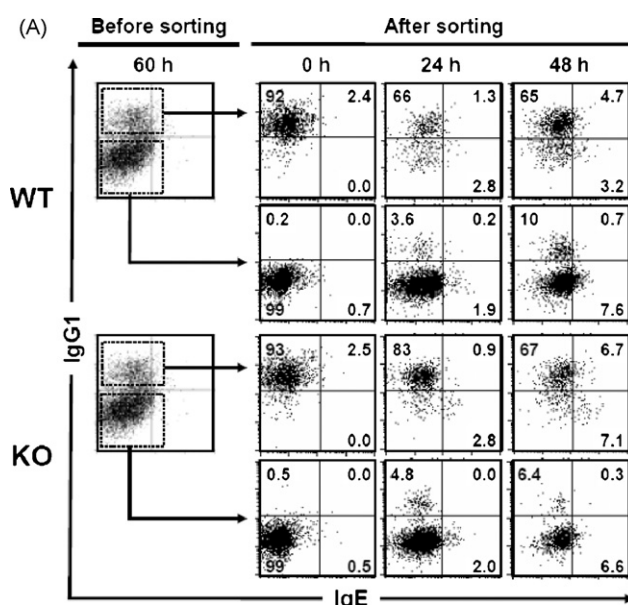


Fig. 2. Differentiation of IgG1⁺ B cells to IgG1⁺IgE⁺ B cells in the culture of naive B cells stimulated with anti-IgM and anti-CD40 plus IL-4. Purified splenic B cells from Bcl6-WT and Bcl6-KO mice were cultured with anti-IgM Ab and anti-CD40 Ab plus IL-4 for 60 h. IgG1⁺ and IgG1[−] B cells in the culture were sorted by FACS. (A) Sorted IgG1⁺ and IgG1[−] B cells were further cultured in the presence of IL-4 for 48 h and analyzed expression of IgG1 and IgE on those B cells by a FACS. These results are representative of three independent experiments. The threshold of IgE staining was determined by histograms (data not shown). (B) Amounts of sterile C ϵ , switched C ϵ and mature C ϵ transcript in FACS-isolated Bcl6-KO (open) and Bcl6-WT (closed) B cells were analyzed by real-time RT-PCR.

shows that IgE⁺ and IgG1⁺IgE⁺ B cells differentiated from IgG1⁺ B cells in both Bcl6-WT and Bcl6-KO culture 48 h after re-stimulation. Although IgG1⁺ and IgE⁺ B cells differentiated from IgG1[−] B cells in both Bcl6-WT and Bcl6-KO culture 48 h after re-stimulation, IgG1⁺IgE⁺ B cells were not detected in the Bcl6-WT and Bcl6-KO culture until 48 h after re-stimulation.

We examined the presence of sterile, switched and mature C ϵ transcript in FACS-isolated IgG1⁺ B cells by real-time RT-PCR. The amount of sterile C ϵ transcript in IgG1⁺ Bcl6-KO B cells was similar to that in IgG1⁺ Bcl6-WT B cells (Fig. 2B). Amounts of switched and mature C ϵ transcript were detected in those IgG1⁺ B cells although their amounts in the Bcl6-KO B cells were slightly more than those in the Bcl6-WT B cells.

3.2. Effect of IL-21 on differentiation of IgG1⁺IgE⁺ B cells in the culture of naive B cells stimulated with anti-IgM and anti-CD40 plus IL-4

IgE⁺ B cells are hardly detected in GCs (Erazo et al., 2007) and Tfh cells in GCs produce IL-21 which inhibits C ϵ expression (Suto et al., 2002). To examine the effect of IL-21 on inhibition of IgG1⁺IgE⁺ B cell development, splenic naive B cells from Bcl6-WT and Bcl6-KO mice were stimulated with anti-IgM and anti-CD40 plus IL-4 in the presence of IL-21. Development of IgG1⁺ and IgE⁺ B cells in the culture was analyzed on a FACS. Percentages of IgG1⁺ B cells in Bcl6-KO culture were more than those in Bcl6-WT culture until day 3 of culture (Fig. 3A). IgG1⁺IgE⁺ B cells were not detected in Bcl6-KO culture until day 4 of culture and were slightly detected on day 5 of culture. However, IgG1⁺IgE⁺ B cells were hardly detected in Bcl6-WT culture although IgE⁺ B cells were detected in the culture until day 5 of culture.

