A Role for c-fos/Activator Protein 1 in B Lymphocyte Terminal Differentiation


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Expression of B lymphocyte-induced maturation protein 1 (Blimp-1) transcription factor is essential for promoting B cell differentiation into plasma cells. However, a critical transcription factor for Blimp-1 expression in activated B cells is unclear. When splenic B cells were stimulated with CD40 ligand (CD40L) and IL-4, terminal differentiation was induced in the B cells from c-fos transgenic (H2-c-fos) mice but barely in those from control littermates and from c-fos-deficient mice. AP-1 family and Blimp-1 mRNAs were transiently induced in the control B cells, and overexpression of c-Fos induced a sufficient amount of Blimp-1 for terminal differentiation in the H2-c-fos B cells. When normal and c-fos-deficient B cells were stimulated with LPS, a sufficient amount of Blimp-1 for terminal differentiation was induced in those B cells. However, expression of c-fos/AP-1 family mRNAs in LPS-stimulated normal B cells was similar to that of normal B cells stimulated with CD40L and IL-4. EMSA and chromatin immunoprecipitation assays using the AP-1-binding DNA sequence in the murine Blimp-1 promoter region demonstrated that AP-1-binding activity in nuclear protein of LPS-stimulated normal B cells was prolonged more than that in normal B cells stimulated with CD40L and IL-4. Furthermore, the percentage of CD138⁺ B cells within germinal center B cells in the spleen and the number of Ab-forming cells in the bone marrow of H2-c-fos mice was larger than that of control mice 12 days after immunization. Thus, although c-Fos is not essential for Blimp-1 expression, c-Fos/AP-1 positively regulates Blimp-1 expression and terminal differentiation of activated B cells. The Journal of Immunology, 2005, 174: 7703–7710.

The transcriptional regulator B lymphocyte-induced maturation protein 1 (Blimp-1) is a 98-kDa protein containing five Krippel-type zinc fingers that confer sequence-specific DNA binding (1). Blimp-1 has been postulated to be a master regulator of B cell terminal differentiation. In the BCL-1 lymphoma model of differentiation from a mature B cell to a plasma cell, ectopic expression of Blimp-1 is sufficient to cause terminal differentiation evidenced by loss of surface Igks, IgM secretion, expression of CD138 (syndecan-1) on the cell surface, and cessation of cell division (1–3). Blimp-1 was also found in a fraction (4–15%) of germinal center B cells in murine spleen and human tonsils (4). In mice, Blimp-1⁺ germinal center B cells express syndecan-1 and cytoplasmic Ig but not Bcl6 (4), and Blimp-1 expression is repressed by Bcl6 (5, 6). The Blimp-1 conditional knockout mice demonstrated that Blimp-1 is required for differentiation of plasma cells and pre-plasma memory B cells (7). Thus, these data support a model in which Blimp-1 expression is critical for commitment to a plasma cell, rather than a memory cell, fate. Since Bcl6 suppresses cytokine-driven differentiation of primary B cells to plasma cells by inhibiting STAT3-dependent transcriptional events (6), STAT3 may be an important transcription factor for Blimp-1 expression. The recent report also demonstrated that Bcl6 can be a potent repressor of transcriptional activity mediated by AP-1 factors and that repression of AP-1 function by Bcl6 may be a key mechanism for how Bcl6 regulates Blimp-1 expression (8).

The protooncogene c-fos encodes a nuclear phosphoprotein, c-Fos. c-Fos is in a complex with the product of another protooncogene c-jun (AP-1) regulates expression of the AP-1-binding genes at their transcriptional level (9–12). Since expression of c-fos and c-jun family genes is transiently induced in B cells stimulated with CD40 ligand (CD40L) and IL-4 as an immediate early gene, the function of c-Fos/AP-1 may be implicated in the transduction of signals induced by growth and differentiation factors (13, 14). We generated transgenic mice carrying the murine c-fos gene under the control of the murine MHC gene (H2-Kb) promoter (H2-c-fos) (15). Splenic T and B cells from the mice constitutively express a high level of the exogenous c-fos gene (16). When H2-c-fos mice are immunized with T-dependent Ags, the mice can make the reduced size of germinal centers and fail to generate memory B cells in the spleen (16, 17). These results suggest that the ectopic expression of c-Fos may accelerate commitment of germinal center B cells to a plasma cell fate.

