IκB Kinase Signaling Is Essential for Maintenance of Mature B Cells

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Abstract

Nuclear factor (NF)-κB proteins play crucial roles in immune responses and cellular survival. Activation of NF-κB is mediated by the IκB kinase (IKK) complex, which is composed of two kinases, IKK1 and IKK2, and a regulatory subunit termed NF-κB essential modulator (NEMO). IKK2- and NEMO-deficient mice die at early embryonic stages. We therefore used conditional gene targeting to evaluate the role of these proteins in B cells in adult mice. B lineage–specific disruption of either IKK signaling by deletion of NEMO, or of IKK2-specific signals by ablation of IKK2 activity leads to the disappearance of mature B lymphocytes. We conclude that maintenance of mature B cells depends on IKK-mediated activation of NF-κB.

Key words: NF-κB • IKK • CD19-Cre • B cell subsets • maintenance

Introduction

The NF-κB/Rel transcription factor family regulates the expression of genes playing critical roles in cell activation, proliferation, and survival. In mammals it consists of five different Rel proteins: p65/RelA, c-Rel, RelB, p50/NF-κB1, and p52/NF-κB2; the latter two are generated through proteolytic processing of their respective precursor molecules p105 (p50) and p100 (p52). These subunits form various hetero- or homodimeric complexes, which in most resting cells are kept inactive sequestered to the ankyrin repeats of inhibitory molecules termed inhibitor of NF-κBs (IκBs)* (1). The precursors of NF-κB1 and NF-κB2 also contain ankyrin repeats and therefore can act as inhibitors of Rel proteins (2). Activation of the NF-κB signaling pathway ultimately leads to the phosphorylation and subsequent polyubiquitination and proteasome-mediated degradation of the IκBs, releasing NF-κB that then accumulates in the nucleus where it activates transcription from its target genes, including genes encoding proinflammatory cytokines and chemokines, cell-adhesion molecules and antiapoptotic proteins (3). The inducible phosphorylation of IκB in response to most known stimuli is mediated by the IκB kinase (IKK)-signalsome, a kinase complex consisting of two kinases, IKK1 and IKK2, and a regulatory subunit termed NF-κB essential modulator (NEMO) or IKKγ (4, 5).

Activated Rel proteins are involved in important and diverse functions of B cells, such as proliferation, isotype switching, and cytokine production (6–8). NF-κB is constitutively active in mature B cells indicating that this signaling pathway could be important for B cell maintenance. Indeed early studies using gene targeting technology showed that mice deficient for more than one of the NF-κB subunits have defects in B cell development. Adoptive transfer experiments using fetal liver cells of p50/p65 double knockout mice resulted in absence of B lymphopoiesis. However, when $p50^{-/-}p65^{-/-}$ fetal liver cells were mixed with WT bone marrow (BM) cells they gave rise to mature B cells in the spleen, demonstrating that this phenotype is non cell autonomous (9). More detailed studies using similar adoptive transfer experiments of mixed fetal liver cells showed that $p50^{-/-}$ or $p50^{-/-}p65^{-/-}$ B cells cannot develop into marginal zone (MZ) B cells (10). In p50/p52 double knockout mice B cell development is blocked at the immature transitional stage, shortly after B cells exit from the BM (11). Similarly $RelA^{-/-}c\text{-}Rel^{-/-}$ fetal liver cells fail to give rise to $IgM^{low}IgD^{+}$ mature B cells. $RelA^{-/-}c\text{-}Rel^{-/-}$ immature (IM) B cells undergo accelerated cell death in culture and express abnormally low levels of Bcl-2 (12). Furthermore, B cell–specific inhibition of NF-κB activity by transgenic expression of a trans-domi-

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**Abbreviations used in this paper:* BCR, B cell receptor; BM, bone marrow; BrdU, 5′-bromo-2′-deoxyuridine; FO, follicular; IκB, inhibitor of NF-κB; IKK, IκB kinase; IM, immature; MZ, marginal zone; NEMO, NF-κB essential modulator.

nant form of IκB-α leads to a reduction of mature splenic B cells and recirculating $B220^{high}IgM^{+}$ BM cells (13). Mice deficient for p50 have normal numbers of follicular (FO) B cells (10). It has been shown, however, that the in vivo turnover of $p50^{-/-}$ B cells is increased compared with WT B cells (14, 15). Whether this is due to a defect intrinsic to the B cell lineage or to a defect in other cell types is not clear. Ex vivo isolated p50-deficient B cells die faster than WT B cells, indicating that p50 could play a role in mature B cell survival. However, a direct analysis of whether mature B cells, once generated, depend on NF-κB–mediated survival signals in a cell-autonomous fashion in vivo, has not been performed so far.

Most signals that activate NF-κB converge at the IKK complex. It has been shown that B cells lacking IKK1 activity are short-lived and have a reduced capacity to proliferate. Very few IKK1-deficient B cells develop into $IgD^{+}IgM^{low}$ mature B cells in the spleen (16, 17). IKK1 is dispensable for cytokine-induced activation of NF-κB by degradation of IκB, but is critical for the generation of p52 from its precursor p100 (17). It is, however, controversial whether there is an overall reduction of NF-κB binding activity in IKK1-deficient B cells (16) or whether the maturation defect is solely due to lack of processing of p100 to p52(17). IKK2 and NEMO-deficient mice die at early embryonic stages showing massive liver degeneration (18–23). Studies in embryonic fibroblasts revealed that lack of IKK2 leads to strongly reduced activation of NF-κB in response to proinflammatory cytokines, whereas absence of NEMO completely abolishes this activation (18–23). The *Nemo* gene is located on the X-chromosome and heterozygous NEMO knockout mice develop a progressive skin disease that closely resembles the human X-linked genodermatosis incontinentia pigmenti (IP; references 20, 21, and 24). This disease is characterized by the complete skewing of X-inactivation in peripheral blood leukocytes (25), possibly due to selection against NEMO-deficient leukocytes. Chimeras generated from NEMO-knockout ES cells have no ES cell–derived B and T cells in the blood, indicating that NEMO could be essential for the development or survival of these cells (21). $Ikk2^{-/-}$ radiation chimeras lack peripheral blood B cells (26), similarly to the p50/p65 double knockout radiation chimeras (9), but it is not clear yet whether the inability of IKK2-deficient fetal liver cells to give rise to B cells is a B cell–autonomous phenomenon or not. In transgenic mice expressing a dominant-negative version of IKK2 specifically in B cells, B cells develop normally but show defects in proliferation and antibody responses (27). Thus, whereas IKK1 is important for the generation of mature B cells, the role of IKK2 is controversial and little is known about the importance of NEMO in B cell development. Whether activation of NF-κB through IKK is also essential for maintenance of mature B cells remains unresolved.

