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Impaired B Cell Development and Function in the Absence of IkBNS

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IkBNS has been identified as a member of the IkB family of NF-κB inhibitors, which undergoes induction upon TCR signaling. Mice carrying a targeted gene disruption of IkBNS demonstrate dysregulation of cytokines in T cells, macrophages, and dendritic cells. IkBNS mediates both positive and negative gene regulation, depending on individual cell type and/or cytokine. In this study, we demonstrate an additional role for IkBNS in the B cell lineage. B cells from IkBNS knockout (KO) mice were impaired in proliferative responses to LPS and anti-CD40. IgM and IgG3 Igs were drastically reduced in the serum of IkBNS-depleted spleens of IkBNS KO mice. Ab-secretion in IkBNS KO mice was impaired in IkBNS KO mice, although IkBNS significantly reduced in IkBNS KO B cells. In agreement with this finding, the number of Ab-secretting cells in the spleens of IkBNS KO mice was reduced and production of Ag-specific Igs was lower in IkBNS KO mice after influenza infection as compared with wild-type mice. Additionally, IkBNS KO mice lacked B1 B cells and exhibited a reduction in marginal zone B cells. Thus, IkBNS significantly impacts the development and functions of B cells and plasma cells. The Journal of Immunology, 2011, 187: 3942–3952.

NUCLEAR FACTOR κB (NF-κB) IS A TRANSCRIPTION FACTOR THAT WAS FIRST IDENTIFIED IN B CELLS (1) BUT IS NOW RECOGNIZED AS A MASTER CONTROLLER OF MULTIPLE GENES IN VIRTUALLY EVERY CELL TYPE. IN PARTICULAR, NF-κB PLAYS A KEY ROLE IN THE OVERALL REGULATION OF THE IMMUNE SYSTEM AND THE INFLAMMATORY RESPONSE. NF-κB CONSISTS OF HOMODIMERS OR HETEROdìMMERS FORMED BY FIVE DIFFERENT NF-κB FAMILY MEMBERS (2–5). TRANSCRIPTIONAL CONTROL VIA NF-κB IS EXTREMELY RAPID DUE TO THE UNIQUE MECHANISM OF REGULATION OF NF-κB BY INHIBITORY PROTEINS CALLED IkBs. THE FIRST IkB PROTEIN IDENTIFIED AND BEST CHARACTERIZED, IkBα, Binds NF-κB PROTEINS AND POSITIONS THEM IN THE CYTOPLASM AND AWAY FROM THE NUCLEUS, THEREBY PREVENTING DNA BINDING AND GENE REGULATION (6, 7).

IkBNS demonstrates dysregulation of cytokines in T cells, macrophages, and dendritic cells. IkBNS mediates both positive and negative gene regulation, depending on individual cell type and/or cytokine. In this study, we demonstrate an additional role for IkBNS in the B cell lineage. B cells from IkBNS knockout (KO) mice were impaired in proliferative responses to LPS and anti-CD40. IgM and IgG3 Igs were drastically reduced in the serum of IkBNS KO mice, although IkBNS significantly reduced in IkBNS KO B cells. In agreement with this finding, the number of Ab-secretting cells in the spleens of IkBNS KO mice was reduced and production of Ag-specific Igs was lower in IkBNS KO mice after influenza infection as compared with wild-type mice. Additionally, IkBNS KO mice lacked B1 B cells and exhibited a reduction in marginal zone B cells. Thus, IkBNS significantly impacts the development and functions of B cells and plasma cells. The Journal of Immunology, 2011, 187: 3942–3952.

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Upon triggering of cell surface receptors that signal through NF-κB, including the TCR, BCR, TNFR1/2, or many other input signals, the IkBα protein is phosphorylated by IkB kinase and then ubiquitinated, leading to degradation of IkBα and release of the NF-κB heterodimer, enabling nuclear translocation followed by gene regulation. In turn, IkBα is itself a target of NF-κB regulation such that degradation of IkBα releases NF-κB, which then acts to induce synthesis of new IkBα proteins that can begin another cycle of sequestering NF-κB and shutting down the transcriptional activity. This mechanism of NF-κB activation has been termed the “classical” activation pathway. A pathway activating relB, termed the “nonclassical” pathway, involves the partial proteolysis of p100 to p52, which translocates to the nucleus with relB (reviewed in Refs. 8, 9). Thus, the extremely rapid response to the >150 stimuli that induce NF-κB activity is a result of the release from IkB inhibition of the preexisting NF-κB proteins, and NF-κB activation occurs without the need for transcription or translation, allowing cells to respond very rapidly to cell surface signals.

Five forms of NF-κB proteins have been identified (2–5), but detailed analysis of their specific individual roles has been complicated due to the overlapping tissue distributions and redundant functions of the various homo- and heterodimeric NF-κB pairs. The p65, c-Rel, and relB NF-κB proteins contain transactivation domains capable of activating gene transcription, whereas p50 and p52 lack transactivation domains. Thus, homodimers of p50 and p52 are thought to inhibit gene transcription by blocking κB-binding sites. Strict regulation of NF-κB activation is necessary for proper immune cell function and avoidance of tumor formation (10), and abnormal levels of NF-κB subunits lead to a variety of cancers (reviewed in Ref. 11), including various B cell leukemias (reviewed in Ref. 12). Targeted gene disruption or transgenic overexpression of NF-κB genes has aided more precise delineation of the roles of each of these subunits. In particular, deletion of each of the NF-κB subunits affects the immune response in some way, underscoring the importance of NF-κB in cells of the immune system (reviewed in Ref. 13). The phenotypes of mice

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carrying deletions of NF-κB genes include effects on both T and B cells, but in this study our concentration is on B lymphocytes. Deletion of rela is embryonically lethal, and reconstitution of rag-1−/− or SCID mice with embryonic day 13 rela−/− fetal liver cells demonstrates that RelA is required for mitogen-induced lymphocyte proliferation and isotype switching. Various defects in B cell activation result from the deletion of rel, and isotype switching is also affected, perhaps as a result of reduced transcription through the H chain locus. Isotype switching and Ig secretion are normal when assayed in vitro on relb−/− cells, indicating that the humoral defect of impaired IgG responses to T cell-dependent Ags observed in relb−/− mice is most likely a secondary consequence due to the absence of specific dendritic cell populations. Thus, although normal hemopoiesis does not require any of the other NF-κB proteins, the development of specific dendritic cell populations requires RelB. In nfkbl−/− mice, Ig secretion, Ig switching, and proliferation are impaired, and quiescent nfkbl−/− B cells turnover more rapidly, suggesting that p50 is required for survival of nonactivated B cells. Mice lacking p52/p100 proteins develop normally but display disruption of splenic and lymph node architecture. However, nfkhb2−/− lymphocytes activated in vitro exhibit only mildly impaired proliferative responses coupled with normal Ab or cytokine production. Thus, although the failure of nfkhb2−/− mice to mount a normal T cell-dependent Ab response is associated with an inability to form germinal centers (GCs), intrinsic B cell defects are excluded.