Engagement of CD40 on B cells by its ligand, gp39 or CD154, on activated T cells is a crucial component of cognate T cell help (18). CD40 signaling in B cells triggers a potent proliferative response (19, 20), induces expression of costimulatory and adhesion molecules (21), and mediates clonal expansion and survival within germinal centers (22). Mice deficient in either CD40 or CD40L are
unable to form germinal centers or to make high-affinity, class-switched Abs after immunization with T-dependent Ags (23–25). Several in vitro experiments have also suggested that CD40 signaling is important for promoting B cell differentiation and Ig secretion (26–28). However, stimulation of CD40 on B cells actively inhibits the B cells from differentiating into Ab-forming cells (AFCs) (29–32). This arrest is manifested as a reduction in mRNA levels of secretory Ig gene products as well as the loss of Blimp-1 (32). Thus, a role for CD40 signaling in B cell terminal differentiation is controversial. In this study, we directly tested the role for the ectopic c-Fos in terminal differentiation of B cells activated with CD40L stimulation. When splenic B cells from H2-c-fos mice were cultured with CD40L and IL-4, cell proliferation of these B cells was augmented compared with that of normal control B cells. The H2-c-fos B cells but not the control B cells differentiated into AFCs within 2 days after stimulation. We discuss a critical role for c-Fos/AP-1 in terminal differentiation of B cells activated by CD40L stimulation.

Materials and Methods

Animals and immunization
C57Bl/6CrSlc mice were purchased from Japan SLC. Transgenic mice carrying the mouse c-fos gene under the control of the H-2Kb promoter (H2-c-fos) (15), and c-fos-deficient mice (33) were maintained by heterozygous mating in our animal facilities. These mice were immunized i.p. (H2-c-fos) (15), and c-fos-deficient mice (33) were maintained by heterozygous mating in our animal facilities. These mice were immunized i.p. with 100 μg of alum-precipitated DNP-OVA or with 50 μg of alum-precipitated (4-hydroxy-3-nitrophenylacetetyl-chicken γ-globulin (NP35-CG) (Biosearch Technologies).

Preparation 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 PE-anti-CD16/32 Ab (BD Pharmingen). These cells were subsequently reacted with immunomagnetic beads coated with anti-PE Ab (Miltenyi Biotec). Labeled cells were removed by a MACS system (Miltenyi Biotec). The resulting B cell fraction contained >95% of B220+ B cells.

Cell culture and stimulation of lymphocytes
Purified B cells (1.5 × 10^5/ml) were cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FCS (Intergen), 50 μM 2-ME, 100 μg/ml streptomycin (Wako Chemical), and 100 U/ml penicillin G/potassium (Banyu Pharmaceutical). For B cell activation, CD40L (culture supernatant from anti-CD40L antibodies (Medicin Chemical), and the cells were lysed by SDS lysis buffer with protease inhibitors. ChIP assays were performed as previously described (44). Briefly, formalin-fixed nuclei were allowed to occur at 4°C for 60 min, and the cells were lysed by SDS lysis buffer with protease inhibitors. Chromatin in the lysate was sonicated to an average length of 400–600 bp by the manufacturer’s instruction. Competitive EMSA was done by adding 100-fold molar excess of unlabeled double-stranded oligonucleotides to the mixture. The sequences of the mutant oligonucleotides (two bases mismatch, underlined) were as follows: Mut-1, 5’-TTATTAACCCGATGCAATCGACCACTATGGGACAGAAA-3’ and Mut-2, 5’-TTTCTGATCCATGTGACGTCATCGACCATGTTAAATAA-3’, underlined; AP-1-binding (AP-1) was synthesized and biotinylated using the biotin 3’-end-labeling kit (Pierce). Binding activity of AP-1 to the mAP1BS sequence was determined using the LightShift chemiluminometric EMSA kit (Pierce) and the manufacturer’s instruction. Competitive EMSA was done by adding 100-fold molar excess of unlabeled double-stranded oligonucleotides to the mixture. The sequences of the mutant oligonucleotides (two bases mismatch, underlined) were as follows: Mut-1, 5’-TTATTAACCCGATGCAATCGACCACTATGGGACAGAAA-3’ and Mut-2, 5’-TTTCTGATCCATGTGACGTCATCGACCATGTTAAATAA-3’. EMSA

Double-stranded oligonucleotides corresponding to putative AP-1-binding sequence were made (28 cycles). Primer I, 5’-GGTACGTTGCTCTTGTGCCAGG-3’; Primer II, 5’-TTTCTGATCCATGTGACGTCATCGACCATGTTAAATAATG-3’. Double-stranded oligonucleotides were made from the cDNAs, which were made by reverse-transcribed PCR with specific primers (40–42).

