

TANK is a negative regulator of Toll-like receptor signaling and is critical for the prevention of autoimmune nephritis

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The intensity and duration of immune responses are controlled by many proteins that modulate Toll-like receptor (TLR) signaling. TANK has been linked to positive regulation of the transcription factors IRF3 and NF- κ B. Here we demonstrate that TANK is not involved in interferon responses and is a negative regulator of proinflammatory cytokine production induced by TLR signaling. TLR-induced polyubiquitination of the ubiquitin ligase TRAF6 was upregulated in *Tank*^{-/-} macrophages. Notably, *Tank*^{-/-} mice spontaneously developed fatal glomerulonephritis owing to deposition of immune complexes. Autoantibody production in *Tank*^{-/-} mice was abrogated by antibiotic treatment or the absence of interleukin 6 (IL-6) or the adaptor MyD88. Our results demonstrate that constitutive TLR signaling by intestinal commensal microflora is suppressed by TANK.

Toll-like receptors (TLRs) recognize microbial components and elicit innate as well as adaptive immune responses. Stimulation with TLR ligands induces the production of proinflammatory cytokines and type I interferons in cells of the innate immune system through intracellular signaling cascades^{1–3}. After stimulation, TLRs trigger the recruitment of adaptor molecules containing Toll–interleukin 1 receptor (IL-1R) homology domains. One adaptor, MyD88, is essential for the ‘downstream’ signaling of various TLRs, except for TLR3 (refs. 4–6). MyD88 interacts with the kinase IRAK4, which activates IRAK1 and IRAK2 (ref. 7). In turn, the IRAKs dissociate from MyD88 and interact with TRAF6 (A002312), which acts as a ubiquitin protein ligase. Together with an E2 ubiquitin-conjugating enzyme complex composed of Ubc13 and Uev1A, TRAF6 catalyzes formation of a lysine 63-linked polyubiquitin chain on TRAF6 itself and on the transcription factor NF- κ B modulator NEMO (also called IKK γ). The kinase TAK1 is also recruited to TRAF6 and phosphorylates the kinases IKK β and MEKK6 (ref. 8). Subsequently, the inhibitor of NF- κ B (I κ B) kinase (IKK) complex, composed of IKK α , IKK β and NEMO, is formed. NF- κ B binds to I κ B α in resting cells and is sequestered in the cytoplasm. Phosphorylation of I κ B by the IKK complex leads to its degradation by the ubiquitin-proteasome system, thereby freeing NF- κ B to translocate to the nucleus and activate the expression of genes encoding proinflammatory cytokines. Activation of the mitogen-activated protein kinase cascade is responsible for gene expression induced by the transcription activator AP-1. In plasmacytoid dendritic cells (DCs), MyD88-dependent

signaling activates the production of type I interferons through the transcription factor IRF7 (refs. 1,9).

Many proteins control TLR signaling to ensure that the strength and duration of TLR signals is appropriate for any given immune response. TLRs have been linked to the development of autoimmune diseases, and aberrant activation of innate immunity may contribute to rheumatoid arthritis, inflammatory bowel disease and systemic lupus erythematosus^{10,11}. Endogenous RNA molecules such as U1snRNP can activate autoreactive B cells and DCs through TLR7 (ref. 12). Furthermore, duplication of the *Tlr7* gene accounts for the autoimmune phenotypes associated with Yaa mice (Y chromosome-linked autoimmune accelerator)¹³. TLR9 is also involved in the recognition of immune complexes of DNA and anti-double-stranded DNA (anti-dsDNA) antibodies together with the B cell antigen receptor (BCR)¹⁴. Signaling proteins that inhibit TLR signaling include IRAKM, ST2, SIGIRR, SOCS1, the tumor suppressor CYLD and A20 (refs. 15–21). Cells lacking any one of those proteins produce more proinflammatory cytokines in response to TLR stimulation. Furthermore, mice lacking SOCS1 or A20 have immune disorders that lead to premature death. In addition, the immunosuppressive cytokine IL-10 suppresses colitis development by inhibiting TLR responses^{22–24}. Such studies indicate that negative regulation of TLR signaling is important for coordinated innate immune responses.

