Negative role of TAK1 in marginal zone B-cell development incidental to NF-κB noncanonical pathway activation

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The transcription factor nuclear factor-κB (NF-κB) signaling pathway is crucial in B-cell physiology. One key molecule regulating this pathway is the serine/threonine kinase TAK1 (MAP3K7). TAK1 is responsible for positive feedback mechanisms in B-cell receptor signaling that serve as an NF-κB activation threshold. This study aimed to better understand the correlation between TAK1-mediated signaling and B-cell development and humoral immune responses. Here we showed that a B-cell conditional deletion of TAK1 using mb1-cre resulted in a dramatic elimination of the humoral immune response, consistent with the absence of the B-1 B-cell subset. When monitoring the self-reactive B-cell system (the immunoglobulin hen egg lysozyme/soluble hen egg lysozyme double-transgenic mouse model), we found that TAK1-deficient B cells exhibited an enhanced susceptibility to cell death that might explain the disappearance of the B1 subset. In contrast, these mice gained numerous marginal zone (MZ) B cells. We consequently examined the basal and B-cell receptor-induced activity of NF-κB2 that is reported to regulate MZ B-cell development, and demonstrated that the activity of NF-κB2 increased in TAK1-deficient B cells. Thus, our results present a novel in vivo function, the negative role of TAK1 in MZ B-cell development that is likely associated with NF-κB2 activation. (ImmunoLOGY and Cell Biology (2016) 94, 821–829; doi:10.1038/icb.2016.44)

Activation of the nuclear factor-κB (NF-κB) signaling pathway is known to play an important role in physiological and pathological processes including inflammation, immunity and cell survival.¹⁻³ The phosphorylation and subsequent degradation of the NF-κB inhibitor IκB induced by the IκB kinase (IKK) complex, which is composed of the IKK-α and IKK-β kinases and a regulatory subunit of IKK-γ (NEMO), are central signaling events that lead to the translocation of the NF-κB subunits NF-κB1, RelA and c-Rel to the cell nucleus. This so-called canonical pathway is utilized by a variety of cellular stimuli including proinflammatory cytokines and pathogens. In contrast, the noncanonical pathway activates the alternate NF-κB subunits NF-κB2 and RelB.

B-cell receptor (BCR) signaling also shares this canonical cascade that is pivotal for B-cell development, maintenance, function and pathogenesis.⁴⁻⁵ Consistent with this, genetic mutations of pathway mediators have been reported in B-cell lymphomas.⁶ BCR signaling employs the adapters CARD-containing MAGUK protein 1 (CARMA1, also called CARD11), Malt1 and Bcl-10 that serve as a scaffold for the signaling modules and which activate the IKK signalosome through the phosphorylation of CARMA1 by protein kinase C-β. The signal is further propagated by a member of the MAP3K (mitogen-activated protein kinase (MAPK) kinase kinase) family, TAK1 (MAP3K7), that has been characterized as a key common upstream kinase of IKK in inflammatory and immune signaling pathways.⁵⁻⁷ The positive feedback loop formed by the CARMA1/TAK1/IKK signaling cascade has been shown to generate a unique and dynamic NF-κB activation 'switch-like' activity⁸ that confers a NF-κB activation threshold that might determine antigen response.

The molecular functions of TAK1 in vitro have been intensely investigated using cell lines.⁹ However, the physiological role and development of TAK1 in B lymphocytes remains unclear. Two studies on B-cell conditional TAK1 deletion using CD19-cre elucidated the development of major peripheral subsets, the humoral immune response and BCR-induced IKK/NF-κB activation.¹⁰,¹¹ One group showed that the B-1 B-cell population was reduced, whereas the development of splenic follicular B cells and marginal zone B (MZ B) cells was normal. BCR-mediated IKK/NF-κB activation was not altered, although humoral immune responses were impaired.¹⁰ In contrast, another group showed that the development of B-1 B as well