Isolation of nuclear proteins
Nuclear proteins were isolated from splenic B cells using the method as described previously (43), with slight modification. Briefly, splenic B cells (1 × 10^7) were resuspended in 400 μl of cold hypotonic buffer (10 mM Hepes (pH 7.9), 10 mM KCl, 1.5 mM MgCl2, 0.1 mM EDTA, 0.1% Triton X-100, 1 mM DTT, 100 mM PMSF, and 5 μg/ml aprotinin). Nuclei were collected by centrifugation and disrupted by sonication in 100 μl of immunoprecipitation buffer (1 mM HEPES (pH 7.5), 5 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 0.1% Tween 20, 10% glycerol, 1 mM DTT, 100 mM PMSF, and 5 μg/ml aprotinin) at 4°C. Nuclear extracts were immediately stored at −80°C. The amount of protein was determined using the Bio-Rad protein assay (Bio-Rad).

EMSA

Double-stranded oligonucleotides were made (28 cycles). Primer I, 5’-GGTACGTTGCTCTTGTGCCAGG-3’; Primer II, 5’-TTTCTGATCCATGTGACGTCATCGACCATGTTAAATAATG-3’. Double-stranded oligonucleotides were made from the cDNAs, which were made by reverse-transcribed PCR with specific primers (40–42).

Chromatin immunoprecipitation (ChIP) assay
ChIP assays were performed as previously described (44). Briefly, formaldehyde solution (37%; Fisher Scientific), at a final concentration of 1%, was added directly to B cells (3 × 10^6) after each stimulation. Cross-linking of proteins on chromatin was allowed to occur at 4°C for 2 h, and the cells were lysed by SDS lysis buffer with protease inhibitors. Chromatin in the lysate was sonicated to an average length of 400–600 bp by the manufacturer’s instruction. The immune complexes were incubated with salmon sperm DNA/protein A/agarose/50% slurry for 3 h at 4°C and washed with 2 μg of rabbit polyclonal anti-c-Fos Abs (Santa Cruz Biotechnology) overnight. The immune complexes were incubated with salmon sperm DNA/protein A/agarose/50% slurry with mild shaking for 3 h at 4°C, washed, and eluted. After proteinase K treatment, DNA in samples was phenol extracted and resuspended in 50 μl of TE buffer (10 mM Tris-HCl (pH 7.5) and 1 mM EDTA). DNA solution (2 μl) was used for PCR amplification (28 cycles). PCR products were analyzed by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. The following primers were used: mAP1BS-1, 5’-GGAAAACAGAAACTATTCTTGA-3’; mAP1BS-2, 5’-GAAAACAGAAACTATTCTTGA-3’ and mAP1BS-2, 5’-GAAAACAGAAACTATTCTTGA-3’. Blimp-1 EXPRESSION BY c-fos/AP-1

Northern blot
Northern blotting was performed as described previously (38). Briefly, total RNA (10 μg) was electrophoresed through a 1.0% agarose gel containing formaldehyde and transferred to a nylon membrane (Roche Molecular Biochemicals). The filter was posthybridized for 1 h and hybridized overnight at 50°C in 50% formamide hybridization buffer with 0.5% SDS, 1% blocking reagent, and 15 ng/ml digoxigenin (DIG; Roche Molecular Biochemicals)-labeled probes. Following hybridization, the filter was washed twice for 15 min with 0.1× SSC and 0.1% SDS at 55°C. The DIG-labeled probe was detected with sheep anti-DIG Abs conjugated with alkaline phosphatase. The anti-DIG Ab detection reaction was performed using an enhanced chemiluminescent detection system (Roche Molecular Biochemicals). DIG-labeled probes were made as described previously (17, 39). Other probes (fosB, fra-1, fra-2, Blimp-1, XBP-1, and J chain) were made from the cDNAs, which were made by reverse-transcribed PCR with specific primers (40–42).
Results