The assessment of the role of IKK2 and NEMO in tissues of adult mice is compromised by the early lethality of homozygous knockout mice. We opted to circumvent this problem by using the approach of conditional gene targeting, which allows the deletion of genes in specific cell types of the adult mouse (28). Using B cell–restricted expression of Cre recombinase we specifically ablated NEMO or IKK2 in B lymphocytes in order to investigate the role of these molecules in B cell development and function. We show here that interference with either NEMO or IKK2 activity is incompatible with B cell survival in the spleen.

Materials and Methods

Mice. Mice carrying $Ikk2^{D}$ and $Ikk2\Delta K^{D}$ alleles were generated by crossing $Ikk2^{FL}$ and $Ikk2\Delta K^{FL}$ mice to a Cre-deleter strain (29). All mice were housed in the conventional animal facility of the Institute for Genetics.

Flow Cytometry. Cells obtained from the various lymphoid organs were surface-stained with combinations of fluorochromes (FITC, PE, Cy-Chrome, PerCP, and/or allophycocyanin) or biotin-conjugated mAbs for 20 min on ice. Stainings with biotinylated mAbs were followed by a secondary staining with either Streptavidin-Cy-Chrome (BD Biosciences) or Streptavidin-PERCP (Becton Dickinson). Stained cells were acquired on a FACScan™ or FACSCalibur™ and data were analyzed using CELLQuest™ software (Becton Dickinson). Dead cells were labeled with propidium iodide or Topro-3 (Molecular Probes) and excluded from the analysis.

Monoclonal antibodies R33–24.12 (anti-IgM), 1.3–5 (anti-IgD), RA3–6B2 (anti-B220), and 30F1 (anti-HSA) were prepared and conjugated in our laboratory. Monoclonal antibodies to CD5, TCRβ, CD19, CD21/CD35, CD23, CD43, CD69, Fas, and CD86 were purchased from BD Biosciences.

For cell sorting B cells were purified by MACS (Miltenyi Biotec) and then stained with antibodies against various cell surface markers. B cells of individual B cell subsets were then sorted using a dual laser FACStar™ (Becton Dickinson). MACS-isolated B cells were typically ≥90% pure and sorted B cell subpopulations were ≥95% pure.

5′-Bromo-2′-Deoxyuridine Labeling. Mice were fed with 5′-bromo-2′-deoxyuridine (BrdU; Sigma-Aldrich) in the drinking water (1 mg/ml) for 1 or 1 wk. Splenocytes were stained with FITC-conjugated anti-B220 mAbs (clone RA33.A1.CL6) and fixed with 70% methanol followed by 2% formalin in PBS. Cells were then treated with 1 M HCl/0,5% Tween 20 for 15 min at 37°C followed by 0,1% $Na_2B_4O_7$. Cells were finally stained with a biotin-conjugated anti-BrdU mAb (Alexis Biochemicals) and streptavidin-Cychrome and analyzed with a FACSCalibur™ (Becton Dickinson).

Anti–IL-7 Receptor Antibody Injections. Mice were injected intravenously with 1 mg of anti–IL-7 receptor (anti-IL-7R) mAb (clone A7R34) every other day for 4 wk before sacrifice. Cell suspensions from the various lymphoid organs were analyzed by FACS®. DNA isolated from MACS-purified splenic B cells was analyzed with Southern blotting.

Results

Description of the Conditional Mouse Strains Used in This Study. Mice with conditional *nemo* alleles ($nemo^{FL}$) were generated as described (21). For the *Ikk2* gene we generated two different mouse strains, designated $Ikk2^{FL/FL}$ and $Ikk2\Delta K^{FL/FL}$. The $ikk2^{FL}$ allele was generated by placing exons 6 and 7 of the *Ikk2* gene between loxP sites. Cre-

mediated deletion of these two exons leads to an *ikk2 null* allele that does not produce any IKK2 protein (30).

In the absence of IKK2 IKK complexes consist of NEMO associated with IKK1 homodimers and exhibit inducible kinase activity (5, 31). To eliminate IKK2 kinase activity without disrupting IKK stoichiometry we therefore decided to generate another conditional *ikk2* allele, which allows replacement of IKK2 by a kinase-dead molecule (IKK2ΔK) upon Cre-expression. This allele (*ikk2ΔK^{FL}*) was generated by placing only exon 7 of the *Ikk2* gene between lox P sites. After deletion of exon 7, exons 6 and 8 splice in frame and the resulting mRNA produces a truncated IKK2 protein lacking amino acids 160–189, which include the two serines of the activation loop ($Ser_{177, 181}$) that are essential for the activation of the kinase (32; Fig. 1). This IKK2ΔK protein lacks kinase activity and is incorporated into the IKK complex, but it is expressed at lower levels compared with WT IKK2, presumably due to decreased protein stability (unpublished data). Inflammatory cytokine–induced activation of NF-κB, as measured by the sensitivity of the cells to TNF-induced death and by assaying production of IL-6 in response to IL-1 or LPS, is reduced in *Ikk2ΔK$^{D/D}$* MEFs compared with IKK2 knockout MEFs, but it is not completely abolished as in NEMO-deficient MEFs (unpublished data). The generation and characterization of the IKK2ΔK mutation will be described in detail elsewhere.