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as follicular B and MZ B cells was reduced in addition to a reduction in the activation of IKK/NF-κB, although, conversely, the immune responses were normal.\textsuperscript{11}

We have clearly demonstrated in our previous work that TAK1 is essential for the canonical NF-κB pathway in BCR signaling using mb1 (Cd79a)-cre,\textsuperscript{3} an effective deleter that expresses cre recombinase from the mb1 gene that encodes the Ig-α signaling subunit of the B-cell antigen receptor.\textsuperscript{13} Here, we used these mice in conjunction with the hen egg lysozyme (HEL)-transgenic mouse system to investigate the effect of TAK1 deletion on the survival of autoreactive B cells and splenic B-cell subtypes including transitional B-cell subsets, follicular B cells and MZ B cells. We further investigated the basal and BCR-induced activity of NF-κB2 to determine the role of the NF-κB2 noncanonical pathway in MZ B-cell development in conjunction with TAK1-associated canonical NF-κB2 signaling.

RESULTS

TAK1 is indispensable for immune responses

B cells mediate humoral immunity, in which BCR signaling plays a central role upon encountering an antigen.\textsuperscript{13} To address the influence of TAK1 deletion on biological outcomes related to B cells in vivo, we initially observed basal antibody production following immunization in TAK1\textsuperscript{+/−}Mb1\textsuperscript{cre/+} (TAK1 B-cell knockout (TAK1-bKO)) or control initial antibody titer of all isotypes in TAK1-bKO mice were lower than those of control mb-1 mice (Figure 1a). Neither the immune response to a T-cell dependent antigen (4-hydroxy-3-nitrophenyl (NP) chicken γ-globulin; NP-CGG/alum) nor a T-cell independent type II antigen (NP-Ficoll) was eradicated in TAK1-bKO mice (Figure 1b).

TAK1 is required for cellular responses induced by BCR engagement or lipopolysaccharide or CpG stimulation

We next confirmed cell proliferation, activation markers and survival in response to mitogenic stimuli. As reported previously,\textsuperscript{10,11} cell proliferation and the induction of activation markers in response to the Toll-like receptor ligand lipopolysaccharide, an oligodeoxy-nucleotide (CpG-ODN), CD40 stimulation or anti-IgM crosslinking (Supplementary Figure 1) were significantly blocked in TAK1-bKO B cells. Consistent with the proliferation results, TAK1-bKO B cells exhibited impaired survival in response to anti-IgM, lipopolysaccharide or CpG (Figure 2a). On the other hand, stimulation with B-cell activating factor (BAFF, tumor necrosis factor ligand superfamily, member 1b) or anti-CD40 resulted in normal cell survival. As BAFF predominantly induces the activation of the noncanonical NF-κB pathway and CD40 mediates both the canonical and noncanonical pathways,\textsuperscript{14-16} these results suggest that TAK1 is preferentially required for the canonical NF-κB pathway.

TAK1 deficiency impairs B-1a B-cell development

To understand the role of TAK1 in the development of B cells, we evaluated the cellularity in TAK1-bKO mice using flow cytometry (Figures 2b and c). Although the total number of B220-positive cells in the peritoneal cavity was comparable to that in control mb-1 mice, the B-1 subset (B220\textsuperscript{+}CD5\textsuperscript{−}) was clearly reduced in TAK1-deficient mice (Figure 2b upper panel, and Figure 2c). Because the B-1 population contains the B-1a and B-1b subsets,\textsuperscript{17,18} we next explored these specific populations. B cells from the TAK1-bKO and mb-1 peritoneal cavity were gated into IgM\textsuperscript{hi}IgD\textsuperscript{low} as B-1 cells and into IgM\textsuperscript{lo}IgD\textsuperscript{hi} as B-2 cells (Figure 2b, middle panel); in addition, we observed the expression of B220 and CD5 (Figure 2b, bottom panel). Few B-1a cells (IgM\textsuperscript{hi}IgD\textsuperscript{low}B220\textsuperscript{low}CD5\textsuperscript{+}) in TAK1-bKO mice were found, and although B-1b cells (IgM\textsuperscript{lo}IgD\textsuperscript{low}B220\textsuperscript{+}CD5\textsuperscript{−}), B-2 cells (IgM\textsuperscript{lo}IgD\textsuperscript{hi}) and mature B cells (IgM\textsuperscript{lo}IgD\textsuperscript{hi} B220\textsuperscript{+}CD5\textsuperscript{+}) were observed, their numbers were not significantly increased upon TAK1 deletion (Figure 2c). The major role of B-1a cells is to spontaneously secrete IgM, especially in the circulating IgM;\textsuperscript{19} thus, the observed impaired basal IgM titers (Figure 1a) were consistent with the poor development of B-1a cells in the TAK1-bKO mice. Furthermore, TAK1-bKO animals failed to respond to immunization (Figure 1b), possibly because of the absence of B-1a cells as this subset is required for the rapid and proper production of antibodies to react to T cell-independent antigens.\textsuperscript{17}