With regard to the NF-κB family of proteins no new family members have been identified for some time. In contrast, the family of IκB inhibitors has continued to expand with the identification of IκBNS (14) and IκBζ (15–17). Prior to this, seven NF-κB inhibitor proteins were known: IκBa, IκBβ, IκBε, IκBγ, Bcl-3, p100, and p105 (reviewed in Ref. 2). IκBS and IκBζ show more sequence homology to each other and to Bcl-3 than to IκBa, and, similar to Bcl-3, these proteins appear to be nuclear rather than cytoplasmic. IκBβ (18), Bcl-3 (2, 19, 20), and IκBζ (21–23) have been shown to regulate promoters of cytokines controlled by NF-κB and to be involved in inflammatory responses. Their mechanisms of action are different from those of IκBα since their expression is transcriptionally rather than posttranslationally regulated and is more restricted in cell expression patterns (14–19, 24, 25), the more ubiquitous NF-κB and other IκB family members.

We previously created a knockout (KO) mouse line of IκBNS and demonstrated that T cells in IκBNS KO mice exhibit defective proliferation and cytokine production (23). For example, IκBNS KO T cells produce less IL-2, a key cytokine in survival and activation of T cells during the immune response (23). Additional cytokines, including IFN-γ, are also affected by IκBNS (23). Others have shown independently that IκBNS suppresses the inflammatory cytokines IL-6 and IL-12 in macrophages and dendritic cells (21, 22). In contrast, IκBζ increases expression of IL-6 and IL-12 as well as other inflammatory cytokines (18). Both IκBS and IκBζ bind the p50 NF-κB family member (14, 18). Thus, these newly identified members of the IκB family serve to focus the action of the ubiquitous NF-κB transcription factors. Their tissue specificity explains the mechanism by which various cell surface receptors act through the NF-κB pathway to induce different genes in different cell types.

In this study, we describe the effects of the deletion of IκBNS on B cells. Our initial analysis of the IκBNS KO mouse showed slightly lower total B cell numbers, but the difference was not significant between wild-type (WT) and IκBNS KO mice (23). However, further detailed analysis has revealed a disturbance in individual B lineage subsets of IκBNS KO mice. Furthermore, we find that IκBNS KO B cells are deficient in proliferation, Ig production, and plasma cell differentiation.

### Materials and Methods

#### Mice

IκBNS KO mice on a C57BL/6 (B6) background were established and maintained as described (23). B6 and B6.D2.SJL mice were purchased from Taconic (Germantown, NY). Mice were maintained and bred under specific pathogen-free conditions in the animal facilities of the Dana-Farber Cancer Institute and Keio University under protocols reviewed and approved by the Institutional Animal Care and Use Committees.

#### Abs and flow cytometric analysis

The following mAbs were used: FITC-anti-Igλ1 (A20), FITC-anti-CD19 (1D3), FITC-anti-CD5 (53-7.3), FITC- or PE-Cy7-anti-IgM (R6-60.2), PE-anti-CD45.2 (104), PE-anti-CD21/35 (7C6), PE-anti-CD138 (syndecan-1) (281-2), PE- or allophycocyanin-Cy7-anti-B220 (RA3-6B2), biotin-anti-CD23 (B34), PE-Cy7- or allophycocyanin-streptavidin (BD Biosciences). Single-cell suspensions were prepared in a FACS buffer solution (1% PBS containing 2% FCS and 0.05% NaN3). Cells were pretreated for 10 min at 4°C with anti-FcγRIII (clone 2.4G2; 1 μg/ml) to reduce nonspecific staining. Staining for cell-surface Ag expression was performed at saturating Ab concentrations for 20 min at 4°C. Cells were washed once in FACS buffer solution and incubated with second-step reagent if necessary. A FACSScan (BD Biosciences) and CellQuest software were used for analysis of triple-stained samples, and FACSComp and FlowJo software (Tree Star) were used for five-color samples. Dead cells were excluded from the analysis by forward and side scatter gating. Although B6 mice do not have the IgG2a isotype but, rather, the IgG2c isotype, the anti-IgG2a Ab crossreacts with IgG2c and therefore detects IgG2c in our mice.

#### B cell proliferation assay

Splenic B cells from IκBNS KO or B6 WT mice were purified by negative selection using anti-CD43–conjugated microbeads (Miltenyi Biotec). The CD43− cells were suspended in PBS containing 0.5% BSA, stained with 1 μM CFSE at 37°C for 10 min, washed three times in complete medium, and cultured in the presence of 0.1, 1, or 10 μg/ml LPS (Sigma-Aldrich), 0.1, 1, or 5 μg/ml anti-CD40 (BD Biosciences), or 10 μg/ml anti-IgM (μ-chain) (Jackson ImmunoResearch Laboratories). The cells were harvested on different days as indicated, and CFSE intensity was monitored by flow cytometry. For determination of B cell proliferation by 3Hthy midine incorporation, assays were carried out as previously described (23) and 2 μCi/well [3H]thymidine was added for the last 18 h of culture, and the incorporated radioactivity was measured by a scintillation counter (PerkinElmer MicroBeta 2 LumiJET).

#### Immunizations

To measure the immune response to T-dependent and T-independent Ags, 8- to 12-wk-old mice were inoculated with an i.p. injection of 100 μg 2,4,6-trinitrophenol (TNP)-Ficoll or 75 μg TNP-keyhole limpet hemocyanin (KLH) (Bioresearch Technologies) mixed with Imject Alum (Pierce) as an adjuvant. Serum was collected every 7 d after immunization and analyzed for antibody if necessary. A FACScan (BD Biosciences) and CellQuest software were used for five-color samples. Dead cells were excluded from the analysis by forward and side scatter gating. Although B6 mice do not have the IgG2a isotype but, rather, the IgG2c isotype, the anti-IgG2a Ab crossreacts with IgG2c and therefore detects IgG2c in our mice.

#### Immunohistochemical analysis

For analysis of GC formation, five WT and five IκBNS KO mice received an i.p. injection of 5 × 107 SRBCs (Innovative Research). Mice were sacrificed 5 or 10 d later and spleens examined for peanut agglutinin (PNA) reactivity.

#### Immunohistochemical analysis

Spleens were removed, frozen in Tissue-Tec OCT compound (Sakura Finetek), and subjected to immunofluorescent staining. Cryosections (5 mm thick) were mounted onto slides, air-dried for 1 h, and fixed with ice-cold acetone for 10 min. Sections were stained with tetramethylrhodamine isothiocyanate-anti–mouse IgM (SouthernBiotech) and FITC-MOMA-1 (AbD Serotec). The slides were mounted with low fluorescent glycerol (Invitrogen) and observed with a fluorescent microscope (Olympus).