To delete the loxP flanked *nemo* and *ikk2* alleles in B lymphocytes we used a transgenic mouse strain expressing Cre recombinase under the control of the endogenous CD19 locus (33). This CD19-Cre mouse has been shown to delete loxP flanked alleles specifically in the B cell lineage. The deletion efficiency was shown to be 75–80% in BM and >95% in splenic B cells (33–35). The fact that deletion efficiency is higher in splenic than in BM B cells indicates that Cre-mediated deletion is an ongoing process during B cell development and maturation, leading to the essentially complete deletion of loxP flanked alleles in mature B cells.

Flow Cytometric Analysis of the B Cell Compartment in CD19-Cre/Ikk2$^{FL/D}$, -Ikk2ΔK$^{FL/D}$, and -Nemo$^{FL/Y}$ Mice. Initial FACS® analysis of B cell populations in *Ikk2$^{FL/FL}$*, *Ikk2$^{FL/D}$*, *Ikk2ΔK$^{FL/FL}$*, *Ikk2ΔK$^{FL/D}$*, and *Nemo$^{FL/Y}$* mice did not show any differences compared with controls. To facilitate Cre-mediated deletion, we used mice in which only one loxP flanked allele remains to be deleted (*CD19-Cre/Ikk2$^{FL/D}$*, *-Ikk2ΔK$^{FL/D}$*, and *-Nemo$^{FL/Y}$*) for our experiments. FACS® analysis of B cell populations in these mouse strains revealed very similar pictures. In the BM early B cell development was essentially normal and the only difference found compared with control mice was a 2–4-fold reduction of mature recirculating B cells (Fig. 2, A and B). In the spleen, B cells were strongly reduced leading to an inverted B/T cell ratio (Fig. 3, A and B). The reduction seems to occur mainly in the IgM^{low}IgD^{+} mature B cell population. Splenic B cells were then subdivided into immature (IM: CD21^{low}HSA^{high}), follicu-

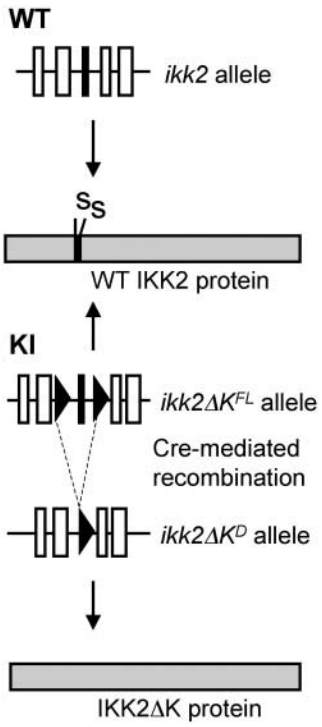

Figure 1. The conditional *ikk2ΔK* allele. Schematic representation of the WT *ikk2* allele, which produces WT IKK2 protein and the *ikk2ΔK* allele, which after deletion of loxP-flanked exon 7 generates a kinase-dead version of IKK2 (IKK2ΔK). Open and filled boxes represent exons, triangles represent loxP sites, and the two serines of the IKK2 activation loop are indicated using the amino acid single letter code.

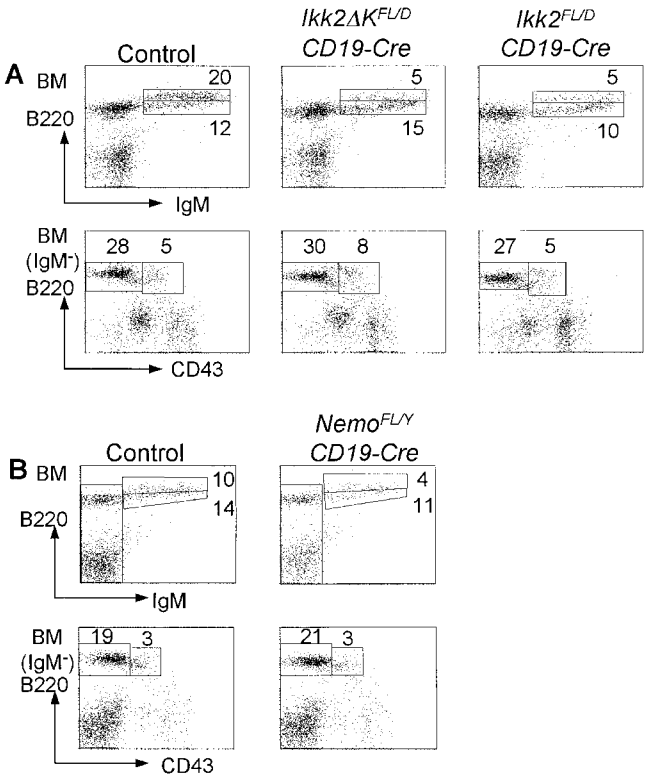

Figure 2. FACS® analysis of BM B cell populations. (A) *CD19-Cre/Ikk2$^{FL/D}$*, Ikk2Δ*K$^{FL/D}$*, and controls and (B) *CD19-Cre/Nemo$^{FL/Y}$* and control mice. Genotypes are as indicated. Cell surface markers are shown as coordinates and gated cell populations are indicated in brackets. The numbers next to boxed lymphocyte populations refer to the percentages of live cells in the lymphocyte gate.