Kinetic curves of total and IgG1⁺ B cell numbers clearly indicate the enhancing effect of IL-21 on proliferation and CSR to IgG1 of those B cells (Fig. 3B). Total and IgG1⁺ B cell numbers in Bcl6-KO culture were more than those in Bcl6-WT culture until day 3 of culture. The peak of total and IgG1⁺ B cell numbers in Bcl6-KO and Bcl6-WT culture was on day 3 and day 5 of culture, respectively. However, IgE⁺ B cell numbers were very small in the Bcl6-KO and Bcl6-WT culture, suggesting IL-21-mediated apoptosis of IgE⁺ B cells.

IL-21 drives differentiation of activated B cells into plasma (Synd⁺) cells through up-regulation of *Blimp1* mRNA (Ozaki et al., 2004), and Blimp1 and Bcl6 are known to repress transcription of the genes encoding each other (Angelin-Duclos et al., 2000; Reljic et al., 2000; Shaffer et al., 2000, 2002; Calame et al., 2003). Thus, development of Synd⁺ B cells in the culture was analyzed. Although percentages of Synd⁺ B cells in Bcl6-KO culture were similar to those in Bcl6-WT culture on day 2 of culture, percentages of Synd⁺IgG1⁺ B cells in Bcl6-KO culture were more than those in Bcl6-WT culture (Fig. 3A). Percentages of Synd⁺ B cells in Bcl6-KO culture were slightly more than those in Bcl6-WT culture after day 3 of culture. The peak of Synd⁺ B cell numbers in both Bcl6-WT and Bcl6-KO culture was on day 3 of culture, and Synd⁺ B cell numbers in Bcl6-KO culture were more than those in Bcl6-WT culture until day 5 of culture (Fig. 3B).

3.3. Effect of Bcl6 on C γ 1 and C ϵ expression in naive B cells stimulated with anti-IgM and anti-CD40 plus IL-4 and IL-21

Development of IgG1⁺ B cells in Bcl6-KO culture was earlier than that in Bcl6-WT culture, suggesting regulatory effect of Bcl6 on CSR to IgG1. Thus, the induction of C γ 1 transcript in splenic naive B cells stimulated with anti-IgM and anti-CD40 plus IL-4 in the presence or absence of IL-21 was analyzed by RT-PCR. C γ 1 transcript was induced in Bcl6-WT B cells stimulated with IL-4 and IL-21 but not in Bcl6-WT B cells with IL-4 in the absence of IL-21 24 h after stimulation, suggesting enhancing effect of IL-21 on C γ 1 expression in B cells stim-