#### Surface and intracellular Ig staining

Cells were stained for surface Ig with biotinylated Abs and streptavidin-allophycocyanin. Purified anti-mouse Ig Abs were then used to block surface Ig. The membrane-labeled cells were treated with fixation and permeabilization reagents (BD Pharmingen) and then stained with FITC-labeled anti-Ig diluted in permeabilization medium (0.05% saponin, 2%
FBS, PBS). The cells were washed and resuspended in PBS containing 2% FBS and immediately analyzed on a flow cytometer.

Influenza virus inoculation
Mice received an i.p. injection of 10^4 multiplicity of infection (MOI) influenza A virus (PR8). Fifteen days after the inoculation, serum was collected to measure the virus-specific Ab levels, and mice were intranasally infected with 10^6 MOI PR8. Survival was monitored for 20 d after the infection.

Serum Ig titration
Ig isotype levels of serum were measured by ELISA with 2.5 μg/ml anti-IgG, IgM, IgD, and IgA-coated immunoplates and HRP-conjugated mouse Ig isotype-specific Abs (SouthernBiotech). For TNP-specific Abs, the plates were precoated with 10 μg/ml TNP-BSA. The TMB substrate set (BD Pharmingen) was used, and absorbance at 450 nm was measured. For detection of influenza-specific Ab, plates coated overnight with 100 μl 10^6 MOI/ml UV-inactivated influenza virus in PBS were used.

Adaptive transfer of bone marrow cells
Bone marrow cells were prepared from the tibiae and femurs of 2- to 3-month-old WT or IκBNS KO mice. T and B cells were eliminated from total bone marrow cells by CD4-, CD8-, and B220-negative separation using magnetic beads. Bone marrow cells (2 × 10^6) were injected i.v. injected into irradiated (900 rad) Ly5.1-congenic mice (B6.Ly5.1). Six weeks after the injection, recipient mice were sacrificed, and spleen, peripheral blood, and peritoneal cavity cells were FACs analyzed for donor-derived Ly5.2^+ cells. For competitive reconstruction assays of B cell subsets, one million bone marrow cells prepared from B6.Ly5.1 and IκBNS KO mice were mixed and transferred together into 900 rad irradiated B6 Ly5.1 mice.

Reconstitution of B and T cells in Rag-2−/−deficient mice
B cells were purified from splenocytes of WT and IκBNS KO mice by positive selection of B220-expressing cells using anti-B220-conjugated MACS magnetic beads (Miltenyi Biotec). T cells were purified from lymph nodes of WT mice by depleting B cells with anti-B220-conjugated magnetic beads. FACs analysis between B220, CD4, and CD8 staining showed that B cell preparations contained >98% B220^+ cells, whereas T cell preparations contained >95% CD4^+ and CD8^+ cells. The purified B cells from WT and IκBNS KO mice were mixed with T cells from WT mice (1 × 10^6 each) and injected i.v. into rag-2−/− mice. The reconstituted mice were challenged with 75 μg alum-precipitated TNP-KLH given i.p. 24 h after the transfer. Serum was collected every 7 d after immunization, and the spleen was harvested on day 14 postimmunization.

ELISPOT assay for Ab-secreting cells
Nitrocellulose membranes were coated with anti-IgG Ab at 2.5 μg/ml in PBS for 2 h and then blocked with 5% BSA in PBS for 4 h, both at room temperature. Single-cell suspensions were prepared from spleens, RBCs were depleted, and the cells were incubated with the membranes in 10% FBS, 50 μM 2-μE, and RPMI 1640 at 37°C for 18 h. Membranes were washed in PBS and incubated with HRP-conjugated goat anti-mouse IgG or IgM for 1 h at room temperature followed by detection using an ECL kit (GE Healthcare/Amersham Biosciences). The results are expressed as the mean of Ig-secreting cells per 10^6 spleen cells for three to five experiments.

Semiquantitative RT-PCR analysis
Semiquantitative RT-PCR analysis was performed to compare the relative mRNA levels of μM, μs, γM, and γS in stimulated B cells from WT and IκBNS KO mice. Purified splenic B cells from B6 or IκBNS KO mice were incubated with LPS (5 μg/ml) alone or in combination with IL-4 (10 ng/ml) for 3–5 d and RNA was isolated using TRizol reagent (Invitrogen). One microgram total RNA was used for cDNA synthesis (Roche). Semiquantitative RT-PCR was performed using β-actin or hypoxanthine phosphoribosyltransferase (HPRT) as an internal control. Primers for μM and μS (26) and for germline and postswitch γM and γS (27) were as previously described.

Real-time PCR
RNA isolated using TRizol from purified WT or IκBNS KO B cells was used for both semiquantitative and quantitative real-time PCR analysis. Real-time PCR was performed using the Applied Biosystems TaqMan Universal PCR Master Mix. TaqMan Gene Expression premade probes were used for Blimp-1, Xbp1, Irf4, and β-actin. Probes for IκBNS were custom ordered and sequences are available upon request. The expression level of β-actin was used to normalize the template input. When the relative gene expression levels in WT and IκBNS KO B cells were compared, the mean value of WT B cells was set at 1. Assays were performed in triplicate.

Statistical analysis
The results are shown as the mean ± SD of values obtained from three to five separate experiments. For proliferation, results of a single representative experiment are provided. ELISA data were analyzed by the Student’s t-test to assess the significance of the differences between IκBNS KO and WT groups.

Results
Altered B cell compartments in the absence of IκBNS
We had previously noted a modest reduction in the number of B cells in the spleen and lymph nodes of the IκBNS KO mice. This was confirmed after backcrossing the IκBNS KO mice onto a B6 background for 11 generations. Analyses of cells isolated from the lymph node and spleen of B6 and IκBNS KO mice using an Ab against the B cell marker B220 further demonstrated that the percentage of B cells is lower in the IκBNS KO spleen and lymph node compared with WT mice. This reduction was even more dramatic in the blood of these animals (Fig. 1A). Furthermore, within the IκBNS KO B220^+ population, the distribution of IgM was skewed compared with WT mice. The geometric mean fluorescence intensity of IgM was 216 on IκBNS KO peripheral blood B220^+ cells and 37 on WT B cells (Fig. 1A, Supplemental Fig. 1). These observations led us to perform a more detailed analysis of IκBNS KO B cells as follows.

