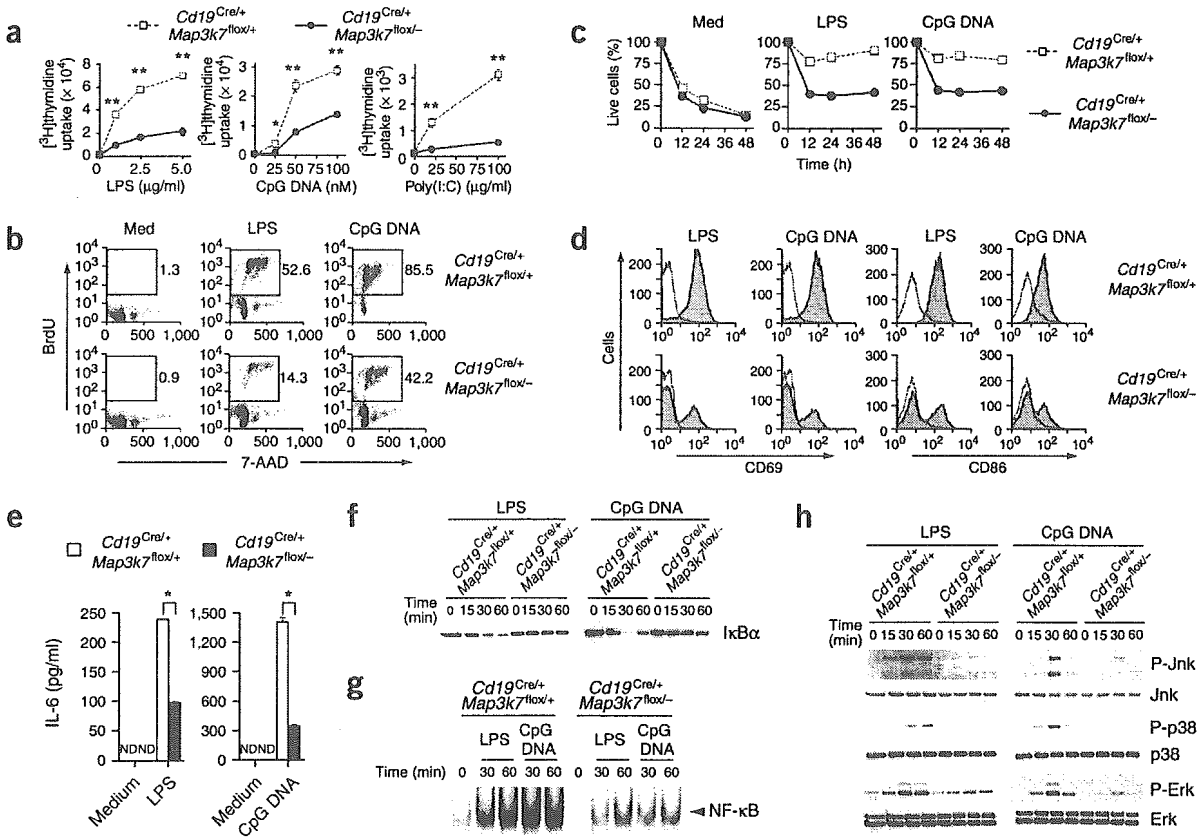

Figure 5 Impaired B cell activation in response to TLR ligands in *Cd19*$^{Cre/+}$*Map3k7*$^{flox/-}$ mice. (**a**) Proliferation of purified splenic B cells treated 48 h with various stimuli (horizontal axes), assessed by [^{3}H]thymidine incorporation. Data are mean ± s.d. of triplicate cultures. *, $P < 0.05$ and **, $P < 0.005$, versus TAK1-deficient cells (Student's *t*-test). (**b**) Cell cycle profiles of B cells left untreated (Med) or after *in vitro* stimulation with 5 μg/ml of LPS or 100 nM CpG DNA. Cells were labeled with BrdU and were analyzed by flow cytometry 24 h after stimulation. Numbers beside boxed areas indicate percentage of cells in S phase. (**c**) Defective survival of *Cd19*$^{Cre/+}$*Map3k7*$^{flox/-}$ cells. B cells were stimulated with 5 μg/ml of LPS or 100 nM CpG DNA. Viability of cells was assessed by staining with annexin V–indocarbocyanine followed by flow cytometry (time, horizontal axes). (**d**) Surface expression of activation markers. B cells were left unstimulated (open) or were stimulated for 24 h with 5 μg/ml of LPS or 100 nM CpG DNA (filled). Cells were then stained with anti-CD69 or anti-CD86. (**e**) ELISA of IL-6 production by B cells stimulated with LPS (20 μg/ml) or CpG DNA (2 μM). Data are mean ± s.d. of triplicate samples of one representative of three independent experiments. *, $P < 0.005$, versus TAK1-deficient cells (Student's *t*-test). (**f**) Immunoblot of IκBα degradation by B cells in response to LPS (20 μg/ml) or CpG DNA (2 μM). (**g**) EMSA of NF-κB DNA-binding activity in nuclear extracts of purified splenic B cells treated (time, above lanes) with LPS (20 μg/ml) or CpG DNA (2 μM). (**h**) Immunoblot of lysates of B cells stimulated (time, above lanes) with LPS (20 μg/ml) or CpG DNA (2 μM). Antibodies, right margin. All results are representative of three different experiments.

