

IKK-induced NF- κ B1 p105 proteolysis is critical for B cell antibody responses to T cell-dependent antigen

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The importance of I κ B kinase (IKK)-induced proteolysis of NF- κ B1 p105 in B cells was investigated using *Nfkb1^{SSAA/SSAA}* mice, in which this NF- κ B signaling pathway is blocked. *Nfkb1^{SSAA}* mutation had no effect on the development and homeostasis of follicular mature (FM) B cells. However, analysis of mixed bone marrow chimeras revealed that *Nfkb1^{SSAA/SSAA}* FM B cells were completely unable to mediate T cell-dependent antibody responses. *Nfkb1^{SSAA}* mutation decreased B cell antigen receptor (BCR) activation of NF- κ B in FM B cells, which selectively blocked BCR stimulation of cell survival and antigen-induced differentiation into plasmablasts and germinal center B cells due to reduced expression of Bcl-2 family proteins and IRF4, respectively. In contrast, the antigen-presenting function of FM B cells and their BCR-induced migration to the follicle T cell zone border, as well as their growth and proliferation after BCR stimulation, were not affected. All of the inhibitory effects of *Nfkb1^{SSAA}* mutation on B cell functions were rescued by normalizing NF- κ B activation genetically. Our study identifies critical B cell-intrinsic functions for IKK-induced NF- κ B1 p105 proteolysis in the antigen-induced survival and differentiation of FM B cells, which are essential for T-dependent antibody responses.

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Abbreviations used: BAFF, B cell activation factor; BCR, B cell antigen receptor; FM, follicular mature; GC, germinal center; HEL, hen egg lysozyme; I κ B, inhibitor of NF- κ B; IKK, I κ B kinase; NF- κ B, nuclear factor κ B; PC, plasma cell; MZ, marginal zone; NP-CGG, 4-Hydroxy-3-nitrophenylacetyl hapten conjugated to chicken gammaglobulin; SRBC, sheep red blood cells; TD, T cell-dependent.

NF- κ B transcription factors, which are composed of dimers of Rel polypeptides, regulate gene expression by binding to κ B elements in the promoters and enhancers of target genes (Ghosh et al., 1998). Inactive NF- κ B dimers are sequestered in the cytoplasm of unstimulated cells by interaction with proteins of the inhibitor of NF- κ B (I κ B) family, which includes I κ B α , I κ B β , I κ B ϵ , and NF- κ B2 p100. After appropriate agonist stimulation, the canonical NF- κ B signaling pathway stimulates the I κ B kinase (IKK) complex, which is composed of IKK1 (IKK α) and IKK2 (IKK β) kinases and the regulatory ubiquitin-binding protein NEMO (IKK γ), to phosphorylate I κ B α (Karin and Ben-Neriah, 2000). This promotes K48-linked ubiquitination of I κ B α and subsequent degradation by the proteasome, releasing associated NF- κ B1 p50-RelA and NF- κ B1 p50-c-Rel dimers to translocate into the nucleus and modulate gene expression. The proteolysis of both I κ B β and I κ B ϵ is controlled by the IKK complex in a

similar fashion. A subset of NF- κ B agonists activates an alternative NF- κ B signaling pathway, which induces IKK1 to phosphorylate NF- κ B2 p100 promoting its partial proteolysis by the proteasome to produce p52, which is principally associated with RelB (Beinke and Ley, 2004).

Most of our knowledge about the specific functions of NF- κ B activation in mature B cells is based on in vitro experiments with purified splenic B cells from mice deficient in specific Rel proteins (Kaileh and Sen, 2012). These studies have suggested important roles for canonical NF- κ B activation in B cell growth, proliferation, and survival after B cell antigen receptor (BCR) stimulation (Grumont et al., 1999; Grumont et al., 1998, 2002). Whole animal studies have also demonstrated a requirement

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for NF- κ B family members in the B cell response to antigen. For example, NF- κ B1 or c-Rel deficiency diminishes the antibody response, whereas compound NF- κ B1 and c-Rel deficiency results in a complete block (Pohl et al., 2002). However, because both NF- κ B1 and c-Rel have essential roles in dendritic cells and T cells (Gerondakis and Siebenlist, 2010a), it has remained unclear whether NF- κ B activation in B cells is required for optimal antibody responses.

The cell-intrinsic functions of canonical NF- κ B activation in B cell physiology in vivo have been investigated genetically by conditional deletion of components of the IKK complex in the B cell lineage, using a CD19-Cre driver mouse strain. Although ablation of either IKK2 or NEMO does not affect B cell development in the BM, it does lead to the disappearance of mature B lymphocytes (Pasparakis et al., 2002; Li et al., 2003). In line with this, mature B cells fail to accumulate in the periphery in the combined absence of c-Rel and RelA (Grossmann et al., 2000). Similarly, mice with mutations in components of the alternative NF- κ B signaling pathway, which regulates NF- κ B2 p100 proteolysis to p52, are also deficient in mature B cells, whereas B cell development in the BM is largely unaffected (Gerondakis and Siebenlist, 2010b; Kaileh and Sen, 2012). The alternative pathway is activated downstream of the receptor for B cell activation factor (BAFF), which promotes peripheral B cells survival and determines the size of the B cell compartment (Mackay et al., 2010), and CD40 (Kaileh and Sen, 2012). Together these genetic studies have established that NF- κ B activation has a critical role for the development and/or homeostasis of mature B cells. However, the requirement for NF- κ B activation to maintain normal mature B cell numbers has precluded the use of conditional knockout strains lacking IKK subunits in B cells to determine the B cell-intrinsic function of NF- κ B activation in humoral immunity (Pasparakis et al., 2002; Li et al., 2003; Derudder et al., 2009).

NF- κ B1 p105 functions as a cytoplasmic I κ B through binding to preformed NF- κ B dimers via its C-terminal ankyrin repeat region, and to Rel monomers via its N-terminal Rel homology domain (Savinova et al., 2009). NF- κ B1 p105 is also the precursor of p50, which is produced constitutively by partial proteolysis (processing) of p105 by the proteasome after its monoubiquitination on multiple lysine residues by an unknown E3 ligase (Kravtsova-Ivantsiv et al., 2009). Activation of the canonical NF- κ B pathway induces IKK2 to phosphorylate two serines in the C-terminal PEST region of p105 (Lang et al., 2003; Yang et al., 2012), creating a binding site for SCF β -TrCP E3 ligase, which catalyzes the subsequent K48-linked polyubiquitination of p105 and proteolysis by the proteasome. This can result either in the complete degradation of p105, releasing associated Rel subunits, or the processing of p105 to p50, both of which can potentially modulate NF- κ B-dependent transcription (Srisanktharajah et al., 2009; Yang et al., 2012). In addition, IKK-induced p105 proteolysis is an essential step in the activation of the p105-associated MEK1/2 kinase TPL-2, which regulates ERK-1/2 MAP kinase activation in TLR-stimulated macrophages (Beinke et al., 2004; Waterfield et al., 2004; Yang et al., 2012). The role of

IKK-induced proteolysis of NF- κ B1 p105 in regulating the development and function of B cells, via NF- κ B and/or TPL-2, remain unknown.

In the present study, the effect of blocking IKK phosphorylation of NF- κ B1 p105 in B cells was investigated using *Nfkb1^{SSAA/SSAA}* knock-in mice, which express mutant p105^{SSAA} that is resistant to signal-induced proteolysis due to mutation of the IKK2-target serines to alanines (Srisanktharajah et al., 2009). We found that *Nfkb1^{SSAA}* mutation had no effect on B cell development in the BM, or on the number of follicular mature (FM) B cells in the spleen or LNs. However, analysis of mixed BM chimeras in which the *Nfkb1^{SSAA}* mutation was restricted to B cells revealed that *Nfkb1^{SSAA}* FM B cells were completely unable to mount an immune response to a T cell-dependent (TD) antigen, failing to develop into antigen-specific plasmablasts or germinal center (GC) B cells. In contrast, TPL-2 deficiency in FM B cells did not affect a TD antibody response. The *Nfkb1^{SSAA}* mutation was found to decrease BCR activation of NF- κ B in FM B cells, which prevented BCR signaling promoting cell survival, but had minimal inhibitory effects on BCR-induced growth and proliferation. Significantly, we also discovered that *Nfkb1^{SSAA}* mutation reduced NF- κ B-dependent induction of the IRF4 transcription factor after BCR ligation, which prevented antigen-induced differentiation of FM B cell to plasmablasts and GC B cells. All of the inhibitory effects of *Nfkb1^{SSAA}* mutation on FM B cell function were overcome by normalizing NF- κ B activation genetically. Our results demonstrate that the regulation of NF- κ B activation in B cells by IKK-induced proteolysis of NF- κ B1 p105 is essential for antibody responses to TD antigens.

RESULTS

Nfkb1^{SSAA} mutation does not affect steady-state numbers of mature follicular B cells

To investigate the role of IKK-induced p105 proteolysis in B cell development and homeostasis, we analyzed B cell subpopulations in the BM, secondary lymphoid organs (spleen and lymph nodes), and peritoneal cavity of *Nfkb1^{SSAA/SSAA}* (*Nfkb1^{SSAA}*) and WT mice by flow cytometry. B cell development in the BM was similar between both genotypes, with similar proportions and absolute numbers of pro-B cells, pre-B cells, and immature B cells (Fig. 1 A and Fig. S1 A). The numbers of mature recirculating B cells in the BM were also unaffected by *Nfkb1^{SSAA}* mutation. Total numbers and proportions of B cells in spleen and peripheral lymph nodes, respectively, were equivalent in *Nfkb1^{SSAA/SSAA}* and WT mice (Fig. 1, B and C; and Fig. S1, B and C). Numbers of splenic FM B cells were also similar. In contrast, marginal zone (MZ) B cells number in the spleen were substantially reduced by *Nfkb1^{SSAA}* mutation, whereas the numbers of transitional type 1 (T1) and type 2 (T2) B cells were fractionally increased (Fig. 1 B). The proportion of B1a cells in the peritoneal cavity was also significantly reduced in *Nfkb1^{SSAA/SSAA}* mice compared with WT (Fig. 1 D and Fig. S1 D). However, the fractions of peritoneal B1b and B2 cells were similar between genotypes.

In summary, mutation of the IKK target serines on NF- κ B1 p105 did not alter the overall number of peripheral mature B cells, in marked contrast to deletion of IKK2 or NEMO in the B cell lineage that results in substantially reduced numbers of such cells (Pasparakis et al., 2002). However, MZ B cell numbers were significantly reduced by *Nfkb1^{SSAA}* mutation, in line with the known importance of NF- κ B for the production of this subset of B cells in the spleen (Pillai and Cariappa, 2009). Mixed BM chimeras demonstrated that the reduction in MZ

B cells resulted from a B cell-autonomous effect of the *Nfkb1^{SSAA}* mutation and confirmed that *Nfkb1^{SSAA}* mutation did not affect FM B cell numbers (Fig. 1 E).

