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Krüppel-Like Factor 4 Regulates B Cell Number and Activation-Induced B Cell Proliferation[1]

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Abstract

Krüppel-like factor 4 (Klf4) is a transcription factor and functions in regulating cell differentiation, cell growth, and cell cycle. Although *Klf4* is expressed in lymphocytes, its function in lymphocytes is unknown. In this study, we report that the levels of *Klf4* expression were low in pro-B cells and continuously increased in pre-B and in mature B cells. Upon activation, *Klf4* was rapidly decreased in mature B cells after 2 h of activation. A modest decrease in numbers of pre-B cells in bone marrow and mature B cells in spleen was observed in *Klf4*-deficient mice. In the absence of *Klf4*, fewer B cells entered the S phase of the cell cycle and completed cell division in response to the engagement of BCR and/or CD40 in vitro. Furthermore, the delay in entering the cell cycle is associated with decreased expression of cyclin D2 in B cells that lack *Klf4* expression. We then demonstrated that Klf4 directly bound to the promoter of cyclin D2 and regulated its expression. These findings demonstrate that Klf4 regulates B cell number and activation-induced B cell proliferation through directly acting on the promoter of cyclin D2.

Homeostasis of B cells is regulated by the processes of cell proliferation and death. Cell proliferation induced by engagement of the BCR and costimulatory receptors such as CD40 requires successful transition through different cell cycle phases and checkpoints (1). Upon receiving the activation signal, the decision of resting B cells to enter the cell cycle reflects a balance between growth-promoting and growth-inhibitory regulators (2). D-type cyclins are key regulators for the G_1/S transition checkpoint and are primary targets for proliferation signals. Among the three members of D cyclins, cyclin D2 is the main D-type cyclin expressed in mature mouse B cells and plays an important role in cell proliferation after BCR-mediated proliferation (2-5). However, the transcriptional control of cyclin D2 expression in B cells is not completely understood.

Krüppel-like factor 4 (Klf4)[3] is a zinc-finger transcription factor that regulates cell proliferation and differentiation (6-8). One unique feature of Klf4 is its function in cell cycle and growth depending on the surrounding context (9). Klf4 induces cell cycle arrest at the G_1/S boundary in untransformed cells while acting as an oncogene by repressing p53 in transformed cells expressing RAS^{V12} (7). In addition, Klf4 induces oncogenic transformation in the absence of

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[3]Abbreviations used in this paper: Klf4, Krüppel-like factor 4; PI, proliferation index; ChIP, chromatin immunoprecipitation.

functional cyclin D1 and the cyclin-dependent kinase inhibitor, $p21^{WAF1/Cip1}$ (10). A recent report shows that *Klf4* is expressed in several types of human B cell lymphoma/leukemia lineages (11), and overexpression of Klf4 can induce cell cycle arrest and apoptosis in the G_1 phase of the cell cycle of transformed pro/pre-B cell lines. Pro/pre-B cells that overexpress Klf4 display increased expression of $p21^{CIP1}$, and decreased expression of c-Myc and cyclin D2, indicating that Klf4 might regulate cell cycle in B cell malignancies. However, the physiological role of Klf4 in normal B cells has not yet been examined.

To determine the role of Klf4 in B cells, we generated mice deficient for Klf4 in B cells ($bKlf4^{-/-}$) and studied their response to activation-induced proliferation. In this study, we report that $bKlf4^{-/-}$ mice had a modest reduction of pre-B cells in bone marrow and in mature B cells in spleen; B cells in the absence of Klf4 exhibited a defect in the BCR-mediated proliferation response. We demonstrate that this proliferation defect is due to the inability of cyclin D2 up-regulation in B cells from $bKlf4^{-/-}$ mice, and that Klf4 directly binds to the promoter and regulates cyclin D2 expression. These findings suggest that Klf4 plays an important role in regulating activation-induced B cell proliferation.

Materials and Methods

Generation of B cell-specific-Klf4-knockout mice

Klf4-loxP mice (12) were crossed with CD19-Cre transgenic mice (13) to generate $bKlf4^{-/-}$ mice in which the *Klf4* allele is specifically deleted in B cells. The deletion of the *Klf4* gene in B cells was confirmed by Southern blotting and by RT-PCR. The breeding was conducted between $bKlf4^{+/-}$ mice and genotyping was done by PCR around 4 wk of age. $bKlf4^{-/-}$ and $bKlf4^{+/+}$ littermate mice were used in the experiments at 8–16 wk of age. Maintenance and experiments of mice were in accordance with the National Institutes of Health policies for animal care and use.

Isolation and stimulation of B cells

Pro- and pre-B cells were isolated from bone marrow by cell sorting (Mo-Flo) based on the expressions of $CD43^+/CD24^-/B220^+$ for pro-B cells and $CD43^-/CD24^+/B220^+$ for pre-B cells. Spleen B cells were isolated by a negative depletion method using a B cell isolation kit (Miltenyi Biotec) according to the manufacturer's instruction. The purity of these isolated cells was >95%. Freshly isolated splenic B cells were resuspended in RPMI 1640 medium containing 10% FBS, 2 mM L-glutamine, 50 μM 2-ME, and 10 U/ml penicillin/10 μg/ml streptomycin (Invitrogen Life Technologies), and 24 μg/ml AffiniPure goat anti-mouse IgM μ-chain specific (Jackson ImmunoResearch Laboratories) alone or in combination with 5 μg/ml anti-mouse CD40 (clone 1C10; eBioscience) or with LPS (10 μg/ml; Sigma-Aldrich) were added. Stimulated B cells were harvested at specified time for mRNA, protein, and other analyses.