TANK (also known as I-TRAF) has been identified as a TRAF-binding protein^{25,26}. Among the six reported TRAF family members, TRAF1, TRAF2, TRAF3, TRAF5 and TRAF6 interact with TANK^{25–28}.

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Received 10 February; accepted 16 June; published online 9 August 2009; doi:10.1038/ni.1771

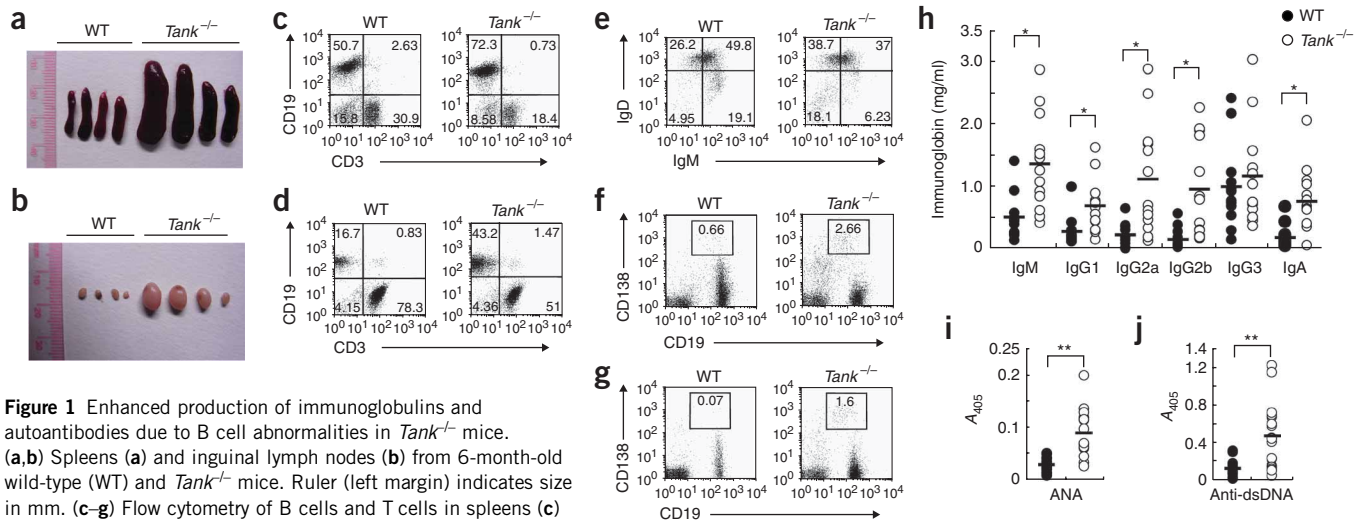


Figure 1 Enhanced production of immunoglobulins and autoantibodies due to B cell abnormalities in *Tank*^{-/-} mice. (a,b) Spleens (a) and inguinal lymph nodes (b) from 6-month-old wild-type (WT) and *Tank*^{-/-} mice. Ruler (left margin) indicates size in mm. (c–g) Flow cytometry of B cells and T cells in spleens (c) and lymph nodes (d), expression of IgM and IgD on splenic B cells (e), and expression of CD138 and CD19 on cells in spleens (f) and lymph nodes (g) from wild-type and *Tank*^{-/-} mice. Numbers in quadrants and outlined areas indicate percent cells in each. (h) Enzyme-linked immunosorbent assay (ELISA) of basal titers of immunoglobulin isotypes in serum from unimmunized 3-month-old wild-type and *Tank*^{-/-} mice. (i,j) ELISA of antinuclear antibodies (ANA; i) and anti-dsDNA antibodies (j) in serum from 12-month-old wild-type and *Tank*^{-/-} mice. A₄₀₅, absorbance at 405 nm. Each symbol represents an individual mouse; small horizontal lines indicate the mean (h–j). **P* < 0.005 and ***P* < 0.001, versus *Tank*^{-/-} cells (h–j;). Data are representative of at least five experiments (a,b); three independent experiments (c–g); or single experiments with a total of 13 (h) or 12 (i,j) mice per genotype (h–j).