Nfkb1^{SSAA/SSAA} B cells cannot mount an antibody response to a TD antigen

Measurement of steady-state levels of Ig in serum revealed significantly reduced IgM (Fig. 1 F), consistent with the reduction in MZ B cells in the spleen and B1a cells in the

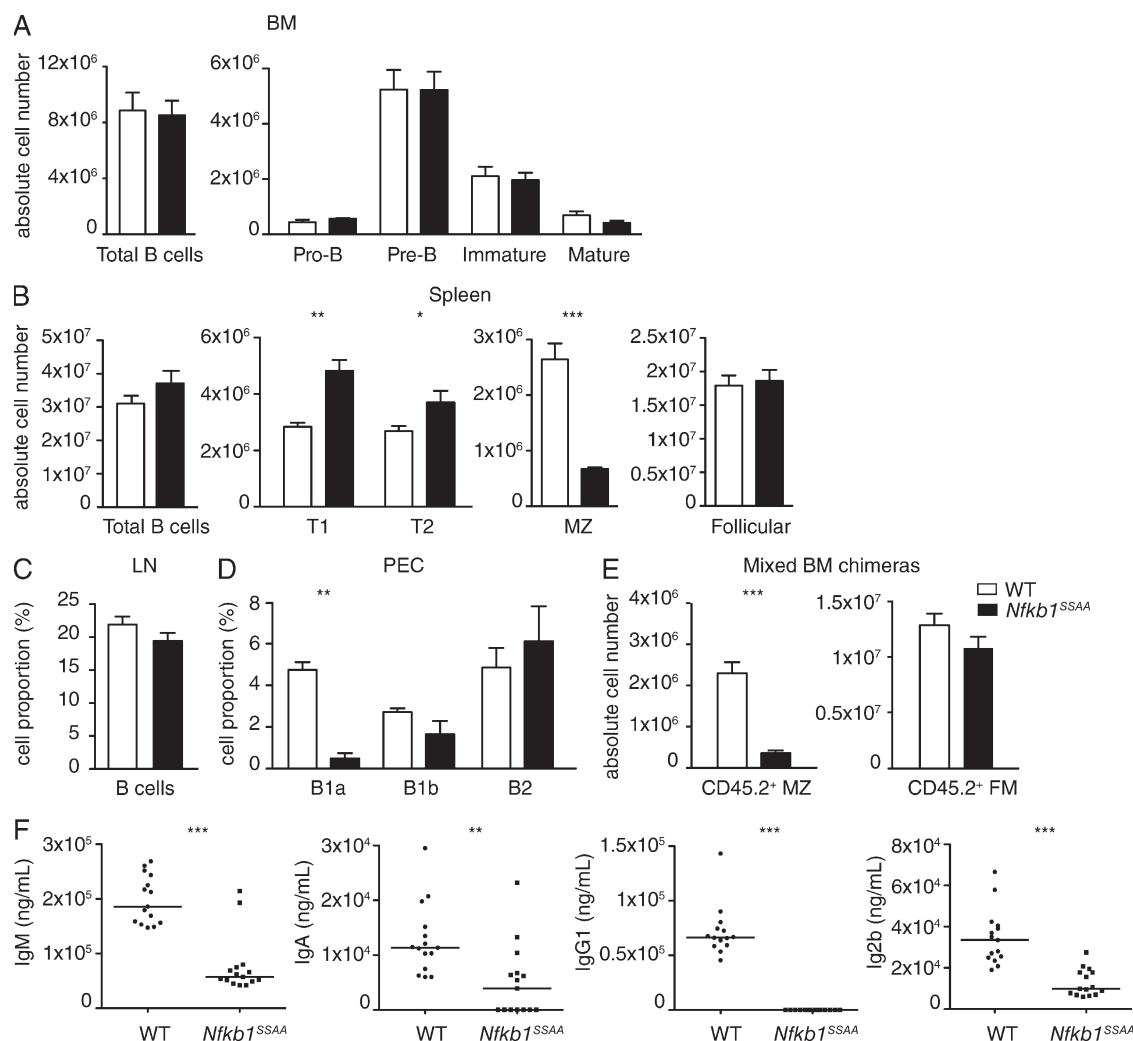


Figure 1. *Nfkb1^{SSAA/SSAA}* mice have normal numbers of FM B cells. (A–D) Flow cytometric analysis of B cell populations in WT and *Nfkb1^{SSAA/SSAA}* mice from the indicated organs. The gating strategies used are shown in Fig. S1. (A) Absolute cell numbers of pro-B (B220⁺CD19⁺IgD[−]IgM[−]CD2[−]), pre-B (B220⁺CD19⁺IgD[−]IgM[−]CD2⁺), immature mature B (B220⁺CD19⁺IgD[−]IgM⁺CD2⁺), and mature B cells (B220⁺CD19⁺IgD⁺IgM⁺CD2⁺) in the BM (mean ± SEM; *n* = 10–11 mice/genotype). (B) Absolute cell numbers (mean ± SEM; *n* = 10–11 mice/genotype) of total B cells (IgM⁺IgD⁺), immature B cells (IgD^{lo}B220⁺AA4.1⁺) separated into transitional T1 B cells (IgM^{hi}CD23[−]), and T2 B cells (IgM^{hi}CD23⁺), and mature B cells (IgD^{hi}B220⁺AA4.1[−]) separated into FM B cells (IgM^{hi}CD23⁺) and MZ B cells (IgM^{hi}CD23[−]) in the spleen. (C) Percentages of B cells (IgM⁺CD19⁺) in peripheral lymph nodes (pools of single cervical, axillary and inguinal nodes; mean ± SEM; *n* = 10–11 mice/genotype). (D) Proportion of B1a (B220⁺CD19⁺CD5⁺CD23[−]), B1b (B220⁺CD19⁺CD5[−]CD23[−]), and B2 (B220⁺CD19⁺CD5[−]CD23⁺) cells in the peritoneal cavity (mean ± SEM; *n* = 5–7 mice/genotype). In A–D, results are representative of at least three independent experiments. (E) WT or *Nfkb1^{SSAA/SSAA}* CD45.2⁺ BM cells were mixed with WT CD45.1⁺ BM cells at a 1:1 ratio, and transferred into sublethally irradiated *Rag1^{−/−}* mice. After 8-wk reconstitution, the absolute number of CD45.2⁺ MZ B cells was assessed by flow cytometry. FM B cells were used as the control population. Graphs show absolute cell numbers (mean ± SEM; *n* = 14 mice/genotype), results are representative of two independent experiments. (F) Graphs show ELISAs for IgM, IgA, IgG1, and IgG2b, assaying sera from naive WT and *Nfkb1^{SSAA/SSAA}* mice. *n* = 18 mice per genotype. PEC, peritoneal cavity. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

peritoneum (Zhang, 2013). Serum titers of IgA and both IgG1 and IgG2b were also significantly decreased in unimmunized mice, raising the possibility that one or several aspects of antibody responses might also be impaired by *Nfkb1*^{SSAA} mutation. Because we were interested in investigating the role of NF- κ B1 p105 proteolysis in B cell function in immunity, we focused our subsequent experiments on the effects of *Nfkb1*^{SSAA} mutation on TD antibody responses. It has previously been shown that RBP-J-deficient mice, which lack MZ B cells, are still able to generate normal antibody responses to chicken gammaglobulin (CGG; Tanigaki et al., 2002). The reduction in

MZ B cells in *Nfkb1*^{SSAA/SSAA} mice, therefore, should not contribute significantly to TD antibody responses, although this might decrease antibody responses to T cell-independent antigens. Consequently, any detected effects of *Nfkb1*^{SSAA} mutation on antibody responses to a TD antigen were likely to result from alterations in the function of FM B cells (Oracki et al., 2010), as their numbers were normal in *Nfkb1*^{SSAA/SSAA} mice (Fig. 1 B).

To investigate the B cell-intrinsic effects of *Nfkb1*^{SSAA} mutation on FM B cell function, we generated mixed BM chimeras. BM cells from μ MT^{-/-} mice, lacking B cells (Kitamura

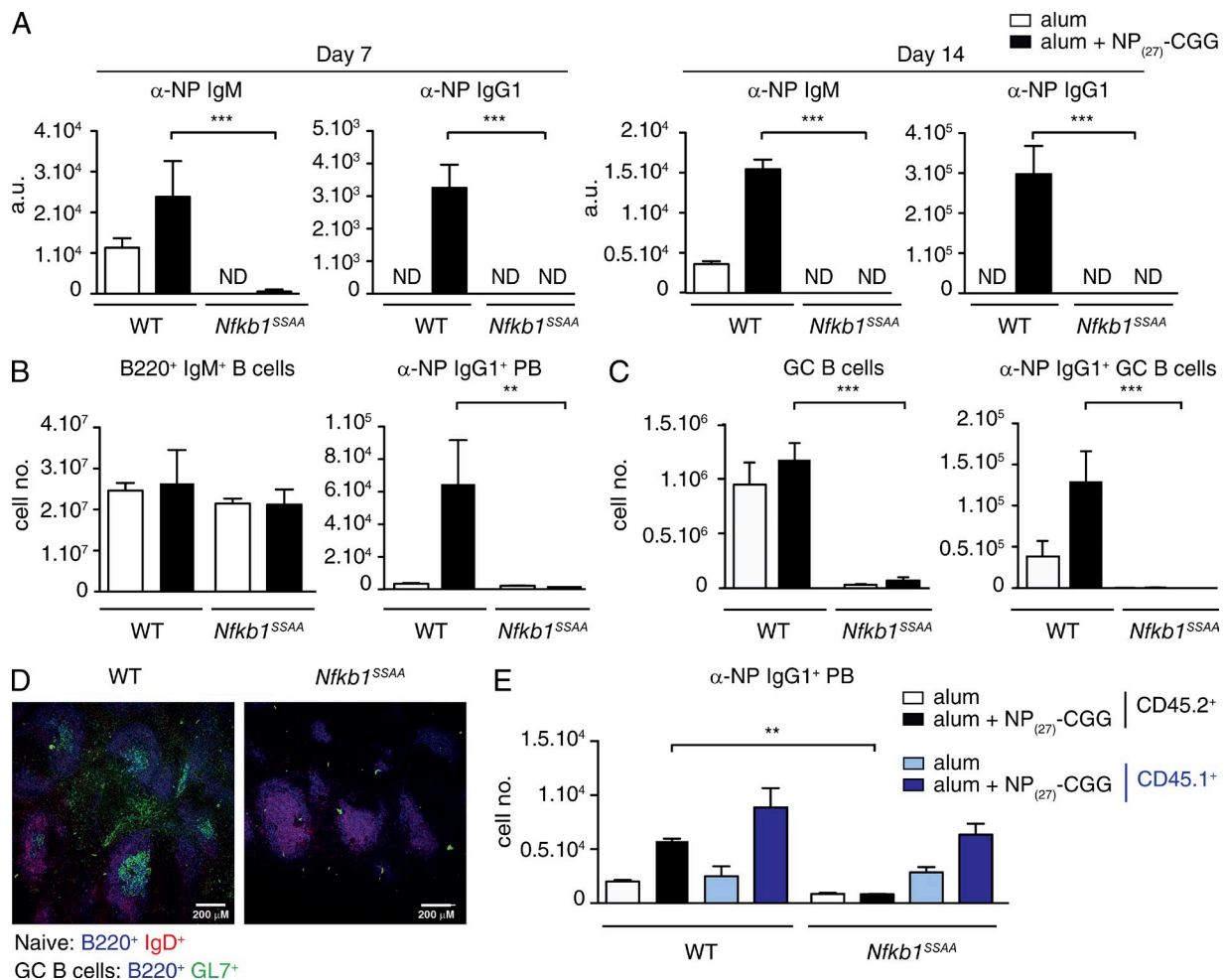


Figure 2. *Nfkb1*^{SSAA/SSAA} mutation blocks the B cell antibody response to a TD antigen. WT or *Nfkb1*^{SSAA/SSAA} BM cells were mixed with μ MT^{-/-} BM cells in a 1:4 ratio, and transferred into sublethally irradiated Rag1^{-/-} mice. After 8 wk, chimeras were immunized with NP₂₇-CGG alum or PBS alum controls. (A) Serum antibody response to NP₂₇-CGG immunization of WT or *Nfkb1*^{SSAA/SSAA} mixed BM chimeras assessed 7 and 14 d after challenge. Data show mean NP-specific serum IgM and IgG1 levels (\pm SEM) measured by ELISA. (B) Flow cytometric analysis of total naive B cells (IgM⁺B220⁺; left) and α NP-IgG1⁺ plasmablasts (PB; B220⁺IgM⁺IgD⁻CD138⁺; right) in the spleens of chimeras 7 d after NP₂₇-CGG immunization (mean absolute number \pm SEM). (C) Flow cytometric analysis of total GC B cells (IgM⁺B220⁺IgD⁻PNA^{hi}GL7⁺; left) and α NP-IgG1⁺ GC B cells (right) in chimeras 14 d after NP₂₇-CGG immunization (mean absolute number \pm SEM). (D) Confocal microscopy of spleen sections from WT or *Nfkb1*^{SSAA/SSAA} chimeras immunized with NP₂₇-CGG (14d). GC formation was measured by staining with anti-IgD (red), anti-B220 (blue) and GL7 (green). In A–D, results are representative of at least three independent experiments with $n = 5$ mice per genotype. (E) WT or *Nfkb1*^{SSAA/SSAA} CD45.2⁺ BM cells were mixed with WT CD45.1⁺ and μ MT^{-/-} BM cells in a 1:1:3 ratio, and transferred into sublethally irradiated Rag1^{-/-} mice. After an 8-wk reconstitution, chimeras were immunized with NP₂₇-CGG alum or alum alone. Graph shows the flow cytometric analysis of α NP-IgG1⁺ plasmablasts (PB), 7 and 14 d after NP₂₇-CGG immunization. Results are representative of at least two independent experiments with $n = 5$ –6 mice per genotype. **, $P < 0.01$; ***, $P < 0.001$.

et al., 1991), were mixed at 4:1 ratio with BM cells from WT or *Nfkb1^{SSAA/SSAA}* mice. These were then transferred into irradiated *Rag1^{-/-}* mice, producing chimeric mice in which all B cells had a WT (*Nfkb1^{+/+}*) or *Nfkb1^{SSAA/SSAA}* genotype, whereas 80% of the other hematopoietic cells had a WT genotype. After reconstitution for 8 wk, chimeric mice were then injected with NP₂₇-CGG precipitated in alum or alum-only control. *Nfkb1^{SSAA}* mutation totally blocked the production of serum anti-NP IgM and IgG1 at 7 and 14 d (Fig. 2 A), although the numbers of splenic B cells were equivalent between WT and *Nfkb1^{SSAA/SSAA}* chimeric mice (Fig. 2 B, left). *Nfkb1^{SSAA}* mutation also prevented the generation of anti-NP-specific plasmablasts (Fig. 2 B, right) and anti-NP-specific GC B cells (Fig. 2 C). Furthermore, immunofluorescence analysis of spleen sections demonstrated the absence of GC structures in *Nfkb1^{SSAA/SSAA}* chimeras after NP₂₇-CGG-alum immunization (Fig. 2 D), which is consistent with a reduction in total GC B cell numbers (Fig. 2 C, left). Analysis of allotype-marked mixed BM chimeras confirmed that the effect of *Nfkb1^{SSAA}* mutation on generation of anti-NP-specific plasmablasts was B cell intrinsic (Fig. 2 E).