Sensitivity to cell death is increased in TAK1-deficient self-reactive B cells

B-1 B cells develop, in part, through the positive selection of self-reactivity.\textsuperscript{20} To understand the self-reactivity of TAK1-deficient B cells, we crossed the TAK1-bKO mice with soluble HEL/HEL-Ig transgenic mice,\textsuperscript{21} a well-characterized system in which the BCR transgene recognizes HEL as self. However, a change in the receptor expression profiles between HEL-Ig and HEL-lg in the presence of TAK1-bKO was not observed (Figures 3a and b). The double transgene (shEL/HEL-lg (wHEL)) yielded a phenotype of down-modulated IgM but retained its expression of the total transgene-encoded receptor (heavy chain of IgM and IgD (IgH)) as compared with that of the reported HEL-lg single transgene-encoded receptor. In contrast, the combination of defective TAK1 expression with wHEL exhibited reduced expression of IgM; notably, the total transgene IgH level was also significantly decreased (Figures 3a and b). To address the cause of this reduction, we investigated the survival of wHEL B cells. As shown in Figures 3c and d, the spontaneous cell death of B220\textsuperscript{+} gated cells (Supplementary Figure 2) in TAK1-deficient wHEL mice was significantly increased, suggesting that the enhanced susceptibility of TAK1-bKO mice to cell death reduces the B-1a B-cell subset.

MZ B cells are augmented by TAK1 deletion

We next examined whether TAK1 deletion affected B-cell maturation in the spleen. The number of splenic B220-positive cells was comparable between mb-1 and TAK1-bKO mice (Figures 4a and b). Splenic B cells are mainly composed of three subsets: transitional (T1−3), MZ and follicular.\textsuperscript{18,22,23} Transitional B cells are characterized by the phenotype of their surface receptors: AA4.1 (CD93)+ IgM\textsuperscript{hi}CD23\textsuperscript{−} as T1, AA4.1\textsuperscript{+}IgM\textsuperscript{hi}CD23\textsuperscript{−} as T2 and AA4.1\textsuperscript{+}IgM\textsuperscript{low}CD23\textsuperscript{+} as T3 cells. The number of T2 and T3 B cells was markedly reduced in TAK1-bKO mice (Figures 4a and b), whereas the cell numbers of AA4.1\textsuperscript{+}CD21\textsuperscript{med}CD23\textsuperscript{−} Fol and IgM\textsuperscript{hi}IgD\textsuperscript{hi}CD21\textsuperscript{med} FoII were normal. Conversely, AA4.1\textsuperscript{+}CD21\textsuperscript{hi}CD23\textsuperscript{−} MZ B cells were augmented in TAK1-bKO animals. In addition, the MZ precursor, defined as IgM\textsuperscript{hi}IgD\textsuperscript{hi}CD21\textsuperscript{hi}, was found in large numbers.