Flow cytometry analysis

Single-cell suspensions of bone marrow cells and splenocytes were incubated with different combinations of the following Abs against: CD4-FITC, CD8-PE, CD19-TC, CD24-FITC, CD43-PE, B220-Tri-color, IgM-PE (Invitrogen Life Technologies). Data were collected on the FACSCalibur and analyzed using CellQuest Pro software (BD Biosciences).

Cell cycle analysis

Cell cycle analysis was conducted using the propidium iodide staining kit (BD Biosciences) according to the manufacturer's instruction. The data were collected using a FACSCalibur and analyzed using MultiCycle software (Phoenix Flow Systems).

[^{3}H]Thymidine incorporation assays

Freshly isolated B cells were plated in triplicate at 2×10^5 cells/well in 96-well plates and stimulated with 24 μg/ml goat anti-mouse IgM, μ-chain specific (Jackson ImmunoResearch Laboratories) alone or in combination with 5 μg/ml anti-mouse CD40 or LPS for 48 h and then 1 μCi of [^{3}H]thymidine was added to each well. Cells were harvested 20 h later and [^{3}H] thymidine incorporation was measured.

CFSE assay

Freshly isolated B cells were labeled with 2.5 μM CFSE (Invitrogen Life Technologies) at 37° C for 10 min and washed twice with RPMI 1640 containing 10% FCS. After stimulation for 2 and 3 days, the CFSE profiles were evaluated by flow cytometry and the proliferation index (PI) was calculated using MODFIT software (Verity Software House).

Quantitative RT-PCR

The procedure of quantitative RT-PCR was previously described (14). Primers were designed using PRISM Primer Express 2.0 (Applied Biosystems) and made by Integrated DNA Technologies. Primers sequences are Klf4: 5′-GGTGCAGCTTGCAGCAGTAA-3′ and 5′-AAAGTCTAGGTCCAGGAGGTCGTT-3′; CcnD2: 5′-GCGTGCAGAAGGACATCCA-3′ and 5′-CCTCACAGACCTCTAGCATCCA-3′; CcnD3: 5′-ACGACTTCCTGGCCTTGATTC-3′ and 5′-CAAAGCCTGCCGGTCACT-3′; CdknA1 (p21): 5′-GGTGGGCCCGGAACAT-3′ and 5′-GCGCTTGGAGTGATAGAAATCTG-3′; c-Myc: 5′-GGGCCAGCCCTGAGCCCCTAGTG-3′ and 5′-ATGGAGATGAGCCCGACTCCGACC-3′; L32: 5′-ACCA GTCAGACCGATATGTGAAAA-3′ and 5′-CGCACCCTGTTGTCAATGC-3′; β-actin: 5′-AGGTCATCACTATTGGCAACGA-3′ and 5′-AGGA TTCCATACCCAAGAAGGAA-3′. The real-time PCR was conducted in 96-well plates in a 25 μl total volume using SYBR green PCR mix kit (Bio-Rad) under the manufacturer's condition. The specificity of the PCR product was confirmed by gel (2% agarose) electrophoresis. Each RT-PCR was repeated twice and the mean was used in the figures. The threshold cycle values obtained from each reaction were normalized to the average of ribosomal protein L32 and β-actin at each correlating time point.

Western blot

Standard Western blots were performed using the following Abs: cyclin D2 (1:200; Abcam), cyclin D3 (1:2,000; Cell Signaling Technology), p21 (1:500; BD Biosciences), and β-actin (1:10,000; Sigma-Aldrich) as a loading control, followed by HRP-linked anti-mouse IgG (1:5,000; GE Healthcare). Signals were detected using the ECL detection system (GE Healthcare) according to the manufacturer's instructions. Five different samples were analyzed for each Ab. The quantitation was conducted by collecting images (FluorChem; Alpha Innotech) and calculated using UN-SCAN-IT software (Silk Scientific). The intensity values were normalized to β-actin and the mean intensity values were presented.

Chromatin immunoprecipitation (ChIP) assay

The procedure of the ChIP assay was previously described (14). In brief, 2 million freshly isolated spleen B cells from C57BL/6 mice were incubated with 1% formaldehyde at 37°C for 10 min and washing twice with cold PBS containing protease inhibitors (Complete; Roche). The cells were lysed with lysis buffer, and the chromosomal DNA was then sheared with sonication (Sonic Dismembrator Model 100; Fisher Scientific). To eliminate nonspecific binding, Dynabeads protein G was first incubated with cell lysate and washed, then cell lysates were separated into three parts: 1) input, 2) immunoprecipitate with 2 μg of monoclonal anti-Klf4 (ABNOVA), and 3) immunoprecipitate with an isotype-matched control IgG. DNA from these three fractions were isolated and used for real-time PCR. Three pairs of primers were

used: CcnD2_p2 (−233 to −372 bp): 5′-AAGCCTCCGAAGTTAGAGAGCAC-3′ and 5′-CTCCCCATCCAGCCCCGC-3′; CcnD2_p3 (−501 to −638 bp): 5′-AAGCACCCCTTTCTCCAACATC-3′ and 5′-GCAACCTCGCCAAACCAGG-3′; and CcnD2_p4 (−766 to −922 bp): 5′-AGCCAAACCTAAACCCTCCCTCTC-3′ and 5′-ACGCAGGAAAAACCCGCTTC-3′. The data were derived from two independent experiments and presented as the percentage of input value.

Statistical analysis

The Student *t* test was used to analyze the statistical differences between *bklf4*$^{+/+}$ and *bklf4*$^{-/-}$ mice, and *p* values <0.05 were considered significant.