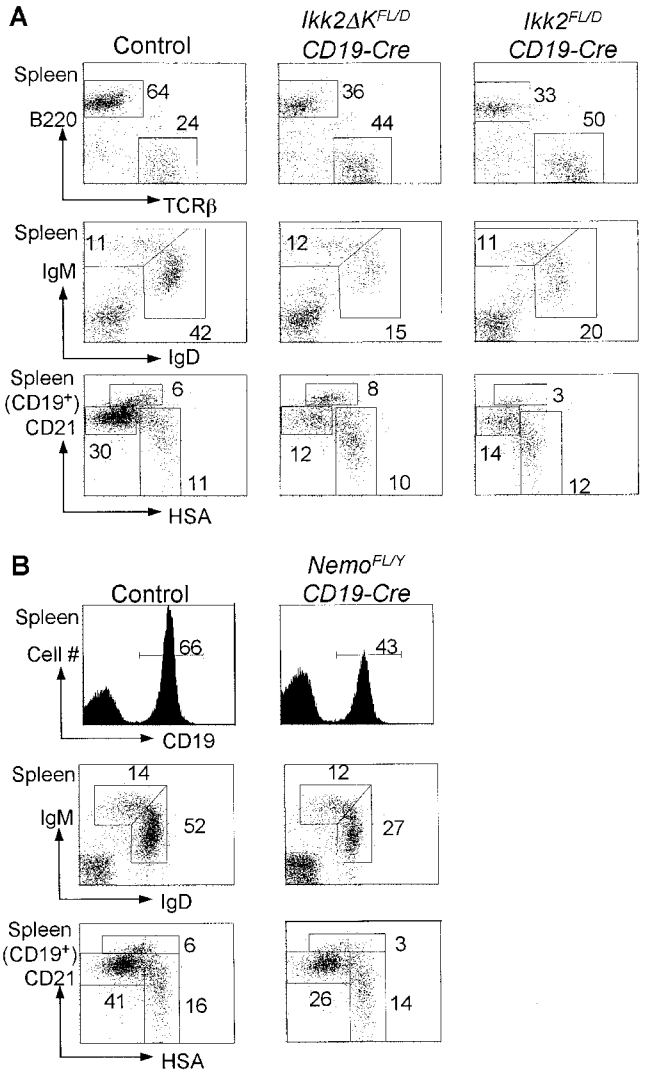

Figure 3. FACS® analysis of splenic B cell populations. (A) *CD19-Cre/Ikk2*$^{FL/D}$, *Ikk2ΔK*$^{FL/D}$, and controls and (B) *CD19-Cre/Nemo*$^{FL/Y}$ and control mice. Genotypes are as indicated. Cell surface markers are shown as coordinates and gated cell populations are indicated in brackets. The numbers next to boxed lymphocyte populations refer to the percentages of live cells in the lymphocyte gate.

lar (FO: CD21^{int}HSA^{low}) and marginal zone (MZ: CD21^{high}HSA^{int}) B cells according to CD21 and HSA expression (36). This analysis revealed that compared with controls the FO B cells are the most diminished B cell population in the spleens of *CD19-Cre/Ikk2*$^{FL/D}$, *-Ikk2ΔK*$^{FL/D}$, and *-Nemo*$^{FL/Y}$ mice (Fig. 3, A and B). Lymph node B cells and both B1 and B2 cells in the peritoneal cavity of these mice were also strongly reduced compared with control mice (unpublished data).

Reduction of Absolute B Cell Numbers in Mice with B Cell–specific Ablation of NEMO or IKK2. FACS® analysis revealed a strong reduction of mature B cells in mice with B cell–specific ablation of NEMO or IKK2. Calculation of the absolute cell numbers of individual B cell populations in the spleens of *CD19-Cre/Ikk2*$^{FL/D}$, *-Ikk2ΔK*$^{FL/D}$, and *-Nemo*$^{FL/Y}$ mice revealed a 3–4-fold decrease in the total number of splenic B cells in all mutant mouse strains compared with controls (Fig. 4 A). FO and MZ B cells were most severely affected (Fig. 4, C and D). Analysis of IM B cells revealed a different picture in the three different mouse strains. This population was not significantly affected in *CD19-Cre/Ikk2*$^{FL/D}$ mice, while a clear reduction was observed in *CD19-Cre/Nemo*$^{FL/Y}$ mice and *CD19-Cre/Ikk2ΔK*$^{FL/D}$ mice showed an intermediate picture (Fig. 4 B).

Preferential Loss of Splenic B Cells of the Deleted Genotype in CD19-Cre/Ikk2$^{FL/D}$, *-Ikk2ΔK*$^{FL/D}$, *and -Nemo*$^{FL/Y}$ *Mice.* The strong decrease of peripheral B cell numbers in mice with B cell specific ablation of NEMO or IKK2 activity suggests that inhibition of IKK signaling interferes with the development or persistence of B lymphocytes. However, all different subpopulations of B cells are present in *CD19-Cre/Ikk2*$^{FL/D}$, *-Ikk2ΔK*$^{FL/D}$, and *-Nemo*$^{FL/Y}$ mice, although in reduced numbers. One possible explanation for this could be that the remaining cells persist because they have escaped deletion of the loxP flanked alleles.

To investigate this hypothesis we performed Southern blot analysis of DNA isolated from FACS®-purified BM and splenic B cells populations from these three strains of mice. This analysis revealed that between 64–76% of BM IgM$^-$ B cells had deleted the loxP-flanked alleles (Fig. 5, A–C). This result is in agreement with the previously reported deletion efficiency of the CD19-Cre transgene in the BM (33) and with deletion seen in *CD19-Cre/Ikk2*$^{FL/WT}$ mice, in which deletion of the loxP-flanked allele has no effect due to IKK2-expression from the remaining WT allele (Fig. 5 D). Between 64–69% of the IgM$^+$ BM B cells isolated from *CD19-Cre/Ikk2*$^{FL/D}$, *-Ikk2ΔK*$^{FL/D}$, and *-Nemo*$^{FL/Y}$ mice carried the deleted genotype, compared with an 87% deletion of the loxP-flanked allele in *CD19-Cre/Ikk2*$^{FL/WT}$ mice. This indicates that IKK-deleted IgM$^+$ BM B cells are counterselected, in accordance with the reduced number of recirculating B cells in the BM of these mice. Whereas deletion was very efficient in splenic IM *CD19-Cre/Ikk2*$^{FL/D}$ and *-Ikk2ΔK*$^{FL/D}$ B cells (>90%), only 44–66% of FO B cells were found to have deleted the loxP flanked alleles (Fig. 5, A and B). In *CD19-Cre/Ikk2*$^{FL/WT}$ mice deletion was 87 and 95% for IM and FO B cells, respectively (Fig. 5 D). The finding that within the FO B cell compartment there are less cells with the deleted genotype than in the immature compartment suggests that B cells lacking IKK2 activity are counterselected as they move on to become more mature FO B cells (Fig. 5, A and B). In *CD19-Cre/Nemo*$^{FL/Y}$ mice deletion in IM splenic B cells was less efficient than in the IKK2 loxP flanked alleles (~70% compared with >90%) and was not further reduced in FO B cells (Fig. 5 C). This indicates that the cells that delete the *nemo*^{FL} allele are already counter-selected at the immature B cell stage. A more dramatic effect is seen in MZ B cells. In all three mouse strains very few cells with deleted alleles (≤12%) were found within the marginal zone B cell pop-