but not Jnk downstream of Bcl10 in BCR signaling. Mice lacking Bcl10, CARD11 or MALT1 are reported to have defects in the development of B-1 B cells and B cell activation. That prompted us to hypothesize that TAK1 may be recruited to the Bcl10 complex to activate Jnk. Therefore, we examined the association of TAK1 and Bcl10 using the human B cell line WEHI-231. When the cells were stimulated with antibody to IgM (anti-IgM), Bcl10 was immunoprecipitated together with TAK1 and with CARD11 (**Fig. 6i**). In contrast, TAK1 failed to precipitate together with Bcl10 in LPS-stimulated cells (**Fig. 6i**). These results suggest that TAK1 is recruited to the Bcl10 complex after BCR stimulation and is involved in Bcl10-mediated Jnk activation in B cells.

TAK1 is required for *in vivo* immune responses

We further investigated the involvement of TAK1 in humoral immune responses. The serum immunoglobulin concentrations of all isotypes except IgM were lower in *Cd19*$^{Cre/+}$*Map3k7*$^{flox/-}$ B cells than in *Cd19*$^{Cre/+}$*Map3k7*$^{flox/+}$ B cells (**Fig. 7a**). To induce humoral immune responses, we challenged *Cd19*$^{Cre/+}$*Map3k7*$^{flox/-}$ and littermate *Cd19*$^{Cre/+}$*Map3k7*$^{flox/+}$ mice with the T cell–dependent antigen nitrophenol conjugated to chicken γ-globulin or with the T cell–independent type II antigen trinitrophenol conjugated to Ficoll. The production of antigen-specific IgG1 in response to the T cell–dependent antigen was considerably impaired in *Cd19*$^{Cre/+}$*Map3k7*$^{flox/-}$ mice compared with that of control *Cd19*$^{Cre/+}$*Map3k7*$^{flox/+}$ mice, whereas IgM titers were similar in both groups of mice (**Fig. 7b**). Similarly, IgG3 production of *Cd19*$^{Cre/+}$*Map3k7*$^{flox/-}$ mice injected with trinitrophenol-Ficoll was impaired compared with that of control mice (**Fig. 7c**). This might have been due to the reduction in B-1 B cells in *Cd19*$^{Cre/+}$*Map3k7*$^{flox/-}$ mice, as B-1 B cells are the chief mediators of the T cell–independent response[24]. Impaired activation of B cells may also contribute to the defect in the istype switching. These results show that TAK1 is required for the appropriate induction of humoral immune responses.