Results

Klf4 expression is regulated in B cells during development and activation

To examine the expression of *Klf4* in B cells, we isolated pro-B cells to pre-B cells from bone marrow and mature B cells from spleen and measured levels of *Klf4* mRNA by real-time quantitative RT-PCR. We found that levels of *Klf4* expression were low in pro-B cells, increased in pre-B cells, and reached highest in mature B cells (Fig. 1*A*). Compared with pro-B cells, mature B cells expressed at least seven times more *Klf4*. To further examine *Klf4* expression after activation, we stimulated mature B cells from spleen with anti-IgM (anti-μ-specific Ab) in vitro. We found that *Klf4* levels rapidly down-regulate after 2 h of stimulation and reached the lowest level after 48 h stimulation (Fig. 1*B*). These findings suggest that expression of *Klf4* increases with B cell maturation and decreases after activation.

bKlf4$^{-/-}$ mice have a modest decrease in percentage/number of pre-B cells in bone marrow and of mature B cells in spleen

To further evaluate the function of Klf4, we generated mice that deleted the *Klf4* gene specifically in B cells by crossing *Klf4-loxP* mice (12) with CD19-Cre transgenic mice (13) B cells isolated from the *bKlf4*$^{-/-}$ mice deleted the *Klf4* gene only in B cells, but not in other cells as confirmed by Southern blot and PCR (Fig. 2, *A* and *B*). In addition, we demonstrated the loss of *Klf4* at the mRNA level in B cells from *bKlf4*$^{-/-}$ mice by real-time RT-PCR (Fig. 2*C*). The *bKlf4*$^{-/-}$ mice were fertile and had no apparent abnormalities. Analysis of B cells in bone marrow from *bKlf4*$^{-/-}$ mice showed a modest increase of pro-B cells but it did not reach statistical significance (Fig. 3*A*, Table I). In contrast, a modest and statistical significant decrease of the percentage of pre-B cells (11% reduction, $p = 0.001$) was observed in *bKlf4*$^{-/-}$ mice (Fig. 3*A*, Table I). In spleen, a similar modest and statistical significant decrease in percentage (9% reduction, $p < 0.05$) and in numbers (25% reduction, $p < 0.05$) of mature B cells was observed (Fig. 3, *B* and *C*, Table I). The percentage of B1 B cells in the peritoneal cavity was not changed between *bKlf4*$^{-/-}$ mice and their controls (Table I). Together, these findings suggest that Klf4 may participate in the regulation of B cell development and homeostasis.

Klf4 involves activation-induced proliferation

To determine whether Klf4 regulates B cell proliferation, we compared DNA synthesis and cell division of B cells between *bKlf4*$^{-/-}$ and *bKlf4*$^{+/+}$ mice in response to anti-IgM alone, or anti-IgM plus anti-CD40, or LPS stimulation in vitro. [^{3}H]Thymidine incorporation was used for evaluating DNA synthesis; B cells from *bKlf4*$^{-/-}$ mice showed significantly reduced [^{3}H] thymidine uptake compared with B cells from *bKlf4*$^{+/+}$ mice under stimulation conditions ($p < 0.05$ for both anti-IgM alone, $n = 10$ and for anti-IgM/anti-CD40, $n = 5$) but not under LPS stimulation ($p = 0.11$, $n = 3$) (Fig. 4*A*). CFSE-tracking dye was used to examine activation-induced cell division by comparing PI. PI is defined by the total number of cells after culture

divided by the computed number of original parent cells, and was significantly lower in B cells from *bKlf4*$^{-/-}$ than from *bKlf4*$^{+/+}$ mice ($p < 0.05$ for anti-IgM alone, $n = 9$ and $p < 0.01$ for anti-IgM/anti-CD40, $n = 14$) (Fig. 4*B*). Together, these findings suggest that Klf4 is involved in BCR-mediated, activation-induced B cell proliferation.

Klf4 is required for the progression from the G_1 to the S phase of B cell cycle

To further investigate the precise defects in activation-induced proliferation in *bKlf4*$^{-/-}$ B cells, the cell cycle status of B cells after activation was compared between *bKlf4*$^{-/-}$ and *bKlf4*$^{+/+}$ mice. More B cells remained in G_1 phase and fewer B cells in S phase from *bKlf4*$^{-/-}$ mice than from *bKlf4*$^{+/+}$ mice after 48 h of anti-IgM (Fig. 5) and after anti-IgM/anti-CD40 stimulations (data not shown). This finding suggests that Klf4 regulates cell cycle progression from G_1 to S phase in B cells and the absence of Klf4 results in partial blockade of B cell progression from G_1 to S phase.

Klf4 is required for cyclin D2 induction in B cell activation-induced cell cycle

To understand the mechanisms underlying the defects of G_1 to S phase progression, we examined the expression levels of four cell cycle and proliferation-related genes ($p21^{WAF1/Cip1}$, cyclin D2, cyclin D3, and c-Myc). At the mRNA level, $p21^{WAF1/Cip1}$ and cyclin D2 were expressed lower in freshly isolated and stimulated B cells from *bKlf4*$^{-/-}$ mice than from the control mice but cyclin D3 and c-Myc were expressed at similar levels (Fig. 6*A*). At the protein level, cyclin D2 was lower in B cells at resting and after 24-h stimulation from *bKlf4*$^{-/-}$ mice than from control mice but was similar after 48-h stimulation between *bKlf4*$^{-/-}$ and the control mice (Fig. 6*B*). The protein levels of cyclin D3 and p21 were similar in resting and activated B cells from *bKlf4*$^{+/+}$ and *bKlf4*$^{-/-}$ mice (Fig. 6*B*). The low expression of cyclin D2 at resting B cells and the slow up-regulation in 24-h stimulated B cells from *bKlf4*$^{-/-}$ mice may explain the declined proliferation due to the defects of G_1- to S-phase transition.