c-Fos augments proliferation of splenic B cells stimulated with CD40L and IL-4

The number of B220⁺ B cells in the spleen of H2-c-fos mice was similar to that of control littermates (17), and the amount of CD40 on H2-c-fos B cells was also similar to that on control B cells (data not shown). These B cells were stimulated with various doses of CD40L in the presence or absence of IL-4. Cell proliferation of the B cells was measured by DNA synthesis on day 2 of culture. The response of H2-c-fos B cells was markedly augmented compared with that of control B cells at any doses of CD40L examined (Fig. 1A). We have shown that H2-c-fos B cells stimulated with LPS proliferated more than LPS-activated normal B cells (45) and those results were repeated in Fig. 1B. The plateau responses of H2-c-fos and control B cells stimulated with CD40L and IL-4 were almost the same as those of H2-c-fos and control B cells stimulated with LPS, respectively. These results suggest that the amount of c-Fos may decide the intensity of signaling in activated B cells to proliferate.

c-Fos augments terminal differentiation of B cells stimulated with CD40L and IL-4

CFSE-labeled H2-c-fos B cells were cultured with CD40L and IL-4 for 3 days, and cell surface expression of CFSE and several differentiation markers including CD138 (syndecan-1) on these B cells were analyzed on a FACS. As shown in Fig. 2A, the H2-c-fos B cells such as the control B cells divided up to five cell divisions within 3 days after stimulation, suggesting that the ectopic c-Fos does not accelerate cell cycle progression of B cells stimulated with CD40L and IL-4.

Expression of IgM and IgD on the H2-c-fos and control B cells was reduced gradually as cell division progressed, and the reduction of surface IgM expression on the H2-c-fos B cells was slightly slower than that on the control B cells (Fig. 2B). However, the amount of IgD on the H2-c-fos B cells was clearly detected on some of the H2-c-fos B cells divided more than three cell divisions but not on the control B cells. Since the reduction of surface IgD expression and the induction of syndecan-1 expression on the H2-c-fos B cells were faster than those on the control B cells, the ectopic c-Fos might accelerate terminal differentiation of B cells stimulated with CD40L and IL-4.

Then we examined kinetics of syndecan-1 expression on H2-c-fos B cells after stimulation with CD40L and IL-4. Syndecan-1⁺ B cells were clearly detected in both H2-c-fos and control B cell cultures from day 2 of culture and the numbers in both cultures increased thereafter, although the percentages in the H2-c-fos culture were larger than those in the control culture (Fig. 3A). Kinetics of syndecan-1⁺ B cells developed in the H2-c-fos culture was compared with that in H2-c-fos B cell culture stimulated with LPS. The kinetics was almost similar between them and also similar to that in control B cell culture stimulated with LPS.

We examined kinetics of IgM-AFCs developed in these B cell cultures. No IgM-AFC (<1 of 10⁵ B cells) was detected in these B cell cultures before stimulation (Fig. 3B). More than 10⁷ IgM-AFCs of 10⁵ B cells were detected in H2-c-fos B cells stimulated with CD40L and IL-4 from day 2 after stimulation. The number in the H2-c-fos culture increased and reached the plateau from day 3 of culture. Although the number of IgM-AFCs in control B cell culture stimulated with CD40L and IL-4 was not >10⁷ IgM-AFCs of 10⁵ B cells within 4 days of culture, these small numbers were

FIGURE 1. c-Fos augments cell proliferation of splenic B cells stimulated with CD40L. Splenic B cells from H2-c-fos (solid line) and wild-type control (broken line) mice were cultured with various concentrations of CD40L in the presence or absence of IL-4 (A) or with various doses of LPS (B). Cell proliferation was measured by [³H]thymidine uptake on day 2 of culture. Results represent means and variations (SD) from triplicate cultures. The data presented are representative of three independent experiments.