The results in this section demonstrated an essential role for IKK-induced proteolysis of NF- κ B1 p105 within FM B cells for their participation in TD antibody responses.

Nfkb1^{SSAA} mutation reduces BCR activation of NF- κ B

Our earlier work demonstrated that blockade of IKK-induced p105 proteolysis by *Nfkb1^{SSAA}* mutation can impair the activation of both TPL-2 and NF- κ B in macrophages (Yang et al., 2012). Therefore, the inhibitory effects of *Nfkb1^{SSAA}* mutation on TD antibody responses could have resulted from reduced TPL-2 and/or NF- κ B activation in B cells. To investigate whether TPL-2 had important B cell-intrinsic functions in TD antibody responses, mixed BM chimeras were generated in which B cells lacked expression of TPL-2 (Dumitru et al., 2000). Immunization of these chimeric mice with NP₂₇-CGG revealed that TPL-2-deficient B cells produced a TD antibody response equivalent to that of WT controls (Fig. 3). This

suggested that the failure of *Nfkb1^{SSAA/SSAA}* FM B cells to mount an antibody response to NP₂₇-CGG did not result from defective TPL-2 signaling, and was likely due to altered NF- κ B activation.

To investigate this possibility, we determined the effect of *Nfkb1^{SSAA}* mutation on NF- κ B activation in purified splenic FM B cells in vitro. Steady-state protein levels of RelA, RelB, and c-Rel were comparable between *Nfkb1^{SSAA/SSAA}* and WT FM B cells (Fig. 4 A). However, similar to our previous findings with macrophages (Yang et al., 2012), *Nfkb1^{SSAA}* mutation increased the steady-state concentration of NF- κ B1 p105, while NF- κ B1 p50 was reduced. *Nfkb1* mRNA levels were comparable between *Nfkb1^{SSAA/SSAA}* and WT FM B cells (unpublished data), implying that these changes in p105 and p50 protein levels resulted from *Nfkb1^{SSAA}* mutation blocking constitutive p105 phosphorylation by IKK. Consistent with that hypothesis, unstimulated WT FM B cells had small amounts of phosphorylated p105, that increased after stimulation with anti-IgM, CD40L and LPS (Fig. 4 B and not depicted). In addition, steady-state levels of NF- κ B2 p52 were increased in *Nfkb1^{SSAA/SSAA}* B cells compared with WT, whereas levels of p100 were fractionally reduced (Fig. 4 A), suggesting that *Nfkb1^{SSAA}* mutation increased constitutive activation of the NF- κ B alternative pathway.

Nuclear translocations of RelA and p50, after stimulation with anti-IgM for 1 h, were clearly decreased in *Nfkb1^{SSAA/SSAA}* FM B cells compared with WT cells (Fig. 4 C). Accordingly, NF- κ B ELISAs demonstrated that *Nfkb1^{SSAA}* mutation impaired BCR activation of p50 and RelA binding activity at 1 h (Fig. 4 D). Although nuclear translocation of c-Rel was not obviously affected after 1h BCR stimulation, c-Rel binding activity was fractionally reduced by *Nfkb1^{SSAA}* mutation (Fig. 4, C and D). However, *Nfkb1^{SSAA}* mutation had a more pronounced effect on the second wave of c-Rel activation that results from prolonged BCR stimulation (Damdinsuren et al., 2010), detected at 12 h (Fig. 4 D). Both p50 and RelA nuclear translocations and binding activities were reduced by *Nfkb1^{SSAA}* mutation after 4-h CD40L stimulation. In contrast, CD40L-induced

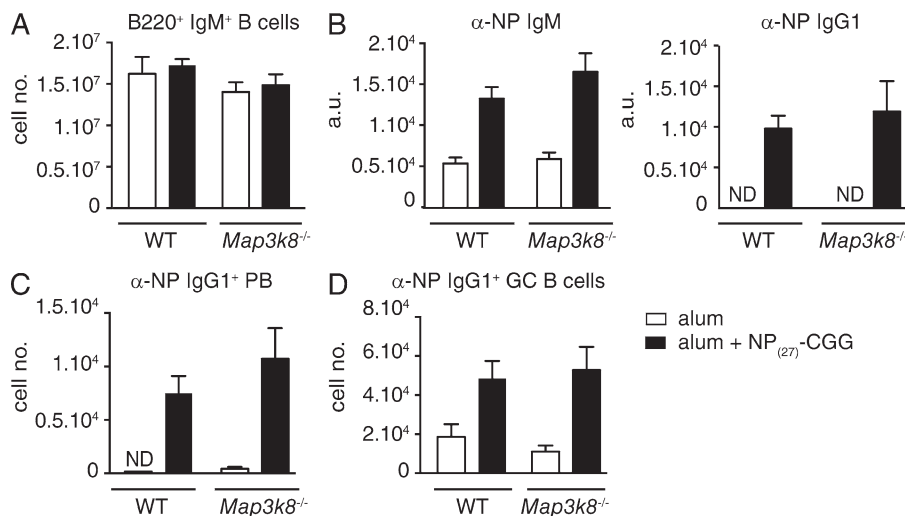


Figure 3. TPL-2 is not required for a B cell antibody response to TD antigen. WT or *Map3k8^{-/-}* BM cells were mixed with *μMT^{-/-}* BM cells in a 1:4 ratio, and transferred into sublethally irradiated *Rag1^{-/-}* mice. After 8 wk, chimeras were immunized with NP₂₇-CGG alum or PBS alum controls. (A) Flow cytometric analysis of total naive B cells (IgM⁺B220⁺). (B) α-NP IgM and IgG1 serum antibody levels were quantified by ELISA (mean ± SEM), 14 d after NP₂₇-CGG immunization. (C) Splenic α-NP IgG1⁺ plasmablasts (PB; B220⁺IgM⁺IgD⁻CD138⁺) 7 d after NP₂₇-CGG immunization (mean absolute number ± SEM). (D) Splenic α-NP IgG1⁺ GC B cells 14 d after NP₂₇-CGG immunization (mean absolute number ± SEM). All results are representative of two independent experiments (4–5 mice per genotype each).

nuclear translocations and NF- κ B binding activities of c-Rel, RelB, and NF- κ B2 p52 were similar between *Nfkb1*^{SSAA/SSAA} and WT FM B cells. Thus, the major effects of *Nfkb1*^{SSAA} mutation in FM B cells were to reduce the early activation of canonical NF- κ B subunits p50 and RelA, after stimulation of either BCR or CD40, and the late activation of c-Rel after prolonged BCR cross-linking. p50, RelA and c-Rel each co-immunoprecipitated with p105, suggesting that *Nfkb1*^{SSAA} mutation reduced activation of these Rel subunits, in part, by p105SSAA retention in the cytoplasm (Fig. 4 E).

Nfkb1^{SSAA} mutation prevents BCR augmentation of FM B cell survival

Optimal TD antibody responses depend on the clonal expansion and survival of antigen-stimulated B cells (Oracki et al., 2010).

Furthermore, in vitro studies with purified splenic B cells from Rel-deficient mice have suggested that NF- κ B promotes the cell growth, proliferation and survival of mature B cells after BCR stimulation (Gerondakis and Siebenlist, 2010a; Kaileh and Sen, 2012). As NF- κ B activation was impaired by *Nfkb1*^{SSAA} mutation, we next compared the growth, proliferation and survival of *Nfkb1*^{SSAA/SSAA} FM B cells with WT cells. *Nfkb1*^{SSAA} mutation did not affect FM B cell growth (Fig. 5 A), and only modestly decreased the fraction of FM B cells that divided following anti-IgM stimulation (Fig. 5 B). However, *Nfkb1*^{SSAA} mutation prevented BCR ligation increasing FM B cell survival (Fig. 5 C). CD40-induced proliferation and survival were similar between *Nfkb1*^{SSAA/SSAA} and WT FM B cells (Fig. 5, B and C). The survival defect of *Nfkb1*^{SSAA/SSAA} FM B cells after BCR cross-linking was rescued by co-stimulation with CD40L (Fig. 5 D).

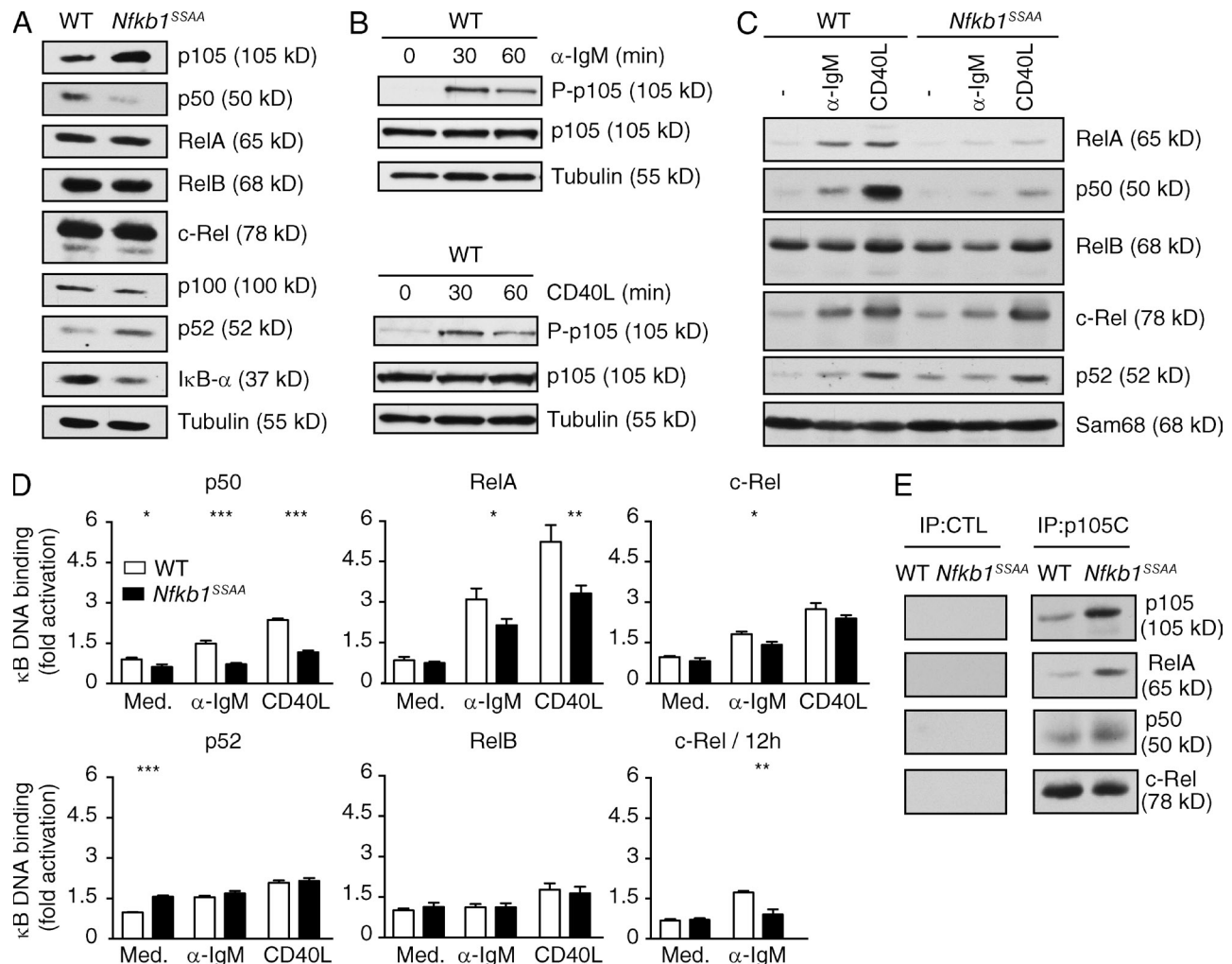


Figure 4. Effects of *Nfkb1*^{SSAA} mutation on NF- κ B activation in FM B cells. (A) Lysates of purified splenic FM B cells from WT or *Nfkb1*^{SSAA/SSAA} mice were immunoblotted for the indicated proteins. (B) Purified splenic FM B cells from WT mice were stimulated with α -IgM or CD40L, and whole-cell extracts immunoblotted. (C) Purified splenic FM B cells were stimulated with α -IgM (1 h) or CD40L (4 h). Nuclear fractions were immunoblotted. (D) Nuclear fractions were prepared as in C, and NF- κ B binding of the indicated Rel subunits was determined by ELISA (mean \pm SEM). To assay late activation of c-Rel, FM B cells were stimulated with anti-IgM for 12 h. (E) p105 was immunoprecipitated (IP) from lysates of purified splenic FM B cells. Associated Rel proteins were detected by immunoblotting. Pre-immune serum was used for control immunoprecipitations (CTL). All results are representative of at least three separate experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