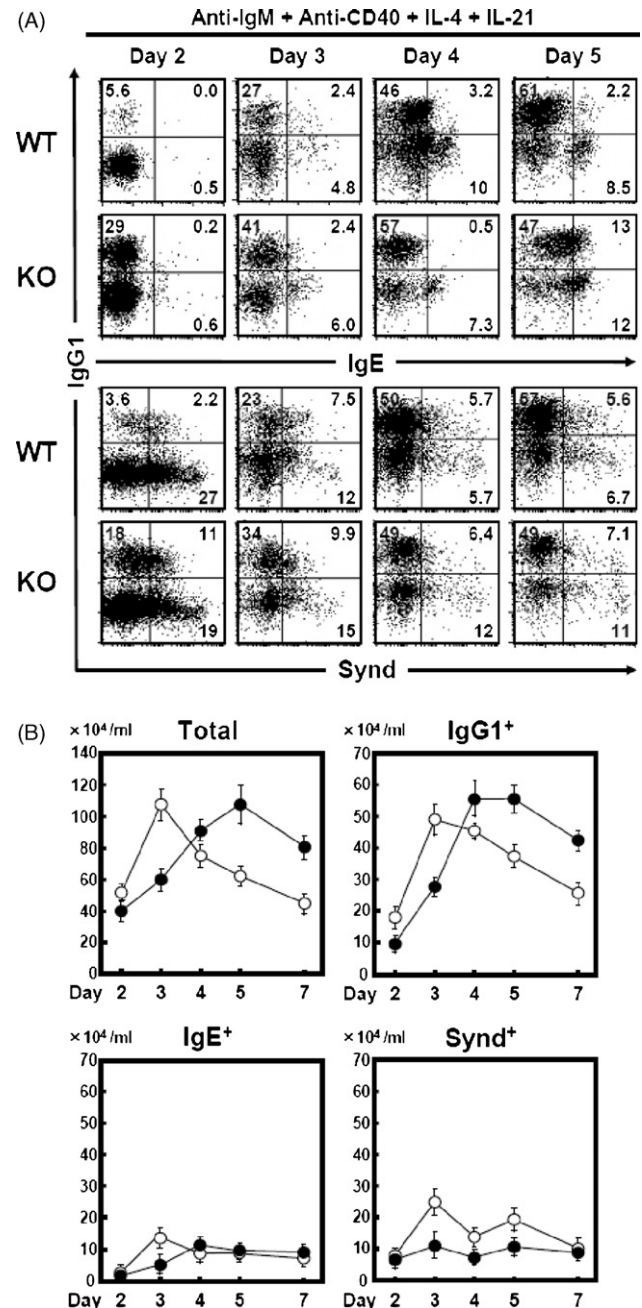


Fig. 3. Effect of IL-21 on differentiation of IgG1⁺IgE⁺ B cells from naive B cells stimulated with anti-IgM and anti-CD40 plus IL-4. Purified splenic B cells from Bcl6-WT or Bcl6-KO mice were cultured with anti-IgM Ab and anti-CD40 Ab plus IL-4 and IL-21 for 7 days. (A) Expression of IgG1, IgE and syndecan on activated B cells was analyzed by a FACS. The number in each corner indicates the percentage of B cells in each square. The threshold of IgE staining was determined by histograms (data not shown). (B) Kinetic curves of total, IgG1⁺, IgE⁺ and Synd⁺ B cell numbers in the culture of Bcl6-WT (closed circle) and Bcl6-KO (open circle) B cells. Results represent means and variations (S.D.) from triplicate culture. These results are representative of three independent experiments.

ulated with IL-4 (Fig. 4A). Although C γ 1 transcript was not detected in activated Bcl6-KO B cells until 12 h after stimulation, C γ 1 transcript was induced in activated Bcl6-KO B cells 24 h after stimulation irrespective of IL-21 stimulation. Relative amounts of C γ 1 transcript were calculated from those of β -actin