Klf4 directly regulates cyclin D2 expression in B cells

To determine whether Klf4 directly regulates cyclin D2 expression, we applied ChIP with a mAb against Klf4. Based on the consensus Klf4-binding sequence (CACCC) (15), there were three potential sites located at the two regions (−336 to −340 bp and −622 to −626/−631 to −635 bp) (16) (Fig. 7*A*). Three pairs of primers were designed to cover these two putative-binding sites and one no-binding site as a control. We found that Klf4-binding sites had higher levels of binding (PCR region −233 to −372 bp and −501 to −638 bp) compared with anti-Klf4 Ab and a control Ab. There was no difference in the region containing no Klf4-binding site (region −776 to −922 bp) between anti-Klf4 Ab and control (Fig. 7*B*). These findings demonstrate that Klf4 directly binds to the promoter of cyclin D2 and regulated its expression.

Discussion

In this report, we analyzed the function of transcription factor Klf4 in B cells. We found that the levels of *Klf4* increased with B cell development and reached peak at resting mature B cells. In the absence of Klf4, we found that a modest but significant decrease in the percentage of pre-B cells in bone marrow and in the number of mature B cells in spleen of *bKlf4*$^{-/-}$ mice. Furthermore, mature B cells from *bKlf4*$^{-/-}$ mice had significant slow proliferation in response to BCR stimulations than those from wild-type mice. This proliferation defect was manifested by the partial blockade at the G_1 to S cell cycle phase junction, which is in part due to the failure of up-regulation of cyclin D2 in B cells lacking Klf4. Finally, we demonstrated that Klf4 directly bound to the promoter of cyclin D2 and facilitated its expression. Together, these findings demonstrate that Klf4 plays an important role in B cell development and in activation-induced B cell proliferation.

Although the mechanism is not completely understood, the number of B cells in mice is tightly maintained (17). We found a significant reduction of pre-B cells in bone marrow and mature B cells in spleen in the absence of Klf4 but there was no decrease of pro-B cells in bone marrow. As the CD19-cre-mediated deletion occurs during pro-B cell to pre-B cell stages and *Klf4* was expressed in relatively low levels in pro-B cells, it remains to be determined whether Klf4 plays any significant role in the pro-B cells. It is interesting to note that cyclin D2 has a role in regulation of B cell numbers (18,19). In cyclin D2-deficient mice, there was a reduction of B-1 B cells and B cell progenitors but no obvious change in the number of spleen B cells. In this study, we show a decrease of pre-B cells and mature B cells, but not pro-B cells nor B-1 B cells, in *bKlf4*$^{-/-}$ mice. Although a reduced expression of cyclin D2 was found in resting and 24-h stimulated B cells of *bKlf4*$^{-/-}$ mice, the decrease of different types of B cells between *bKlf4*$^{-/-}$ mice and cyclin D2-deficient mice suggests that Klf4 may regulate a broad range of genes including cyclin D2 that may be involved in control of B cell numbers. Interestingly, we did not find a compensatory increase of cyclin D3 in B cells from bKlf4$^{-/-}$ mice or a decrease in proliferation in response to LPS. Together, these findings suggest that Klf4 regulates cyclin D2 but not cyclin D3 through a BCR-mediated activation pathway. Thus, it is necessary to identify additional Klf4 target genes that are involved in regulation of B cell number.

Activation-induced cell proliferation is a multiple-step process that contains many checkpoints. In the absence of Klf4, we found reduced DNA synthesis measured by [^{3}H]thymidine uptake along with an increase in G_1-phase cells and a reduction in the number of cells that underwent cell divisions after in vitro stimulation. Together, these findings suggest that Klf4 acts as a positive regulator in BCR-mediated B cell proliferation. Furthermore, the levels of cyclin D2, but not p21, were decreased in the absence of Klf4. Inability of up-regulation of cyclin D2 provides a partial explanation for the reduced cell proliferation in B cells that lack Klf4. Interestingly, overexpression of Klf4 in transformed pro-/pre-B cell lines causes cell cycle arrest of these transformed pro-/pre-B cell lines (11). This suggests that the consequence of Klf4 and its target gene expressions is dependent on the physiological makeup of the cells. As demonstrated in tumor cells, Klf4 can be either a tumor suppressor or an oncogene depending on the cellular conditions or “context” (20). Thus, understanding the availability and function of Klf4 target genes in B cells will help to elucidate the role of Klf4 in regulation of cell cycle and growth of B cells.