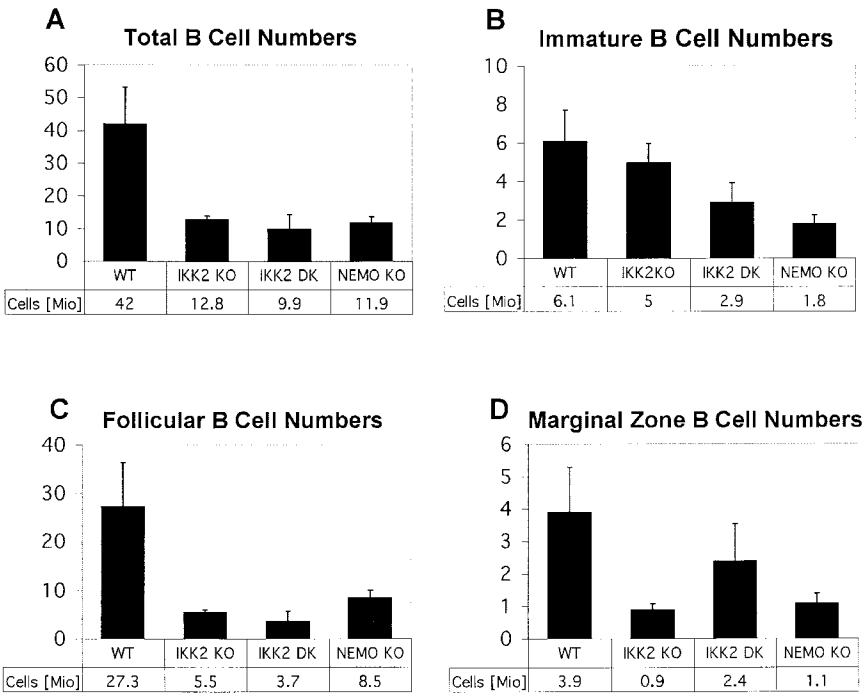

Figure 4. Reduction of B cell numbers in *CD19Cre/IKK*-conditional mice. Absolute numbers of B cell subpopulations in the spleens of *CD19-Cre/Ikk2*$^{FL/D}$, *CD19-Cre/Ikk2ΔK*$^{FL/D}$, and *CD19-Cre/Nemo*$^{FL/Y}$ compared with control mice are shown. (A) Total B cell numbers, (B) IM B cells, (C) follicular B cells, and (D) marginal zone B cells. Bar charts showing the absolute cell numbers for the indicated B cell population in the spleen for each of the following mouse strains: CT (controls), IKK2 KO (*CD19-Cre/Ikk2*$^{FL/D}$), IKK2 DK (*CD19-Cre/Ikk2ΔK*$^{FL/D}$), and NEMO KO (*CD19-Cre/Nemo*$^{FL/Y}$). For each group three to eight mice were analyzed. Error bars indicate standard deviation. The absolute cell numbers for each population were calculated by multiplying the percentage of each B cell subset of all live splenocytes obtained from the FACS® analysis with the total number of live cells recovered from the perspective spleen.

ulation, whereas in *CD19-Cre/Ikk2*$^{FL/WT}$ mice deletion was virtually complete, suggesting that IKK activity is essential for the development or maintenance of these cells (Fig. 5, A–D).

Based on the above data we envisage the following scenario: ongoing deletion continuously leads to the generation of B cells that have deleted the respective gene, but still retain enough protein to stay alive. With time and through turnover of endogenous mRNAs and proteins these cells lose their ability to signal via the IKK complex and die. Continuous influx of newly generated B cells from the BM and continuous Cre-mediated deletion of the loxP flanked alleles leads to the deletion pattern observed in B cells of *CD19-Cre/Ikk2*$^{FL/D}$, *-Ikk2ΔK*$^{FL/D}$, and *-Nemo*$^{FL/Y}$ mice. This scenario predicts a higher turnover rate of mutant compared with control B cells.