DISCUSSION

Here we generated TAK1-deficient mice and a mouse strain with conditional expression of a *Map3k7* allele. *In vitro* studies have

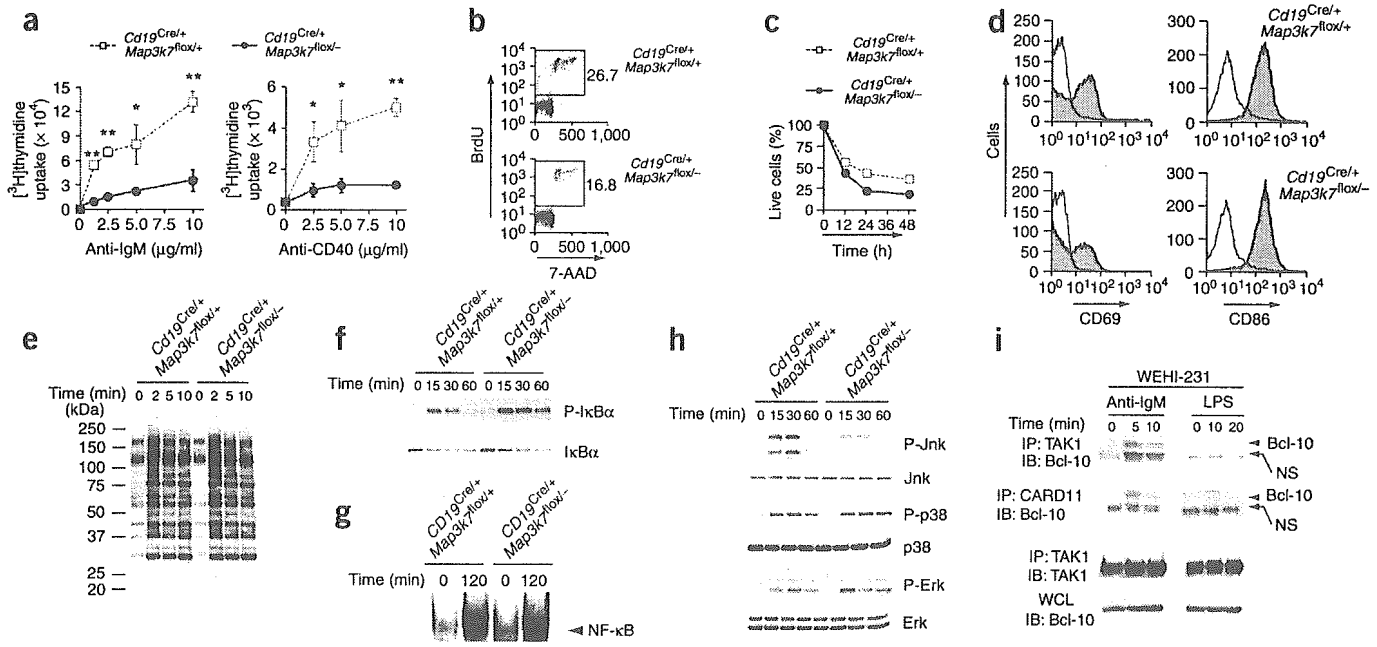

Figure 6 Impaired B cell activation by crosslinking of BCRs in *Cd19*$^{Cre/+}$*Map3k7*$^{flox/-}$ mice. (**a**) Proliferation of purified splenic B cells stimulated for 48 h (stimuli, horizontal axes), assessed by [^{3}H]thymidine incorporation. Data are mean ± s.d. of triplicate cultures. *, $P < 0.05$ and **, $P < 0.005$, versus TAK1-deficient cells (Student's *t*-test). (**b**) Cell cycle profiles of B cells stimulated with 5 μg/ml of anti-IgM. Cells were labeled with BrdU and were analyzed by flow cytometry 24 h after stimulation. Numbers beside boxed areas indicate percentages of cells in S phase. (**c**) Viability of B cells stimulated with 5 μg/ml of anti-IgM, assessed by staining with annexin V–indocarbocyanine followed by flow cytometry (time, horizontal axis). (**d**) Flow cytometry of purified splenic B cells left unstimulated (open) or stimulated for 24 h with 5 μg/ml of anti-IgM (filled) and then stained with anti-CD69 or anti-CD86. (**e**) Total tyrosine phosphorylation of B cells stimulated with 20 μg/ml of anti-IgM (time, above lanes). (**f**) Immunoblot of IκBα degradation in B cells in response to 20 μg/ml of anti-IgM. (**g**) EMSA of NF-κB DNA-binding activity in nuclear extracts from purified splenic B cells stimulated for 2 h with 20 μg/ml of anti-IgM. (**h**) MAPK activation in BCR-stimulated B cells stimulated with 20 μg/ml of anti-IgM (time, above lanes). (**i**) Association between TAK1 and Bcl10. WEHI-231 cells (1.5×10^8) were stimulated with 20 μg/ml of anti-IgM or 20 μg/ml of LPS (time, above lanes); cell lysates were immunoprecipitated (IP) with anti-TAK1 or anti-CARD11 and immunoprecipitates or whole-cell lysates (WCL) were analyzed by immunoblot (IB; antibodies, left margin). All results are representative of three different experiments. NS, nonspecific band.