1. B cells consist of a number of different subpopulations, including terminally differentiated plasma cells, GC B cells, and marginal zone (MZ) B cells. In the periphery, surface IgM^high B cells contain immature or “transitional” B cells and MZ B cells. Therefore, we further analyzed the developmental status of B cells in the absence of IκBNS. As shown in Supplemental Fig. 2, the IκBNS KO has a lower percentage of IgM^high IgM^low cells, which contain the most mature B cells, but B cells from the IκBNS KO spleen have slightly more CD93 (AA4.1^+ positive immature B cells (7.0% in IκBNS WT, 8.2% in IκBNS KO at 2–3 months of age; n = 3). Examination of MZ B cells (B220^+CD21^highCD23^low) revealed a deficit of this population in the spleens of 4-wk-old IκBNS KO mice (Fig. 1B). However, as 10-mo-old IκBNS KO mice have MZ B cells, the development of this population seems delayed rather than blocked in the absence of IκBNS. Histological analysis of frozen sections from the spleens of WT and IκBNS KO mice was performed to further examine MZ B cells. In both WT and IκBNS KO mice, the MZ could be observed as a layer outside of the B cell-rich follicle separated by a layer of MOMA-1^+ MZ-attachment macrophages. The MZ area of IκBNS KO spleens was reduced compared to WT in young mice (Supplemental Fig. 3). Additionally, IκBNS KO mice lacked B1 B cells, a B220^low population found mainly in the peritoneal and pleural cavities (28) (Fig. 1C). Neither the B1a (CD5^+ B1a) nor B1b (CD5− B1b) cells were detected in the absence of IκBNS.

When bone marrow cells from IκBNS KO mice were adaptively transferred to irradiated Ly5.1^+ congenic WT hosts, the donor-derived Ly5.2^+ cells recapitulated the MZ B cell deficiency observed in the IκBNS KO mice (Fig. 2A), demonstrating that this developmental defect is inherent to the IκBNS KO cells. Additionally, we noted reduced generation of GC (Fas^+PNA−) B cells when bone marrow cells were derived from IκBNS KO (Fig. 2A). These results are confirmed in competitive reconstitution assays. When donor cells consisted of Ly5.1^+ WT plus Ly5.2^+ IκBNS KO bone marrow cells, the resulting B220^+ CD21^highCD23^low MZ cells and Fas^−PNA+ GC cells were Ly5.1^+.
and thus derived from WT but not IκBNS KO bone marrow (Fig. 2B). Furthermore, analysis of peritoneal cavity cells after adoptive transfer of WT or IκBNS KO bone marrow showed that CD5+ and CD5−B220low cells were not repopulated when the donor cells were from IκBNS KO mice (Fig. 2A).

IκBNS KO B cells are altered in proliferation
The expression of IκBNS is induced by TCR-mediated signals in T cells (23) and TLR-mediated signals in macrophages and dendritic cells (21, 22). We therefore examined the ability of various stimuli to induce IκBNS expression in B cells. Purified splenic B cells were exposed to LPS, anti-CD40, and anti-IgM. Using real-time PCR we demonstrated that IκBNS RNA expression is induced upon stimulation with each of these mitogens (Fig. 3A).

Splenic B cells stimulated with LPS, anti-CD40, and anti-IgM increased expression of IκBNS within 0.5–6 h, and RNA expression peaked at ∼2 h. The increased RNA levels led to increased IκBNS protein levels (Fig. 3B).

Given that we observed a defect in proliferation of the IκBNS KO T cells (23) and that known B cell mitogens induce IκBNS expression, we next analyzed the proliferation of IκBNS KO B cells. As seen in Fig. 3C, proliferation measured by incorporation of [3H]thymidine in response to LPS and anti-CD40 is reduced in IκBNS KO B cells whereas the response to anti-IgM is slightly higher. This experiment was performed five times and the response of the IκBNS KO B cells to IgM was consistently equivalent or higher. The alterations in proliferation in the IκBNS KO B cells were further confirmed using flow cytometry and

**FIGURE 1.** Altered B cell profiles in IκBNS KO mice. A, IκBNS KO mice exhibit a reduction in the percentage of B220+ B cells in the periphery. Cells prepared from the lymph nodes, spleens, or blood of 9-mo-old B6 or IκBNS KO mice were examined for B220 and IgM surface expression by FACs. Numbers in boxes indicate the percentage of B220+ cells, and the mean fluorescence intensity of IgM–FITC on B220+ cells is given for each sample. B, Abnormal generation of MZ B cells in IκBNS KO mice. B220+ splenic B cells from 4-wk-old or 10-mo-old WT and IκBNS KO mice were examined for CD21 and CD23 by flow cytometry. Young IκBNS KO mice show a reduced percentage of CD21highCD23low MZ B cells. C, IκBNS KO mice lack B1 B cells in the peritoneal cavity. Peritoneal cavity cells isolated from WT or IκBNS KO mice were analyzed for B220+CD5−B220low cells. Three age-matched animals were examined in each set of experiments.

**FIGURE 2.** A developmental defect of MZ, GC, and B1 B cells is an intrinsic property of IκBNS KO B cells. A, T- and B-depleted bone marrow cells (2 × 10⁶) from WT or IκBNS KO mice (Ly5.2) were adoptively transferred to lethally irradiated (900 rad) B6Ly5.1 hosts, and Ly5.2+ donor-derived cells were examined at 6 wk posttransfer. IκBNS KO-derived bone marrow produced reduced CD21highCD23low MZ and PNAhighFas+ GC B cells after gating on B220+ cells. Additionally, KO-derived bone marrow failed to repopulate B1a (CD5−B220int/low) and B1b (CD5−B220low) cells. Two independent experiments were performed and similar results obtained. B, Competitive reconstitution of B cell populations in bone marrow chimera mice. T- and B-depleted bone marrow cells (2 × 10⁶) from WT (Ly5.1) or IκBNS KO mice (Ly5.2) were adoptively transferred to lethally irradiated (900 rad) B6Ly5.1 hosts and donor-derived B220+ cells were examined as described in A. In the upper panel, spleen cells from one bone marrow chimera show Ly5.1 WT- and Ly5.2 KO-derived populations, although fewer KO cells are present. WT Ly5.1+ bone marrow-derived cells repopulated both MZ (CD21highCD23low) and GC (PNA−Fas−) subsets, whereas IκBNS KO Ly5.2+ bone marrow-derived cells did not. Two recipient animals of each type were analyzed.
BrdU cell cycle analysis. Cells were pulsed with BrdU, then stained with anti-BrdU Ab and 7-aminoactinomycin D. Percentages of BrdU+ S-phase cells are indicated. Two independent experiments were performed and similar results obtained.

Transfer to polyvinylidene difluoride membranes, I were treated for 2 h with the indicated reagent or left untreated (None). Nuclear lysates (50 μg) were incubated with the indicated reagent or left untreated (None). Nuclear lysates (50 μg) were incubated with the indicated reagent or left untreated (None). Nuclear lysates (50 μg) were incubated with the indicated reagent or left untreated (None). Nuclear lysates (50 μg) were incubated with the indicated reagent or left untreated (None). Nuclear lysates (50 μg) were incubated with the indicated reagent or left untreated (None). Nuclear lysates (50 μg) were incubated with the indicated reagent or left untreated (None). Nuclear lysates (50 μg) were incubated with the indicated reagent or left untreated (None). Nuclear lysates (50 μg) were incubated with the indicated reagent or left untreated (None). Nuclear lysates (50 μg) were incubated with the indicated reagent or left untreated (None). Nuclear lysates (50 μg) were incubated with the indicated reagent or left untreated (None).