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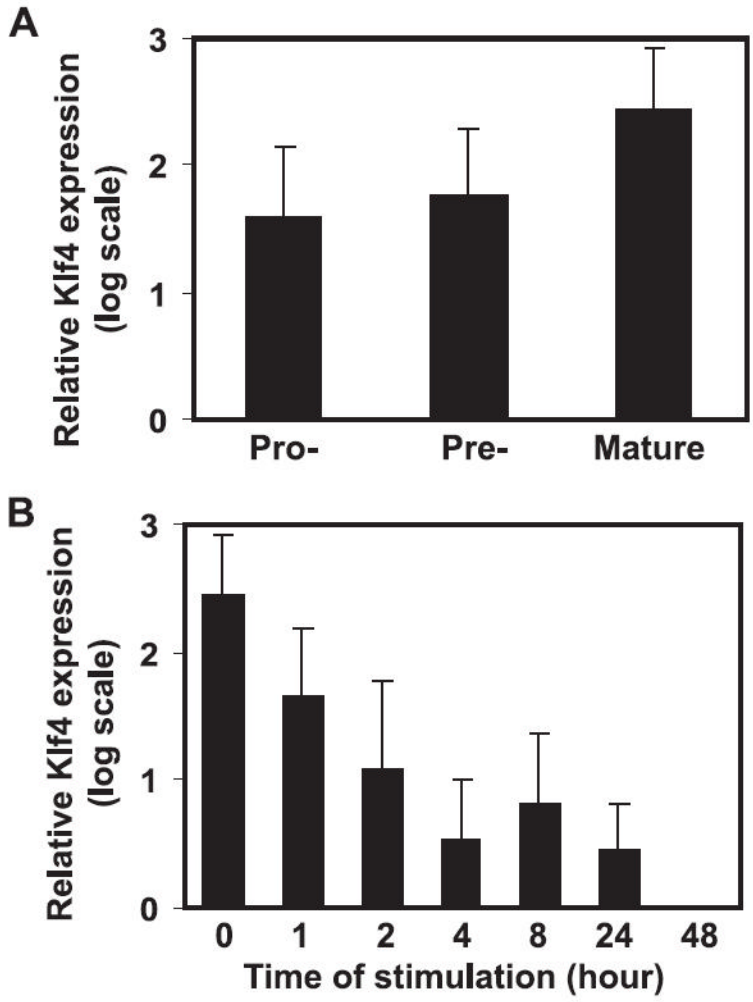

FIGURE 1.

Klf4 expresses in pro-, pre-, and mature B cells. *A*, Expression of Klf4 during B cell development. Pro- and pre-B cells were isolated from bone marrow; mature B cells were isolated from spleen. *B*, Kinetic expression of Klf4 in mature B cells after stimulation with anti-IgM. The levels of *Klf4*, as measured by real-time quantitative RT-PCR and normalized to the mean of *β*-actin and L32, rapidly decrease after 2-h stimulation to undetectable at 48 h poststimulation. Data are presented as the mean and SEM of four independent experiments.

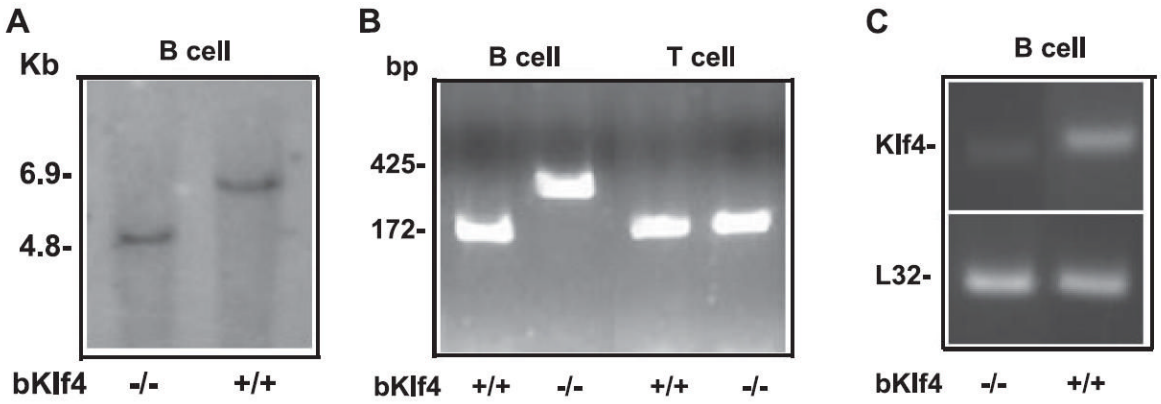

FIGURE 2.

The *Klf4* gene is deleted in B cells of *bKlf4*$^{-/-}$ mice. *A*, Deletion of *Klf4* exon2 and exon3 in B cells from *bKlf4*$^{-/-}$ mice. A 4.8-kb band represents deletion of the *Klf4* gene in B cells from *bKlf4*$^{-/-}$ mice and a 6.9-kb band represents the wild-type *Klf4* gene in B cells from *bKlf4*$^{+/+}$ mice by Southern blot hybridization. *B*, Specific deletion of *Klf4* in B cells but not T cells from *bKlf4*$^{-/-}$ mice. *Klf4* gene deletion resulted in a 450-bp band only in B cells from *bKlf4*$^{-/-}$ mice but not in B cells from *bKlf4*$^{+/+}$ mice by PCR. In contrast, a 175-bp band can be detected in B and T cells from *bKlf4*$^{+/+}$ mice and T cells from *bKlf4*$^{-/-}$ mice by PCR. *C*, Loss of Klf4 mRNA expression in B cells from spleen of *bKlf4*$^{-/-}$ mice by RT-PCR. L32 is a ribosomal protein that served as a control.