suggested that TAK1 has an important function in IL-1R and TNFR signaling by forming a complex with TAB1 and TAB2 (refs. 11,14,15). However, mice lacking TAB2 have normal IL-1β responses[18]. The function of TAB1 in the IL-1R signaling is still unclear, although involvement TAB1 in TGF-β signaling has been reported in studies of TAB1-deficient mice[19]. It is possible TAB3, a homolog of TAB2, functions redundantly in TAB2-deficient mice[16,17,25,26]. In contrast, TAK1-deficient MEFs showed considerably impaired responses, including activation of NF-κB and MAPKs in response to IL-1β stimulation. However, IL-1β-induced production of IL-6 or activation of NF-κB was not completely abrogated in TAK1-deficient MEFs, indicating IL-1R activates both TAK1-dependent and TAK1-independent signaling pathways.

The involvement of TAK1 in TNFR signaling is controversial. Initially, TAK1 was reported to bind TRAF6 but not TRAF2, an important mediator of TNFR signaling[8]. However, other *in vitro* studies have shown that TAK1 regulates TNF-induced NF-κB activation[11,26]. Our results have demonstrated that TAK1 is critical for activation of both NF-κB and MAPKs in response to TNF. In addition, stimulation with TNF alone induced massive cell death in *Map3k7*$^{-/-}$ MEFs. In wild-type cells, TNF stimulation activates NF-κB and MAPK pathways, which mediate cell survival and proliferation by expressing target genes such as those encoding inhibitor of apoptosis and Bcl-2 family members[1]. TNF simultaneously activates the apoptotic pathway by recruiting Fas-associated death domain and caspase 8, followed by activation of caspase 3. This pathway does not require protein synthesis[1]. It is believed that the balance between life and death signals determines the fate of the cell. TNF kills wild-type cells when protein synthesis is inhibited. Given that TNF-induced NF-κB and MAPK activation was considerably impaired in *Map3k7*$^{-/-}$ MEFs, cells lacking TAK1 might fail to induce survival genes that protect cells from TNF-induced cell death.

The function of TAK1 in TLR signaling is less well understood. As B cells express various TLRs and are activated in response to pathogen-associated molecular patterns, we examined the involvement of TAK1 in TLR signaling using the B cell system as a model. Although most TLRs as well as IL-1R share MyD88 as an adaptor for triggering intracellular signaling, several other adaptor molecules such as TRIF contribute to the TLR signaling pathways[3]. Responses to TLR3, TLR4 and TLR9 ligands were considerably impaired in TAK1-defeicient B cells. Moreover, activation of MAPKs and NF-κB in response to TLR4 and TLR9 ligand was consistently impaired, although the activation was not completely abrogated even in the absence of TAK1. As TLR9 signaling depends completely on MyD88, it is obvious that TAK1 is important for MyD88-dependent signaling pathways. TLR4 activates MyD88-dependent and TRIF-dependent pathways. Both signaling pathways can activate NF-κB and MAPKs, although the time course differs. The MyD88-dependent pathway governs early activation of these signaling molecules, whereas the TRIF-dependent pathway is responsible for sustaining the activation[27,28]. In B cells, both MyD88 and TRIF are required for TLR4-induced proliferative response. In TLR4 signaling, the activation of NF-κB and MAPKs was considerably