Induction of IκB was determined by Western blot. Arrows indicate two isoforms. HC, H chain of the immunoprecipitating IgG. C, Splenic B cells (CD43+) were incubated for 48 h in the presence of media only (None) or the indicated reagents (0.1, 1, or 10 μg/ml LPS; 0.1, 1, or 5 μg/ml anti-CD40; 10 μg/ml anti-IgM). Proliferation was measured by [3H]thymidine incorporation. *p < 0.01. D, BrdU cell cycle analysis. Cells were pulsed with BrdU, then stained with anti-BrdU Ab and 7-aminoactinomycin D. Percentages of BrdU+ S-phase cells are indicated. Two independent experiments were performed and similar results obtained.

CFSE labeling (data not shown). The lower proliferative response for LPS and anti-CD40 in IκBNS KO B cells results from reduced S-phase progression in the absence of IκBNS (Fig. 3D). Signaling induced by LPS, anti-CD40, and anti-IgM involves the NF-κB pathway (reviewed in Ref. 4), consistent with the fact that disruption of IκBNS impacts the proliferative response in B cells. Because all three agents induce expression of IκBNS, the fact that the proliferation induced by anti-IgM is not reduced in the IκBNS KO seems to be a contradiction. However, as described above and as shown in Fig. 1, IκBNS KO B cells exhibited an altered distribution of IgM compared with WT B cells. The increased number of IgM molecules on a cell may mask a decreased proliferative response to the crosslinking of IgM in the IκBNS KO B cells.

Defective Ig production in IκBNS KO mice

Because a primary function of B cells is to produce Ab, we determined the level of IgG in sera from IκBNS KO and WT mice (Fig. 4A). Levels of IgM and IgG3 were dramatically lower in the IκBNS KO mice and levels of IgG2c appeared slightly reduced. IgG1 and IgG2b Ig levels were equal to those in WT mice, however. Although the surface levels of IgM appeared higher on the IκBNS KO B cells, less IgM was secreted in vivo. To examine the possibility that the regulation of membrane versus secretory IgM production was defective in IκBNS KO B cells, we performed flow cytometric analysis of surface versus intracellular IgM in B cells purified from the spleens of WT and IκBNS KO mice. Whereas cytoplasmic IgM was virtually undetectable in stimulated and unstimulated IκBNS KO B cells, the surface IgM levels were much higher compared with WT (Supplemental Fig. 4), suggesting a defect in IgM secretion. Both the lack of B1 B cells and the delayed differentiation of MZ B cells as described above, two major cellular sources of serum IgM (28, 29), as well as a decreased level of the secreted form of IgM mRNA (Fig. 4B) likely account for the low serum IgM in IκBNS KO mice.

The production of classes of Igs other than IgM requires switching at the DNA level, and we therefore investigated switching using an in vitro assay. Spleen B cells were exposed to LPS or LPS plus IL-4 to induce production of IgG3 or IgG1, respectively (Fig. 4C). Measurement of both germline and post-switch transcription in B cells was carried out by PCR using primers designed to produce distinct DNA products arising from either the germline or recombined DNA configurations. Treatment of both WT and IκBNS KO B cells with LPS or LPS plus IL-4 resulted in increased transcription of activation-induced cytidine deaminase (AID) to an equal extent relative to the HPRT control (Fig. 4C, lower panel). AID is required for the process of IgH class-switch recombination (CSR) (27), and the equal induction of this enzyme in both WT and IκBNS KO B cells implies that the process of class switching has been successfully initiated, at least with respect to this enzyme. Transcription from the germline configuration was approximately equal for the WT and IκBNS KO B cells, and the level of transcription from the germline configuration was increased by the addition of LPS plus IL-4 for IgG1 and by addition of LPS alone for IgG3 in both WT and IκBNS KO cells (Fig. 4C). The level of transcription from the post-switch configuration of IgG1 appeared to be reduced in the IκBNS KO cells, whereas transcription from the post-switch IgG3 configuration was undetectable. The defect in class switching was confirmed when anti-CD40 was used to stimulate IgG3 and IgG1 class switching in the presence and absence of IL-4, respectively (Supplemental Fig. 5). Thus, the absence of IκBNS dramatically impaired class switching to the IgG3 H chain gene as reflected in the lack of detectable IgG3 in the IκBNS KO mouse serum.

Given these deficiencies, are the IκBNS KO mice capable of mounting a humoral immune response? We performed ELISA assays to measure the TNP-specific IgM and IgG produced by IκBNS KO and WT mice before and after i.p. immunization with TNP-Ficoll, a type II T-independent Ag. At 1 and 2 wk post-immunization, IκBNS KO mice produced no detectable TNP-
mice are on a B6 background: one mouse was WT, four mice were I
functions, we performed adoptive transfer assays into B6.Rag-2
assessed in cDNA prepared from cells cultured in the presence and absence
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The finding that there are fewer ASCs in the spleens of IxkBNS KO mice led us to examine the process of plasma cell differentiation in IxkBNS KO B cells. B cells from spleens were cultured for 5 d in the presence of LPS to induce plasma cell differentiation. Proliferation was followed using CFSE staining, whereas plasma cell differentiation was measured by CD138 (syndecan-1) expression. As shown in Fig. 7A, LPS-stimulated plasma cell differentiation was decreased in IxkBNS KO B cells. Addition to the culture of IL-4, IFN-γ, or TGF-β in combination with LPS resulted in production of IgG1∗, IgG2c∗, or IgG2b∗ cells, respectively, in the culture as determined by FACS of cell surface IgG (Supplemental Table I). Cells incubated with LPS alone expressed surface IgG3 (Supplemental Table I). The percentage of IgG3∗ or IgG2c∗ cells was lower in the IxkBNS KO cultures than in the WT cultures, but the percentages of IgG1∗ or IgG2b∗ cells were approximately equivalent (Supplemental Table I). However, the percentage of CD138∗ cells was 4- to 6-fold lower in the IxkBNS KO cultures under every culture condition (Fig. 7B), indicating that IxkBNS KO B cells were defective in plasma cell differentiation.

The differentiation of plasma cells involves a well-studied transcriptional program (reviewed in Refs. 32, 33), including Prdm-1 (Blimp-1), Xbp1, and Irf4. Given the role of IxkBNS in gene regulation, we used real-time PCR to examine the levels of expression of these transcription factors during LPS-stimulated culture of B cells from WT and IxkBNS KO mice. Splenic B cells from WT and IxkBNS KO mice were stimulated with LPS for 24, 48, and 72 h. RNAs prepared at these time points were examined for levels of Blimp-1, Xbp1, and Irf4. As shown in Fig.

