

# I $\kappa$ B Kinase $\alpha$ Is Essential for Mature B Cell Development and Function

By Tsuneyasu Kaisho,\*<sup>‡</sup> Kiyoshi Takeda,\*<sup>||</sup> Tohru Tsujimura,<sup>§</sup>  
Taro Kawai,\*<sup>||</sup> Fumiko Nomura,\*<sup>||</sup> Nobuyuki Terada,<sup>§</sup>  
and Shizuo Akira\*<sup>||</sup>

From the \*Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan; the <sup>‡</sup>Institute for Advanced Medical Sciences and the <sup>§</sup>Department of Pathology, Hyogo College of Medicine, Nishinomiya, Hyogo 663-8501, Japan; and <sup>||</sup>Core Research for Evolution Science and Technology (CREST), Japan Science and Technology Corporation, Tokyo 101-0062, Japan

## Abstract

I $\kappa$ B kinase (IKK)  $\alpha$  and  $\beta$  phosphorylate I $\kappa$ B proteins and activate the transcription factor, nuclear factor (NF)- $\kappa$ B. Although both are highly homologous kinases, gene targeting experiments revealed their differential roles in vivo. IKK $\alpha$  is involved in skin and limb morphogenesis, whereas IKK $\beta$  is essential for cytokine signaling. To elucidate in vivo roles of IKK $\alpha$  in hematopoietic cells, we have generated bone marrow chimeras by transferring control and IKK $\alpha$ -deficient fetal liver cells. The mature B cell population was decreased in IKK $\alpha$ <sup>-/-</sup> chimeras. IKK $\alpha$ <sup>-/-</sup> chimeras also exhibited a decrease of serum immunoglobulin basal level and impaired antigen-specific immune responses. Histologically, they also manifested marked disruption of germinal center formation and splenic microarchitectures that depend on mature B cells. IKK $\alpha$ <sup>-/-</sup> B cells not only showed impairment of survival and mitogenic responses in vitro, accompanied by decreased, although inducible, NF- $\kappa$ B activity, but also increased turnover rate in vivo. In addition, transgene expression of *bcl-2* could only partially rescue impaired B cell development in IKK $\alpha$ <sup>-/-</sup> chimeras. Taken together, these results demonstrate that IKK $\alpha$  is critically involved in the prevention of cell death and functional development of mature B cells.

Key words: gene targeting • B cells • I $\kappa$ B kinase  $\alpha$  • germinal center • nuclear factor  $\kappa$ B

## Introduction

The nuclear factor (NF)<sup>1</sup>- $\kappa$ B family of transcription factors plays critical roles in the activation of inflammatory immune responses (1, 2). In resting cells, NF- $\kappa$ Bs associate with I $\kappa$ Bs and are retained in the cytoplasm as inactive forms. Proinflammatory stimuli, such as LPS, IL-1, or TNF- $\alpha$ , cause phosphorylation and degradation of I $\kappa$ Bs, which lead to NF- $\kappa$ B activation. The protein kinases that phosphorylate I $\kappa$ B have been identified by three indepen-

dent groups (3–7). Two I $\kappa$ B kinases (IKKs), IKK $\alpha$  and IKK $\beta$ , are main components of the I $\kappa$ B kinase complex and homologous with each other in the amino acid structures. Both contain a kinase domain, a leucine zipper, and a helix-loop-helix. However, gene targeting experiments revealed that they function differentially depending on the tissue. IKK $\alpha$  is essential for limb patterning and for epidermal keratinocyte proliferation and differentiation (8–10). Meanwhile, IKK $\beta$ -deficient mice showed a massive apoptosis of hepatocytes (11–13). Furthermore, IKK $\beta$ , but not IKK $\alpha$ , was found to be essential for NF- $\kappa$ B activation by proinflammatory cytokines.

B lineage cells also contain NF- $\kappa$ B activity, which can be augmented by LPS or CD40 ligation. All known mammalian members of the NF- $\kappa$ B/Rel transcription factor family, including p50(NF- $\kappa$ B1), p52(NF- $\kappa$ B2), p65(RelA), RelB, and c-Rel, are expressed in B cells (14–18). Gene targeting of these components caused various levels of impairment of B cell generation and function (19–28). How-

Address correspondence to Shizuo Akira, Department of Host Defense, Research Institute for Microbial Disease, Osaka University, Yamadaoka 3-1, Suita, Osaka 565-0871, Japan. Phone: 81-6-6879-8302; Fax: 81-6-6879-8305; E-mail: sakira@biken.osaka-u.ac.jp

<sup>1</sup>Abbreviations used in this paper: B6, C57BL/6; BCR, B cell receptor; BM, bone marrow; BrdU, 5-bromo-2'-deoxyuridine; BTK, Bruton's tyrosine kinase; CG, chicken  $\gamma$ -globulin; FDC, follicular dendritic cell; GC, germinal center; HE, hematoxylin and eosin; HSA, heat stable antigen; IKK, I $\kappa$ B kinase; NF, nuclear factor; NP, 4-hydroxy-3-nitrophenylacetyl; PB, peripheral blood; PNA, peanut agglutinin; RAG, recombination activating gene; tg, transgenic.

ever, because IKK $\alpha$ - and IKK $\beta$ -deficient mice died in an early neonatal period, it is unclear whether IKK $\alpha$  or IKK $\beta$  is involved in NF- $\kappa$ B activation in B lineage cells.

To investigate how IKK $\alpha$  is involved in lymphocytes, we have established bone marrow (BM) chimeras with a transfer of fetal liver cells from mice obtained by intercrossing IKK $\alpha$ <sup>+/-</sup> mice. IKK $\alpha$ <sup>-/-</sup> chimeras showed a reduction of mature B cell population, impairment of basal and Ag-specific Ig production, and disruption of splenic microarchitecture including germinal center (GC) formation. Our results revealed the critical roles of IKK $\alpha$  in peripheral B cell survival and maturation.

## Materials and Methods

**Generation of BM Chimeras.** Generation of IKK $\alpha$ <sup>-/-</sup> mice was described previously (10). H2K-bcl-2 transgenic (tg) mice overexpressing Bcl-2 (29) were provided by Dr. I. Weissman (Stanford University, Stanford, CA). IKK $\alpha$ <sup>+/-</sup> mice were intercrossed to obtain embryos with IKK $\alpha$ <sup>+/+</sup>, IKK $\alpha$ <sup>+/-</sup>, or IKK $\alpha$ <sup>-/-</sup> genotype. bcl-2-tg-IKK $\alpha$ <sup>-/-</sup> embryos were obtained from bcl-2-tg-IKK $\alpha$ <sup>+/-</sup>  $\times$  IKK $\alpha$ <sup>+/-</sup> mating. For each chimera, 5–10  $\times$  10<sup>6</sup> fetal liver cells from the embryos at day 13.5–15.5 of gestation were transferred intravenously into a recombination activating gene (RAG)2-deficient C57BL/6 (B6) mice (30) that had received 12 Gy from an x-ray irradiation system, MBR-1520R (Hitachi Medical Corporation) before transfer, as described previously (31). The recipient mice were given 1 mg/ml neomycin sulfate and 1,000 U/ml polymyxin B in their drinking water after irradiation and analyzed 6–10 wk after reconstitution.