NF-κB2 activity is enhanced in TAK1-deficient B cells

The selective accumulation of MZ B cells in TAK1-bKO mice prompted us to consider the possibility that the noncanonical pathway was hyper- or constitutively activated, as it has been previously reported that constitutive activation of the noncanonical pathway promotes MZ B-cell development.\textsuperscript{24} To further explore this phenomenon, we performed immunoblotting to observe the activity of the noncanonical NF-κB components NF-κB2 and RelB in response to BCR engagement (Figure 5a). In mb-1 control cells, the cytosolic fraction of the NF-κB2 precursor p100 was processed to the activated p52 form; this was increased by anti-IgM (anti-μ) stimulation at 5 min, whereas p100 was diminished 10 min after stimulation.
In TAK1-bKO cells, p100 was apparently decreased before stimulation, whereas p52 was increased by degrees in response to BCR crosslinking. Although the p52 in the mb-1 and TAK1-bKO nuclear fractions did not show these relative particular behaviors, the basal level of p52 and its DNA-binding potency was clearly enhanced in TAK1-bKO cells (Figures 5b and c, respectively).

As the loss of canonical NF-κB signaling leads to enhanced processing of p100,25,26 and RelB is constantly associated with p100,27 we postulated that the accumulation of nuclear RelB might be caused by the higher processing of p100 in the absence of TAK1. To confirm this, we observed the NF-κB-inducing kinase (NIK)-mediated phosphorylation of NF-κB2 at Ser 866 and 870 that regulates its processing.28 Notably, NF-κB2 was constitutively phosphorylated and NIK protein stability was increased in TAK1-bKO mice (Figure 5d).

Furthermore, augmented mRNA expression of Pim2, the specific target gene of the noncanonical pathway,29 was seen in a previous report of TAK1-KO microarray analysis.8 To confirm this finding, we performed quantitative PCR. Accordingly, Pim2 mRNA expression in TAK1-bKO was found to be increased over basal levels and in response to BCR stimulation, at least across the examined time span, compared with the mb-1control (Figure 5e). These lines of evidence indicated that the lack of TAK1 leads to enhanced activation of the noncanonical NF-κB2 pathway. In supporting of this model, an increase in the number of recirculating B cells (B220hiCD43−) in the bone marrow was also observed in TAK1-bKO mice (Figures 5f and g), likely because the noncanonical pathway is important for the regulation of these cells.30 Thus, an accumulation of MZ B cells among TAK1-deficient B cells was assumed to be caused by enhanced activation of the NF-κB noncanonical pathway.

**DISCUSSION**

We previously highlighted the molecular mechanisms of BCR signaling in vitro, revealing that the CARMA1/TAK1/IKK cascade forms a positive feedback loop during BCR signaling to produce ‘all or nothing’ NF-κB activation.31–33 This positive feedback is considered to be an intrinsic B-cell mechanism that determines whether a threshold should be activated, possibly allowing B cells to discriminate against self-antigens. The analysis and potential manipulation of this signaling cascade is a promising approach to develop therapeutic strategies for autoimmune and immunodeficiency disorders and...
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**Figure 2** TAK1 is required for cell survival following stimulation and for B-1a B-cell development. (a) B cells were isolated from mb-1Cre/TAK1F/F and mb-1Cre/TAK1+/+ mouse spleens. Survival was monitored after stimulation for 72 h with anti-IgM (anti-μ, 10 μg/ml−1), anti-CD40 (2 μg/ml−1), BAFF (200 ng/ml−1), lipopolysaccharide (LPS; 5 μg/ml−1), CpG (100 μμ) or with a combination of anti-IgM (10 μg/ml−1) and either BAFF (40 ng/ml−1) or anti-CD40 (2 μg/ml−1). The survival percentages are presented as the mean±s.d. (n=4). Results are from two similar experiments. Asterisks indicate one-way analysis of variance (ANOVA) results where **P<0.01, ****P<0.001 versus mb-1Cre/TAK1F/F. (b) Subpopulations in the peritoneal cavity. B cells from the peritoneal cavity of mb-1Cre/TAK1F/F and mb-1Cre/TAK1+/+ mice were stained with antibodies to B220, CD5, IgM and IgD and were subjected to flow cytometric analysis. Numbers in the boxes indicate the percentages of B cells in the gated populations. (c) Cell numbers of B220+, B-1a, B-1b, mature and B-2 B cells. Results represent the mean±s.d. of three mice for each genotype. Asterisks indicate one-way ANOVA results where ****P<0.001 versus mb-1Cre/TAK1F/F.