FIGURE 4. IxkBNS KO mice manifest B cell defects in serum Ig levels and IgG3-switch recombination. A, Basal Ig type levels in the sera from naive IxkBNS KO (−) mice and age-matched WT/HET mice (+) were measured by ELISA. Ig levels <0.1 μg/ml were plotted on the base line. The serum IgA levels of IxkBNS KO mice were similar to those of WT mice (data not shown), p < 0.05 for IgG2c; p < 0.01 for IgM and IgG3. All mice are on a B6 background: one mouse was WT, four mice were IxkBNS heterozygous, and four mice were IxkBNS KO. B, Splenic B cells (CD43−) from WT and IxkBNS KO mice were stimulated in vitro with LPS (5 μg/ml) alone or LPS plus cytokines (10 ng/ml) as indicated for 4 d. Semi-
quantitative RT-PCR analysis of μM, μS, and HPRT mRNA was performed. C, Germline and postswitch transcripts for IgG1 and IgG3 were assessed in cDNA prepared from cells cultured in the presence and absence of LPS alone or in combination with IL-4, respectively. Lower panels, AID and HPRT expression before and after stimulation with LPS or LPS plus IL-4 were examined. AID expression confirms that switch recombination processes have initiated and HPRT expression confirms equivalent loading of RNA in each sample. For B and C, two independent experiments were performed and similar results were obtained.

specific IgM or IgG3, whereas such Abs were detected in WT mice at both time points (Fig. 5A). Using TNP-KLH as a T-
dependent Ag, we next measured the TNP-specific IgM and IgG1 produced by IxkBNS KO and WT mice 1 and 2 wk after immunization. As shown in Fig. 5B, the IxkBNS KO mice produced little, if any, TNP-specific IgM. Production of Ag-specific IgG1 appeared to be delayed by a week, although the final levels of IgG1 were equivalent to those of WT mice. Because we previously showed that the absence of IxkBNS also alters T cell functions, we performed adoptive transfer assays into B6.Rag-2−/− mice using purified B cells from WT or IxkBNS KO spleen mixed with WT T cells. Upon immunization with TNP-KLH, the production of IgM by IxkBNS KO B cells remained decreased, showing that the decreased production of IgM was a B cell-
intrinsic defect (Supplemental Fig. 6). However, in the presence of WT T cells, production of IgG1 from IxkBNS KO B cells was equivalent to that of WT B cells, indicating that the defective IxkBNS T cells altered the production of IgG1 by the IxkBNS KO B cells.

The humoral response to infection in the absence of IxkBNS was assayed using a murine-adapted influenza A virus, PR8. WT and IxkBNS KO mice were immunized i.p. and then infected intranasally ~2 wk later with influenza virus. The survival curve in Fig. 5C shows that IxkBNS KO mice were more severely affected by influenza A, with 50% succumbing to infection. The asterisk in Fig. 5C indicates the single death of one WT mouse after receiving a sublethal viral challenge. Ag-specific Ab production in response to influenza immunization was then determined (Fig. 5D). Whereas IxkBNS KO mice were deficient in production of influenza-specific Abs of the IgM, IgG1, and IgG2b classes (data not shown), the most significant deficiency was in IgG2c Ig, as shown in Fig. 5D. This is noteworthy as this class of Igs affords a prominent protective response against influenza A in the murine system (30, 31).

To examine the B cell response histologically, we assayed GC formation using immunization with SRBCs in WT and IxkBNS KO mice (Fig. 5E). Five days after immunization, spleens were removed and sections prepared by staining with H&E followed by PNA staining to visualize GCs where core-1 glycans are O-linked without sialic acid adducts and, hence, are PNA-reactive. Although GCs were clearly evident in the spleens of WT mice, no GC formation was detected in the spleens of the five IxkBNS KO mice examined. Given the fact that the TNP-KLH IgG1 response appeared delayed, this experiment was repeated and GC formation examined at 10 d after immunization. Again, no GC formation was observed in the IxkBNS KO mice whereas areas of PNA reactivity were clear in the WT mice (data not shown).

IxkBNS KO B cells exhibit reduced plasma cell differentiation

One path of B cell differentiation culminates in the production of plasma cells, which are terminally differentiated, nondividing Ab-secreting cells (ASCs). Determination of the ex vivo number of ASCs from the spleens of WT and IxkBNS KO mice showed that in the IxkBNS KO spleen there were fewer B cells secreting all of the types of the Igs assayed, but the most dramatic differences were in IgM-, IgG3-, and IgG2b-producing splenic cells. Fig. 6 shows results of an individual ELISPOT assay and statistics compiled from three to five experiments in spleen (Fig. 6B) and bone marrow (Fig. 6C). Note that IgM and IgG3 ASCs were reduced in both organs.

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Similar results were obtained in both experiments. C, Twelve age-matched (8- to 12-wk-old) male WT and IκBNS KO mice were immunized i.p. with influenza virus PR8 at an MOI of $1 \times 10^3$. Fifteen days after immunization, mice were intranasally infected with $1 \times 10^6$ PR8 virus and survival was followed. Day 1 on the survival graph is the day of infection. D, Virus-specific IgG2c Abs were determined by ELISA in WT and IκBNS KO mice before immunization (Prebleed) and 15 d postimmunization (PI). Filled circles represent WT samples; open circles represent IκBNS KO samples. The asterisk in C and D denotes one WT mouse that succumbed to viral infection and did not produce IgG2c in response to PR8. At 15 d postimmunization, $p < 0.01$ for WT versus KO. E, Spleen sections were prepared from WT and IκBNS KO mice 5 d after i.p. injection of SRBCs. Sections were stained with PNA and with H&E. Original magnification $\times 20$. Arrows indicate PNA+ areas in active GCs in WT mice. IκBNS KO mice did not develop active GCs. Five WT and five KO mice were examined with similar results.