**Flow Cytometric Analysis.** Single cell suspensions were incubated first with anti-CD16/32 to minimize nonspecific staining and stained with cocktails of mAbs conjugated to FITC, PE, or biotin for 20 min at 4°C. The biotinylated Abs were developed with streptavidin conjugated to PE or Cychrome. All mAbs, except PE-labeled anti-IgD (Southern Biotechnology Associates, Inc.), were purchased from BD PharMingen. Flow cytometric analysis was performed using a FACSCalibur™ (Becton Dickinson) with CELLQuest™ software (Becton Dickinson).

**Analysis for Cell Survival.** Cells were cultured at 2  $\times$  10<sup>6</sup> per ml in 24-well plates for the indicated periods with or without 25  $\mu$ g/ml LPS (055:B5; Difco) or 0.5  $\mu$ g/ml anti-CD40 (BD PharMingen). Harvested cells were stained with PE-B220 and further stained with annexin-V-FLUOS staining kit (Boehringer) by following the manufacturer's protocol. Stained cells were analyzed with a FACSCalibur™.

**Lymphocyte Activation in Culture.** Splenic B cells were purified by depletion of non-B cells with a Magnetic Cell Sorter (MACS; Miltenyi Biotec). In brief, splenocytes were first stained with biotinylated anti-CD43 mAb and incubated with streptavidin microbeads. Then CD43<sup>-</sup> cells were purified on MACS AS column and used as splenic B cells (32). Splenic T cells were also purified by MACS with biotinylated anti-CD8 mAb, and CD4 and streptavidin microbeads. The resulting B and T cell preparations contained >90% B220<sup>+</sup> cells and 95% CD3<sup>+</sup> cells, respectively, as determined by FACS®. The B cells (10<sup>5</sup> cells/well) from chimeras were cultured in complete RPMI 1640 (RPMI 1640 medium supplemented with 10% FCS, 2-ME, penicillin, and streptomycin) with or without 25  $\mu$ g/ml LPS, 0.5  $\mu$ g/ml anti-CD40, 0.5  $\mu$ g/ml anti-CD40 plus 200 U/ml IL-4, or 25  $\mu$ g/ml LPS plus 0.5  $\mu$ g/ml anti-CD40. After 48 h, they were pulsed with 0.2  $\mu$ Ci of [<sup>3</sup>H]thymidine (NEN Life Science Products) and

cultured for a further 15 h. [<sup>3</sup>H]Thymidine incorporation was measured with a liquid scintillation counter, Top Count (Packard Instrument Co.).

**Labeling Cells with 5-Bromo-2'-deoxyuridine.** Chimeric mice were fed with light-protected drinking water containing 1 mg/ml 5-bromo-2'-deoxyuridine (BrdU; Wako) for 4 d. BrdU-labeled cells were detected by flow cytometric analysis with FITC-conjugated anti-BrdU Ab, 3D4 (BD PharMingen). In brief, splenic cells were stained with PE-B220 and fixed with PBS containing 1% paraformaldehyde and 0.01% Tween 20 for 24 h at 4°C. Cells were washed and incubated for 1 h at 37°C in 40 mM Tris-HCl, pH 8.0, containing 10 mM NaCl, 6 mM MgCl<sub>2</sub>, and 50 Kuniz units of DNase I (Sigma-Aldrich). Then cells were washed and incubated with FITC-anti-BrdU or FITC-conjugated control Ab in the presence of 0.5% Tween 20. Stained cells were analyzed with a FACSCalibur™.

**Electrophoretic Mobility Shift Assay.** Splenic B (CD43<sup>-</sup>) cells from IKK $\alpha$ <sup>+/+</sup> and IKK $\alpha$ <sup>-/-</sup> mice were incubated with or without 25  $\mu$ g/ml LPS or 0.5  $\mu$ g/ml anti-CD40 for 1.5 h. Then, whole cell lysates were prepared, and NF- $\kappa$ B DNA binding activities were analyzed as described previously (33). For supershift analysis, Abs for each NF- $\kappa$ B component (Santa Cruz Biotechnology, Inc.) were incubated with the lysates before the addition of an NF- $\kappa$ B oligonucleotide probe.

**Reverse Transcriptase PCR for A1.** Purified splenic B cells were incubated with or without 25  $\mu$ g/ml LPS or 0.5  $\mu$ g/ml anti-CD40 for 2 h. Extraction of total RNAs, reverse transcription, and PCR analysis were performed as described previously (31). Primers for A1 were as follows: sense primer, 5'-TCATGCATATCCACTCCCTGGCTGAGC-3'; and antisense primer, 5'-GTCCTGTCATCTGCAGAAAAGTCAGCC-3'.

**Serum Ig Level and T Cell-dependent Immune Responses.** Serum Ig isotype concentrations of RAG2-deficient chimeras were analyzed by ELISA as described (34). Abs and standard Igs were purchased from Southern Biotechnology Associates, Inc. For T cell-dependent immune responses, mice were immunized with 100  $\mu$ g/head of alum-precipitated chicken  $\gamma$ -globulin (CG) coupled to 4-hydroxy-3-nitro-phenylacetyl (NP) and bled at the indicated days. The serum titers of NP-specific IgM and IgG1 were determined by ELISA as described (34). Statistical analysis was performed with the unpaired Student's *t* test.

**Histological Analysis of Splenic Sections.** Mice were killed 14 d after immunization with NP-CG, and the spleens were removed promptly. Each spleen was divided into two pieces, one piece for hematoxylin and eosin (HE) stain and the other for immunohistochemistry. For HE stain, spleen tissues were fixed in 10% buffered formalin, pH 7.2, and embedded in paraffin. Deparaffinized sections (4  $\mu$ m thick) were then stained with HE. For immunohistochemistry, freshly dissected spleens were covered with Tissue-Tek OCT compound (Miles, Inc.) and quickly frozen in liquid nitrogen. Frozen sections (4  $\mu$ m thick) were then fixed with ice cold acetone, and incubated in 3% H<sub>2</sub>O<sub>2</sub> in 50% methanol for 30 min to inactivate internal peroxidase. After washing with PBS, the sections were incubated with normal goat serum or normal horse serum (Vector Laboratories) to block nonspecific binding of Abs, and subsequently with the following reagents: anti-B220 (BD PharMingen), anti-IgD (Southern Biotechnology Associates, Inc.), biotin-conjugated peanut agglutinin (PNA; Seikagaku kogyo), follicular dendritic cell (FDC)-M1 (reference 35; a gift from Dr. M.H. Kosco-Vilbois, Serono Pharmaceutical Research Institute, Geneva, Switzerland), biotinylated F4/80 (Serotec), MOMA-1 (Serotec), antisialoadhesin (Serotec), or rabbit anti-BST-1 serum (reference 36; a gift from Drs. T. Hirano and K.