NF- κ B was previously shown to maintain the viability of BCR-activated B cells by inducing the expression of *Bcl-xl* and *A1* (*Bfl-1*) genes (Grumont et al., 1999; Köntgen et al., 1995). Expression of *Mcl-1*, which is essential for survival of mature B cells (Hettmann et al., 2003) and plasma cells (Peperzak et al., 2013), may also be regulated by NF- κ B (Buggins et al., 2010). To gain further insight into how IKK-induced p105 proteolysis regulates B cell survival, we determined the expression of these genes in purified FM B cells from *Nfkb1^{SSAA/SSAA}* and WT FM mice. *Nfkb1^{SSAA}* mutation significantly reduced BCR up-regulation of *Bcl-xl*, *A1*, and *Mcl-1* mRNAs (Fig. 5 E). CD40 induction of *Bcl-xl* and *Mcl-1* mRNAs was also reduced in *Nfkb1^{SSAA/SSAA}* FM B cells compared with WT. In contrast, CD40 up-regulation of *A1* mRNA was similar between genotypes, which may explain why CD40-induced FM B cell survival was not altered by *Nfkb1^{SSAA}* mutation.

Together these results suggested that failure of the BCR to up-regulate Bcl-2 family proteins in *Nfkb1^{SSAA/SSAA}* FM B cells increased apoptosis, and might explain the block in TD antibody responses caused by *Nfkb1^{SSAA}* mutation. To investigate this hypothesis, we crossed *Nfkb1^{SSAA/SSAA}* mice with a transgenic mouse strain that overexpresses *Bcl-xl* in the B cell lineage (Takahashi et al., 1999). In vitro experiments demonstrated that Bcl-xl normalized *Nfkb1^{SSAA/SSAA}* FM B cell survival after BCR stimulation (Fig. 6 A). Nevertheless, chimeric mice in which the B cell compartment was generated from the BM of *Bcl-xl Nfkb1^{SSAA/SSAA}* mice were still unable to

mount an IgG1 antibody response to NP₂₇-CGG, while the IgM anti-NP response was substantially reduced compared with WT *Bcl-xl* chimera controls (Fig. 6 B). The recovery of transplanted FM B cells from MD4 *Nfkb1^{SSAA/SSAA}* mice, which express a transgenic BCR specific for HEL (hen egg lysozyme; Goodnow et al., 1988), was increased by Bcl-xl expression (Fig. 6 C). However, MD4 *Bcl-xl Nfkb1^{SSAA/SSAA}* B cells did not differentiate into plasmablasts or GC B cells after immunization with HEL-SRBC, in contrast to MD4 *Bcl-xl Nfkb1^{+/+}* control B cells under the same conditions (Fig. 6 C and Fig. S2). Thus, *Nfkb1^{SSAA}* mutation did not block TD antibody responses solely by reducing BCR-induced FM B cell survival.

Nfkb1^{SSAA} mutation does not block B cell migration to the follicle T cell zone or antigen presenting function

The antigen-specific interaction between B cells and helper T cells in secondary lymphoid organs is an essential step in TD humoral immune responses (Cyster, 2005). T cells provide a second signal via CD40 ligand, which promotes B cell survival and proliferation, and prevents B cell paralysis following BCR stimulation (Goodnow et al., 2010). Our observation that *Nfkb1^{SSAA}* mutation did not affect CD40L induction of FM B cell proliferation or survival (Fig. 5, B and C), and that CD40 co-stimulation rescued the BCR-induced survival defect of *Nfkb1^{SSAA/SSAA}* FM B cells in vitro (Fig. 5 D) raised the possibility that these cells failed to receive a signal from CD4⁺ T cells after NP₂₇-CGG immunization.

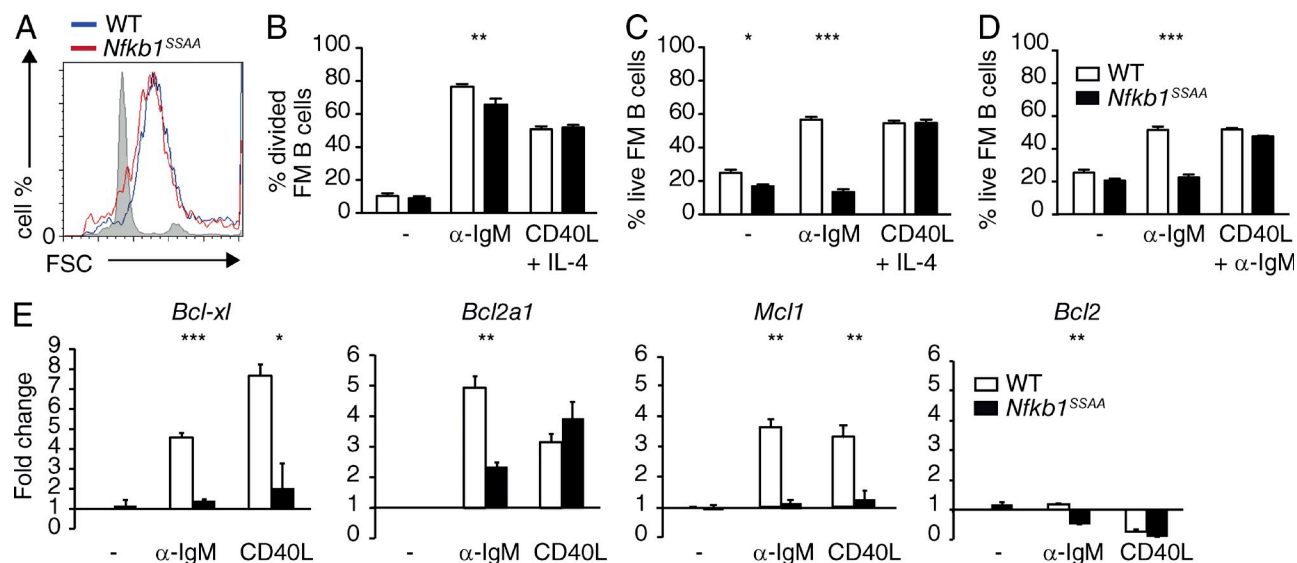


Figure 5. Effects of *Nfkb1^{SSAA}* mutation on FM B cell survival and proliferation. (A) Flow cytometric analysis of cell growth (forward scatter) on purified FM B cells cultured in presence of α -IgM, for 24 h. Unstimulated WT FM B cells are represented in gray. (B) Purified splenic FM B cells plus α -IgM, CD40L, and IL-4 or control medium (—) were cultured for 68 h. The fraction of FM B cells that had divided was determined by flow cytometric analysis of CFSE dilution (mean \pm SEM; triplicates). (C and D) Purified splenic FM B cells were cultured for 48 h with α -IgM, CD40L plus IL-4, α -IgM plus CD40L and IL-4, or control medium (—). The fraction of live FM B cells was determined by flow cytometric analysis of 7AAD staining (mean \pm SEM; triplicates). (E) Triplicate cultures of purified splenic FM B cells were stimulated with α -IgM for 1 h or with CD40L for 4 h. Levels of specific mRNAs were determined by quantitative RT-PCR. Data are normalized to *Hprt1* mRNA levels and represent mean fold change (\pm SEM) relative to unstimulated WT cells. All results are representative of at least three independent experiments (3 mice/genotype each). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

After binding antigen, efficient FM B cell interaction with CD4⁺ T cells is facilitated by BCR up-regulation of CCR7, which induces FM B cell migration to the B cell zone-T cell zone boundary of the follicle in response to the T zone-expressed CCR7 ligand, CCL21 (Pereira et al., 2010). CCR7 is an NF- κ B-regulated gene (Krappmann et al., 2004), but *Nfkb1*^{SSAA} mutation did not impair the up-regulation of CCR7 expression on FM B cells following BCR ligation (Fig. 7 A). Furthermore, anti-IgM-induced mRNA expression of Epstein-Barr virus-induced molecule 2 (EBI-2), another NF- κ B-regulated gene (Glynne et al., 2000) encoding a cytokine which facilitates the initial uniform distribution of B cells along the B/T border (Gatto et al., 2009), was also unaffected by *Nfkb1*^{SSAA} mutation (Fig. 7 B). Consistent with these data, anti-IgM-stimulated CD45.2⁺ *Nfkb1*^{SSAA/SSAA} FM B cells migrated to the follicle-T cell zone border after transfer into wild-type CD45.1⁺ recipients, similar to their WT counterparts (Fig. 7 C). These results suggest that a failure to co-localize with T cells did not explain the defective function of *Nfkb1*^{SSAA/SSAA} B cells in TD antibody responses. However, we cannot exclude some contribution of the NF- κ B1 signaling pathway in this process, as in vitro stimulation before cell transfer may not completely replicate the in vivo environment.

For productive T cell-B cell cooperation in TD antibody responses, B cells must capture external antigens and present them as peptide fragments on MHC class II molecules to CD4⁺ T cells (Yuseff et al., 2013). It was therefore possible that while *Nfkb1*^{SSAA} mutation did not inhibit T cell-B cell co-localization, the antigen presenting function of FM B cells

was impaired. A model system was used to assay this (Huntington et al., 2006), in which OVA was targeted to the BCR by treating *Nfkb1*^{SSAA/SSAA} and WT FM B cells sequentially with biotinylated anti-IgM and antibiotin OVA-FITC. Antigen-pulsed B cells were then co-cultured with CTV-labeled OTII CD4⁺ T cells, which recognize OVA-derived peptides presented on I-Ab class II molecules. Flow cytometric analysis of OTII CD4⁺ T cell proliferation indicated that *Nfkb1*^{SSAA/SSAA} FM B cells were able to process and present OVA peptides in an active process requiring viable cells as efficiently as WT cells (Fig. 7 D). In accordance with these data, BCR up-regulation of CD86 and MHC class II was equivalent between *Nfkb1*^{SSAA/SSAA} and WT FM B cells (unpublished data). The absence of a TD antibody response in *Nfkb1*^{SSAA/SSAA} chimeric mice, therefore, did not result from a block in the ability of FM B cells to present antigen.