The response to the thymus-dependent antigen involves certain processes such as T-cell assistance via CD40 signaling.24,14 As CD40-induced functions are largely dependent on the NF-κB canonical pathway, cell proliferation and the expression of activation markers significantly decreased in TAK1-bKO animals. However, residual responses and normal survival of TAK1-bKO B cells are likely because CD40 employs TAK1-independent multi-pathways including the NF-κB noncanonical, phosphatidylinositol-3-kinase (PI3K)/Akt and MAPK pathways, although its biological role in CD40 signaling is relatively unestablished.41,42 In other processes such as affinity maturation and germinal center formation, the strength of BCR toward the canonical NF-κB pathway is important.4,14 Thus, the severely impaired response to the thymus-dependent antigen in TAK1-deficient B cells was considered to be in accordance with these known processes.

Overall, the observations in this study are clearcut compared with those from previous reports.10,11 Although the exact reasons for the differences between the study results are not evident, we consider that a reasonable explanation would be the difference in efficiency of the gene deletion. We used mb1-cre mice, in which the recombination efficiency is reported to reach 99% in splenic B cells compared with ~80% obtained with CD19-cre lines.12 In addition, as the difference...
CD19/PI3K signaling. The synergistic effects of haploinsufficiency occur because the noncanonical pathway might compensate for severe canonical pathway defects as this has previously been observed in some cases. In addition, in p100-deficient mice that lack the inhibitory portion of NF-kB but express active p52, the numbers of MZ and MZ precursor B cells were markedly elevated. In our work, we did not observe obvious alterations in the survival response to BAFF stimulation (Figure 2), and the expression of its receptors (Supplementary Figure 3). Thus, we considered that the BAFF receptor-mediated signal was unlikely to contribute to the TAK1-deficient phenotype.

It has been proposed that Bcl-3 negatively regulates NF-kB-dependent transcription such as proinflammatory gene expression, whereas Bcl-3-deficient B cells exhibit an increase in MZ B-cell numbers. Therefore, we examined the effects of TAK1 deletion on Bcl-3 nuclear localization (Supplementary Figure 4). In response to BCR stimulation, the nuclear translocation of Bcl-3 was reduced, implying the involvement of TAK1 in the Bcl-3 translocation signal. However, we failed to determine a link between TAK1-mediated and Bcl-3 activating signals owing to limited information on the function of Bcl-3 in B cells or in association with BCR signaling; in addition, the means by which Bcl-3 controls MZ B-cell development remain undetermined.

Alternately, we inferred that a possible MZ B-cell expansion under TAK1 deficiency occurs because the noncanonical pathway might compensate for severe canonical pathway defects as this has previously been observed in some cases. In addition, in p100-deficient mice that lack the inhibitory portion of NF-kB but express active p52, the numbers of MZ and MZ precursor B cells were markedly elevated. Furthermore, MZ B cells are able to develop and accumulate in mice lacking the p50 component of the NF-kB-mediated transcription complex. Here, we showed that the basal and BCR-induced activities of noncanonical NF-kB signaling were higher in TAK1-deficient B cells (Figure 5). We also noted an incremental elevation of mRNA expression of the PIM2 kinase that represents the target gene of the noncanonical pathway. In addition, the numbers of recirculating B cells, which are thought to be dependent on the noncanonical pathway, also increased. Together, these data supported our concept that a defect of the NF-kB canonical pathway consequent to