Ishihara, Osaka University). For negative controls, rabbit pre-immune serum or isotype-matched rat IgGs were used. After washing with PBS, sections were further incubated with biotin-conjugated, goat anti-rabbit IgG (Vector Laboratories) or rabbit anti-rat Igs (Dako). Immunoreacted cells were then visualized by using a Vectastain ABC Elite kit (Vector Laboratories) and diaminobenzine tetrahydrochloride (Sigma-Aldrich). The sections were lightly counterstained with hematoxylin.

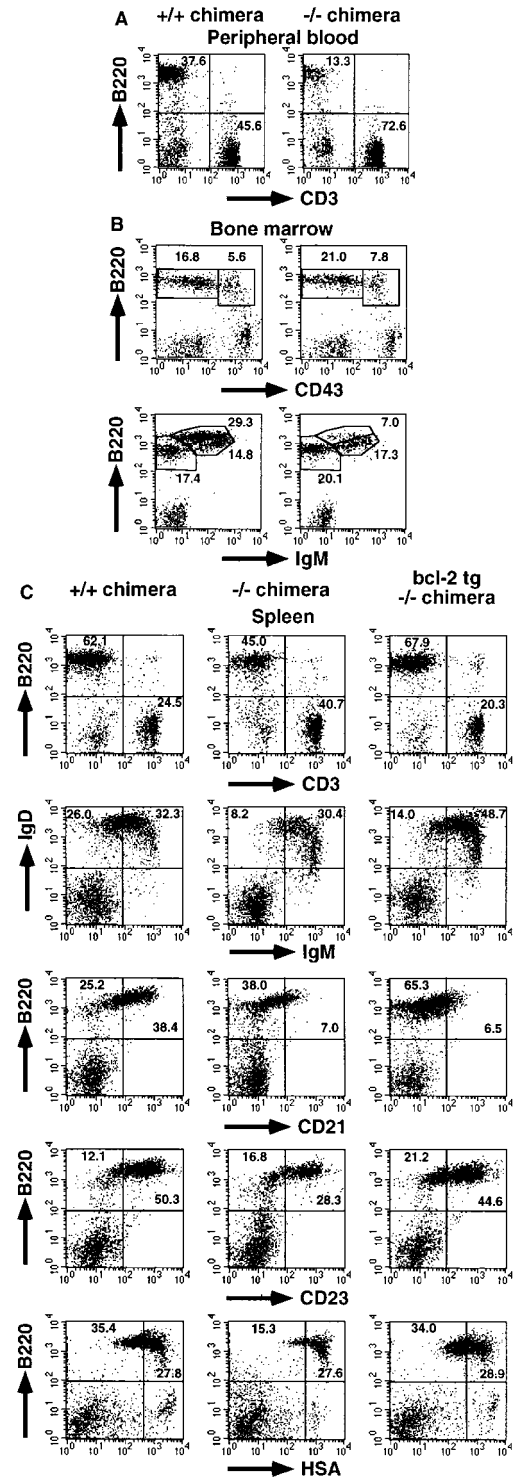
## Results

**Decrease of Mature B Cell Population in  $IKK\alpha^{-/-}$  Chimeras.**  $IKK\alpha$ -deficient mice die in an early neonatal period (8–10). To analyze roles of  $IKK\alpha$  in hematopoietic cells, BM chimeras were established and analyzed for lymphocyte populations in various organs with flow cytometry (Fig. 1). In the peripheral blood (PB),  $B220^+$  cells in  $IKK\alpha^{-/-}$  chimeras significantly decreased compared with those in  $IKK\alpha^{+/+}$  chimeras. Concomitant with decrease in the B cell population, the  $CD3^+$  T cell population increased in  $IKK\alpha^{-/-}$  chimeras (Fig. 1 A). Also in the spleen, decrease of  $B220^+$  and increase of  $CD3^+$  T cells were observed in  $IKK\alpha^{-/-}$  chimeras (Fig. 1 C). The results from  $IKK\alpha^{+/-}$  chimeras were similar to  $IKK\alpha^{+/+}$  chimeras (data not shown). Mean total spleen cell numbers from 18 control ( $IKK\alpha^{+/+}$  and  $+/-$ ) and 19  $-/-$  chimeras were  $2.1 \times 10^7$  and  $1.2 \times 10^7$ , respectively. Therefore, the increase of  $CD3^+$  T cell population percentages is not because of an increase of its absolute numbers. Furthermore, CD4 versus CD8 staining of thymus and spleen revealed no significant differences between  $IKK\alpha^{+/+}$  and  $IKK\alpha^{-/-}$  chimeras, indicating that T cell development proceeds normally in the absence of  $IKK\alpha$  (data not shown).

Next, we analyzed splenic B cell maturation status in chimeras (Fig. 1 C). Peripheral B cell development proceeds from immature  $IgM^{high}IgD^{high}$  to mature  $IgM^{low}IgD^{high}$  cells (37, 38). The decrease of B cell numbers in  $IKK\alpha^{-/-}$  chimeras was more prominent in  $IgM^{low}IgD^{high}$  cells than in  $IgM^{high}IgD^{high}$  cells. Given the decrease of total spleen cell numbers, the  $IgM^{high}IgD^{high}$  cell population in  $IKK\alpha^{-/-}$  chimeras was also reduced approximately twofold. B cell maturation is also characterized by the upregulation of CD21 and CD23 and the downregulation of heat stable antigen (HSA; reference 39). This marker analysis (Fig. 1 C), together with IgM versus IgD staining, clearly demonstrates that mature B cells were more severely decreased than immature B cells in the spleen of  $IKK\alpha^{-/-}$  chimeras.

We also analyzed early B cell development in the BM (Fig. 1 B). The population size of pro-B ( $CD43^+IgM^-B220^+$ ), pre-B ( $CD43^-IgM^-B220^+$ ), and immature B ( $CD43^-IgM^+B220^{low}$ ) cells was comparable between  $IKK\alpha^{+/+}$  and  $IKK\alpha^{-/-}$  chimeras. However, mature recirculating B ( $CD43^-IgM^+B220^{high}$ ) cells (40) significantly decreased in  $IKK\alpha^{-/-}$  chimeras (7.0%) compared with those in  $IKK\alpha^{+/+}$  chimeras (29.3%).