Nfkb1^{SSAA} mutation blocks the differentiation of FM B cells to plasma cells

After antigen exposure and T cell help, proliferating B cells differentiate into either short-lived extrafollicular plasma cells or GC B cells, which subsequently generate plasma cells that secrete high-affinity antibody and persist for a lifetime. Although GC did not develop after NP₂₇-CGG immunization of *Nfkb1*^{SSAA/SSAA} chimeric mice (Fig. 2 C), the lack of anti-NP IgM and IgG1 at 7d suggested that the inhibitory effect of *Nfkb1*^{SSAA} mutation might precede GC formation. The complete lack of NP₂₇-specific IgM plasma cells in *Nfkb1*^{SSAA/SSAA} chimeric mice raised the possibility that

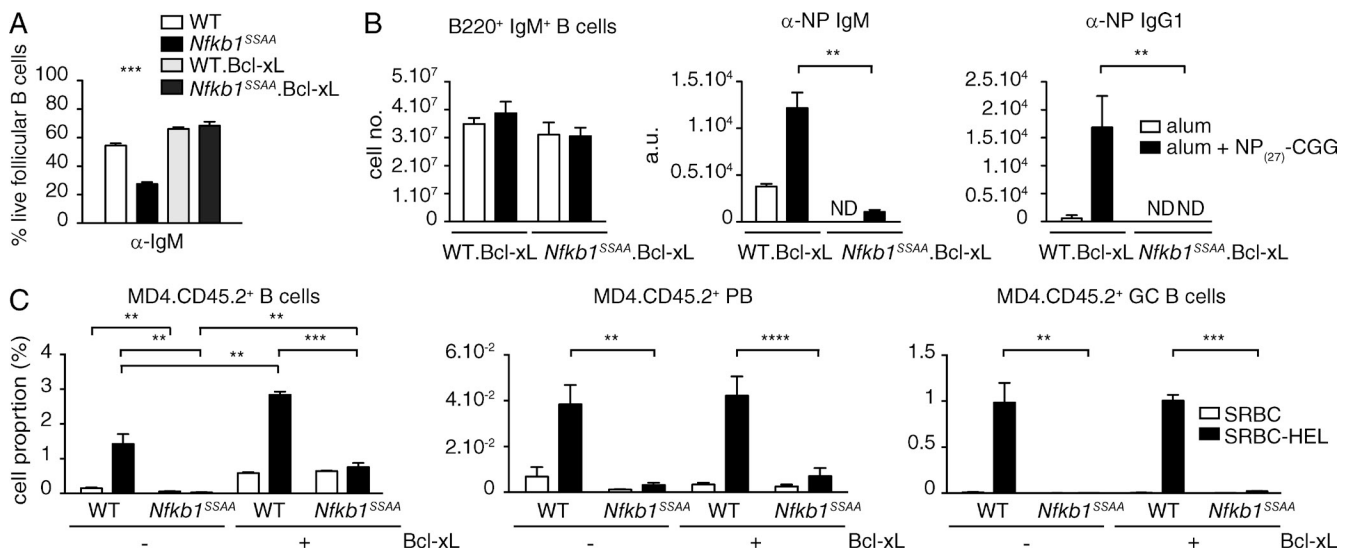


Figure 6. Bcl-xl overexpression does not rescue the TD antibody response of *Nfkb1*^{SSAA/SSAA} FM B cells. (A) The fraction of live FM B cells purified from mice of the indicated genotypes was determined as in Fig. 5 C (mean ± SEM). (B) Mixed radiation chimeras were made as in Fig. 2. LH graph show flow cytometric analysis of splenic B220⁺ IgM⁺ B cells. Central and RH graphs show anti-NP ELISAs of sera (mean ± SEM) 14 d after NP₂₇-CGG immunization. Results are representative of three independent experiments (4–5 mice per genotype). (C) Mean percentages (±SEM) of transferred CD45.2⁺ cells differentiating to plasmablasts (B220⁺IgM⁺IgD⁺CD138⁺CD45.2⁺; middle) and GC B cells (B220⁺PNA⁺GL7⁺CD45.2⁺; right) in the spleens of CD45.1⁺ WT hosts after 6d immunization with SRBC-HEL or unconjugated SRBC. Results are representative of two independent experiments ($n = 6$ mice per condition). Gating strategies used are shown in Fig. S2 (A and B). **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Nfkb1^{SSAA} mutation might affect plasmablast differentiation of FM B cells.

Plasma cell differentiation depends on high expression of the transcription factors BLIMP-1 (B lymphocyte-induced maturation protein 1) and IRF4 (interferon regulatory factor 4). The transcription of *Prdm1* (encoding BLIMP-1) and *Itf4* genes is regulated by NF- κ B (Grumont and Gerondakis, 2000; Saito et al., 2007; Morgan et al., 2009), although the role of NF- κ B activation in plasma cell differentiation has not been investigated. The failure to generate antigen-specific plasmablasts in *Nfkb1*^{SSAA/SSAA} chimeric mice suggested that *Nfkb1*^{SSAA} mutation might prevent antigen-stimulated FM B cells differentiating into plasmablasts by affecting the expression of these transcription factors. To initially investigate this, qRT-PCR was used to determine *Prdm1* and *Itf4* mRNA levels in cultured FM B cells. We found that *Nfkb1*^{SSAA} mutation significantly reduced the mRNA levels of both genes induced by stimulation with anti-IgM (Fig. 8 A). LPS induction of *Itf4* mRNA was also decreased in *Nfkb1*^{SSAA/SSAA} FM B cells compared with WT controls, which correlated with a defect in LPS activation of NF- κ B (unpublished data). In accordance with these latter data, flow cytometric analyses demonstrated that *Nfkb1*^{SSAA} mutation prevented FM B cells (from mice transgenic for Bcl-xl to maintain cell survival) from up-regulating IRF4 protein and differentiating into B220^{lo}CD138⁺ plasmablasts secreting IgM after stimulation with LPS plus IL-4 (Fig. 8, B and C). *Nfkb1*^{SSAA} mutation also prevented LPS plus IL-4 induced switching to IgG1 (Fig. 8 D), consistent with the critical role of IRF4 in class switch recombination (Sciammas et al., 2006). Importantly, the inhibitory effect of *Nfkb1*^{SSAA/SSAA} mutation on plasmablast differentiation was confirmed in vivo. Purified FM B cells from MD4 Bcl-xl *Nfkb1*^{SSAA/SSAA} mice did not differentiate into plasmablasts after transfer to WT recipient mice and concomitant immunization with HEL-SRBC, which contrasted markedly with the MD4 Bcl-xl *Nfkb1*^{+/+} control B cells under the same conditions (Fig. 6 C and Fig. S2). This defect in MD4 Bcl-xl *Nfkb1*^{SSAA/SSAA} B cells correlated with impaired up-regulation of IRF4 (Fig. 8 E and Fig. S3 A) and down-regulation of IgD (Fig. S3 A). Transient expression of *Itf4* is essential for up-regulation of *Bcl6* and generation of GC B cells (Ochiai et al., 2013). In line with this, *Nfkb1*^{SSAA} mutation blocked antigen-induced up-regulation of BCL6 (Fig. 8 F and Fig. S3 B) and the formation of GC B cells (Fig. 6 C and Fig. S2 B).

To determine whether the reduction of *Itf4* and/or *Prdm1* mRNA levels caused by *Nfkb1*^{SSAA} mutation was responsible for the block in plasma cell differentiation, WT and *Nfkb1*^{SSAA/SSAA} FM B cells were transduced with recombinant retroviruses encoding BLIMP-1 or IRF4. Restoring IRF4 expression increased the fraction of *Nfkb1*^{SSAA/SSAA} FM B cells that differentiated into plasmablasts sixfold after restimulation with LPS and IL-4 in vitro, whereas BLIMP-1 expression had little effect (Fig. 8 G). Furthermore, retroviral expression of IRF4 in purified FM B cells from MD4 Bcl-xl *Nfkb1*^{SSAA/SSAA} mice rescued antigen-induced generation of GC B cells after

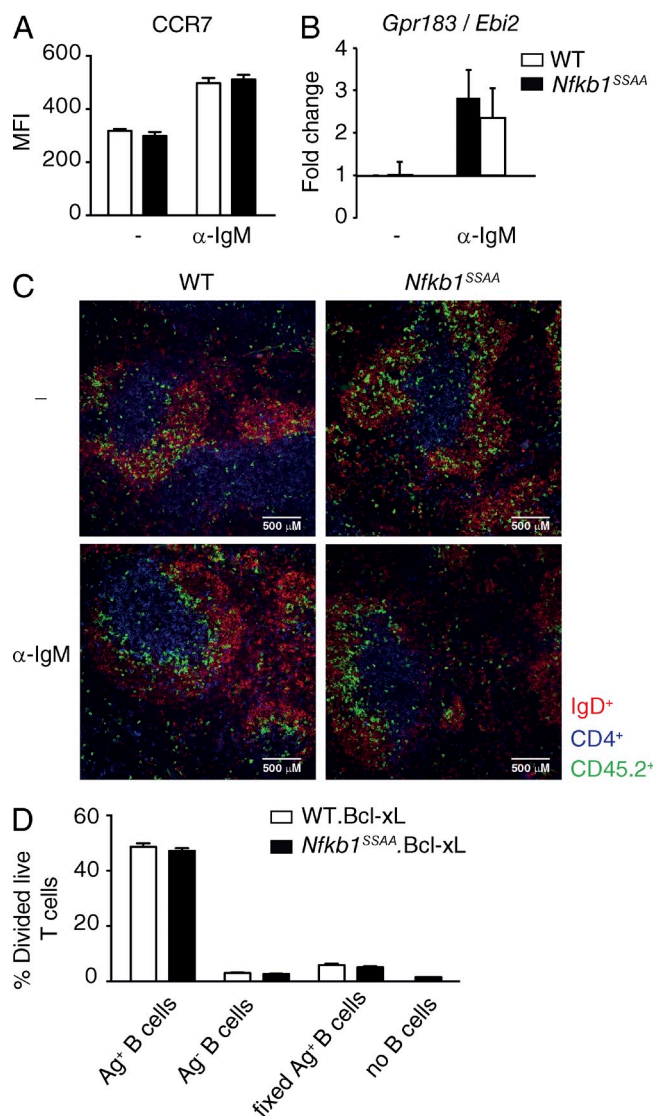


Figure 7. *Nfkb1*^{SSAA/SSAA} B cells migrate normally to follicle T cell zone. (A) Flow cytometric analysis of CCR7 surface expression on purified FM B cells, cultured \pm α -IgM for 6 h. Graphs represent CCR7 mean fluorescent intensity (\pm SEM) of live B cells (7AAD⁻B220⁺). (B) Triplicate cultures of purified splenic FM B cells cultured \pm α -IgM for 1 h. Expression of *Ebi-2* mRNAs was determined by quantitative RT-PCR. Data were normalized to *Hprt1* mRNA levels and represent mean fold change (\pm SEM) relative to unstimulated WT cells. All results are representative of at least two independent experiments done, with 3 mice/genotype each. (C) Splenic distribution of WT and *Nfkb1*^{SSAA/SSAA} CD45.2⁺ B cells, prestimulated in vitro for 1 h with α -IgM or control buffer (-), 5.5 h after transfer in to CD45.1⁺ WT hosts. Transferred B cells were detected by staining for CD45.2 (green), whereas host B cells and T cells were stained with antibodies to IgD (red) and CD4 (blue), respectively. Views are representative of at least two mice of each type. (D) Purified antigen-pulsed (Ag⁺) or not pulsed (Ag⁻) B cells were co-culture with purified CTV-labeled OTII CD4⁺ T cells for 72 h. The fraction of OTII CD4⁺ that had divided was determined by flow cytometric analysis of CTV dilution (mean \pm SEM; triplicates). All results are representative of at least two independent experiments done, with 3 mice/genotype each.