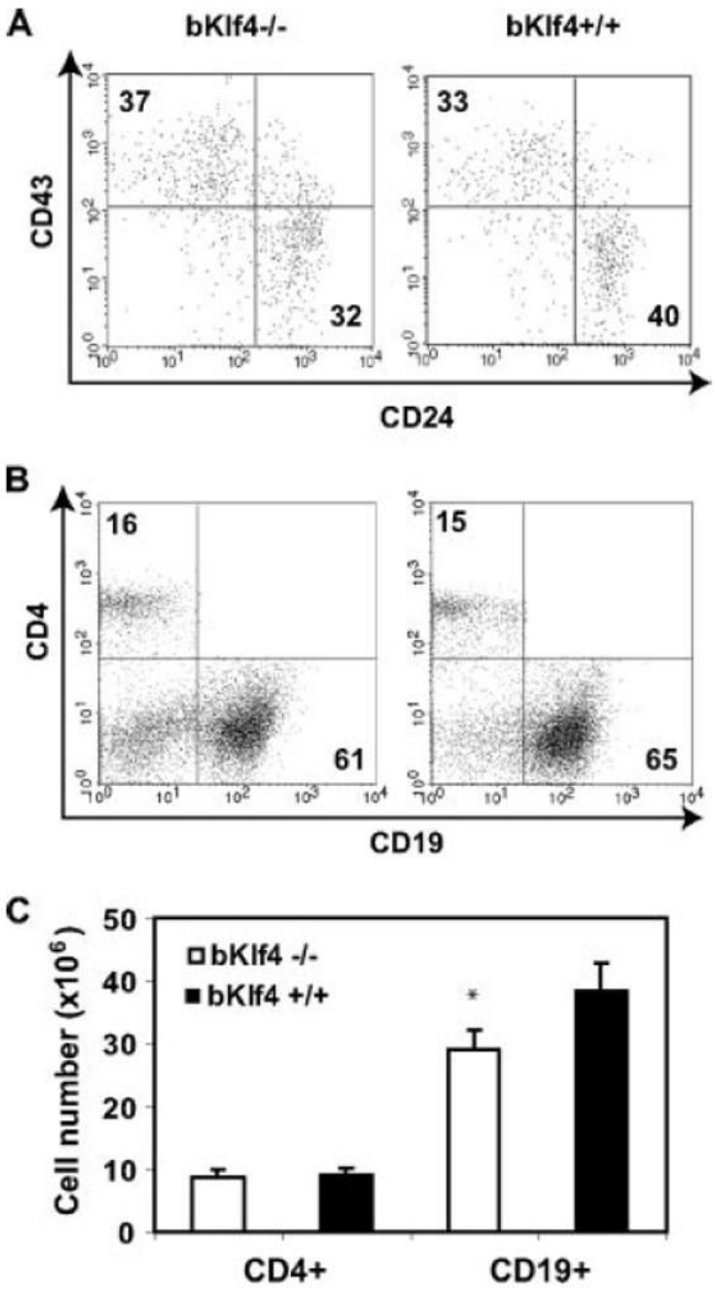

FIGURE 3.

Modest loss of pre-B cells and mature B cells is observed in *bKlf4*$^{-/-}$ mice. *A*, Decrease of pre-B, but not pro-B, cells in bone marrow of *bKlf4*$^{-/-}$ mice. A representative dot plot of CD43 and CD24 staining of B220^{+} bone marrow cells from *bKlf4*$^{-/-}$ and *bKlf4*$^{+/+}$ mice. The numbers indicate the mean percentage of cells. *B*, Decrease of mature B cells in spleen of *bKlf4*$^{-/-}$ mice. A representative dot plot of CD19 and CD4 staining of splenocytes from *bKlf4*$^{-/-}$ and *bKlf4*$^{+/+}$ mice. *C*, Reduction of number (25%) of mature B cells but not CD4 T cells in spleen in *bKlf4*$^{-/-}$ mice. Data were presented as mean ± SEM (n = 12); *, $p < 0.05$.

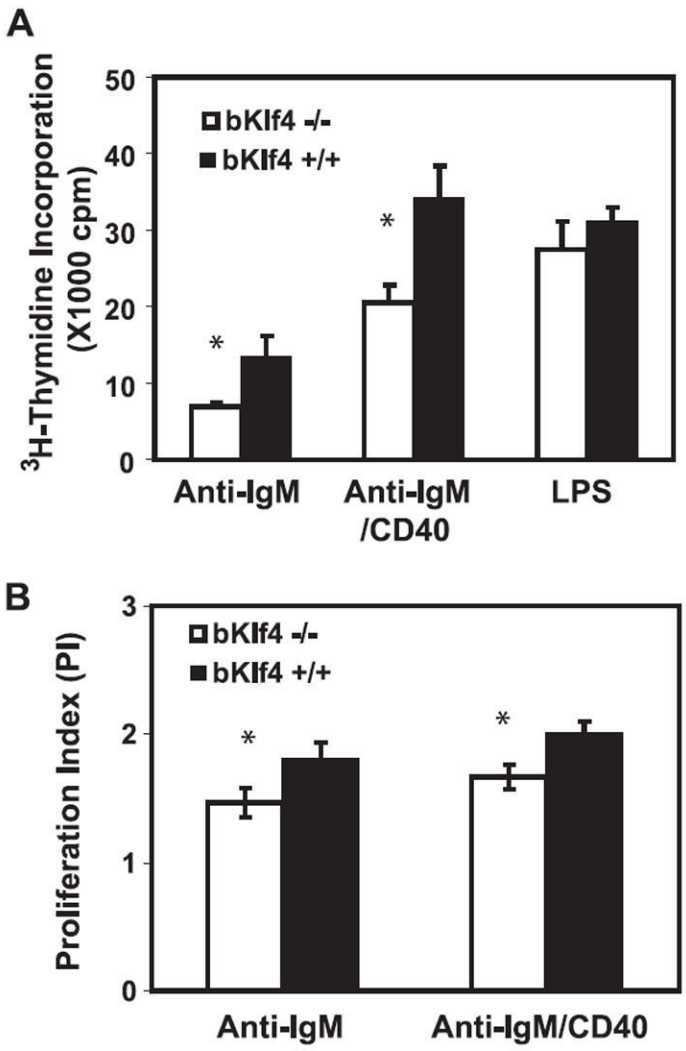

FIGURE 4.