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**Figure 3** Increased cell death sensitivity in TAK1-deficient self-reactive B cells. For flow cytometric analysis, spleen cells were recovered from HEL-lg/TAK1F/F, HEL-lg/mb1-cre/TAK1F/F, sHEL/HEL-lg/TAK1F/F and sHEL/HEL-lg/mb1-cre/TAK1F/F mice. (a) Cells were stained with anti-mouse IgM and biotin-conjugated lysozyme (top) or anti IgM and IgD recognizing the a-allotype encoded by a transgene (bottom). (b) Percentages of IgM\(^+\)/HEL-binding\(^+\) (bind), IgM\(^+\)/HEL-binding\(^+\), IgM\(^+\)/IgD\(^+\), IgM\(^+\)/IgD\(^+\) and total Ig-heavy chain (IgH)\(^+\) B cells. Data represent the mean ± s.d. of three individual mice. Asterisks indicate one-way analysis of variance (ANOVA) results where \( * \) \( P < 0.05 \), \( ** \) \( P < 0.01 \) versus sHEL/HEL-lg/TAK1F/F. (c) B220\(^+\) cells were gated (Supplementary Figure 2) and subjected to 7-amino-actinomycin D (7AAD) and anti-IgM staining. Boxes within boxes indicate the 7AAD-positive fraction as the dying population. (d) Percentages of the dying population in each genotype. Data represent the mean ± s.d. of three individual mice. Asterisks indicate one-way ANOVA where \( *** \) \( P < 0.005 \) versus sHEL/HEL-lg/TAK1F/F.
TAK1 deletion causes compensatory noncanonical pathway activation that promotes MZ B-cell accumulation. Because BCR induced the activation of noncanonical pathway components independent of TAK1 (Figure 5), it is possible that TAK1 has a negative role in the noncanonical pathway as an intrinsic function. TAK1 has been suggested to negatively contribute to disease regulation in neutrophils and hepatocytes.25,26,58 In accordance with this, mutual control between the canonical and noncanonical pathways has been recently investigated by several laboratories.25,26,58

In this study, we identified higher levels of nuclear RelB in TAK1-deficient cells, suggesting a possibility that the defect of the CARMA1/TAK1/IKK signaling cascade caused by TAK1 deficiency might lead to a failure of MALT1-mediated RelB cleavage, thereby resulting in nuclear RelB accumulation. Notably, the inhibition of MALT1 protease activity further increased the amount of nuclear RelB (Supplementary Figure 5A). However, the cleaved form of RelB was normally observed in TAK1-bKO cells (Supplementary Figure 5B). We note that these results are not mutually exclusive, as RelB cleavage is not dependent on IKK activity27 and MALT1 protease inactive mutant mice exhibit a reduced MZ B-cell population.59,61

The loss of IKKx, IKKβ or NF-kB1 has been shown to be associated with enhanced NF-κB2 and p100 processing,25,26 similar to that observed in TAK1-deficient B cells, suggesting that canonical NF-kB signaling regulates the activation of the noncanonical NF-kB pathway. This enhanced processing of p100 in IKKx-deficient cells is well correlated with NIK (Map3k14) protein abundance.25 A higher degree of NIK stabilization was also found in TAK1-deficient cells (Figure 5d). Furthermore, Bcl10 has been implicated in the control of NIK activation.52 Collectively, we considered that the elevation in p100 processing diminished the function of the inhibitor of NF-kB (IkBα),26 thus allowing translocation of RelB to the nucleus, because p100 has been shown to be consistently associated with RelB.27 Although the mechanisms underlying the molecular linkage between these signaling modules connecting the NF-κB canonical and noncanonical pathways should be further investigated, our data provide information that clarifies the profound role of TAK1 in vivo and also reveal another aspect of its function, the negative role of the CARMA1/TAK1/IKK signaling cascade toward the NF-κB noncanonical pathway. Together, these findings provide important clues for the derivation of new therapeutic disease strategies and facilitate our understanding of these complex signal networks.