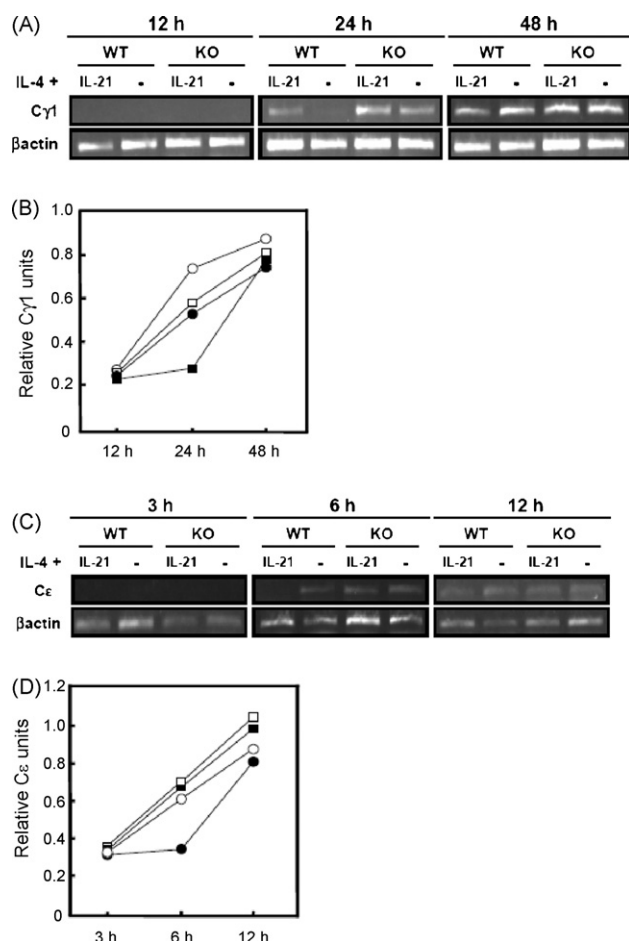


Fig. 4. Effect of Bcl6 on C γ 1 and C ϵ expression in naive B cells stimulated with anti-IgM and anti-CD40 plus IL-4 and IL-21. Purified splenic B cells from Bcl6-WT and Bcl6-KO mice were stimulated with IL-4 in the presence or absence of IL-21 for 48 h. Total cellular RNA was prepared from these B cells and RT-PCR for C γ 1 (A and B) and C ϵ (C and D) transcript as well as β -actin (as a control) was performed. (A) Expression of C γ 1 germline transcript in activated B cells was demonstrated. Shown are representative data from three independent experiments. (B) Numbers of relative units of amounts of C γ 1 compared with those of β -actin signals as 1.0. Bcl6-WT (closed) and Bcl6-KO (open) B cells were stimulated with IL-21 (circle) or without IL-21 (square). The data are presented as a representative of three independent experiments. (C) Expression of C ϵ germline transcript in activated B cells was demonstrated. Shown are representative data from three independent experiments. (D) Numbers of relative units of amounts of C ϵ compared with those of β -actin signals as 1.0. Bcl6-WT (closed) and Bcl6-KO (open) B cells were stimulated with IL-21 (circle) or without IL-21 (square). The data are presented as a representative of three independent experiments.

signals as 1.0. Amounts of C γ 1 transcript in Bcl6-KO B cells were more than those in Bcl6-WT B cells 24 h after stimulation and those became similar to each other 48 h after stimulation (Fig. 4B).

C ϵ transcript was induced in Bcl6-KO and Bcl6-WT B cells stimulated with IL-4 in the absence of IL-21 6 h after stimulation. However, although C ϵ transcript was induced in Bcl6-KO B cells stimulated with IL-4 and IL-21, that was not detected in Bcl6-WT B cells stimulated with IL-4 and IL-21 6 h after stimulation (Fig. 4C). Relative amounts of C ϵ transcript in Bcl6-KO B cells stimulated with IL-4 and IL-21 were more than those in Bcl6-

WT B cells with IL-4 and IL-21 6 h after stimulation and those became similar to each other 12 h after stimulation (Fig. 4D). These results suggest that IL-21-mediated suppression of C ϵ expression is Bcl6-dependent.

3.4. Effect of IL-21 on apoptosis of IgE⁺ B cells developed in the culture of naive B cells stimulated with anti-IgM and anti-CD40 plus IL-4

Splenic naive B cells from Bcl6-WT and Bcl6-KO mice were stimulated with anti-IgM and anti-CD40 plus IL-4 in the presence or absence of IL-21. In order to detect apoptotic cells in the culture, Annexin V staining of those B cells was performed 4 days after stimulation by a FACS. Percentages of Annexin V⁺ B cells in Bcl6-WT and Bcl6-KO culture with IL-4 and IL-21 were more than those with IL-4 in the absence of IL-21 (Fig. 5A). In IgG1 staining, many IgG1⁺ and IgG1⁻ B cells in Bcl6-WT and Bcl6-KO culture with IL-4 and IL-21 were Annexin V⁺. Since IgG1⁻ B cell population contained IgE⁺ B cells, these results suggested that apoptosis occurs in both IgG1⁺ and IgE⁺ B cells. In IgE staining, however, percentages of Annexin V⁺ IgE⁺ B cells in Bcl6-WT and Bcl6-KO culture with IL-4 and IL-21 were similar to those with IL-4 in the absence of IL-21. These results suggest that apoptosis occurs mainly in class-switched IgG1⁺ B cells and class switching B cells to IgE.