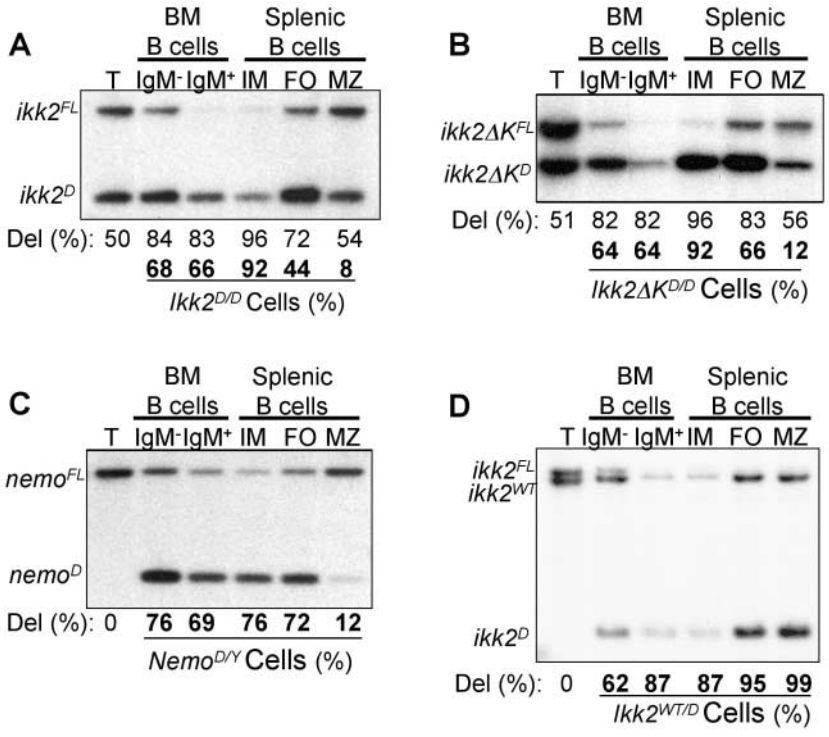

Figure 5. Counterselection against *Ikk*$^{D/D}$, *Ikk2ΔK*$^{D/D}$, and *Nemo*$^{D/Y}$ B cells in the spleen. Southern blot analyses of sorted splenic B cell populations of mice of the indicated genotypes are shown. (A) *CD19-Cre/Ikk2*$^{FL/D}$ mice, (B) *CD19-Cre/Ikk2ΔK*$^{FL/D}$ mice, (C) *CD19-Cre/Nemo*$^{FL/Y}$ mice, (D) *CD19-Cre/Ikk2ΔK*$^{FL/WT}$ mice. T, tail; IgM$^-$ (CD19$^+$B220$^+$IgM$^-$: pro- and pre-B cells); IgM$^+$ (CD19$^+$B220$^+$IgM$^+$ B cells); IM (CD19$^+$CD21^{low}HSA^{high}: immature B cells); FO (CD19$^+$CD21^{int}HSA$^-$: follicular B cells); MZ (CD19$^+$CD21^{high}HSA^{int}: marginal zone B cells). The percentage of deleted alleles and cells of the deleted genotype is given below the blots. Each blot is a representative example of at least four mice analyzed. Del (%), percent of deleted alleles.

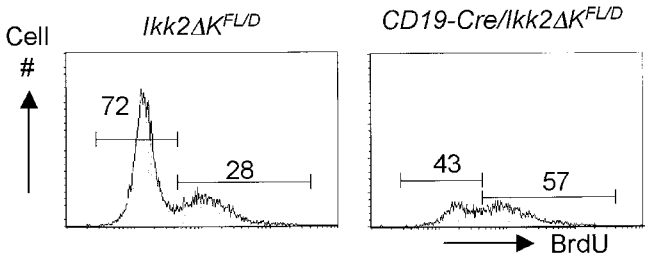

Figure 6. Increased B cell turnover in the spleens of *CD19-Cre/Ikk2ΔK$^{FL/D}$* mice compared with control mice. Analysis of BrdU incorporation by splenic B cells of mice of the indicated genotypes after 1 wk of BrdU administration in the drinking water. Numbers indicate percentages of BrdU-positive and BrdU-negative B cells.

CD19-Cre/Ikk2ΔK$^{FL/D}$ and CD19-Cre/Nemo$^{FL/Y}$ B Cells Have Higher In Vivo Turnover Rates than WT Control B Cells. To test this hypothesis we determined the percentage of BrdU-labeled B cells in the spleens of *CD19-Cre/Ikk2ΔK$^{FL/D}$* and control mice that had been fed with BrdU for 1 wk (Fig. 6). In *CD19-Cre/Ikk2ΔK$^{FL/D}$* 50 to 60% of B lineage splenocytes had incorporated BrdU into their DNA within this period of time, whereas in control mice only 30% of these cells were BrdU-positive (Table I). This result shows that B cell turnover in the spleen of *CD19-Cre/Ikk2ΔK$^{FL/D}$* mice is significantly faster than in WT animals. Similarly, in *CD19-Cre/Nemo$^{FL/Y}$* mice twice as many splenic B lymphocytes incorporated BrdU as B cells of control mice, demonstrating that B cells in *CD19-Cre/Nemo$^{FL/Y}$* mice also have an increased turnover rate (unpublished data). These results suggest that IKK mutant splenic B cells have a shorter half-life then control B cells.

Blockade of De Novo B Cell Generation in CD19-Cre/Ikk2ΔK$^{FL/D}$ Mice Leads to Disappearance of IKK2-deficient B Cells from the Spleen. During B cell development peripheral B cells are continuously generated from the BM. We decided to evaluate whether *CD19-Cre/Ikk2ΔK$^{FL/D}$* B lineage lymphocytes that underwent Cre-mediated deletion can persist in the spleen by blocking influx of IM B cells from the BM. This was achieved by injection of antagonistic monoclonal antibodies against the IL-7R. This treatment blocks B cell development at the pro-B cell stage and thus abolishes the influx of IM B cells from the BM into the periphery (37).

After four weeks of anti–IL-7R antibody administration FACS® analysis demonstrated that B cell development in the BM and the influx of newly generated B cells into the spleen is indeed inhibited in antibody-treated mice (Fig. 7 A). Southern blot analysis of DNA isolated from purified splenic B cells of these mice revealed the absence of cells with *Ikk2ΔK$^{D/D}$* genotype (Fig. 7 C), whereas in untreated *CD19-Cre/Ikk2ΔK$^{FL/D}$* mice 50 to 80% of the CD19-positive splenic B cells are of the deleted genotype (Fig. 7 B). This finding validates the view that the B cells with two deleted *ikk2ΔK* alleles that are found in the spleens of *CD19-Cre/Ikk2ΔK$^{FL/D}$* mice are cells that have only recently undergone Cre-mediated recombination and still retain IKK2 protein. As soon as the influx from the BM is blocked, no new B cells having freshly acquired the deleted genotype enter the spleen from the BM. Within a period of 4 wk all B cells lacking IKK2 activity disappear from the spleen and only cells that have escaped Cre-mediated deletion of the loxP flanked alleles persist. These results demonstrate that IKK-mediated NF-κB activity is essential for the survival of mature B cells.