**Enhanced Cell Death of In Vitro  $IKK\alpha^{-/-}$  B Cells.** NF- $\kappa$ B activity has been shown to mediate antiapoptotic activity in various cells including B cells (41, 42). We have ex-



**Figure 1.** Mature B cell decrease in  $IKK\alpha^{-/-}$  RAG2-deficient B6 chimeras. Single cell suspensions from (A) PB, (B) BM, and (C) spleen were stained with the indicated Abs and analyzed using a FACSCalibur™ with CELLQuest™ software. The percentages of the quadrants or enclosed areas are indicated by numbers. For BM, triple color analysis was performed, and CD43 versus B220 and IgM versus B220 profiles are shown for  $IgM^-$  and  $CD43^-$  lymphoid cells, respectively. In C, data from  $IKK\alpha^{-/-}$  chimeras with transgene expression of *bcl-2* are also shown. Four independent experiments were performed with similar results. One representative experiment is shown.

amined survival of B lineage cells in both  $IKK\alpha^{+/+}$  and  $IKK\alpha^{-/-}$  chimeras with annexin V binding activity. PB from the chimeras was cultured in vitro in the absence of mitogens for the indicated periods and analyzed for their annexin V binding activity with FACS® (Fig. 2, A and B). Before culture (0 h), percentages of  $B220^{+}annexin^{+}$  cells in PB were higher in  $IKK\alpha^{-/-}$  chimeras ( $3.3/7.1 + 3.3 = 31.7\%$ ) than in  $IKK\alpha^{+/+}$  chimeras ( $4.3/4.3 + 26.2 = 14.1\%$ ) (Fig. 2, A and B).  $B220^{+}annexin^{+}$  cells reached >95% of the total  $B220^{+}$  cells in  $IKK\alpha^{-/-}$  chimeras after 24 h of culture, whereas they were 63.4 and 83.7% in  $IKK\alpha^{+/+}$  chimeras after 24 and 48 h of culture, respectively. Likewise, survival of splenic B cells was severely impaired in  $IKK\alpha^{-/-}$  chimeras before and throughout the culture period (Fig. 2, A and B).

Mitogens such as LPS or anti-CD40 can induce B cell activation by inducing NF- $\kappa$ B activation. At 48 h, annexin<sup>+</sup> B cell percentages of LPS-stimulated  $IKK\alpha^{+/+}$  splenocytes (57.3%) were less than those of unstimulated ones (68.9%; Fig. 2 B). However, LPS could not decrease annexin<sup>+</sup> B cell percentages in  $IKK\alpha^{-/-}$  chimeras. Furthermore, after 48 h of stimulation with anti-CD40, annexin<sup>+</sup> B cell percentages in  $IKK\alpha^{+/+}$  splenocytes decreased to 21.2%, while those in  $IKK\alpha^{-/-}$  splenocytes, although slightly reduced, remained at 71.3%. Thus, at any culture periods, with or without mitogens, cell death was significantly augmented in  $IKK\alpha^{-/-}$  B cells.

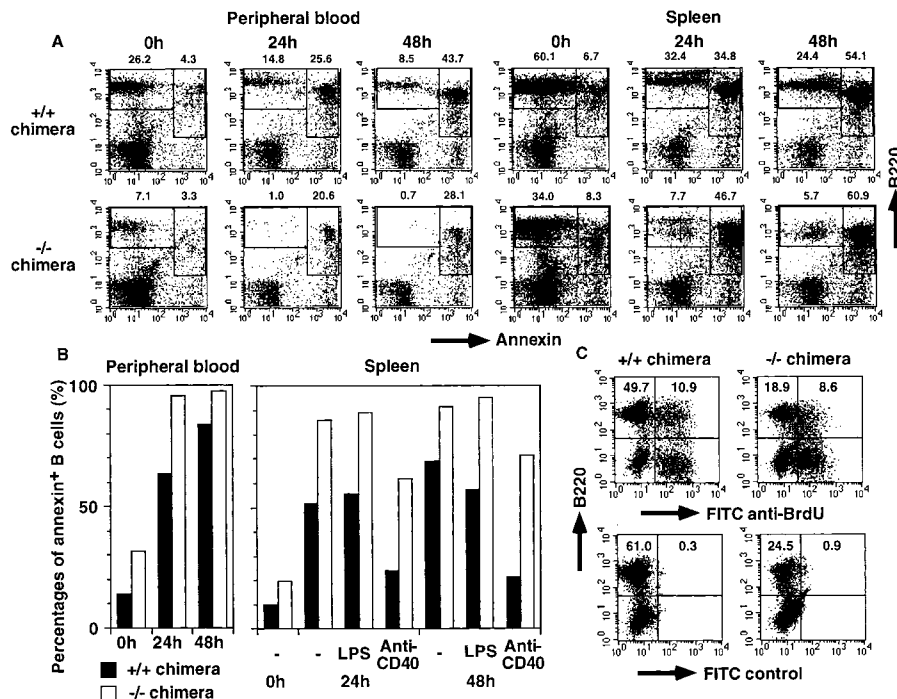
**Increased Turnover of In Vivo  $IKK\alpha^{-/-}$  B Cells.** In vivo B cell turnover rate was evaluated in chimeras. BrdU-labeled  $B220^{+}$  cells were 10.9 and 8.6% in the spleens of  $IKK\alpha^{+/+}$  and  $IKK\alpha^{-/-}$  chimeras, respectively (Fig. 2 C). Considering the decrease of B cell percentages in  $IKK\alpha^{-/-}$  chimeras, it can be reasonably assumed that about two

times more percentages of B cells were labeled with BrdU in  $IKK\alpha^{-/-}$  chimeras than in  $IKK\alpha^{+/+}$  chimeras. The results clearly suggest that B cell turnover in vivo is enhanced in the absence of  $IKK\alpha$ .