METHODS

Mice and immunization
TAK1<sup>−/−</sup> mice and mb-1 cre have been described previously.8,10,12 HEL-lg transgenic mice (C57BL/6 MD4) and soluble HEL (sHEL) transgenic mice (C57BL/6 MLS) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). All mice were 8–12 weeks of age. We generated HEL-lg/TAK1<sup>−/−</sup>, HEL-lg/mb1-cre/TAK1<sup>−/−</sup>, sHEL/HEL-lg/TAK1<sup>−/−</sup> and sHEL/HEL-lg/mb1-cre/TAK1<sup>−/−</sup> mice. For immunization, mice were administered 100 μg NP-CGG in alum or 50 μg NP-Ficoll intraperitoneally (Biosearch Technologies, Novato, CA, USA). For the second immunization, mice were administered 50 μg NP-CGG intraperitoneally without an adjuvant. Mice were maintained under specific pathogen-free conditions, and all protocols were approved by the RIKEN Animal Committee.

Enzyme-linked immunosorbent assay
NP-specific IgM, IgG1 or IgG3 titers were measured using enzyme-linked immunosorbent assay with NP11-bovine serum albumin-coated plates and detected with a horseradish peroxidase-conjugated goat antibody against mouse IgM, IgG1 or IgG3 (Southern Biotech, Birmingham, AL, USA). The wells were developed with the tetramethylbenzidine substrate (KPL Inc., Gaithersburg, MD, USA), and the absorbance was measured at 450 nm. Antigen-specific antibody titers were determined by interpolation of the dilution factor to a...
linear absorbance value from a standard curve generated by serial dilution of the serum.

Flow cytometry
Mice were killed by CO2 asphyxiation and lymphocytes were isolated from the spleens and bone marrow from TAK1−/−Mb1cre/+ or control TAK1+/+Mb1cre/+ mice by cell strainer (70 μm) and were stained with B220 and IgM antibodies and were subjected to flow cytometric analysis. The numbers in the boxes indicate the percentages of B cells in the gated populations.

Cell culture and reagents
Mice were killed by CO2 asphyxiation and lymphocytes were isolated from the spleens and bone marrow from TAK1−/−Mb1cre/+ or control TAK1+/+Mb1cre/+ mice by cell strainer (70 μm) and were stained with B220 and IgM antibodies and were subjected to flow cytometric analysis. The numbers in the boxes indicate the percentages of B cells in the gated populations. (e) Pim2 mRNA expression. The relative expression (a.u.) is standardized using the housekeeping gene Hprt. Results represent the mean ± s.e.m. of two independent experiments of a three-mouse pool for each genotype. Asterisks indicate one-way ANOVA results where *P < 0.05 versus mb-1Cre/TAK1+/+. (f) B cells from the bone marrow of mb-1Cre/TAK1+/+ and mb-1Cre/TAK1−/− mice were stained with B220 and IgM antibodies and were subjected to flow cytometric analysis. The data were obtained from two independent experiments. Asterisks indicate one-way ANOVA results where *P < 0.05 versus mb-1Cre/TAK1+/+.

Proliferation and cell survival assessment
B-cell proliferation was determined using a Cell Counting Kit-8 (Dojindo Labs, Kumamoto, Japan) following the manufacturer’s protocol, and the absorbance at 450 nm was measured using a microplate reader (Bio-Rad, Richmond, CA, USA) as previously described.65 Cell survival was evaluated by flow cytometry following propidium iodide or 7-amino-actinomycin D staining. The survival percentage indicated the propidium iodide- or 7-amino-actinomycin D-negative population.