Discussion

We investigated the role of IKK-induced NF-κB activity in B cells by conditional inactivation of IKK2 and NEMO. B cell specificity was achieved by crossing mice carrying conditional NEMO and IKK2 alleles (*Ikk2$^{FL/D}$*, *Ikk2ΔK$^{FL/D}$*, and *Nemo$^{FL/Y}$*) to CD19-Cre mice. All three mutations similarly lead to a strong reduction of B cell

Table I. *Increased B Cell Turnover of CD19-Cre/Ikk2ΔK$^{FL/D}$ Splenic B Cells Compared to Control B Cells*

Control B cells				*CD19-Cre/Ikk2ΔK$^{FL/D}$* B cells			
BrdU$^+$		BrdU$^-$		BrdU$^+$		BrdU$^-$	
%	×10^6	%	×10^6	%	×10^6	%	×10^6
28	7.9	72	20	51	4.9	49	4.6
28	8.2	72	20	60	3.2	40	2.1
31	9.8	69	22.3	57	2.3	43	1.7
29	12	71	29.3	49	2.2	51	2.3
32	12.1	68	25.7				
31	9.7	69	21.8				
29.8 ± 1.7	10 ± 1.8	**70.2 ± 1.7**	23.2 ± 3.7	**54.3 ± 5.1**	3.2 ± 1.3	**45.8 ± 5.1**	2.7 ± 1.3

BrdU-incorporation by *CD19-Cre/Ikk2ΔK$^{FL/D}$* and control splenic B cells was measured after 1 wk of BrdU administration. The proportions of BrdU-positive (BrdU$^+$) and negative (BrdU$^-$) B cells of total splenic B cells are shown (%). The absolute B cell numbers for these fractions are shown in millions (×10^6). The last row indicates medium numbers and standard deviations.

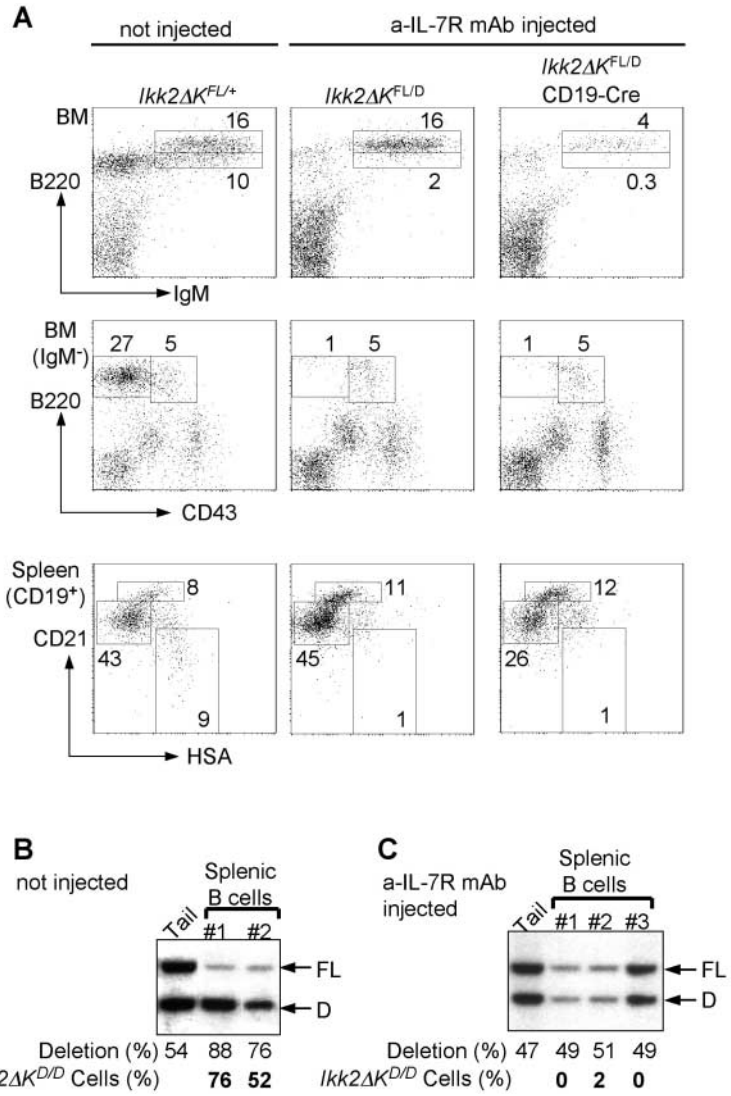

Figure 7. Block of B cell influx from the BM in *CD19-Cre/Ikk2ΔK$^{FL/D}$* mice leads to disappearance of B cells of the deleted genotype in the spleen. (A) Verification of the block in B cell development after injection of anti–IL-7R antibodies by FACS® analysis. Noninjected control mice are compared with mice that had received injections of anti–IL-7R antibodies for 4 wk. The genotypes are as indicated above and cell surface markers are shown as coordinates and gated cell populations are indicated in brackets. The numbers next to boxed lymphocyte populations refer to the percentages of live cells in the lymphocyte gate. (B) Southern blot analysis of Cre mediated deletion in total splenic B cells from two different (#1, #2) *CD19-Cre/Ikk2ΔK$^{FL/D}$* mice. The results are representative of six different mice. (C) Southern blot analysis of Cre mediated deletion in total splenic B cells from three different (#1–3) *CD19-Cre/Ikk2ΔK$^{FL/D}$* mice that received anti–IL-7R antibody injections for 4 wk.

numbers in mutant mouse strains. *CD19-Cre/Ikk2$^{FL/D}$*, *-Ikk2ΔK$^{FL/D}$*, or *-Nemo$^{FL/Y}$* mice have 3–4-fold reduced B cells in the spleen and at least twofold reduced numbers in recirculating BM B cells, lymph node B cells, and peritoneal cavity B1 and B2 cells. Southern blot analysis of sorted B cell populations revealed that whereas deletion efficiency in IgM$^-$ BM B cells is as high as reported for CD19-Cre mice and comparable to deletion observed in *CD19-Cre/Ikk2$^{FL/WT}$* mice, IgM$^+$ BM B cells are already counterselected, most likely reflecting counterselection against recirculating B cells. In the transition from IM to FO B cells the percentage of B cells with deleted alleles is reduced, indicating that mature B cells lacking IKK activity are counterselected. In the case of *CD19-Cre/Ikk2$^{FL/D}$* and *-Ikk2ΔK$^{FL/D}$* mice >90% of the IM B cells have deleted the floxed allele, whereas in follicular cells, which presumably originate from the immature transitional cells, the proportion of cells carrying the deletion ranges from 40 to 70%, clearly demonstrating counterselection of *Ikk$^{D/D}$* and *Ikk2ΔK$^{D/D}$* B cells. In *CD19-Cre/Nemo$^{FL/Y}$* mice, *Nemo$^{D/Y}$* IM B cells are already counterselected, as evidenced by Southern blot and cell counting. The fact that deletion of NEMO, in contrast to the deletion of IKK2, seems to lead to counterselection of cells already within the immature B cell compartment may be explained in two ways. One explanation is that in the absence of IKK2 some IKK-induced NF-κB activation can still occur while loss of NEMO completely blocks IKK activity. Thus, in *CD19-Cre/Ikk2$^{FL/D}$* mice low levels of remaining NF-κB activity could be sufficient to sustain development of B cells to the IM stage. In the *CD19-Cre/Nemo$^{FL/Y}$* mice the complete absence of NF-κB activity may lead to the loss of IM B cells. Additionally or alternatively different turnover rates of IKK2 and NEMO mRNA and protein could play a role. The relatively long half-life of IKK2 (38) might ensure that most B lineage cells of *CD19-Cre/Ikk2$^{FL/D}$* mice develop into FO B cells before they are devoid of this protein. The half-life of NEMO could be shorter than that of IKK2, leading to a rapid depletion of NEMO protein from IM B cells that have deleted the *nemo^{FL}* allele, resulting in the loss