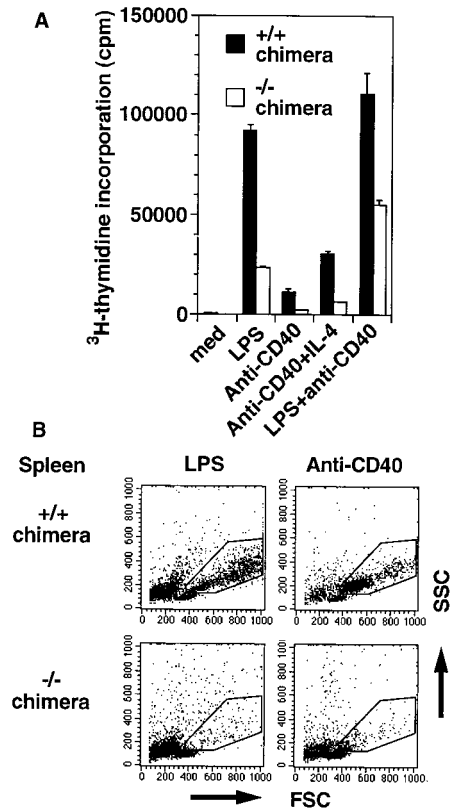
**Impaired Mitogenic Responses of  $IKK\alpha^{-/-}$  B Cells.** We have analyzed splenic B cell responses to LPS and anti-CD40 with thymidine incorporation (Fig. 3 A). The incorporation of  $IKK\alpha^{-/-}$  B cells stimulated with LPS or anti-CD40 was ~5–10 times less than that of  $IKK\alpha^{+/+}$  B cells. Simultaneous stimulation with LPS and anti-CD40 could enhance the proliferation of  $IKK\alpha^{-/-}$  B cells, but not to the same level as  $IKK\alpha^{+/+}$  B cells (Fig. 3 A). Furthermore, blast formation by LPS or anti-CD40 was severely attenuated in  $IKK\alpha^{-/-}$  B cells (Fig. 3 B). In contrast, thymocytes and splenic T cells derived from  $IKK\alpha^{-/-}$  chimeras showed normal proliferative responses to T cell mitogens such as IL-2 plus anti-CD3 or IL-2 plus Con A (data not shown).

**Impaired NF- $\kappa$ B Activation in  $IKK\alpha^{-/-}$  B Cells.** We next analyzed NF- $\kappa$ B activity in  $IKK\alpha^{+/+}$  and  $IKK\alpha^{-/-}$  splenic B cells with or without LPS or anti-CD40 (Fig. 4). Although the mitogens could enhance the NF- $\kappa$ B DNA binding activity in both  $IKK\alpha^{+/+}$  and  $IKK\alpha^{-/-}$  B cells, the magnitude of the activity was decreased in  $IKK\alpha^{-/-}$  B cells (Fig. 4 A). Anti-CD40-induced NF- $\kappa$ B activation was more severely impaired than the LPS-induced one in  $IKK\alpha^{-/-}$  B cells.

To determine the components of NF- $\kappa$ B complexes, we performed a supershift analysis by using specific Abs (Fig. 4 B). In  $IKK\alpha^{+/+}$  B cells, anti-p50 supershifted nearly all the LPS-induced complexes, while anti-p65 did parts of them. Hence, the remaining complex in  $IKK\alpha^{+/+}$  B cells with anti-p65 treatment seems to correspond mainly to p50/p50



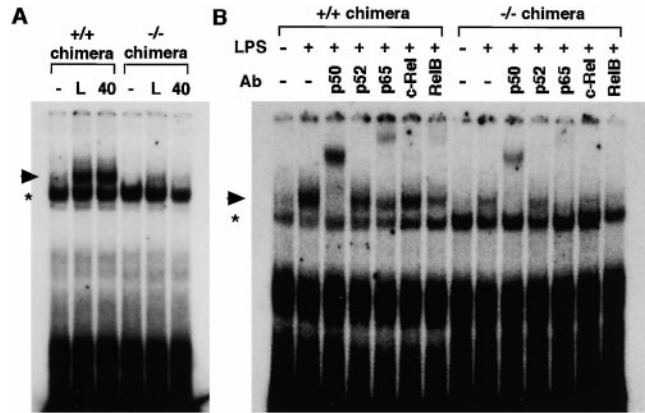
**Figure 2.** Impaired survival in vitro and increased turnover in vivo of  $IKK\alpha^{-/-}$  B cells. (A and B) PB and splenocytes from  $IKK\alpha^{+/+}$  and  $IKK\alpha^{-/-}$  RAG2-deficient B6 chimeras were cultured in complete RPMI 1640 in the absence or presence of mitogens for the indicated periods and stained with FITC-annexin V and PE-B220. Percentages of annexin<sup>+</sup> B cells were calculated by dividing percentages of  $B220^{+}annexin^{+}$  cells by those of total B ( $B220^{+}annexin^{+}$  and  $B220^{+}annexin^{-}$ ) cells and shown as bar graphs in B. (C) Increased B cell turnover in  $IKK\alpha^{-/-}$  chimeras. The turnover of splenic B cells was determined by BrdU incorporation. Numbers represent percentages of the quadrants. Experiments were independently performed three times with similar results. One representative experiment is shown.



**Figure 3.** Impaired mitogenic responses of  $IKK\alpha^{-/-}$  B cells. (A) Splenic B cells from  $IKK\alpha^{+/+}$  and  $IKK\alpha^{-/-}$  RAG2-deficient B6 chimeras were cultured in the absence or presence of mitogens for 72 h and analyzed for their [ $^3$ H]thymidine incorporation. The data indicate means  $\pm$  SD of triplicate samples of one representative experiment. (B) Splenocytes from  $IKK\alpha^{+/+}$  and  $IKK\alpha^{-/-}$  RAG2-deficient B6 chimeras were cultured with LPS or anti-CD40 for 48 h. Forward scatter (FSC) and side scatter (SSC) of harvested cells were analyzed by FACS<sup>®</sup>. Blasts are indicated by enclosed areas. Experiments were independently performed three times with similar results. One representative experiment is shown.

homodimer. The results showed that LPS can mainly induce p50/p50 homodimer and p50/p65 heterodimer as NF- $\kappa$ B complexes. In addition, other components also contributed to NF- $\kappa$ B binding activity to some extent because anti-p52, -c-Rel, and -RelB Abs partially inhibited the binding. As observed in  $IKK\alpha^{+/+}$  B cells, the detectable NF- $\kappa$ B complex in  $IKK\alpha^{-/-}$  B cells showed a similar supershift pattern, indicating that the composition of residual NF- $\kappa$ B complexes in  $IKK\alpha^{-/-}$  B cells is not grossly different from that in  $IKK\alpha^{+/+}$  B cells.

**Decreased Basal Level of A1 Expression in  $IKK\alpha^{-/-}$  B Cells.** NF- $\kappa$ B is involved in the expression of antiapoptotic genes. For example, c-Rel is essential for not only basal but also mitogen-induced expression of A1 (43). A1 expression remains low during BM B cell development, but is upregulated 10-fold as cells mature into a long-lived peripheral B cell stage, suggesting that constitutive A1 expression is critically involved in resting mature B cell survival (44). Therefore, we examined A1 gene expression in  $IKK\alpha^{+/+}$  and  $IKK\alpha^{-/-}$  B cells with or without mitogens. LPS or anti-CD40 could enhance A1 gene expression not

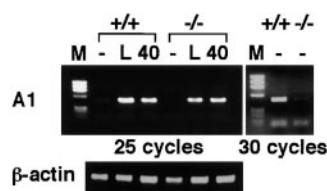


**Figure 4.** NF- $\kappa$ B DNA binding activity in  $IKK\alpha^{+/+}$  and  $IKK\alpha^{-/-}$  B cells. (A) Splenic B cells were stimulated with or without 25  $\mu$ g/ml LPS (L) or 0.5  $\mu$ g/ml anti-CD40 (40) for 1.5 h. The whole cell lysates were prepared and NF- $\kappa$ B activity was determined by electrophoretic mobility shift assay. (B) LPS-induced NF- $\kappa$ B complexes were analyzed by supershift analysis. Specific NF- $\kappa$ B complexes are indicated by arrowheads. \*Nonspecific binding. Similar results were obtained from three independent experiments.

only in  $IKK\alpha^{+/+}$  B cells but also in  $IKK\alpha^{-/-}$  B cells (Fig. 5). However, basal expression of A1 was lower in  $IKK\alpha^{-/-}$  B cells than in  $IKK\alpha^{+/+}$  B cells.