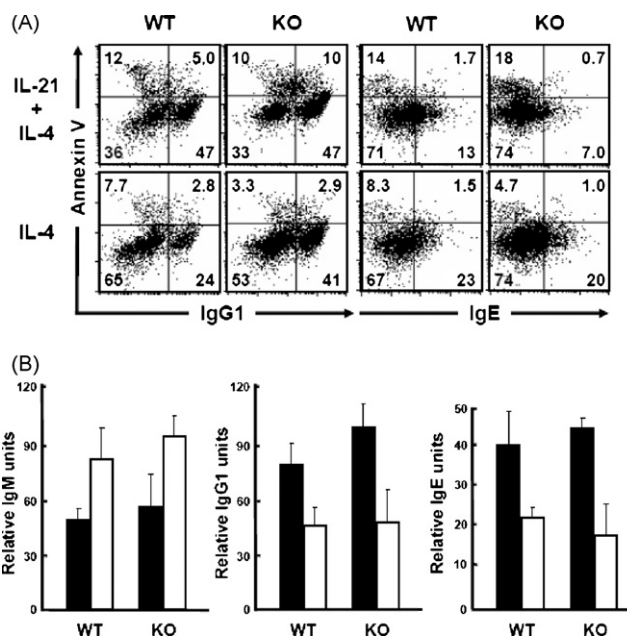


Fig. 5. Effect of IL-21 on apoptosis of IgE⁺ B cells developed in the culture of naive B cells stimulated with anti-IgM and anti-CD40 plus IL-4 and IL-21. (A) Purified splenic B cells from Bcl6-WT and Bcl6-KO mice were cultured with IL-4 in the presence or absence of IL-21 for 4 days. Staining for IgG1, IgE and Annexin V of B cells was analyzed on day 4 by FACS. The threshold of IgE staining was determined by histograms (data not shown). (B) Purified splenic B cells from Bcl6-WT and Bcl6-KO mice were stimulated with IL-4 in the presence (open bar) or absence (closed bar) of IL-21 for 3 days (for IgM) or 7 days (for IgG1 and IgE). Amounts of IgM, IgG1 and IgE in the culture supernatants were determined by CBA. Results represent means and variations (S.D.) from three independent experiments.

Amounts of IgM, IgG1 and IgE in the culture supernatants on day 3 (for IgM) or on day 7 (for IgG1 and IgE) of culture were determined by CBA. Amounts of IgM in Bcl6-WT and Bcl6-KO culture with IL-4 and IL-21 were enhanced compared with those with IL-4 in the absence of IL-21, although the amount in the Bcl6-WT culture was similar to that in the Bcl6-KO culture (Fig. 5B). IgG1 and IgE were readily detected in the supernatants from Bcl6-WT and Bcl6-KO culture with IL-4 in the absence of IL-21 and there was no significant difference between them. However, the addition of IL-21 in the culture reduced IgG1 and IgE production in Bcl6-WT and Bcl6-KO culture.