FIGURE 2. c-Fos accelerates differentiation of splenic B cells stimulated with CD40L and IL-4. CFSE-labeled splenic B cells were stimulated with CD40L (1/8 dilution) and IL-4 for 3 days. A, Cell division of the activated H2-c-fos (c-fos) and wild-type control (WT) B cells was analyzed on FACS. The solid lines in the 0 division show the histograms of CFSE-labeled B cells without stimulation for 3 days. B, Expression of IgM, IgD, and syndecan-1 on the activated H2-c-fos (open histograms) and wild-type control (filled histograms) B cells was analyzed. The data presented are representative of two independent experiments.
increasing from day 2 of culture and reached the plateau on day 3 of culture. Furthermore, \(10^5\) IgM-AFCs were detected in both H2-c-fos and control B cell cultures stimulated with LPS on day 1 of culture. These numbers increased and reached the plateau on day 2 of culture. Since the plateau numbers of IgM-AFCs in both H2-c-fos and control cultures stimulated with LPS were almost the same as those of H2-c-fos culture stimulated with CD40L and IL-4, the induction rate of IgM-AFCs in H2-c-fos B cells stimulated with CD40L and IL-4 was comparable to those in H2-c-fos and control B cells stimulated with LPS.

A role of c-Fos in terminal differentiation of B cells was further examined using c-fos-deficient mice. We analyzed syndecan-1 expression in c-fos-deficient B cells 4 days after stimulation with CD40L and IL-4. Syndecan-1 expression was clearly detected in these B cells.

Both c-fos-deficient and normal B cell cultures, although the percentage in the c-fos-deficient B cell culture was smaller than that in the normal B cell culture (Fig. 3C). However, the percentages of syndecan-1 expressing cells developed in B cell cultures stimulated with LPS were similar between c-fos-deficient and normal B cells.

**FIGURE 3.** c-Fos augments terminal differentiation of splenic B cells stimulated with CD40L and IL-4. Splenic B cells from H2-c-fos, c-fos-deficient, and wild-type control mice were cultured with CD40L (1/8 dilution) and IL-4 or with LPS (3 \(\mu\)g/ml) for 4 days. A, Expression of syndecan-1 on the activated B cells from H2-c-fos (c-fos) and wild-type control (WT) mice was analyzed on each day of culture. The numbers indicate the percentages of CD19dull and syndecan-1high cells in each oval. These results are representative of two independent experiments. B, IgM-AFCs in the B cell cultures from H2-c-fos (■) and wild-type control (○) mice were detected by ELISPOT assays. Results represent means and variations (SD) from triplicate cultures. The data presented are representative of two independent experiments. C, Expression of syndecan-1 on the activated B cells from c-fos-deficient (c-fos-KO) and wild-type control (WT) mice was analyzed. The numbers indicate the percentages of CD19dull and syndecan-1high cells in each oval. These results are representative of two independent experiments.

**FIGURE 4.** Blimp-1 expression is induced in H2-c-fos B cells stimulated with CD40L and IL-4. Splenic B cells from H2-c-fos (c-fos) and wild-type control (WT) mice were cultured with CD40L (1/8 dilution) and IL-4 (A) or with LPS (3 \(\mu\)g/ml; B) for 3 days. Expression of Blimp-1 and other genes related with B cell terminal differentiation in the activated B cells was analyzed on each day of culture by Northern blot. \(\beta\)-actin mRNA was used as an amount control of mRNA. The data presented are representative of two independent experiments.
c-Fos/AP-1 activity is prolonged in B cells activated with LPS

When we examined expression of these genes in normal B cells stimulated with LPS, Blimp-1 expression was transiently induced within 1 h after stimulation, down-regulated until 6 h after stimulation, and then up-regulated from 12 h after stimulation and increased thereafter (Fig. 5B). Bcl6 expression was transiently induced in the B cells within 1 h after stimulation and the peak was between 3 and 6 h after stimulation and down-regulated thereafter. These results suggest that AP-1 induces the transient Blimp-1 expression in activated B cells within 3–6 h after stimulation. However, Blimp-1 expression in the LPS-stimulated B cells between 12 and 72 h after stimulation cannot be explained by the mRNA expression of AP-1 family genes.