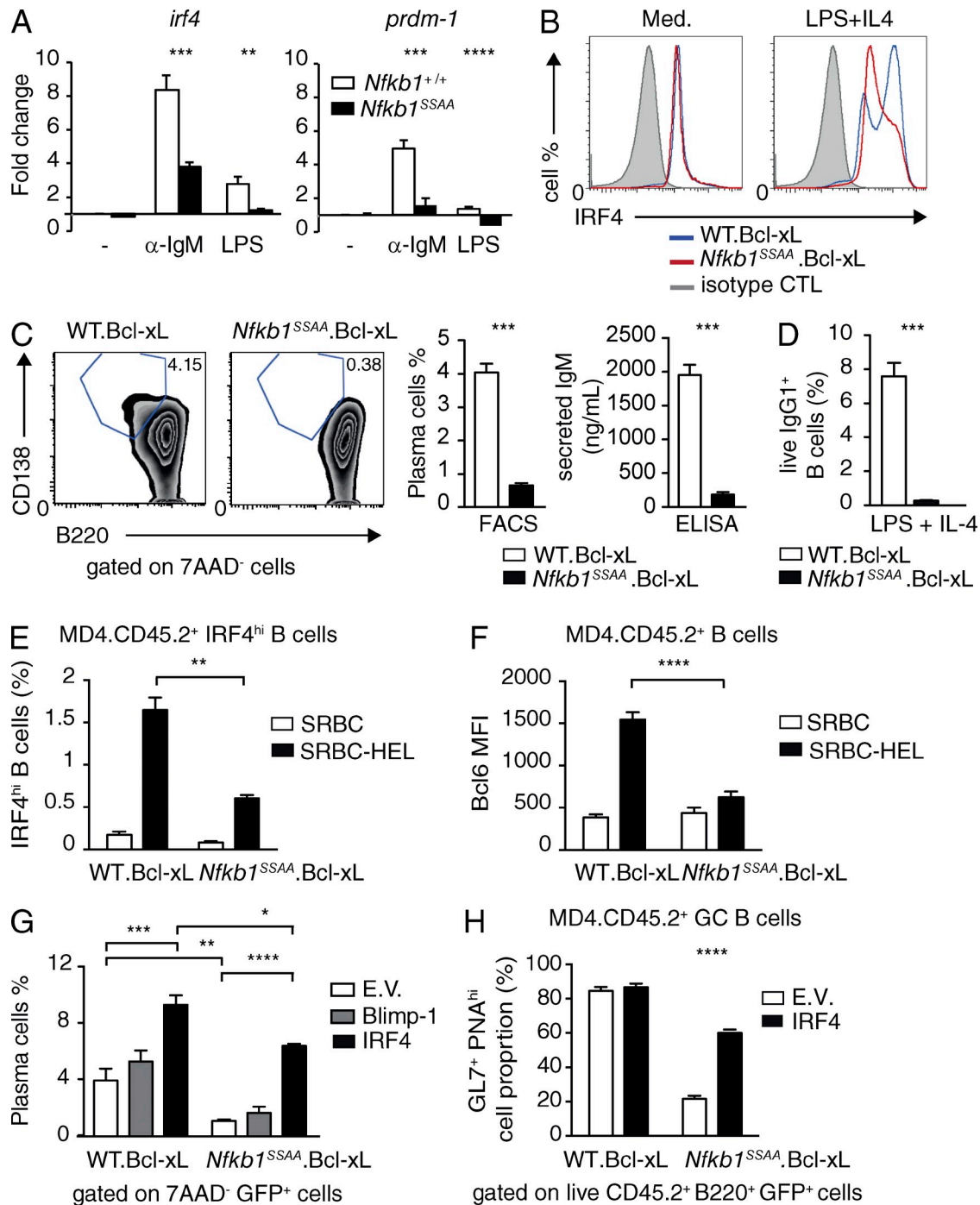


Figure 8. *Nfkb1*^{SSAA} mutation impairs plasma cell differentiation. (A) Purified splenic FM B cells were cultured (triplicates) with α -IgM for 1 h or with LPS for 3 h. Expression of *Irfa* and *Prdm-1* mRNAs was determined by quantitative RT-PCR. Data were normalized to *Hprt1* mRNA levels and represent mean fold change (\pm SEM) relative to unstimulated WT cells. All results are representative of at least three independent experiments (3 mice/genotype each). (B) Histograms show intracellular IRF4 staining in live purified FM B cells cultured \pm LPS and IL-4 (40 h). (C) FM B cells were stimulated with LPS and IL-4 for 4 d. CD138⁺B220^{lo} plasma cells among the live (7AAD⁻) lymphocytes were quantified by flow cytometry. (middle) Mean percentages of plasma cells (\pm SEM). (right) IgM secretion as quantified by ELISA (mean \pm SEM). (D) Purified FM B cells were stimulated with LPS and IL-4 for 3 d. Fractions of IgG1⁺ live B cells (B220⁺7AAD⁻) were determined by flow cytometry (\pm SEM; E) Graphs show the mean percentages (\pm SEM) of transferred CD45.2⁺ cells up-regulating intracellular IRF4 expression in the spleens of CD45.1⁺ WT hosts after 6d immunization with SRBC-HEL or unconjugated SRBC. These results are representative of four independent experiments ($n = 6$ mice per condition). The gating strategies used are shown in Fig. S3 A. (F) Mean fluorescent intensities (MFI) of intracellular Bcl6 in transferred CD45.2⁺ B cells after 6-d immunization with SRBC-HEL or unconjugated SRBC. Results are representative of three independent experiments ($n = 6$ mice per condition). Gating strategies used are shown in Fig. S4 B.

transfer to WT recipient mice (Fig. 8 H and Fig. S4). Insufficient plasmablasts were generated in chimeric mice for quantitation. Together, these results suggested that the inhibitory effect of *Nfkb1^{SSAA}* mutation on TD antibody responses resulted partly from reduced BCR-mediated up-regulation of *Irf4* mRNA, which blocked differentiation of FM B cells to plasmablasts and GC B cells.

Increasing p50 levels overcomes the inhibitory effect of *Nfkb1^{SSAA}* mutation on TD B cell antibody responses

Our in vitro experiments indicated that a major effect of *Nfkb1^{SSAA}* mutation with respect to NF- κ B was to reduce the steady-state levels and activation of NF- κ B1 p50 in FM B cells. To investigate the role of reduced p50 levels in the function of *Nfkb1^{SSAA/SSAA}* FM B cells, *Nfkb1^{SSAA/SSAA}* mice were crossed with *Nfkb1^{ΔCT/ΔCT}* mice, which express elevated amounts of p50 compared with WT cells and no p105 (Ishikawa et al., 1998), as described previously (Yang et al., 2012). For comparison with these *Nfkb1^{SSAA/ΔCT}* mice, *Nfkb1^{+/-}* and *Nfkb1^{SSAA/-}* strains were also generated. *Nfkb1^{SSAA/ΔCT}* FM B cells expressed higher amounts of p50 compared with *Nfkb1^{+/-}* cells, whereas p50 levels were very low in *Nfkb1^{SSAA/-}* FM B cells (Fig. 9 A). The steady-state concentrations of p105^{SSAA} were similar between *Nfkb1^{SSAA/-}* and *Nfkb1^{SSAA/ΔCT}* FM B cells. NF- κ B ELISAs revealed reduced BCR activation of p50, RelA, and c-Rel in *Nfkb1^{SSAA/-}* FM B cells compared with *Nfkb1^{+/-}* cells (Fig. 9 B). However, increasing p50 levels with the *Nfkb1ΔCT* allele normalized BCR activation of all three Rel subunits.

BCR-induced survival was reduced in *Nfkb1^{SSAA/-}* FM B cells in comparison with *Nfkb1^{+/-}* controls (Fig. 9 C), as expected. In contrast, survival of *Nfkb1^{SSAA/ΔCT}* FM B cells after BCR cross-linking was similar to *Nfkb1^{+/-}* controls. BCR-induced proliferation was similar between the three genotypes (unpublished data), in line with the small inhibitory effect of homozygous *Nfkb1^{SSAA}* mutation on anti-IgM-induced cell division (Fig. 5 B). *Nfkb1^{SSAA}* mutation reduced both survival and proliferation of FM B cells after TLR4 stimulation, and these inhibitory effects were rescued by the *Nfkb1^{ΔCT}* allele (Fig. 9 C and not depicted). The impairment by *Nfkb1^{SSAA}* mutation of IRF4 up-regulation, differentiation to CD138⁺ B220^{lo} plasmablasts and switching to IgG1 after LPS and IL-4 stimulation of FM B cells were also overcome by the *Nfkb1^{ΔCT}* allele (Fig. 9, D–F). These results showed that the reduced survival, plasmablast differentiation and class switch recombination of FM B cells in vitro caused by *Nfkb1^{SSAA}* mutation were rescued by increasing p50 levels with the *Nfkb1^{ΔCT}* allele, which normalized NF- κ B activation.

Finally, to investigate the importance of p50 in the inhibitory effect of *Nfkb1^{SSAA}* mutation on TD antibody responses, we generated mixed BM chimeras and then immunized with NP₂₇-CGG. The titers of anti-NP IgM and IgG1 in the serum, as well as the number of anti-NP IgG1 plasma cells and GC B cells in the spleens of chimeras reconstituted with *Nfkb1^{SSAA/-}* BM, were significantly reduced compared with *Nfkb1^{+/-}* control chimeras (Fig. 9 G and not depicted). In contrast, no significant differences were detected between chimeras generated with *Nfkb1^{+/-}* and *Nfkb1^{SSAA/ΔCT}* BM cells. Therefore, rescuing NF- κ B activation by increasing p50 levels with the *Nfkb1ΔCT* allele overcame the inhibitory effect of *Nfkb1^{SSAA}* mutation on the FM B cell antibody response to a TD antigen.

DISCUSSION

Analyses of conditional knockout mouse strains lacking IKK2 or NEMO expression in the B cell lineage have demonstrated that canonical NF- κ B activation is critical for mature B cell generation or homeostasis (Pasparakis et al., 2002; Li et al., 2003). However, this has prevented using such mouse strains for investigation of the potential role for canonical NF- κ B activation in B cells for antibody production during an immune response. In the present study, we used *Nfkb1^{SSAA/SSAA}* mice, in which the development and homeostasis of peripheral FM B cells were normal, to investigate the role of NF- κ B activation in FM B cells in humoral immunity. *Nfkb1^{SSAA}* mutation selectively inhibits the IKK signaling pathway that regulates NF- κ B activation by inducing NF- κ B1 p105 proteolysis (Srisankharajah et al., 2009). Our results demonstrate for the first time that IKK-induced p105 proteolysis is required for optimal BCR activation of NF- κ B in B cells, and that this is essential for generation of a TD antibody response, specifically regulating the survival of FM B cells and their differentiation to plasmablasts and GC B cells after BCR stimulation.

Nfkb1^{SSAA} mutation reduced activation of p50 and RelA after both BCR and CD40 stimulation. Activation of c-Rel was also significantly reduced in *Nfkb1^{SSAA/SSAA}* FM B cells compared with WT, particularly after prolonged BCR stimulation. Immunoprecipitation and immunoblotting demonstrated that p50, RelA, and c-Rel were able to interact with p105. The inhibitory effect of *Nfkb1^{SSAA}* mutation on the activation of these Rel subunits, therefore, probably resulted from their retention in the cytoplasm by p105^{SSAA}. In addition, steady-state levels of p50 were reduced in *Nfkb1^{SSAA/SSAA}* FM B cells compared with WT controls, suggesting that *Nfkb1^{SSAA}* mutation reduced p105 processing to p50, similar to the situation we previously described in macrophages (Yang et al.,

(G) FM B cells were transduced with recombinant retroviruses. The fraction of GFP⁺ cells differentiating to plasma cells (B220^{lo}IgD⁺CD138⁺) after stimulation with LPS and IL-4 was quantified by flow cytometry (\pm SEM). Results are representative of three independent experiments (3 mice/genotype each). (H) FM B cells were transduced with the indicated retroviruses (E.V., empty vector). The fraction of transferred GFP⁺ CD45.2⁺ cells differentiating to GC B cells (B220⁺PNA^{lo}GL7⁺CD45.2⁺) after 6-d immunization with SRBC-HEL or unconjugated SRBC are shown. Results are representative of two independent experiments (n = 4–6 mice per condition). The gating strategies used are shown in Fig. S5. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