Klf4 involves in activation-induced B cell proliferation. *A*, Stimulation induced DNA synthesis of B cells from *bKlf4*$^{-/-}$ and *bKlf4*$^{+/+}$ mice. B cells were isolated from spleen and stimulated in vitro with anti-IgM alone or anti-IgM plus anti-CD40 (anti-IgM/CD40) Abs or LPS (10 μg/ml) for 48 h and added [^{3}H]thymidine for 20 h. B cells from *bKlf4*$^{-/-}$ show a significant reduction of [^{3}H]thymidine uptake compared with control under stimulation conditions: anti-IgM alone ($p < 0.05$, $n = 10$) or anti-IgM/CD40 ($p < 0.05$, $n = 5$) but not under LPS ($p = 0.11$, $n = 3$). *B*, CFSE-tracking dye measuring proliferation of B cells from *bKlf4*$^{-/-}$ and *bKlf4*$^{+/+}$ mice. Low PI of B cells from *bKlf4*$^{-/-}$ mice compared with *bKlf4*$^{+/+}$ mice under both stimulation conditions ($p < 0.05$). Anti-IgM, $n = 9$; anti-IgM/CD40, $n = 14$.

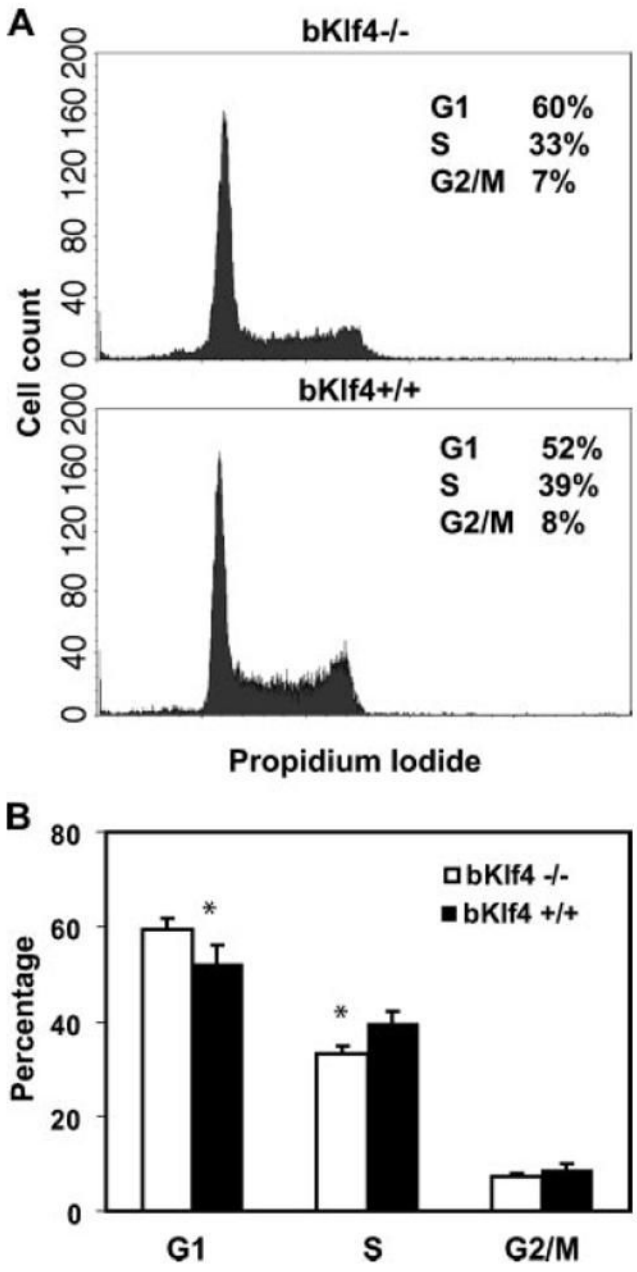

FIGURE 5.

Klf4 regulates the G_1 to S progression of cell cycle. *A*, Representative histograms of propidium iodide staining profile of spleen B cells from *bKlf4*$^{-/-}$ and *bKlf4*$^{+/+}$ mice stimulated with anti-IgM/CD40 for 48 h were presented. *B*, More B cells from *bKlf4*$^{-/-}$ mice remain in G_1 phase than those from *bKlf4*$^{+/+}$ mice at 48 h after stimulation with anti-IgM/CD40 ($p < 0.05$, $n = 8$). Data were represented as mean ± SEM.