Western blot analysis
Western blot analysis was performed as described previously.8 For NF-κB activity, nuclear and cytoplasmic fractions were prepared following as follows. Cells were lysed with lysis buffer containing 50 mM Tris-HCl, 137.5 mM NaCl, 10% glycerol, 5 mM EDTA and a proteinase inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Cell lysates were incubated on ice for 15 min. insoluble nuclei were separated by centrifugation at 12,000 r.p.m. for 15 min at 4 °C. The supernatant was used as the cytoplasmic fraction. For whole-cell lysates, cells were solubilized in lysis buffer (0.5% NP-40, 0.5% Triton X-100) and were then resuspended in 1% NP-40 lysis buffer and used as the nuclear fraction. For whole-cell lysates, cells were solubilized in lysis buffer (0.5% NP-40, 0.5% Triton X-100, 20 μM EDTA, 150 mM NaCl, 12.5 mM β-glycerophosphate, 1.5 mM MgCl2, 2 mM EGTA and 10 mM NaF) supplemented with protease and phosphatase inhibitors (Roche) and the post-nuclear removal lysate was used.

Figure 5 Enhanced activity of NF-κB2 in TAK1-deficient B cells. (a–e) Purified B cells from spleens stimulated with or without anti-IgM (anti-μ, 10 μg ml−1) antibody at specific time periods. Fractionated cell samples, cytoplasmic (Cyt) and nuclear (Nuc) fractions were subjected to western blotting with the indicated antibodies (a, b and d). The data were obtained from two independent experiments. (c) DNA-binding activity of NF-κB2. Binding activity was measured as described in the Methods section. Data are displayed as a fold change comparison of control mb-1Cre/TAK1+/+ samples and the mean ± s.d. of three individual assays. Asterisks indicate one-way analysis of variance (ANOVA) results where ****P < 0.001 versus mb-1Cre/TAK1+/+. (d) Whole-cell lysates were subjected to western blotting with anti-phospho-NF-κB2 (P-NF-κB2) or anti-NIK antibodies. The data were obtained from two independent experiments. (e) Pim2 mRNA expression. The relative expression (a.u.) is standardized using the housekeeping gene Hprt. Results represent the mean ± s.e.m. of two independent experiments of a three-mouse pool for each genotype. Asterisks indicate one-way ANOVA results where *P < 0.05 versus mb-1Cre/TAK1+/+. (f) B cells from the bone marrow of mb-1Cre/TAK1+/+ and mb-1Cre/TAK1−/− mice were stained with B220 and IgM antibodies and were subjected to flow cytometric analysis. The numbers in the boxes indicate the percentages of B cells in the gated populations. (g) Cell numbers of pro (B220+CD43−), pre (B220+CD43−IgM+ (imm)) and recirculating (B220+CD43+ (re-cir)) B cells of the described genotype. Results represent the mean ± s.d. of three mice for each genotype. Asterisks indicate one-way ANOVA results where *P < 0.05 versus mb-1Cre/TAK1+/+.
The DNA-binding activity of NF-xB was analyzed using TransAM NF-xB Kits (Active Motif, Carlsbad, CA, USA) following the manufacturer's protocol, and the absorbance at 450 nm was measured using a microplate reader (Bio-Rad).

Quantitative PCR
Total RNA from murine splenic B cells purified by CD43<sup>+</sup> cell depletion was collected using the NucleoSpin RNA kit (Macherey-Nagel GmbH & Co., Düren, Germany) and subjected to complementary DNA synthesis and quantitative PCR using the KOD SYBR<sup>®</sup> qPCR kit (ToyoBo Life Science, Osaka, Japan) according to the manufacturer's instructions. PCR cycling conditions were as follows: 40 cycles of 10 s at 98 °C, 10 s at 60 °C and 30 s at 68 °C. The primers used to detect the transcripts were as follows: Pmo2 (5'-CAG CTT TGCG AGG CAA ACC TTC-3') and 5'-GAA GAG ATC CTG AGC AGG AGC CAT ATG-3'); and Hprt (as a housekeeping gene for normalization) (5'-CAG GGT TAG TAT CGA TGA TGA ACC-3' and 5'-GCA TCT CCT TGA CAT CTC GAG-3').

Statistical analysis
Data are presented as the mean ± s.d. Statistical analysis was performed with Student's t-test and one-way analysis of variance using Microsoft Excel (Redmond, WA, USA).

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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