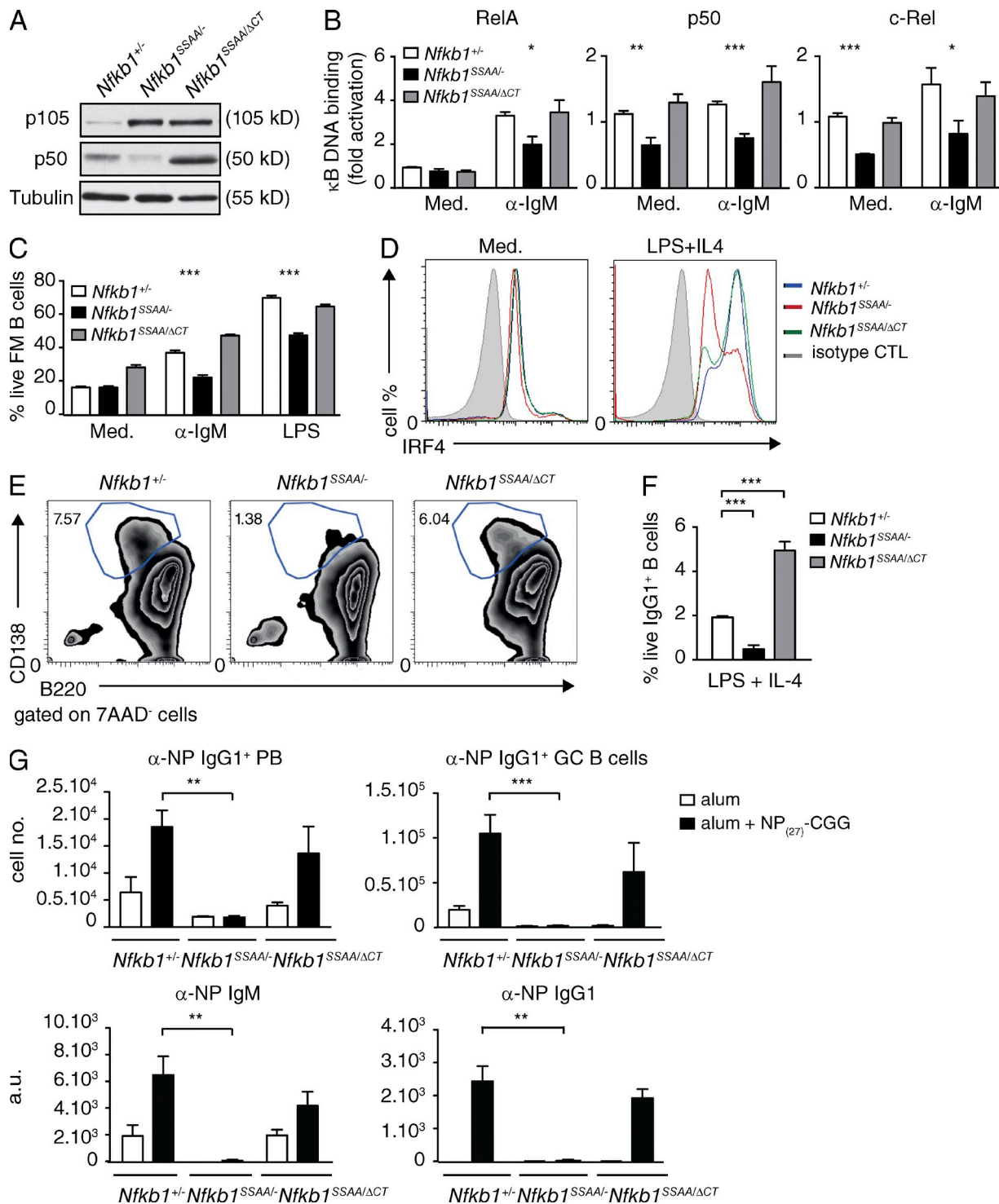


Figure 9. Increasing p50 levels overcomes the inhibitory effect of *Nfkb1*^{SSAAI} mutation on FM B cell function. (A) Lysates of purified splenic FM B cells were immunoblotted. (B) Binding of the indicated Rel subunits to an NF-κB oligonucleotide was determined by ELISA (mean ± SEM). (C) The fractions of live FM B cells were determined as described in Fig. 5 C (mean ± SEM). (D) Intracellular IRF4 in live purified FM B cells ± LPS and IL-4 (40 h) was quantified by flow cytometry. (E) Plasma cell differentiation was monitored as in Fig. 8 C. (F) Purified FM B cells were stimulated with LPS and IL-4 for 3 d. The fractions of IgG1⁺ live B cells (B220⁺7AAD⁻) were determined by flow cytometry (±SEM). (G) Mixed radiation chimeras were made as in Fig. 2. Naive B (B220⁺, IgM⁺) cells and αNP⁺ IgG1⁺ plasmablasts (PB) and GC B cells were quantified by flow cytometry (mean absolute number ± SEM) 7 and 14 d after NP₂₇-CGG immunization, respectively. α-NP IgM and IgG1 serum antibody levels were quantified by ELISA (mean ± SEM) 14 d after NP₂₇-CGG immunization. All results are representative of three independent experiments (4–5 mice per genotype each). *, P < 0.05; **, P < 0.01; ***, P < 0.001.

2012). The *Nfkb1*^{ΔCT} allele overcame the inhibitory effect of *Nfkb1*^{SSAA} mutation on BCR activation of p50, RelA, and c-Rel, presumably by increasing p50 expression to a degree that the concentration of p50 exceeded p105 (p105:p50 [*Nfkb1*^{SSAA/ΔCT}] = 0.63 ± 0.03; p105:p50 [*Nfkb1*^{SSAA/-}] = 4.04 ± 0.3). Consequently, p105^{SSAA} became saturated with p50 monomers and homodimers, with which it preferentially binds (Moorthy and Ghosh, 2003), and no longer able to block BCR activation of RelA and c-Rel monomers and p50/RelA and p50/c-Rel heterodimers. Remarkably, the *Nfkb1*^{ΔCT} allele also normalized FM B cell survival and plasmablast differentiation in vitro, and TD antibody responses in vivo, demonstrating that the inhibitory effects of *Nfkb1*^{SSAA} mutation on FM B cell function resulted from impaired NF-κB activation.

Nfkb1^{SSAA} mutation did not affect RelB and p52 NF-κB-binding activity in CD40L-stimulated cells, presumably because these subunits are predominantly associated with NF-κB2 p100 (Solan et al., 2002). Inability to inhibit activation of the alternative NF-κB pathway probably explains why *Nfkb1*^{SSAA} mutation did not affect *A1* mRNA up-regulation and the survival of FM B cells after CD40 stimulation, which activates both canonical and alternative NF-κB signaling pathways (Rickert et al., 2011). CD40 co-stimulation overcame the BCR-induced survival defect of *Nfkb1*^{SSAA/SSAA} FM B cells in vitro. However, although these cells could co-localize and presumably interact with CD40 on T cells in the spleen after BCR cross-linking, they still failed to mount an antibody response to NP₂₇-CGG. This suggests that defective survival alone did not block the TD antibody response of *Nfkb1*^{SSAA/SSAA} FM B cells. In line with this, transgenic overexpression of Bcl-xL, which restored in vitro BCR-induced survival of *Nfkb1*^{SSAA/SSAA} FM B cells to normal, did not rescue antibody responses to a TD antigen.

Nfkb1^{SSAA} mutation blocked the late activation of c-Rel after BCR cross-linking with anti-IgM, and impaired BCR induction of *Irf4* mRNA, a c-Rel-regulated gene (Grumont and Gerondakis, 2000). The up-regulation of IRF4 protein expression in MD4 B cells by HEL antigen in vivo was also prevented by *Nfkb1*^{SSAA} mutation, confirming the physiological significance of these findings. IRF4 is a critical transcription factor for the induction of plasma cell differentiation and Ig class switch recombination (Sciammas et al., 2006; De Silva et al., 2012). Consistent with this, *Nfkb1*^{SSAA} mutation reduced the ability of LPS plus IL-4 to induce IRF4 expression in FM B cells, impairing differentiation to IgM-secreting plasmablasts and switching to IgG1. The induction of plasmablast differentiation of MD4 B cells in vivo by HEL was also prevented by *Nfkb1*^{SSAA} mutation. In addition, retroviral expression of IRF4 rescued plasma cell differentiation of *Nfkb1*^{SSAA/SSAA} FM B cells stimulated with LPS and IL-4, confirming a functional effect of reduced IRF4 in these cells. These experiments establish *Irf4* as a physiologically important transcriptional target of the NF-κB1 signaling pathway in B cell antibody responses, and demonstrate for the first time the critical role of NF-κB activation in plasmablast differentiation.

CD19-Cre *Irf4*^{fl/fl} mice have a substantially reduced GC response, and residual GCs form only with B cells in which the *Irf4* allele has not been deleted (Ochiai et al., 2013). IRF4, therefore, plays an essential and B cell autonomous role in GC formation, regulating the expression of the key GC transcription factor *Bcl6* (Goodnow et al., 2010; Ochiai et al., 2013). Antigen-stimulated *Nfkb1*^{SSAA/SSAA} FM B cells did not up-regulate *Bcl6* or differentiate into GC B cells during a TD immune response. NF-κB signaling activity is very low in GC B cell centroblasts (Shaffer et al., 2001; Basso et al., 2004). However, IRF4 is required for the initiation, but not maintenance of the GC state (Klein et al., 2006; Ochiai et al., 2013). This suggests that *Nfkb1*^{SSAA} mutation may have prevented the initiation of GC formation by inhibition of NF-κB-dependent transcriptional up-regulation of *Irf4*. Consistent with this hypothesis, retroviral expression of IRF4 significantly increased antigen-induced differentiation of MD4 Bcl-xL *Nfkb1*^{SSAA/SSAA} B cells. The failure to induce IRF4 in *Nfkb1*^{SSAA/SSAA} B cells was a feature of agonist of the BCR and TLR4, but not CD40 (unpublished data). As TLR signaling is not required for antibody responses to antigens delivered in alum (Gavin et al., 2006), this suggests that the immune response defects detected were primarily caused by impaired BCR-mediated signaling.

In this study, we investigated the role of IKK-induced NF-κB1 p105 proteolysis in mature B cells, using *Nfkb1*^{SSAA/SSAA} mice in which this IKK signaling pathway is blocked (Srisankharajah et al., 2009). *Nfkb1*^{SSAA} mutation prevented FM B cells from mounting an antibody response to a TD antigen by selectively blocking BCR-induction of BCL-2 family proteins and IRF4, which impaired FM B cell survival and antigen-induced differentiation of FM B cells to plasmablasts and GC B cells, respectively. Our study demonstrates that the regulation of NF-κB activation in mature B cells by IKK-induced NF-κB1 p105 proteolysis is essential for TD humoral immune responses.

MATERIALS AND METHODS

Mouse strains. Mouse strains were bred in the specific pathogen-free animal facility of the National Institute for Medical Research (NIMR; London, UK). 7–12-wk-old mice were used for all experiments. *Rag1*^{-/-} (Mombaerts et al., 1992), *μMT*^{-/-} (Kitamura et al., 1991), and HEL-specific BCR transgenic mice (MD4; Mason et al., 1992) and *Nfkb1*^{SSAA/SSAA} (Srisankharajah et al., 2009), *Nfkb1*^{-/-} (Sha et al., 1995), and *Map3k8*^{-/-} (Dumitru et al., 2000) mouse strains have been previously described and were all fully backcrossed on to a C57BL/6 background. *Nfkb1*^{+/+}, *Nfkb1*^{SSAA/-} and *Nfkb1*^{SSAA/ΔCT} (Ishikawa et al., 1998) mice were generated by intercrossing *Nfkb1*^{SSAA/SSAA}, *Nfkb1*^{ΔCT/ΔCT}, and *Nfkb1*^{-/-} strains. *Nfkb1*^{SSAA/SSAA}.*Bcl-xL* and *Nfkb1*^{+/+}.*Bcl-xL* mice experimental mice were generated by intercrossing Bcl-xL transgenic mice (Takahashi et al., 1999) with *Nfkb1*^{SSAA/SSAA} and C57BL/6 mouse strains, respectively. These were subsequently intercrossed with MD4.*Rag1*^{-/-} mice to generate MD4.*Rag1*^{+/+}.*Nfkb1*^{SSAA/SSAA}.*Bcl-xL* and MD4.*Rag1*^{+/+}.*Nfkb1*^{+/+}.*Bcl-xL* mice.

This study was conducted following authorization by the UK Home Office, under relevant Project Licence authority.

BM chimeras and B cell adoptive transfer. BM cells were harvested from femora and tibiae of the indicated donor mice, and resuspended in ACK lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, 100 μM EDTA) to

lyse red blood cells. Cells ($4\text{--}5 \times 10^6$ per mouse) were then transferred by intravenous injection into sublethally irradiated *Rag1*^{−/−} mice (500 rads; Cs source). For competition experiments, BM cell suspensions from CD45.1⁺ C57BL/6 were mixed with either *Nfkb1*^{SSAA/SSAA} or wild-type *Nfkb1*^{+/+} CD45.2⁺ BM cells, at a 1:1 ratio. For mixed BM chimeras, sublethally irradiated *Rag1*^{−/−} mice were reconstituted with *Nfkb1*^{SSAA/SSAA} or wild-type *Nfkb1*^{+/+} BM cells mixed at 1:4 ratio with μ MT^{−/−} BM cells. Chimeric mice were immunized 8 wk after reconstitution.