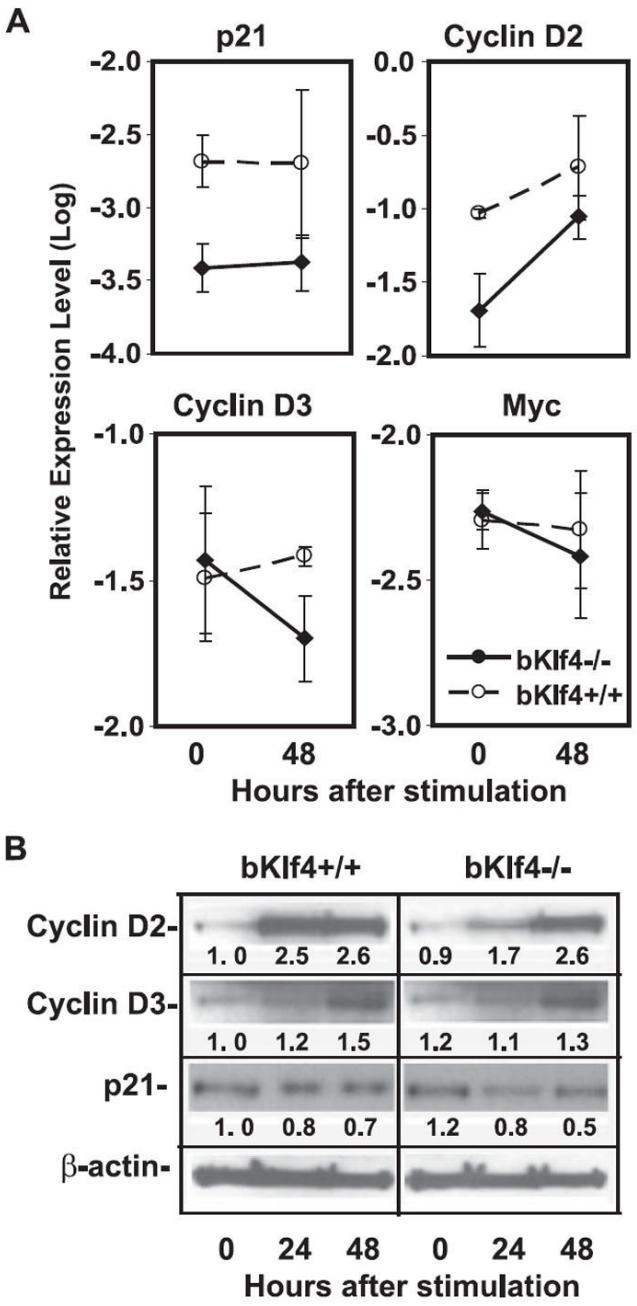

FIGURE 6.

Klf4 regulates cyclin D2 expression. *A*, Expression of cell cycle and proliferation-related genes. Lower mRNA levels of cyclin D2 and p21 in B cells from *bKlf4*$^{-/-}$ than from *bKlf4*$^{+/+}$ mice ($p < 0.05$, $n = 4$) but no significant differences in mRNA levels of cyclin D3 and *c-myc* in B cells between *bKlf4*$^{-/-}$ and *bKlf4*$^{+/+}$ mice. *B*, Decreased expression of cyclin D2 protein in B cells from *bKlf4*$^{-/-}$ mice but no obvious differences of p21 and cyclin D3 proteins between B cells from *bKlf4*$^{-/-}$ and *bKlf4*$^{+/+}$ mice after anti-IgM stimulation. A representative image of one set of samples from five independent experiments was shown. The mean intensity values of each band were presented.

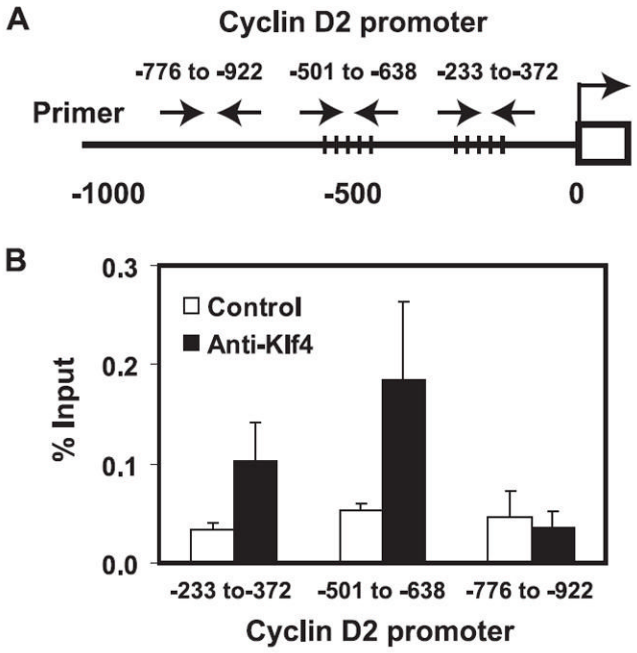

FIGURE 7.

Klf4 directly binds to the promoter of the *cyclin D2* gene. *A*, A diagram of the *cyclin D2* gene promoter. The dotted lines cross the promoter region indicate the putative Klf4-binding sites. The arrows indicate the primers used for PCR. *B*, Klf4 bound specifically to the promoter of cyclin D2. ChIP assay and quantitative PCR were conducted with an anti-Klf4 mAb and an isotype control mAb. Three different regions of the promoter were examined: two putative-binding sites (−336 to −340 and region −626 to −635) showed 2.4- and 2.6-fold higher precipitated DNA by anti-Klf4 Ab than by the control Ab, but no difference in precipitated DNA between two Abs in the region that has no Klf4-putative binding site (−776 to −922). Data were represented as mean ± SEM (n = 3).

Table I

B cell subsets in *bKlf4*$^{-/-}$ mice

Organs	Cell Types	Phenotype	*bKlf4*$^{-/-}$	*bKlf4*$^{+/+}$	Significance (*p*)[a]
Bone marrow	B cell	B220^{+}	26.4 ± 2.8	25.4 ± 2.8	0.405
	Pro-B cell	B220^{+}CD43^{+}	37.4 ± 1.9	33.2 ± 1.8	0.058
	Pre-B cell	B220^{+}CD24^{+}	31.8 ± 1.7	40.2 ± 1.4	0.001
Spleen	Mature B cell	CD19^{+}	60.7 ± 1.9	64.7 ± 1.7	0.030
Peritoneum	B1 B cell	B220^{+}CD5^{+}	25.7 ± 2.5	25.1 ± 4.4	0.448

[a]Bone marrow, $n = 12$; Spleen, $n = 16$; Peritoneum, $n = 8$.