**Partial Rescue of Impaired B Cell Development in  $IKK\alpha^{-/-}$  Chimeras by Bcl-2 Expression.** To assess whether the prevention of  $IKK\alpha^{-/-}$  B cell apoptosis can restore peripheral B cell development,  $IKK\alpha^{-/-}$  chimeras expressing the *bcl-2* transgene were established and analyzed by FACS<sup>®</sup> (Fig. 1 C). Total spleen cell numbers of *bcl-2*-tg- $IKK\alpha^{-/-}$  chimeras ( $8.9 \times 10^7$ ,  $n = 5$ ) were prominently increased compared with  $IKK\alpha^{-/-}$  chimeras. CD3 versus B220 staining revealed that Bcl-2 expression corrected the ratio of B to T cells in  $IKK\alpha^{-/-}$  chimeras. Next, B cell maturation status was analyzed with several markers. Although percentages of  $IgM^{low}IgD^{high}$  and  $IgM^{high}IgD^{high}$  cell populations increased, the ratio of  $IgM^{low}IgD^{high}$  to  $IgM^{high}IgD^{high}$  cell population in *bcl-2*-tg- $IKK\alpha^{-/-}$  chimeras was not significantly different from  $IKK\alpha^{-/-}$  chimeras. Furthermore, in *bcl-2*-tg- $IKK\alpha^{-/-}$  chimeras, CD21 expression on B220<sup>+</sup> cells remained as low as  $IKK\alpha^{-/-}$  chimeras. However, CD23 upregulation and HSA downregulation were restored by Bcl-2 expression. Thus, *bcl-2* transgene expression could enhance B lineage cell expansion, but only partially restore B cell maturation in the absence of  $IKK\alpha$ .

**Figure 5.** Decrease of basal A1 gene expression in  $IKK\alpha^{-/-}$  B cells. A1 expression was analyzed in  $IKK\alpha^{+/+}$  or  $IKK\alpha^{-/-}$  B cells with or without LPS (L) or anti-CD40 (40) stimulation through reverse transcription PCR analysis. Basal A1 gene expression was determined by amplification with 30 cycles. As controls, data for  $\beta$ -actin expression are shown. M,  $\Phi \times 174$ /HaeIII digest marker.



**Decreased Serum Ig Levels and Impaired T Cell-dependent Immune Responses in *IKK $\alpha$ <sup>-/-</sup>* Chimeras.** We further investigated immune functions of *IKK $\alpha$ <sup>+/+</sup>* and *IKK $\alpha$ <sup>-/-</sup>* chimeras. Basal production of all Ig isotypes in *IKK $\alpha$ <sup>-/-</sup>* chimeras was reduced to 1/50 or less of those in *IKK $\alpha$ <sup>+/+</sup>* chimeras ( $P < 0.0001$ , Fig. 6 A). Furthermore, T cell-dependent immune responses were severely impaired in *IKK $\alpha$ <sup>-/-</sup>* chimeras because Ag-specific IgM and IgG1 levels of *IKK $\alpha$ <sup>-/-</sup>* chimeras were  $<1/10$  of *IKK $\alpha$ <sup>+/+</sup>* chimeras (Fig. 6 B, anti-NP IgM at day 7,  $P < 0.001$ ; anti-NP IgM at day 14 and IgG1 at day 7 and 14,  $P < 0.0001$ ).

Histological analysis was performed to evaluate the GC formation and splenic microarchitecture 14 d after immunization (Fig. 7). HE, B220, and IgD staining revealed the well-developed GC with clear B cell areas in control chimeras. In contrast, in *IKK $\alpha$ <sup>-/-</sup>* chimeras, poor GC formation was revealed with HE stain, although B220<sup>+</sup> or IgD<sup>+</sup> cells clearly formed B cell areas. Remarkably, PNA<sup>+</sup> cell clusters, which represent GC B cells, were not detected at all in the spleen of *IKK $\alpha$ <sup>-/-</sup>* chimeras.

To further investigate the alterations of the splenic microarchitecture in *IKK $\alpha$ <sup>-/-</sup>* chimeras, tissue sections were also stained with markers for FDCs or macrophages (Fig. 7 B). FDC clusters, defined by FDC-M1 (35), were readily detected in *IKK $\alpha$ <sup>+/+</sup>* chimeras but not in *IKK $\alpha$ <sup>-/-</sup>* chimeras. A stromal cell antigen, BST-1/Bp-3, is expressed on

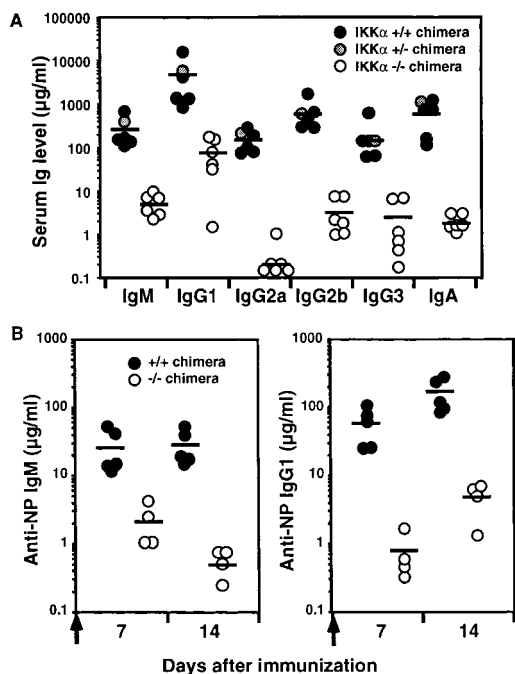
stromal cells in the T zone and follicles (45–47), which include precursors of FDCs (48). Expression patterns of BST-1/Bp-3 were equivalent in *IKK $\alpha$ <sup>+/+</sup>* and *IKK $\alpha$ <sup>-/-</sup>* chimeras, suggesting that there are FDC precursors in *IKK $\alpha$ <sup>-/-</sup>* chimeras. Meanwhile, marginal metallophilic macrophages, identified by MOMA-1 or an antisialoadhesin Ab, were clearly detected in *IKK $\alpha$ <sup>+/+</sup>* chimeras but not in *IKK $\alpha$ <sup>-/-</sup>* chimeras. Furthermore, F4/80<sup>+</sup> red pulp macrophages in *IKK $\alpha$ <sup>+/+</sup>* chimeras were clearly excluded into the red pulp, while some F4/80<sup>+</sup> cells in *IKK $\alpha$ <sup>-/-</sup>* chimeras migrated into the white pulp. Collectively, these histological findings clearly indicate that *IKK $\alpha$*  is critical not only for GC formation but also for establishing the splenic microarchitecture.