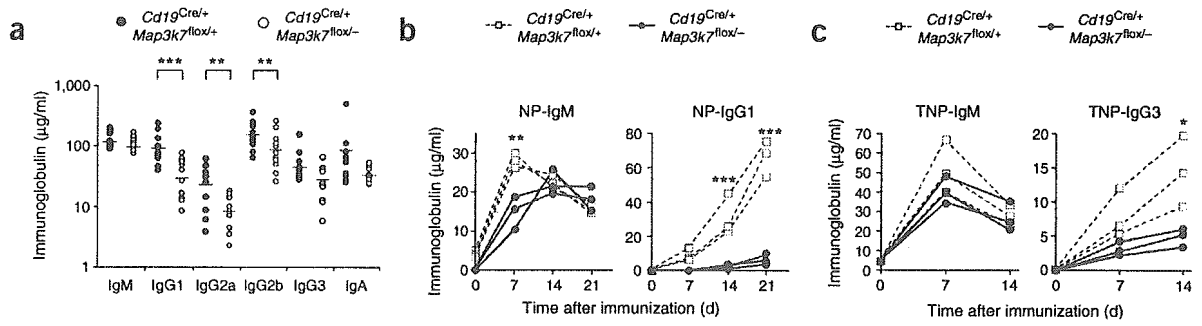

Figure 7 Impaired immune responses in $Cd19^{Cre/+}Map3k7^{flox/-}$ mice. (a) Reduced basal immunoglobulin titers in $Cd19^{Cre/+}Map3k7^{flox/-}$ mice. Immunoglobulin isotypes were measured by ELISA in the sera of nonimmunized 8-week-old $Cd19^{Cre/+}Map3k7^{flox/+}$ mice ($n = 12$) or $Cd19^{Cre/+}Map3k7^{flox/-}$ mice ($n = 12$). Results are from individual mice. (b) Impaired T cell–dependent antibody responses in $Cd19^{Cre/+}Map3k7^{flox/-}$ mice. Mice were immunized with nitrophenol–chicken γ-globulin, and nitrophenol (NP)–specific IgM and IgG1 production was measured by ELISA 7, 14 and 21 d after immunization. Results are from three (of five) representative mice per genotype. (c) Impaired T cell–independent type II antibody responses in $Cd19^{Cre/+}Map3k7^{flox/-}$ mice. Mice were immunized with trinitrophenol-Ficoll, and trinitrophenol (TNP)–specific IgM and IgG3 production was measured 7 and 14 d after immunization. Results are from three (of five) representative mice per genotype. *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.005$, versus TAK1-deficient cells (Student's *t*-test).

impaired in TAK1-deficient B cells at all time points examined, suggesting that MyD88-dependent and TRIF-dependent activation of these molecules depends entirely on TAK1.

Notably, TAK1 is also critical for B cell proliferation as well as Jnk activation in response to BCR crosslinking. Nevertheless, the activation of NF-κB and induction of NF-κB target genes induced by BCR-crosslinking was not impaired in TAK1-deficient B cells. In BCR signaling, a complex of CARD11, Bcl10 and MALT1 transduces signals to NF-κB and MAPKs downstream of protein kinase C-β[22]. CARD11 recruits Bcl10 to lipid rafts after stimulation. Bcl10 targets NF-κB essential modulator for K63-linked polyubiquitination through Ubc13 and MALT1 and activates NF-κB[29]. B cells from *Card11*$^{-/-}$, *Bcl10*$^{-/-}$ or *Malt1*$^{-/-}$ mice are reported to have defects in BCR signaling[30–36]. CARD11 is required for the activation of both NF-κB and Jnk[32]. Furthermore, *Bcl10*$^{-/-}$ B cells fail to activate NF-κB in response to BCR crosslinking. In contrast, B cells deficient in MALT1 (paracaspase) show impaired activation of NF-κB but not Jnk[34], indicating that Jnk is activated in a CARD11- and Bcl10-dependent, MALT1-independent signaling pathway. Given that TAK1 phosphorylates IKKs and MKK6 in IL-1β signaling, it is plausible that TAK1 is activated downstream of the CARD11-Bcl10-MALT1 complex and phosphorylates MAPKKs. In fact, we found that TAK1 interacted with Bcl10 in response to BCR crosslinking, indicating that TAK1 is recruited to the Bcl10 complex after BCR stimulation to induce Jnk activation. Although published work has shown that RNA interference–mediated knockdown of TAK1 in Jurkat cells results in diminished NF-κB activation in TCR signaling[23], our study has demonstrated that TAK1 is dispensable for NF-κB activation, at least in BCR signaling. That earlier report also showed that TRAF6 functions downstream of Bcl10 and MALT1 to activate NF-κB in TCR signaling[23]. Given that TAK1 interacts with Bcl10 in response to BCR but not LPS stimulation, it is likely that TAK1 is activated by Bcl10 without the intervention of TRAF6 in BCR signaling. As TAK1 is required for the activation of Jnk but not NF-κB, MALT1-mediated activation of the IKK complex probably occurs independently of TAK1. These results collectively indicate that in B cells, CARD11 and Bcl10 might activate TAK1 and MALT1 to regulate MAPKKs and IKKs, respectively.