To analyze B cell positioning in the spleen after BCR stimulation, $8\text{--}10 \times 10^6$ splenic purified B cells from either *Nfkb1*^{SSAA/SSAA} or wild-type mice were cultured in vitro with or without anti-IgM (10 μ g/ml; Jackson ImmunoResearch Laboratories) for 1 h (Pereira et al., 2009). Cells were then transferred into CD45.1⁺ C57BL/6 hosts by intravenous injection, and spleens removed 5.5 h later for immunofluorescence analysis.

To monitor plasma cell differentiation and GC B cell formation in vivo, $8\text{--}10 \times 10^6$ splenic purified B cells from *MD4.Rag1*^{+/−}.*Nfkb1*^{SSAA/SSAA}.*Bcl-xL* and *MD4.Rag1*^{+/−}.*Nfkb1*^{+/+}.*Bcl-xL* were cotransferred with $0.5\text{--}1.10^9$ HEL-conjugated or control sheep red blood cells into CD45.1⁺ C57BL/6 hosts by intravenous injection (Reif et al., 2002; Kelly et al., 2011). 6 d after transfer, recipient mice were culled and spleens were removed for flow cytometric analysis.

Assay of antibody responses. For TD antibody responses, mixed BM chimeras were immunized i.p. with 50 μ g NP₂₇-CGG in PBS (Biosearch Technologies) precipitated in alum (Imject; Thermo Fisher Scientific; 3:1 ratio). Alum mixed with PBS was used as a control. The production of NP-specific and total immunoglobulins (IgM and IgG1) was monitored by ELISA, using 96-well Maxisorp ELISA plates pretreated with 50 μ l/well of 5 μ g/ml NP₁₄-BSA (Biosearch Technologies). Antibody isotypes and subclasses were detected with horseradish peroxidase-conjugated mouse IgM and subclass IgG-specific antibodies (Southern Biotech).

Histological analyses. Cryosections (7–10 μ m) of spleen were prepared, dried, and fixed with acetone before immunofluorescent analysis (Turner et al., 1997). GC B cells were stained using GL7-FITC mAb (Ly77, BD), revealed with rabbit anti-FITC (Alexa Fluor 488 Signal-Amplification kit; Invitrogen), IgD-PE (11-26c; eBioscience), and B220-Alexa Fluor 647 (RA3-6B2, BD). For B cell migration assays, CD45.2-FITC (104, eBioscience), CD4-Alexa Fluor 647 (ABD Serotec), and IgD-PE (11-26c; eBioscience) were used to identify transferred B cells. Spleen sections were analyzed by immunofluorescence microscopy using either a Leica confocal microscope TSP-SP2 (Leica) or a DeltaVision RT Imaging System (Applied Precision Inc.).

Flow cytometry. Single-cell suspensions were generated from peripheral lymph nodes, spleen, BM, or peritoneal wash via gentle homogenization through nylon mesh filters (70 μ m; BD). Erythrocytes in BMs and spleens were lysed with ACK lysis buffer before staining, and cell concentrations were determined using a Casy Counter (Scharfe Instrument Systems). CD1d-PE and CD1d-bio (clone 1B1); CD21-FITC (eBio8D9); CD23-PE (B3B4); B220-FITC, B220-PE, B220-APC, and B220-eFluor450 (RA3-6B2); CD93-APC (AA4.1); IgM-PECy7 (II/41); IgD-FITC, IgD-eFluor450, IgD-PE, and IgD-bio (11-26); Ly5.1-FITC, and Ly5.1-APC (A20); Ly5.2-FITC, and Ly5.2-APC-eFluor780 (104), CD43-bio (eBioR2/60), IgG1-APC (X56), Gr1-bio (RB6-8C5), CD11b-Bio (M1/70); CD11c-Bio (N418); CD5-PE (53-7.3); CD19-FITC (MB19-1); TCR β -FITC (H57-597); and CCL19-Fc fusion protein (plus anti-human Fc-PE secondary to stain CCR7) were obtained from eBioscience. CD4-PerCP (RM4-5), B220-PerCP (RA3-6B2), and CD8-PerCP (53-6.7) were purchased from BioLegend; CD2-PE (RM2-5), CD9-bio (KMC8), CD21-PE (7G6), Streptavidin-PerCP (554064), CD138-PE (281-2), and GL7-FITC (Ly77) were obtained from BD; PNA-Bio (FL-1075) was purchased from Vector Laboratories; and CD19-APC (RM705) and CD23-APC (MCD2305) were purchased from Life Technologies. For thymus-dependent (TD) antibody response assays, NP₂₇-BSA (Biosearch Technologies) was labeled with the lynx rapid rpe or percp antibody conjugation kits (AbDserotec) according to

the manufacturer's protocol. Intracellular IRF4 was detected using IRF4-PE (eBRG1), after cell permeabilization (Foxp3 Fixation/Permeabilization kit; eBioscience). Live cells (Live/dead Fixable Neat-IR Dead Cell Stain kit; Life Technologies) and extracellular markers were identified by prior staining of intact cells.

Stained cells were analyzed by flow cytometry on a CyAn ADP Analyzer (Beckman Coulter) or FACSCanto II Analyzer (BD). Data analyses were performed with FlowJo 887 software (Tree Star).

B cell isolation and in vitro culture. Splenic follicular B cells were isolated by sequential hypotonic lysis of RBC and magnetic bead separation using a mixture of biotinylated anti-CD43 and anti-CD1d antibodies and Streptavidin-coupled Dynabeads (Life Technologies). Purity of the resulting FM B cell population (B220⁺CD23^{hi}CD21^{lo}) was >96% as assessed by flow cytometry. Cells were cultured at a density of 2×10^6 cells/ml in complete medium (DMEM plus 10% heat inactivated FCS [Lonza], 100 U/ml Penicillin, 100 μ g/ml Streptomycin, 100 μ M nonessential amino acids, 100 mM Na-Pyruvate, and 50 μ M 2-mercaptoethanol), and stimulated with anti-IgM (10 μ g/ml; Jackson ImmunoResearch Laboratories), CD40L (0.5 μ g/ml; R&D Systems), or LPS (1–10 μ g/ml; *Salmonella mimosetta* R595; Alexis Biochemicals) \pm IL-4 (20 ng/ml; PeproTech).

For in vitro survival assays, 3×10^5 B cells were cultured in a 96-well tissue-culture plate in complete medium (200 μ l) with or without stimulation (see above). Cells were harvested 2 d later, stained for a B cell marker (B220 or CD19) resuspended and 7-amino-actinomycin D (7-AAD; Sigma-Aldrich). Cell division was monitored by flow cytometry after labeling of FM B cells with 2 μ M CFSE (CellTrace; Invitrogen) in Dulbecco's PBS (Invitrogen). The frequency of the precursor population triggered into cell division was determined using FlowJo V8.5 proliferation analysis software (Tree Star). To monitor the antigen-presenting function of FM B cells, OVA was targeted to the BCR by treating purified B cells sequentially with biotinylated anti-IgM (0.5 μ g/ml; Jackson ImmunoResearch Laboratories) and anti-biotin OVA-FITC (Miltenyi Biotec). Antigen-pulsed B cells (2×10^6 /ml) were then co-cultured with Cell Trace Violet-labeled OTII CD4⁺ T cells (5 μ M; Life Technologies; 1×10^6 /ml) for 72 h. T cell activation was determined by estimating the ability of T cells to divide. To demonstrate that OVA presentation was actively mediated, antigen-pulsed B cells were fixed in 3% paraformaldehyde before co-culture with T cells.

To monitor plasma cell differentiation, FM B cells (1.5×10^6 /ml) were cultured with LPS (40 μ g/ml) plus IL-4 (20 ng/ml) for 4 d. Plasma cells (B220^{lo}CD138⁺) were identified by flow cytometry. In vitro IgM production was analyzed by harvesting cells after 4-d culture with LPS and IL-4, and replating washed live cells at 10^6 cells/ml in complete medium. Supernatants were collected after an additional 1-d culture, and analyzed by ELISA. The yields of *Nfkb1*^{SSAA/SSAA} and WT FM B cells were similar after reculture.

Retroviral infection. pMSCV-IRES-GFP, pMSCV-IRF4-IRES-GFP, and pMSCV-Blimp-IRES-GFP plasmids (Kallies et al., 2007) were a gift from A. Kallies (The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia). Retroviruses were prepared as previously described using Plat-E packaging cells (Robinson et al., 2007). FM B cells were cultured for 24 h with CD40L and IL-4 before retroviral infection. To analyze the effect of ectopic IRF4 expression on GC B cell formation, purified *MD4.Rag1*^{+/−}.*Nfkb1*^{SSAA/SSAA}.*Bcl-xL* and *MD4.Rag1*^{+/−}.*Nfkb1*^{+/+}.*Bcl-xL* FM B cells were transduced twice with retrovirus, prior to cotransfer with 0.5 to 1.10^9 HEL-conjugated or control sheep red blood cells (SRBC) into CD45.1⁺ C57BL/6 hosts by intravenous injection. Splenic B cells were analyzed by flow cytometry after 6 d.

Protein analyses. For immunoblots of total lysates, FM B cells (3×10^6 cells per point) were lysed RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 2 mM Na₂P₂O₇, 5 mM dithiothreitol, 1 mM Na₃VO₄, protease inhibitors [Roche], 0.5% deoxycholate, 1% Triton X-100, and 0.1% SDS). After centrifugation, lysates (20–25 μ g) were mixed with an equal volume of 2x SDS-PAGE sample buffer. For immunoprecipitation,

cells (5×10^7 per point) were lysed in IP buffer (10 mM Hepes, pH 7.6, 1 mM EGTA, 10 mM KCl, 1 mM dithiothreitol, 20 mM NaF, 1 mM $\text{Na}_4\text{P}_2\text{O}_7$, protease inhibitors, and 0.6% Nonidet-P40). Immunoprecipitations (150 μg of protein extract) were performed as previously described (Kabouridis et al., 1997).

To analyze nuclear translocation of Rel subunits, washed cells were lysed with buffer N (10 mM Hepes, pH 7.6, 0.1 mM EGTA, 10 mM KCl, 1.5 mM MgCl_2 , 1 mM DTT, 20 mM NaF, 1 mM Na-pyrophosphate, protease inhibitors, and 0.2% NP-40). After 2 min, nuclei were pelleted by centrifugation, washed twice in lysis buffer, and nuclear proteins extracted with RIPA buffer. Rel proteins in nuclear extracts (20 μg) were quantified by immunoblotting and laser densitometry, normalizing to SAM68 content.

The p105C antibody to the p105 C terminus, used for p105 immunoprecipitation, has been described previously (Salmerón et al., 2001). p50 antibody was obtained from (Delta Biolabs) and phospho S933 p105 antibody from Cell Signaling Technology. Antibodies to other Rel family proteins, SAM68 and I κ B α , were purchased from Santa Cruz Biotechnology, Inc. Tubulin was detected with TAT-1 α -tubulin mAb (provided by K. Gull, University of Oxford, Oxford, England, UK) and used as loading control protein on immunoblots of total cell lysates.

NF- κ B activation assays. Nuclear extracts were prepared as above, and activation of NF- κ B subunits assayed (2–4 μg per point) using a commercial ELISA kit (TransAm NF- κ B family kit; Active Motif). To increase sensitivity, c-Rel binding was assayed with anti-c-Rel (1 μg per well; sc-71X; Santa Cruz Biotechnology, Inc.). Data were normalized against SAM68 content of nuclear extracts.

Real-time quantitative PCR. RNA from stimulated and unstimulated FM B cells ($5 \times 10^6/\text{ml}$) was isolated using the RNeasy kit, and contaminating DNA removed using RNase-free DNase set (QIAGEN) according to manufacturer's instructions and DNase treated (Roche). cDNA was produced using the SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Life Technologies) and standard protocols. Expression of target genes was determined by real time PCR using a Perkin Elmer ABI Prism 7000 Sequence Detection System, and commercial FAM labeled probes (Applied Biosystems) with the TaqMan Gene Expression Master Mix (Applied Biosystems). Target gene mRNA levels were normalized against *Hprt* mRNA levels.

Statistical analysis. All data analyses were performed using GraphPad software (GraphPad Software Inc.). In vitro data were compared using Student's *t* test (two tailed and unpaired test). For in vivo experiments, all statistical comparisons were performed using the nonparametric two-tailed Mann-Whitney test. Statistically significant differences are indicated as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Online supplemental material. Fig. S1 shows gating strategies for Fig. 1. Fig. S2 shows gating strategies for Fig. 6. Fig. S3 shows gating strategies for Fig. 8 (E and F). Fig. S4 shows gating strategies for Fig. 8 H. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20132019/DC1>.

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