c-Fos/AP-1 activity is prolonged in B cells activated with LPS

The promoter region of murine Blimp-1 gene contains two putative AP-1-binding sequences (mAP1BS-1 and mAP1BS-2). Thus, EMSA was performed to examine binding activity of c-Fos/AP-1 in nuclear proteins of H2-c-fos and control B cells stimulated with CD40L and IL-4 to the mAP1BS. As shown in Fig. 6A, a gel retardation band to the mAP1BS-1 was observed using the nuclear proteins of H2-c-fos and control B cells. This band from control B cells was obviously removed by a nonlabeled probe with the same sequence (WT) as a cold competitor. In contrast, a mutated cold probe (Mut) as a competitor did not inhibit formation of the gel retardation band. Furthermore, the band was removed by the addition of anti-c-Fos Abs, partially by that of anti-JunD Abs, but not by that of control IgG Abs, indicating sequence-specific binding of c-Fos/AP-1 to the mAP1BS-1. The band was still detected in nuclear protein of the H2-c-fos B cells but not in that of the control B cells 24 h after stimulation. The specific band to the mAP1BS-2 was also detected in the nuclear proteins of H2-c-fos and control B cells (data not shown). The AP-1 band to the mAP1BS-1 and to the mAP1BS-2 (data not shown) was detected in nuclear proteins of H2-c-fos and control B cells stimulated with LPS until 24 h after stimulation.

The binding of c-Fos on the mAP1BS-1 and the AP1BS-2 was confirmed by ChIP assays with anti-c-Fos Abs. The mAP1BS-1 was contained in the DNA complexes of H2-c-fos and control B cells 12 h after stimulation with CD40L and IL-4 (Fig. 6B). The mAP1BS-1 was still contained in the DNA complex of the H2-c-fos B cells but not in that of the control B cells 24 h after stimulation. The mAP1BS-2 was also contained in the DNA complexes of H2-c-fos and control B cells (data not shown). The mAP1BS-1 and the mAP1BS-2 (data not shown) were contained in the DNA complexes of H2-c-fos and control B cells stimulated with LPS until 24 h after stimulation. Since the binding activity of c-Fos/AP-1 in nuclear proteins of these activated B cells to the mAP1BS correlated with Blimp-1 expression in these B cells, c-Fos/AP-1 positively regulates Blimp-1 expression in activated B cells.

c-Fos augments terminal differentiation of Ag-specific B cells in vivo

Germinal center formation in the spleen of H2-c-fos mice is perturbed and the perturbation could not be explained by apoptosis of the germinal center B cells (17). These results prompted us to examine the incidence of terminal differentiation in germinal center B cells of H2-c-fos mice. Since a few of syndecan-1− B cells as plasma cell precursors are detected in germinal center B cells after immunization (4), the percentage of syndecan-1− B cells in germinal center B cells was analyzed in the spleen of H2-c-fos mice immunized with NP-CG in alum. Fig. 7A shows that germinal center formation was perturbed in the spleen of H2-c-fos mice 12 days after immunization. The percentage of PNA− B cells in spleen cells of H2-c-fos mice was less than that of control littersmates. The percentage of syndecan-1− B cells in the PNA− B cells of H2-c-fos mice was clearly larger than that of control mice (Fig.
minal center B cells in total spleen cells and those of syndecan-1 immunization were analyzed. The numbers indicate the percentages of germinal center (PNA-encompasses) compared with that of control B cells. Since expression of the endogenous c-Fos augments terminal differentiation in Ag-specific B cells, and cell proliferation of H2-c-fos B cells stimulated with CD40L in the absence of IL-4 was higher than that of control B cells at any doses of CD40L examined. These results suggest that H2-c-fos B cells are more sensitive to CD40L stimulation and that the amount of c-Fos/AP-1 in B cells stimulated with CD40L may decide the intensity of CD40 signaling to proliferate. The ectopic c-Fos also augmented cell proliferation of naive B cells stimulated with CD40L (45). AP-1 activity was detected in both control and H2-c-fos B cells 24 h after LPS stimulation, suggesting that the augmentation of cell proliferation is not due to the prolonged activation of AP-1 in the B cells. These results also suggest that the amount of c-Fos/AP-1 decides the intensity of LPS signaling to proliferate. Thus, a function of c-Fos/AP-1 induced in activated B cells as an immediate early gene product is an amplifier of the initial signals. Further study is required to elucidate a target gene of c-Fos/AP-1 in the signal transduction pathway initiated by CD40L or LPS stimulation.