## Discussion

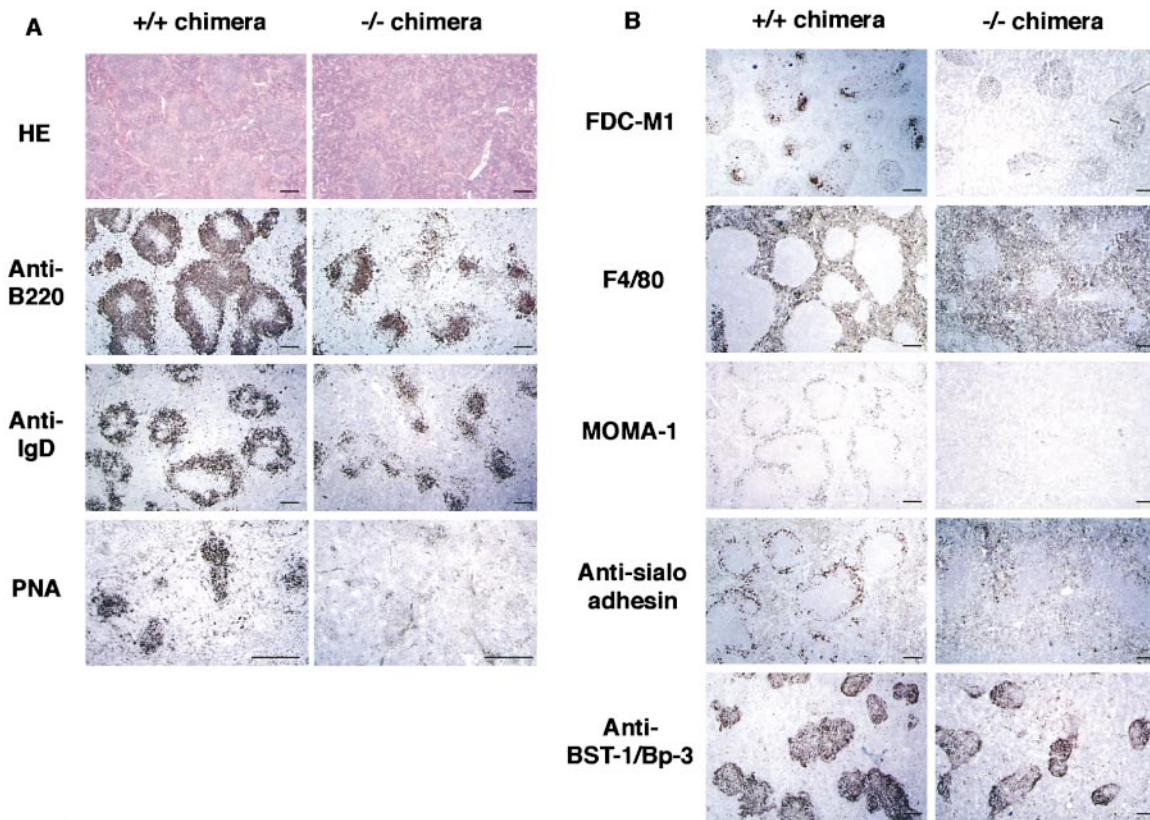
We have generated BM chimeras to elucidate *in vivo* roles of *IKK $\alpha$*  in lymphocytes. Although certain NF- $\kappa$ B subunit-deficient mice showed T cell defects in population size or proliferative responses (24, 25, 28, 49), *IKK $\alpha$ <sup>-/-</sup>* chimeras did not, indicating that *IKK $\alpha$*  is not essential for T cell development or proliferation. This is also consistent with previous findings, including ours, that cytokine- or T cell receptor-induced NF- $\kappa$ B activation is dependent on *IKK $\beta$* , but not on *IKK $\alpha$*  in T lineage cells (10, 50). However, we cannot exclude the possibility that *IKK $\alpha$*  is involved in some T cell functions.

Our results clearly demonstrate that *IKK $\alpha$*  is involved in mature B cell development and function, but not in early B cell development. NF- $\kappa$ B components are developmentally regulated in B lineage cells (14, 15). Gene targeting revealed that an individual NF- $\kappa$ B component plays its own critical roles in a B cell stage-specific manner. For example, BM chimeras transplanted with p50/p65 double knockout fetal liver cells lacked pro-B cells (27), indicating that the p50/p65 heterodimer is essential for early B lymphopoiesis. On the contrary, mutant mice lacking c-Rel showed normal BM B cell development, but manifested impairment of mitogenic responses, basal production of all Ig isotypes, and T cell-dependent immune responses (28). Although p50 is a major component for NF- $\kappa$ B throughout B cell development, p50-deficient mice showed impairment of mature B cell functions: low responses to LPS, reduction of some isotypes of serum Igs (IgG1, IgG2b, IgA, and IgE), and impairment of immune responses (19). Therefore, the phenotype of *IKK $\alpha$ <sup>-/-</sup>* chimeras was more similar to c-Rel- or p50-deficient mice than to p50/p65-deficient mice, in the respect that peripheral, not BM, B cell development is impaired.

Mutant mice lacking other NF- $\kappa$ B components also showed a similar phenotype to *IKK $\alpha$ <sup>-/-</sup>* chimeras. For example, targeting of p52 results in a decrease of B cells, low responses to B cell mitogens, and impaired GC formation (23, 26). An I $\kappa$ B family member, Bcl-3, can function as a potent coactivator with p50/p50 or p52/p52 homodimers (51, 52). Bcl-3-deficient mice also showed decrease of B cells and impairment of GC formation (53, 54). However,



**Figure 6.** Decreased Ig production and impaired T cell-dependent immune responses in *IKK $\alpha$ <sup>-/-</sup>* chimeras. (A) Serum Ig isotype levels in unimmunized, *IKK $\alpha$ <sup>+/+</sup>*, *IKK $\alpha$ <sup>+/-</sup>*, and *IKK $\alpha$ <sup>-/-</sup>* RAG2-deficient B6 chimeras. Serum Ig titers were determined by isotype-specific ELISA. (B) Immune responses to the T cell-dependent Ag, NP-CG. *IKK $\alpha$ <sup>+/+</sup>* and *IKK $\alpha$ <sup>-/-</sup>* RAG2-deficient B6 chimeras were immunized with alum-precipitated NP-CG and bled at the indicated days. NP-specific IgM and IgG1 titers were determined by ELISA. Bars indicate mean values.