Discussion

Our findings that deletion of IκBNS, a member of the IκB family of NF-κB inhibitors, affects the development and function of B cells is consistent with the important role of NF-κB in this cell lineage. As stated above, NF-κB was first identified in B cells (1) and plays a major role in cells involved in inflammation and the immune response. B cells are primarily divided into B1 cells, follicular B cells, and MZ B cells (reviewed in Ref. 34). The origins of each cell type, as well as the transitions that may occur between these cell types, have long been an area of intense analysis. Follicular and MZ B cells are the most abundant mature B cells, with most peripheral B cells being of the follicular type. Follicular B cells are mobile, circulate within the periphery, and are found in the follicles of the splenic white pulp. They participate in both T cell-dependent and T cell-independent immune responses (35). MZ B cells, in contrast, remain at the marginal sinus of the spleen (29, 36), although they migrate to the follicles and back, transporting immune complexes containing Ag to the follicles (37, 38). MZ cells participate in T cell-independent responses and respond rapidly to blood-borne pathogens, a property shared with B1 B cells (29, 39). B1 B cells comprise most of the B cells in the peritoneal and pleural cavities in mice (40, 41) and produce IgM and IgG3 serum Abs (42, 49). The B1 subset is divided into B1a and B1b cells based on the levels of CD5 on these two cell populations. B1a cells, which express CD5, comprise the earliest B cells arising in the neonate to become a self-replenishing population (43, 44) that is the main source of "natural" IgM Abs (44). B1b cells arise from the neonate but are derived from adult bone marrow as well (45, 46). In addition to these developmental differences, B1a and B1b cells differ in function, with B1a contributing to innate immunity and B1b to adaptive immunity (47).

As is true for T cells (23), the absence of IκBNS affects B cell proliferation in response to multiple stimuli, including LPS, anti-CD40, and anti-IgM (Fig. 3). The diminution in LPS-triggered activation in the IκBNS KO is most obvious in $[^{3}H]$thymidine incorporation, whereas that of anti-CD40 is more pronounced in the BrdU experiments. Consistent with this result, each of these stimuli induced IκBNS at both the protein and mRNA level (Fig. 3). LPS also induced IκBNS in gut and peritoneal macrophages (21, 22), presumably acting through the TLR4 (48) in a MyD88-dependent manner (22). However, IκBNS KO B cells sustained a high level of surface IgM expression compared with WT B cells and, thus, even if the signal via each IgM molecule is weaker than in WT B cells, the abundance of IgM on the surface may compensate for the lack of IκBNS. These opposing effects may account for preservation of the anti-IgM signaling in IκBNS KO B cells. Activation via CD40 induces NF-κB, and the reduced
proliferation in the absence of IκBNS is consistent with this. Furthermore, the reduced response to anti-CD40 correlates with the reduction (or delay) in the response of IκBNS KO B cells to T-dependent Ags as shown in Fig. 5.

Production of Ig is also altered in IκBNS KO mice. Measurement of steady-state serum levels (Fig. 4) show that IgM and IgG3 were drastically reduced, IgG2c was somewhat reduced, and IgG1 and IgG2b were approximately equal compared with levels found in WT mice. As B1 cells are the source of serum levels of IgM and IgG3 (reviewed in Ref. 49), the absence of these Igs can be partially explained by the lack of B1 cells in these mice. Additionally, B cells from the IκBNS KO mice produced less secreted IgM as evident by the lack of serum IgM and as assayed by PCR for the secreted versus membrane form of IgM mRNA (Fig. 4B). Given that the reduction in the response appears more profound than the reduction in MZ B cell numbers, perhaps this implies functional defects in residual cells.

CSR to IgG3 at the DNA level was not detected in IκBNS KO B cells (Fig. 4) using an in vitro system that mimics CSR (reviewed in Ref. 50). In these experiments, LPS was used with or without additional cytokines to direct CSR to particular H chain isotypes. However, a low level of IgG3 protein expression was observed (Fig. 7). Presumably, the LPS/cytokine milieu directs germline transcription to distinct isotypes (reviewed in Ref. 51), resulting in modulation of CSR. LPS in combination with IL-4 induces CSR to IgG1 in both WT and IκBNS KO B cells, and induction of AID is equivalent in both cell types, indicating that at least this component of the CSR machinery is operational in IκBNS KO B cells. Clearly a specific effect of deletion of IκBNS is an alteration in IgG3 CSR. This does not appear to be due to a reduction in transcription through the IgG3 locus, as germline transcription before and after LPS addition appears to be equivalent for both WT and IκBNS KO mice (Fig. 4). There is precedence for transcription factors regulating CSR. For example, T-bet acts either by controlling germline transcripts or by mediating IgH locus access to other transcription factors and recombination-inducing machinery (52) and affects T-independent IgG2a production (53). Ikaros was shown to repress transcription of IgG2a and IgG2b, thus increasing transcription from and recombination to other H chain genes (54). Other proteins affecting CSR specificity include TGF-β, which induces Smad and Runx that promote CSR to IgG2b and IgA, and IL-4, which induces the transcriptional activator Stat6 whose activity promotes CSR to IgG1 and IgE (reviewed in Ref. 55). Perhaps more pertinent to our finding of the role of IκBNS in CSR, NF-κB proteins and, in particular, NF-κB p50 have been shown to contribute to isotype specificity in CSR (56–60). In addition to proteins that contribute to the isotype specificity of CSR, the switch region sequences that lie upstream of each Cγ gene also function to mediate Ab class switching (Ref. 61 and references therein), and the roles of the various proteins and switch regions are an area of active investigation. The finding that IκBNS contributes to isotype switching extends further the list of involved proteins.

Along with the observed serum Ig and class-switch phenotypes in the IκBNS KO mice, we found a reduction in ASCs assayed ex vivo from the spleens of IκBNS KO mice (Fig. 6). The pattern of reduction of ASCs corroborates the observed differences in serum Ig values in the IκBNS KO mice. Furthermore, the IκBNS KO mice failed to produce any Ag-specific IgM or IgG3 Abs in response to TNP-Ficoll. Given that T-independent responses are predominantly mediated by MZ B cells (29, 39, 62), the lack of response to TNP-Ficoll correlates with the reduction in MZ B cells in the IκBNS KO mouse. Furthermore, the lack of Ag-specific IgM may result in part from the reduction in MZ B cells but also from the inability of IκBNS KO B cells to produce...
secreted IgM (Fig. 4). This is observed as well in the response to TNP-KLH, a T-dependent Ag, as there is a delayed production of Ag-specific IgG1 but no production of IgM (Fig. 5), although this may also be affected by intrinsic alterations in the IκBNS KO T cells (Supplemental Fig. 6). When WT T cells were mixed with either WT or IκBNS KO B cells and these mixtures were used to reconstitute rag-2<sup>−/−</sup> mice, the IgG1 response was delayed and the response in IκBNS KO mice reached levels similar to that in WT mice, albeit the kinetics of the response were a bit slower in the reconstitution experiment (Supplemental Fig. 6). The phenotype of the reduced secretion of IgM is quite clearly represented in both Fig. 5 and Supplemental Fig. 6, however, and is thus an intrinsic B cell defect. Additionally, flow cytometric analysis of spleen cells in the recipient mice in Supplemental Fig. 6 showed 12.5 ± 1.1% CD138<sup>+</sup>B220<sup>low</sup> plasma cells in recipients of WT B and T cells and 2.8 ± 0.4% CD138<sup>+</sup>B220<sup>low</sup> plasma cells in recipients of IκBNS KO B and T cells (data not shown).