Many investigators have suggested that CD40 signaling induces not only B cell activation and proliferation but also B cell terminal differentiation, particularly in combination with cytokines (20, 28, 46). In contrast, several reports have suggested that CD40L-deficient humans produce normal or elevated levels of serum IgM (47) and that CD40 signaling directly prevents B cell terminal differentiation (29–32, 48). Thus, CD40 signaling is not only insufficient to induce plasma cell formation, but actively arrests B cells at a stage before terminal differentiation. We showed here that terminal differentiation was barely induced in control B cells stimulated with CD40L and IL-4 and that the ectopic c-Fos efficiently induced terminal differentiation of the B cells. Indeed, AP-1 activity was detected in the control B cells and the H2-c-fos B cells until 12 and 24 h after stimulation, respectively. Although stimulation of control B cells with CD40L and IL-4 induced expression of *junB* and *junD* genes until 24 h after stimulation, transcriptional activity of AP-1 composed of a Fos/Jun heterodimer is 25-fold more effective than that of a Jun/Jun homodimer (49), and AP-1 composed of JunB/JunD may not be effective to induce terminal differentiation of the B cells. Thus, the effective AP-1 (c-Fos/Jun) may be a key transcription factor for terminal differentiation of B cells stimulated with CD40L and IL-4.

**Blimp-1** expression was detected in H2-c-fos B cells but not in control B cells stimulated with CD40L and IL-4 on days 1 and 2 after stimulation. Since there are two AP-1-binding sites in the murine **Blimp-1** promoter region and c-Fos binds to these sites in H2-c-fos B cells stimulated with CD40L and IL-4, effective AP-1 composed of a c-Fos/Jun heterodimer may be enough to activate the **Blimp-1** promoter in the B cells. **Blimp-1** expression might be negatively regulated by Bcl6 in control B cells stimulated with CD40L and IL-4 because overexpression of Bcl6 represses transcription of the human **Blimp-1** promoter almost entirely (8). However, Bcl6 is barely induced in either H2-c-fos or control B cells stimulated with CD40L and IL-4. Furthermore, splenic B cells from Bcl6-deficient mice stimulated with CD40L and IL-4 did not differentiate into IgM-AFCs and IgG1-AFCs (data not shown). These results suggest that little induction of **Blimp-1** expression in control B cells stimulated with CD40L and IL-4 is not due to the negative regulatory effect of a transcriptional repressor, Bcl6, but due to the deficiency of an effective transcriptional factor, c-Fos/AP-1.

**Blimp-1** expression and AP-1 activity were detected in both H2-c-fos and control B cells stimulated with LPS from day 1 after stimulation. Although mRNA expression of AP-1 family genes in control B cells stimulated with LPS was similar to that in control stimulation. The cell numbers of H2-c-fos B cells divided more than four divisions were larger than those of control B cells, and cell proliferation of H2-c-fos B cells stimulated with CD40L in the absence of IL-4 was higher than that of control B cells at any doses of CD40L examined. These results suggest that H2-c-fos B cells are more sensitive to CD40L stimulation and that the amount of c-Fos/AP-1 in B cells stimulated with CD40L may decide the intensity of CD40 signaling to proliferate. The ectopic c-Fos also augmented cell proliferation of naive B cells stimulated with CD40L (45). AP-1 activity was detected in both control and H2-c-fos B cells 24 h after LPS stimulation, suggesting that the augmentation of cell proliferation is not due to the prolonged activation of AP-1 in the B cells. These results also suggest that the amount of c-Fos/AP-1 decides the intensity of LPS signaling to proliferate. Thus, a function of c-Fos/AP-1 induced in activated B cells as an immediate early gene product is an amplifier of the initial signals. Further study is required to elucidate a target gene of c-Fos/AP-1 in the signal transduction pathway initiated by CD40L or LPS stimulation.

Many investigators have suggested that CD40 signaling induces not only B cell activation and proliferation but also B cell terminal differentiation, particularly in combination with cytokines (20, 28, 46). In contrast, several reports have suggested that CD40L-deficient humans produce normal or elevated levels of serum IgM (47) and that CD40 signaling directly prevents B cell terminal differentiation (29–32, 48). Thus, CD40 signaling is not only insufficient to induce plasma cell formation, but actively arrests B cells at a stage before terminal differentiation. We showed here that terminal differentiation was barely induced in control B cells stimulated with CD40L and IL-4 and that the ectopic c-Fos efficiently induced terminal differentiation of the B cells. Indeed, AP-1 activity was detected in the control B cells and the H2-c-fos B cells until 12 and 24 h after stimulation, respectively. Although stimulation of control B cells with CD40L and IL-4 induced expression of *junB* and *junD* genes until 24 h after stimulation, transcriptional activity of AP-1 composed of a Fos/Jun heterodimer is 25-fold more effective than that of a Jun/Jun homodimer (49), and AP-1 composed of JunB/JunD may not be effective to induce terminal differentiation of the B cells. Thus, the effective AP-1 (c-Fos/Jun) may be a key transcription factor for terminal differentiation of B cells stimulated with CD40L and IL-4.