**Figure 7.** (A) Impaired GC formation in  $IKK\alpha^{-/-}$  chimeras.  $IKK\alpha^{+/+}$  and  $IKK\alpha^{-/-}$  RAG2-deficient B6 chimeras were immunized with alum-precipitated NP-CG and killed 14 d after injection. Splenic sections were stained with HE or immunostained with B220, anti-IgD, or PNA. (B) Impairment of splenic microarchitecture in  $IKK\alpha^{-/-}$  chimeras. Frozen sections were stained with FDC-M1, F4/80, MOMA-1, or antisialoadhesin mAbs or rabbit anti-BST-1/Bp-3 antiserum. Scale bars, 200  $\mu$ m.

GC formation was restored by the transfer of p52- or Bcl-3-deficient lymphocytes into irradiated RAG1-deficient mice (26). Thus, p52 and Bcl-3 play critical roles in non-BM stromal cells, while  $IKK\alpha$  does in lymphocytes despite their similar phenotype.

Mutant mice established so far lacking a single NF- $\kappa$ B component showed an apparently less severe phenotype than  $IKK\alpha^{-/-}$  chimeras. For example, the B cell population is not decreased in c-Rel- or p50-deficient mice. In addition, not all isotypes of Ig production were impaired and GC formation was observed in p50-deficient mice (19, 26, 53). Double knockout mice manifested more severe phenotype than single knockout mice, exemplified by p50/p52 double knockout mice with severe mature B cell defects (25). Furthermore, tg mice harboring a dominant negative form of I $\kappa$ B, in which function of more than one NF- $\kappa$ B component is presumably attenuated, showed mature B cell decrease as in  $IKK\alpha^{-/-}$  chimeras (55). Given these findings, it can be assumed that  $IKK\alpha$  is critically involved in the activation of several NF- $\kappa$ B components in B cells.

Histologically, B220<sup>+</sup> or IgD<sup>+</sup> staining indicates that  $IKK\alpha$  expression in hematopoietic cells is dispensable for T-B compartmentalization. However,  $IKK\alpha^{-/-}$  chimeras showed loss of GC marker such as PNA or FDC-M1 with normal BST-1/Bp-3 expression. GC formation proceeds

with FDC generation from FDC precursors expressing BST-1/Bp-3 in a mature B cell-dependent manner (48). Therefore, these histological findings are consistent with mature B cell decrease in  $IKK\alpha^{-/-}$  chimeras.

What brings about decrease of mature B cells in the absence of  $IKK\alpha$ ? It is possible that  $IKK\alpha$  is critically involved in survival, mitogenic proliferation, or development in B cells. Cell turnover analysis in vivo and annexin staining in vitro (Fig. 2) clearly indicate enhanced cell death of  $IKK\alpha^{-/-}$  B cells. In addition, impaired mitogenic responses were also observed in  $IKK\alpha^{-/-}$  B cells (Fig. 3). Thus,  $IKK\alpha$  should be critical not only for preventing B cell death but also for B cell mitogenic responses. Similar abnormalities were found in some NF- $\kappa$ B mutant mice. For example, p50<sup>-/-</sup> B cells manifested enhancement of apoptosis (22). Furthermore, B cell mitogenic responses were impaired in mutant mice lacking p50, c-Rel, or Rel-B (19–21, 24, 28).

We further addressed whether  $IKK\alpha$  deficiency can lead to B cell developmental block. To rescue B cells from apoptosis, we have introduced *bcl-2* transgene expression into the  $IKK\alpha^{-/-}$  background. Bcl-2 could restore the ratio of B to T cells, CD23 upregulation, and HSA downregulation, but neither differentiation into IgM<sup>low</sup>IgD<sup>high</sup> cells nor CD21 upregulation in  $IKK\alpha^{-/-}$  chimeras. Partial restoration of

IKK $\alpha^{-/-}$  B cell development with *bcl-2* transgene expression indicates that IKK $\alpha$  is critically involved in peripheral B cell development. It is also possible that the developmental defect caused by IKK $\alpha$  deficiency can make B cells susceptible to apoptosis and hyporesponsive to mitogens.

B cell receptor (BCR) signaling is critical for maintenance of the peripheral B cell population (56). Once B cells lack surface Ig, they die through an apoptotic pathway (56). Mature B cell reduction is also observed in mutant mice lacking BCR signaling molecules such as Bruton's tyrosine kinase (BTK; references 57, 58), B cell linker protein (BLNK/SLP-65; references 59, 60), phosphoinositide 3-kinase p85 $\alpha$  (61, 62), or phospholipase C- $\gamma$ 2 (63, 64). Notably, enhanced apoptosis of B lineage cells was also detected in the *xid* mice carrying the mutation in BTK (38). BTK is essential for coupling the BCR signal to NF- $\kappa$ B activation (65, 66). In addition, BCR signaling can activate phosphoinositide 3-kinase and a serine/threonine kinase, Akt (67), which can induce phosphorylation of IKK $\alpha$  (68). These findings suggest the possibility that IKK $\alpha$  is involved in BCR signaling.

Several lines of evidences suggest that IKK $\beta$ , but not IKK $\alpha$ , is mainly involved in cytokine-induced NF- $\kappa$ B activation (8–13). This seems inconsistent with our finding that NF- $\kappa$ B activation by LPS or anti-CD40 was decreased in IKK $\alpha^{-/-}$  B cells. However, activation is still inducible and sufficient for partial responses to LPS and anti-CD40 (Figs. 2 B and 3 A) and A1 gene induction (Fig. 5), indicating that IKK $\beta$  can still function without IKK $\alpha$ .

The inducible expression of A1 was also observed in p50-deficient B cells, which manifested enhanced apoptosis (22, 43). In contrast, c-Rel-deficient B cells manifested impaired induction of A1 expression but no enhanced apoptosis (22, 43). This might suggest that similar molecular defects, although unidentified, may underlie the augmented cell death of p50- and IKK $\alpha$ -deficient B cells.

In conclusion, this study strongly suggests that IKK $\alpha$  plays crucial roles in the survival, proliferative responses, and maturation of peripheral B cells. The defects caused by IKK $\alpha$  deficiency are most likely intrinsic to B cells, although the possibility cannot formally be excluded that dysfunction of BM-derived, non-B cells also contributes to the phenotype of IKK $\alpha^{-/-}$  chimeras. It is unclear at present which contributes more to the observed B cell defects, disturbed maturation or dysfunction of already matured B cells. Although the molecular mechanism remains to be clarified yet, IKK $\alpha$  is a critical molecule for maintaining the mature B cell population.

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