Although TAK1-deficient B cells failed to proliferate in response to BCR crosslinking, activation of NF-κB was not impaired. Consistent with that finding, the upregulation of cyclin D2 was not altered in TAK1-deficient B cells. However, downregulation of $p27^{Kip1}$ was impaired in TAK1-deficient B cells, suggesting that TAK1-dependent signaling might regulate G1-S progression at the level of $p27^{Kip1}$ degradation. So far, it is not clear whether Jnk alone is responsible for the defect in the proliferation in TAK1-deficient B cells. It is possible that TAK1 regulates the activation of as-yet-unknown signaling pathway(s) in addition to Jnk and that the pathways cooperatively control BCR-mediated proliferation. Additional studies are needed to clarify the molecular mechanisms of cell cycle progression in BCR signaling.

Involvement of TAK1 in early embryogenesis modifying bone morphogenic protein signaling has been suggested[37]. *Map3k7*$^{-/-}$ embryos died at E9.5–E10.5. Mice deficient in genes encoding molecules involved in NF-κB signaling, such as RelA (also called p65) and IKKβ, die *in utero* due to massive liver apoptosis[38–41]. However, *Map3k7*$^{-/-}$ mice die before the initiation of fetal liver development, suggesting that the function of TAK1 in embryonic development is not explained by NF-κB inhibition. TAB1-deficient mice die between E15.5 and E18.5 due to edema and hemorrhage, and TAB2-deficient mice die between E11.5 and E12.5 due to liver apoptosis[18,19]. Thus, the function of TAK1 in embryogenesis might be independent of TAB1 or TAB2. The TAK1-NLK-STAT3 cascade is essential for TGF-β-mediated mesoderm formation in xenopus embryos[42]. Additional studies will be needed to clarify the mechanisms of the involvement of TAK1 in early embryogenesis.

In conclusion, we have shown here that TAK1 is essential for MAPK and NF-κB activation in response to TLR, IL-1R and TNFR stimulation. Consistent with those findings, TAK1-deficient cells failed to activate in response to TLR ligands, IL-1β and TNF. Antigen-induced B cell proliferation as well as immune responses to experimental antigens were considerably impaired in mice with B cell–specific TAK1-deficiency, indicating that TAK1 is involved in both innate and adaptive immunity. These data provide genetic evidence that TAK1 kinase has nonredundant functions in signaling pathways in inflammatory and immune responses.