**Blimp-1** expression was detected in H2-c-fos B cells but not in control B cells stimulated with CD40L and IL-4 on days 1 and 2 after stimulation. Since there are two AP-1-binding sites in the murine **Blimp-1** promoter region and c-Fos binds to these sites in H2-c-fos B cells stimulated with CD40L and IL-4, effective AP-1 composed of a c-Fos/Jun heterodimer may be enough to activate the **Blimp-1** promoter in the B cells. **Blimp-1** expression might be negatively regulated by Bcl6 in control B cells stimulated with CD40L and IL-4 because overexpression of Bcl6 represses transcription of the human **Blimp-1** promoter almost entirely (8). However, Bcl6 is barely induced in either H2-c-fos or control B cells stimulated with CD40L and IL-4. Furthermore, splenic B cells from Bcl6-deficient mice stimulated with CD40L and IL-4 did not differentiate into IgM-AFCs and IgG1-AFCs (data not shown). These results suggest that little induction of **Blimp-1** expression in control B cells stimulated with CD40L and IL-4 is not due to the negative regulatory effect of a transcriptional repressor, Bcl6, but due to the deficiency of an effective transcriptional factor, c-Fos/AP-1.

**Blimp-1** expression and AP-1 activity were detected in both H2-c-fos and control B cells stimulated with LPS from day 1 after stimulation. Although mRNA expression of AP-1 family genes in control B cells stimulated with LPS was similar to that in control
B cells stimulated with CD40L and IL-4, DNA-binding activity of AP-1 in the LPS-stimulated B cells was prolonged more than that in the CD40L-stimulated B cells, suggesting that AP-1 is unstable in the CD40L-stimulated B cells. c-Fos/AP-1 may be dephosphorylated and/or denatured in control B cells stimulated with CD40L and IL-4 within 24 h after stimulation. These results support that c-Fos/AP-1 is a key transcription factor for terminal differentiation of B cells stimulated with CD40L and IL-4. However, terminal differentiation of B cells was induced in c-fos-deficient B cells stimulated with CD40L and IL-4 or with LPS, suggesting the functional redundancy of c-Fos family proteins. Thus, the effective AP-1, including Fos family proteins, may be a key transcription factor for Blimp-1 expression and terminal differentiation of activated B cells.

Functionally effective memory B cells are developed in germinal centers (37), and signaling through CD40 drives germinal center B cells toward a memory cell phenotype by preventing terminal differentiation of these B cells (29, 31). However, the in vitro studies shown here may not support the negative effect of CD40 signaling. CD40 signaling is essential for activated B cells to differentiate into germinal center B cells (22–25), indicating that germinal center B cells are stimulated with CD40L on helper T cells. The germinal center formation is perturbed in H2-c-fos mice after immunization (17) and the percentage of plasma cell precursors (syndecan-1− germinal center B cells) in the spleen of H2-c-fos mice was 2- to 3-fold larger than that of control mice after immunization. Thus, the ectopic c-Fos may compose c-Fos/AP-1 in germinal center B cells and induce Blimp-1 expression in these B cells at an earlier stage, leading to premature generation of plasma cells, which emigrate from the germinal centers. Indeed, the number of IgM-AFCs in the CD40L-stimulated B cells. c-Fos/AP-1 may be dephosphorylated and/or denatured in control B cells stimulated with CD40L and IL-4. However, terminal differentiation of B cells was induced in c-fos-deficient B cells stimulated with CD40L and IL-4 or with LPS, suggesting the functional redundancy of c-Fos family proteins. Thus, the effective AP-1, including Fos family proteins, may be a key transcription factor for Blimp-1 expression and terminal differentiation of activated B cells.

We thank Dr. H. Zhang for discussion, H. Satake for technical assistance, and K. Yagyu and N. Kakinuma for secretarial assistance.

Disclosures
The authors have no financial conflict of interest.

References