IκBNS KO B cells also exhibited a reduced response to influenza virus. WT mice immunized by i.p. injection of influenza successfully withstood influenza infection and produced influenza-specific IgG2c Abs, the major isotype found in mice surviving influenza infections (30, 31). IκBNS KO mice, in contrast, produced less influenza-specific IgG2c and were more susceptible to infection after immunization. The lack of formation of GCs in IκBNS KO mice upon immunization is additional evidence of a functional defect in IκBNS KO B cells and may explain the reduction in the Ag-specific immune response, as the GC is where B cell affinity maturation and class switching occur, resulting in Ag-specific ASCs (reviewed in Ref. 63). Impairment of GC formation is demonstrated by the lack of PNA staining after immunization with a T-dependent Ag, SRBCs (64). The presence of a low level of Ag-specific IgG2c Abs can be explained by the fact that some Ab responses can be generated in the absence of GC formation (65). Note, however, that mice deficient for Bcl-3, an IκB family member to which IκBNS has a strong homology, also fail to develop a GC response and fail to produce influenza-specific Abs, although, in contrast to the IκBNS KO mice, they do produce influenza-specific IgM Abs (66). Additionally, the serum levels of all Ig types were similar between WT and Bcl-3 KO mice.

The GC response is dependent on T cell help provided to B cells through CD40 (reviewed in Ref. 63). We have shown that proliferation of IκBNS KO B cells through CD40 is reduced and the dampening of CD40 signaling in the absence of IκBNS may affect the ability of IκBNS KO B cells to participate in a GC response. Furthermore, B cells express TLRs, which participate in the innate and adaptive immune response (reviewed in Ref. 67). IκBNS plays a role in the downstream signaling of those TLRs that work through MyD88-dependent mechanisms (22). Thus, the absence of IκBNS could affect both innate and adaptive immunity by altering TLR activation. In particular, B1 and MZ B cells respond to TLR ligands in T-independent responses, resulting in production of low-affinity IgM (reviewed in Ref. 67).

Young IκBNS KO mice exhibited a reduced MZ B cell population, but this population reached WT levels after the mice were >6 mo old (Fig. 1 and data not shown). MZ B cell development is blocked in mice carrying a targeted gene deletion of the NF-κB p50 protein (56, 68, 69). We and others have shown that IκBNS interacts with p50 (14, 21), suggesting that this complex and genes regulated by it play a role in MZ B cell development. In addition to NF-κB p50, alterations in the expression of NF-κB p65 (RelA), c-Rel (68) and RelB (70) also affect MZ B cell development.

The absence of IκBNS also affects the development of plasma cells. Plasma cells are a final differentiation state of B cells and are basically an Ig-secreting factory. These cells emerge from a GC reaction and express Ag-specific high-affinity Ig, having undergone somatic hypermutation within the GC. Plasma cells are the source of persistent Ag-specific Ab titers (71) and, thus, the lack of development of plasma cells may contribute to the fact that the IκBNS KO mice have reduced anti-influenza specific Abs. The differentiation of plasma cells has a defined transcriptional program, including Prdm-1/Blimp-1, Xbp1, and Irf4 (reviewed in Refs. 32, 33). Our analysis of these factors in WT and IκBNS KO B cells during in vitro differentiation shows that in the absence of IκBNS, these transcription factors were decreased and in vitro plasma cell differentiation was impaired (Fig. 7). The frequency of ASCs found in the spleen of the IκBNS KO mice was also reduced (Fig. 6). The development and survival of plasma cells in special niches in the bone marrow is only partially understood, and the reduction in expression of Prdm-1/Blimp-1, Xbp1, and Irf4 in the absence of IκBNS suggests that IκBNS may control a point upstream of the plasma cell transcriptional program.

B1 B cells are missing from IκBNS KO mice. The origin of the B1 B cell lineage is controversial (42, 72), and recent findings suggest that signal strength drives B cell fate (73) or that a specific progenitor exists for B1 B cells (46). Altered expression of molecules that affect signal strength through the BCR affect the B cell population in mice. For example, construction of transgenic mice using the LMP2A transgene in place of Igα demonstrated that mice with a high copy number of the transgene, which mimics strong BCR signaling, have higher numbers of B1 cells, whereas mice carrying fewer LMP2A transgenes, consistent with a lower intensity BCR signal, have lower numbers of B1 cells (73). Similarly, more B1 cells are also found in mice that lack negative regulators of BCR signaling (42) or express higher levels of BCR (72, 74). This suggests that IκBNS may play a role in strengthening the BCR signal and that in the absence of IκBNS a reduced BCR signal results in the lack of B1 cells. A similar role for IκBNS was suggested in T cells (23). IκBNS was cloned as a gene that was induced in thymocytes after N15TCRtg rag-2<sup>−/−</sup> mice were injected with VSV8, a negatively selecting peptide for this TCR (14, 75). Positively selecting peptides did not induce expression of IκBNS (75). As negative selection is associated with a strong TCR signal and positive selection is associated with a weaker TCR signal, the expression of IκBNS appeared to correlate with a strong TCR signal. Furthermore, production of IL-2 by T cells is thought to require a strong TCR signal, and in the absence of IκBNS, IL-2 production is reduced (23), again associating IκBNS with receptor signal strength.

The phenotype of p50 KO mice has some commonalities with the phenotype of IκBNS KO mice, which is to be expected, as IκBNS has been demonstrated to interact with p50 (14, 21). Specifically (as reviewed in Ref. 13), p50 KO mice display normal development but are abnormal in immune function. As in the IκBNS KO, p50 KO B cells proliferate poorly to LPS and CD40 and display abnormal H chain switching due to lack of transcription through that region. In fact, p50 and p65 were found to bind the IgG3 switch region, and p50 KO mice are specifically impaired in switching to IgG3 (59, 60, 76) and have a reduced MZ B cell population (68).

In summary, characterization of B cells in our IκBNS KO mouse uncovers a pleiotropic phenotype, which is consistent with other factors that affect NF-κB activity. B cell functions, including Ig production, plasma cell development, and isotype switching, are impaired and particular B cell populations such as B1 cells and MZ B cells are reduced in comparison with WT mice. Although not shown, the development of pro-B and pre-B cells in young adult bone marrow was not affected by IκBNS deletion, arguing
that the function of this regulator is targeted to the mantle zone and follicular B cell compartments, Ig switching processes, and plasma cell development. The phenotype overlaps that of p50 KO mice, correlating with the fact that IκBNS and p50 interact. That the IκBNS KO phenotype also overlaps in part that of the Bcl-3 KO is noteworthy because IκBNS and Bcl-3 are homologous members of the IκB family of NF-κB inhibitors. These results reveal a significant role for IκBNS in development of the B cell arm of the immune system and the humoral response in addition to the already defined role of IκBNS in T cells, macrophages, and dendritic cells.

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Disclosures

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