METHODS

Generation of *Map3k7* mutant mice. Phage clones containing mouse *Map3k7* were isolated by screening of a 129/SvJ genomic library (Stratagene) with a probe corresponding to the 5′ end of mouse TAK1 cDNA. A targeting vector was designed to flank exon 2, containing the sequence encoding the ATP-binding site, with two *lox*P sites. The floxed neomycin-resistance gene fragment was inserted into intron 1 of *Map3k7*. A 1.0-kilobase (kb) *Cla*I–*Bam*HI

fragment was used as the 5′ homology region; a 2.5-kb *Xba*I–*Sal*I fragment, which contains exon 2 of *Map3k7*, was inserted between the two *lox*P sites; and a 6.0-kb *Not*I–*Sac*II fragment was used as the 3′ homology region. The herpes simplex virus thymidine kinase gene was used for negative selection of clones with random integration. A total of 30 μg of *Sac*II-linearized vector was electroporated into E14.1 embryonic stem cells. After positive and negative selection with G418 and ganciclovir, drug-resistant clones were picked up and were screened by PCR and Southern blot analysis. These clones were individually microinjected into blastocysts derived from C57BL/6 mice and were transferred to pseudopregnant females. Matings of chimeric male mice to C57BL/6 female mice resulted in transmission of the floxed allele to the germline. *Map3k7*$^{flox/+}$ or *Map3k7*$^{flox/flox}$ mice were bred with transgenic mouse line carrying the *Cre* transgene under control of the cytomegalovirus immediate early enhancer–chicken β-actin hybrid (*CAG*) promoter[43] to generate the *CAG*$^{Cre/+}$*Map3k7*$^{flox/+}$ (genotype, *Map3k7*$^{+/-}$), which were then intercrossed to generate *CAG*$^{Cre/+}$*Map3k7*$^{flox/flox}$ (genotype, *Map3k7*$^{-/-}$) mice. All animal experiments were done with the approval of the Animal Research Committee of the Research Institute for Microbial Diseases (Osaka University, Osaka, Japan).

Establishment of *Map3k7*$^{-/-}$ MEFs. MEFs were obtained from E13.5 *Map3k7*$^{flox/flox}$ embryos, were immortalized according to a general 3T3 protocol[44] and were cloned. For excision of the floxed genomic fragment containing exon 2, two different clones of *Map3k7*$^{flox/flox}$ MEFs were infected with retrovirus expressing Cre protein together with GFP or were infected with GFP alone (to establish control MEFs). GFP$^+$ cells were sorted by FACSVantage (Becton Dickinson) and then were analyzed by Southern blot and immunoblot to confirm genotype.

Generation of mice with B cell–specific TAK1 deficiency. Mice carrying the *Cre* transgene under control of the *Cd19* promoter[45] were bred with *Map3k7*$^{+/-}$ mice to generate *Cd19*$^{Cre/+}$*Map3k7*$^{+/-}$ mice. These mice were mated with *Map3k7*$^{flox/flox}$ mice; *Cd19*$^{Cre/+}$*Map3k7*$^{flox/+}$ or *Cd19*$^{Cre/+}$*Map3k7*$^{flox/-}$ offspring were used for analysis.

Purification of B cells. Resting B cells were isolated from single-cell suspensions of spleen cells by depletion of CD43$^+$ cells with anti-CD43 magnetic beads (MACS; Miltenyi Biotec). Cell purity was typically more than 95% B220$^+$, as assessed by flow cytometry.

Immunoblot analysis. Cells were lysed in a lysis buffer containing 1.0% Nonidet-P40, 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA and a protease inhibitor 'cocktail' (Roche). Lysates were separated by SDS-PAGE and were transferred onto polyvinylidene difluoride membranes (BioRad). After membranes were blotted with antibodies, proteins on membranes were visualized with an enhanced chemiluminescence system (Perkin-Elmer). Polyclonal anti-TAK1, anti-TAB1, anti-TAB2 and anti-IRAK-1 were as described[8,15,46]. Polyclonal antibody to phosphorylated Jnk (anti-phospho-Jnk), anti-phospho-p38, anti-phospho-Erk and anti-phospho-IκBα were purchased from Cell Signaling. Polyclonal anti-Jnk, anti-p38, anti-Erk, anti-IκBα, anti-p27^{Kip1} and anti–cyclin D2 and monoclonal anti-Bcl10 (clone 331.3) were from Santa Cruz. Monoclonal anti-phosphotyrosine (clone 4G10) was purchased from Upstate Biotechnology. Polyclonal anti-CARD11 was from Alexis Biochemicals.