of cells at this stage. The fact that *CD19-Cre/Ikk2ΔK*$^{FL/D}$ mice display an intermediate phenotype with some loss of deleted IM B cells, as demonstrated by the reduction of the absolute cell numbers of IM B cells in these mice, is compatible with both hypotheses, as the production of IKK2ΔK may lead both to the earlier loss and to more complete inhibition of IKK activity in comparison to the *CD19-Cre/Ikk2*$^{FL/D}$ mice.

The picture seen in MZ B cells differs from that seen in FO B cells. The remaining MZ B cells of the IKK-conditional mutant mice crossed to CD19-Cre mice are nearly completely devoid of the deleted genotype, clearly demonstrating that *Ikk2*$^{D/D}$, *Ikk2ΔK*$^{D/D}$, and *Nemo*$^{D/Y}$ MZ B cells cannot be generated and/or persist. Recent evidence suggests that MZ B cells represent a self-renewing separate subset of B cells (39, 40). In this case cells of the deleted genotype would disappear early during the generation of this lineage and a subset of cells that have lost their ability to delete would be sustained by self-renewal. Another view is that MZ B cells are a highly antigen-selected population of B cells (10, 41). During this selection process all B cells able to undergo Cre-mediated deletion should have deleted the loxP-flanked gene and subsequently disappeared. Thus, the virtual absence of MZ B cells with a deleted genotype in IKK conditional-CD-19Cre mice could be due to the fact that the MZ B cell compartment is not constantly refilled with B cells that recently acquired the deleted genotype as is the follicular compartment. Alternatively, the absence of MZ B cells with deleted *nemo* or *ikk2* alleles could mean that MZ B cells are more dependent on IKK-induced NF-κB activity then other B cell populations. This explanation, which does not exclude the former, is supported by the specific loss of MZ B cells in p50-deficient mice (10).

Mature splenic B cells do not proliferate much (39, 42, 43), so that in a BrdU incorporation experiment over the period of 1 wk most of the BrdU-positive B cells in the spleen represent IM B cells from the BM (42). Roughly twice as many *CD19-Cre/Ikk2ΔK*$^{FL/D}$ and *CD19-Cre/Nemo*$^{FL/Y}$ splenic B cells stain positive for BrdU after 1 wk of BrdU administration compared with control B cells. This means that recently generated B cells constitute a larger fraction of total splenic B cells in mutant compared with control mice despite the reduction in B cell numbers in mutant mice. Thus, *CD19-Cre/Ikk2ΔK*$^{FL/D}$ mice contain a higher percentage of short-lived splenic B cells than do WT mice. These short-lived B cells should be cells that carry two deleted alleles and are on their way to lose their ability to signal through the IKK complex and subsequently die. When B cell influx from the BM is blocked for 1 mo by interfering with IL-7R signaling using injections of anti–IL-7R antibodies, *Ikk2ΔK*$^{D/D}$ B cells completely disappear from the spleens of these mice. The fact that only *Ikk2ΔK*$^{FL/D}$ B cells remain in these *CD19-Cre/Ikk2ΔK*$^{FL/D}$ mice shows that there must be a strong selection for cells in which Cre is either absent or cannot function. FO B cells were shown to have an average calculated half-life of 134 d in the absence of replenishment of the splenic B cell pool from the BM (39). As all of the *Ikk2ΔK*$^{D/D}$ FO B cells disappear after 1 mo, this clearly demonstrates that IKK2 signaling is essential for the maintenance of mature B cells. We therefore conclude that mature B cells need IKK-mediated survival signals.

Based on our experimental findings we envisage the following scenario: deletion of the conditional alleles in mice containing the CD19-Cre transgene is initiated in the BM and continues throughout the later stages of B cell development. B cells that underwent Cre-mediated recombination initially retain sufficient amounts of IKK2 or NEMO protein to allow development into IM B cells and some of these cells progress further into the mature B cells compartments. B cells that escape Cre-mediated deletion develop normally into mature B cell subsets. Thus, the follicular B cell compartment of these mice consists of a mixture of cells that have already deleted and cells that retain loxP-flanked alleles. The fact that blockade of B cell development in the BM for 4 wk leads to the disappearance of all B cells that have deleted the *ikk2ΔK* floxed alleles from the spleen demonstrates that mature B cells cannot survive without IKK2 activity.

It has been speculated that constitutive activation of NF-κB by signaling through the B cell receptor (BCR) is essential for B cell survival (13, 44). In agreement with this idea B cells die by apoptosis upon inducible deletion of the BCR (45). However, it was shown recently that while deletion of Bcl10 in mice selectively abolishes NF-κB activation by the antigen receptor in B cells, Bcl10$^{-/-}$ mice show normal B cell development. This argues against the possibility that NF-κB activation originating from the BCR is needed for B cell survival (46). Therefore mature peripheral B cells seem to depend on NF-κB activating signals other than BCR cross-linking. These signals are likely to be transmitted by interactions of TNF/TNF-R family members such as BAFF/BAFF-R (47).

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