4. Discussion

A major fraction of IgE⁺ B cells develops by sequential CSR from IgM via IgG1 to IgE (Mandler et al., 1993; Cameron et al., 2003), although the sequential CSR was not essential for the production of IgE antibodies (Jung et al., 1994). Recently it has been reported that high-affinity IgE⁺ memory B cells differentiate outside of GCs from IgG1⁺ B cells developed in GCs by the sequential CSR from IgG1 to IgE (Erazo et al., 2007). However, the regulatory mechanism of the sequential CSR from IgG1 to IgE in GC B cells was not determined. We showed that Bcl6 inhibits the sequential CSR from IgG1 to IgE in activated B cells in vitro using Bcl6-WT and Bcl6-TG B cells. This result is supported by the evidence that IgG1⁺IgE⁺ B cells in GCs have a plasma cell phenotype (Erazo et al., 2007) in which the level of Bcl6 is rather low (Angelin-Duclos et al., 2000). The inhibition was transient in activated Bcl6-WT B cells and IgG1⁺IgE⁺ B cells did not develop in Bcl6-TG culture until day 5 of culture. Bcl6 is highly expressed in GC B cells (Cattoretti et al., 1995; Onizuka et al., 1995) and activated Bcl6-TG B cells may mimic GC B cells. Bcl6 has been shown to inhibit C ϵ transcription through the C ϵ promoter (Harris et al., 1999). Thus, the sequential CSR from IgG1 to IgE may be completely inhibited in GC B cells by Bcl6-mediated suppression of C ϵ expression in IgG1⁺ B cells.

Although the induction of C ϵ expression in activated Bcl6-KO B cells is similar to that in activated Bcl6-WT B cells, development of IgG1⁺IgE⁺ B cells (on day 3 of culture) in Bcl6-KO B cell culture was earlier than that (on day 4 of culture) in Bcl6-WT B cell culture. The induction of C γ 1 expression in activated Bcl6-KO B cells is earlier than that in activated Bcl6-WT B cells, indicating that Bcl6 controls C γ 1 expression in B cells stimulated with IL-4. CSR to IgG1 requires C μ and C γ 1 expression in activated B cells and C μ germline transcript is expressed constitutively in splenic B cells (Li et al., 1994). CSR also requires activity of activation-induced cytidine deaminase (Muramatsu et al., 2000), which was induced in those activated B cells from day 2 of culture (data not shown). Since activated B cells proliferated before CSR, this earlier induction of C γ 1 expression in activated Bcl6-KO B cells may reflect to the increased generation of IgG1⁺ B cells in Bcl6-KO culture after day 3 of culture. Since the induction of C ϵ expression (within 6–12 h after stimulation) is earlier than that of C γ 1 expression (within 24–48 h after stimulation) in activated B cells and IgG1⁺IgE⁺ B cells can develop after IgG1⁺ B cell development,

the increased development of IgG1⁺IgE⁺ B cells in Bcl6-KO culture is reflected by the increased development of IgG1⁺ B cells in the culture.

Bcl6 negatively controls C γ 1 expression in B cells stimulated with IL-4. Although Bcl6 negatively regulates C ϵ expression through the C ϵ promoter (Harris et al., 1999), the induction of C ϵ expression in Bcl6-KO B cells was similar to that in Bcl6-WT B cells after IL-4 stimulation. Thus, regulatory activity of Bcl6 is more effective to C γ 1 expression than to C ϵ expression in IL-4-activated B cells. In IL-4-activated Bcl6-KO B cells, the induction of C ϵ expression (within 6 h after stimulation) is also earlier than that of C γ 1 expression (within 24 h after stimulation). This result is supported by the evidence that the promoter regions of C γ 1 and C ϵ germline transcript contain STAT6-binding sequences and the binding affinity of STAT6 to the C ϵ promoter region is 10-fold higher than that to the C γ 1 promoter region (Mao and Stavnezer, 2001). Furthermore, Bcl6 also binds competitively to the STAT6-binding sequence in the C ϵ promoter (Dent et al., 1997; Harris et al., 1999). Thus, the strong binding affinity of STAT6 to the C ϵ promoter region may competitively deny Bcl6 binding to the region and induce its expression within 6 h after stimulation. The binding affinity of STAT6 to the C γ 1 promoter region may not be strong enough to deny Bcl6 binding to the region in activated Bcl6-WT B cells within 24 h after stimulation.