Luciferase reporter assay. HEK293 cells were transiently transfected with 100 ng of either NF-κB (5×) or AP-1 luciferase reporter plasmids, together with a total of 1.0 μg expression vector(s). Then, 48 h later, the luciferase activity in the total cell lysate was measured with the Dual-luciferase reporter assay system (Promega).

Measurement of IL-6 production. MEFs (2×10^4) and purified splenic B cells (5×10^4) were stimulated for 48 h with recombinant mouse IL-1β (R&D Systems) and LPS (Sigma) or with CpG DNA (ODN1668; Hokkaido System Science), respectively. Culture supernatants were collected and IL-6 was measured with an ELISA kit (R&D Systems).

Cell viability. MEFs (2×10^5) were seeded onto six-well plates and were treated for 24 h with various concentrations of recombinant mouse TNF (R&D Systems). Purified splenic B cells (1×10^6) were stimulated with LPS, CpG DNA or anti-IgM (Jackson ImmunoResearch) for various periods. Cell viability was assessed with annexin V–indocarbocyanine (BioVision) and a FACSCalibur (Becton Dickinson).

Electrophoretic mobility-shift assay (EMSA). MEFs (1×10^6) or purified splenic B cells (5×10^6) were treated with stimuli for various periods. Nuclear extracts were purified from cells, were incubated with a probe specific for the NF-κB DNA-binding site, were separated by electrophoresis and were visualized by autoradiography as described[47].

Flow cytometry. Single-cell suspensions were prepared from thymi, bone marrow, spleens and peritoneal cavities of untreated mice. Cells were stained with fluorescein isothiocyanate–, phycoerythrin- or allophycocyanin-conjugated antibodies (Pharmingen) and then were analyzed on a FACSCalibur.

***In vivo* immunization and ELISA.** Mice were immunized intraperitoneally with 50 μg nitrophenol–chicken γ-globulin (Biosearch Technologies) precipitated with Imject alum (Pierce) or with 25 μg trinitrophenol-Ficoll (Biosearch Technologies). Antigen- and isotype-specific antibodies were measured by ELISA in sera collected from peripheral blood at various time points, on plates coated with nitrophenol-BSA or trinitrophenol-BSA. Antibodies to mouse IgM, IgG1, IgG2a, IgG2b, IgG3 and IgA were purchased from Southern Biotechnology.

B cell proliferation assay. Purified splenic B cells (5×10^4) were cultured in 96-well plates for 48 h with various concentrations of LPS, CpG DNA, poly(I:C) (Amersham), anti-IgM or anti-CD40 (Pharmingen). Samples were pulsed with 1 μCi [^{3}H]thymidine for the last 12 h and then ^{3}H uptake was measured with a β-scintillation counter (Packard).

Cell cycle analysis. Cell cycles of B cells were analyzed with the BrdU Flow Kit (Pharmingen) according to the manufacturer's instructions. Cells were cultured with LPS, CpG DNA or anti-IgM for 24 h, were pulsed with 10 μM BrdU for an additional 16 h, were stained with fluorescein isothiocyanate–anti-BrdU and 7-amino-actinomycin D and then were analyzed by flow cytometry.

Microarray analysis. Purified splenic B cells were treated for 4 h with or without anti-IgM (20 μg/ml). Total RNA was extracted with an RNeasy kit (Qiagen), and double-stranded DNA was synthesized from 10 μg of total RNA with the SuperScript Choice System (Invitrogen) primed with a T7-Oligo primer (Affymetrix). This cDNA was used to prepare biotin-labeled cRNA by an *in vitro* transcription reaction done with T7 RNA polymerase in the presence of biotinylated ribonucleotides, according to the manufacturer's protocol (Enzo Diagnostics). The cRNA product was purified with an RNeasy kit and fragmented and was hybridized to Affymetrix mouse expression array A430.2 microarray chips according to the manufacturer's protocol (Affymetrix). The hybridized chips were stained and washed and were scanned with a GeneArray Scanner (Affymetrix).

Accession code. GEO: microarray data, GSE3065.

Note: Supplementary information is available on the Nature Immunology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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