CSR to IgG1 and to IgE depends on IL-4, and IL-21 enhances IL-4-mediated CSR to IgG1 but inhibits IL-4-mediated CSR to IgE. We showed that C γ 1 expression is enhanced in Bcl6-WT B cells activated with IL-4 and IL-21 compared with that with IL-4. This enhancing effect by IL-21 stimulation is Bcl6-independent since C γ 1 expression is also enhanced in Bcl6-KO B cells activated with IL-4 and IL-21. These results indicate that IL-21 induces enhancer molecules for C γ 1 expression. Since the promoter region of C γ 1 germline transcript contains STAT3-binding, STAT6-binding and NF κ B-binding sequences (Warren et al., 1999) and IL-21 activates STAT3 but not STAT6 in mature B cells (Arguni et al., 2006), STAT3 and/or NF κ B may be enhancer molecules for C γ 1 expression in mature B cells activated with IL-4 and IL-21. However, IL-21 inhibits IL-4-mediated CSR to IgE. We showed that the inhibitory effect of IL-21 on C ϵ expression was observed in Bcl6-WT B cells but not in Bcl6-KO B cells stimulated with IL-4 and IL-21, indicating that the IL-21-mediated suppression of C ϵ expression is due to Bcl6.

CSR to IgE is independent of prior switch recombination to any other class (Jung et al., 1994). Indeed, both IgE⁺ and IgG1⁺IgE⁺ B cells simultaneously developed in Bcl6-KO culture on day 3 of culture. However, IgE⁺ B cells but not IgG1⁺ B cells are hardly detected in GCs. Bcl6 inhibits CSR to IgE in GC B cells by repressing C ϵ expression. Bcl6 also inhibits C γ 1 expression in IL-4-activated B cells and CSR to IgG1 may be inhibited completely in GC B cells. Thus, IgG1⁺ B cells in GCs may derive from outside of GCs. Similarly, IgE⁺ B cells developed outside of GCs could also make GC-formation. In GCs, Tfh cells produce large amounts of IL-21 (Wurster et al., 2002; Chtanova et al., 2004) but little IL-4 (Vinesa et al., 2005), and Bcl6 is required for protection of IL-21-induced apoptosis of IgG1⁺ B cells but not for that of IgE⁺ B cells (Tsuruoka et al.,

2007). Furthermore, IL-21 induces apoptosis to IgE⁺ B cells by inducing a proapoptotic factor, Bmf (Harada et al., 2006). Thus, IgE⁺ B cells developed outside of GCs may become apoptosis in GCs by IL-21.

Bcl6 represses the transcription of Blimp1, a gene whose expression is crucial for plasma cell differentiation (Shapiro-Shelef and Calame, 2005), and Synd⁺ B cell numbers and IgM production in Bcl6-KO culture were more than those in Bcl6-WT culture and IL-21 stimulation increased Synd⁺ B cell numbers and IgM production in Bcl6-WT and Bcl6-KO culture. However, IL-21 stimulation did not increase IgG1 and IgE production in Bcl6-WT and Bcl6-KO culture. The IL-21-mediated reduction of IgE production is due to IL-21-induced apoptosis to class switching B cells to IgE and IgE⁺ B cells. The reduction of IgG1 production in Bcl6-KO culture can also be explained by IL-21-induced apoptosis to IgG1⁺ B cells. In Bcl6-WT culture, IL-21 stimulation may maintain IgG1⁺ B cells at the B cell stage by inducing Bcl6 which represses Blimp1 expression in these B cells.

In summary, Bcl6 negatively controls not only C ϵ expression but also C γ 1 expression and can inhibit the sequential CSR from IgG1 to IgE in IL-4-activated B cells. IL-21 stimulation enhanced C γ 1 expression independent of Bcl6 but suppressed C ϵ expression with relation to Bcl6 in IL-4-activated B cells. Thus, Bcl6 stabilizes class switching to IgG1 in activated B cells simultaneously stimulated with IL-4 and IL-21.

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