TANK has been linked to the positive regulation of NF- κ B activation. In addition to TRAF family members, Ikki and TBK1 are TANK-binding partners^{29,30}. These proteins phosphorylate IRF3 and IRF7, which are transcription factors essential for the expression of type I interferon and interferon-inducible genes^{31,32}. TBK1 and Ikki are activated in response to recognition of viruses through TLRs and RNA helicase RIG-I-like receptors (RLRs)^{33,34}. TRAF3 is required for the activation of TBK1 and Ikki downstream of TLRs and RLRs^{35,36}. It has been reported that TANK functions as an adaptor that bridges TRAF3 and TBK1-Ikki and that TANK is required for the production of type I interferon in response to viral infection or TLR stimulation³⁷. However, the functions of TANK *in vivo* have not yet been clarified.

Here we used *Tank*-deficient (*Tank*^{-/-}) mice to demonstrate that TANK is not involved in interferon responses but is a negative regulator of TLR and BCR signaling. Macrophages and B cells from *Tank*^{-/-} mice had more canonical NF- κ B activation in response to stimulation of TLRs and the BCR. TLR-induced polyubiquitination of TRAF6 was upregulated in *Tank*^{-/-} macrophages, which indicates that TANK suppresses TLR signaling by controlling TRAF ubiquitination. *Tank*^{-/-} mice spontaneously developed lupus-like autoimmune nephritis. Autoantibody production in *Tank*^{-/-} mice was abolished in the absence of IL-6 or MyD88 but not in the absence of tumor necrosis factor (TNF). Furthermore, treatment of *Tank*^{-/-} mice with antibiotics resulted in lower autoantibody production, which indicates that IL-6 produced by constitutive TLR stimulation resulting from intestinal commensal microflora is important for the development of disease.

RESULTS

Tank^{-/-} mice develop lupus-like nephritis

To investigate the physiological functions of TANK *in vivo*, we generated *Tank*^{-/-} mice by homologous recombination in embryonic stem cells. We targeted exons 3 and 4 of mouse *Tank* with a neomycin-resistance cassette in embryonic stem cells and established *Tank*^{-/-} mice (Supplementary Fig. 1a). We confirmed homologous recombination of the *Tank* locus by Southern blot analysis (Supplementary Fig. 1b). Expression of *Tank* mRNA and TANK protein was abrogated

in *Tank*^{-/-} macrophages (Supplementary Fig. 1c,d). *Tank*^{-/-} mice were born from interbred *Tank*^{+/-} mice at the expected mendelian ratios and grew normally.

Tank^{-/-} mice had splenomegaly and lymphadenopathy (Fig. 1a,b). Flow cytometry showed a higher percentage of CD19⁺ B cells in the spleen and lymph nodes of *Tank*^{-/-} mice (Fig. 1c,d). Immunoglobulin M-low (IgM^{lo}) IgD^{hi} mature B cells accumulated in the spleen of *Tank*^{-/-} mice (Fig. 1e). The percentage of CD19⁺CD138⁺ plasma cells was also much higher in the spleen and lymph nodes of *Tank*^{-/-} mice (Fig. 1f,g). In contrast, the percentage of Foxp3⁺CD4⁺ regulatory T cells did not differ between wild-type and *Tank*^{-/-} mice (Supplementary Fig. 2a). Consistent with the larger B cell populations, basal serum concentrations of IgM, IgG1, IgG2a, IgG2b and IgA were significantly higher, by 1.2-fold to 6.2-fold, in *Tank*^{-/-} mice than in wild-type mice (Fig. 1h). Notably, we detected antinuclear antibodies and anti-dsDNA antibodies in the serum of *Tank*^{-/-} mice (Fig. 1i,j).

Tank^{-/-} mice began to spontaneously die at 3 months after birth, and about 50% had died by 12 months after birth (Fig. 2a). Histological studies showed that 24-week-old *Tank*^{-/-} mice had glomerulonephritis with mesangial cell proliferation and expansion of the mesangial matrix (Fig. 2b,c). The glomerular structure was devastated in terminally ill *Tank*^{-/-} mice (data not shown), which suggested that renal failure was the cause of death. Although we found infiltration of lymphocytes in the liver and lungs of *Tank*^{-/-} mice, we detected no histological changes in their intestine, heart or joints (data not shown). In addition, we found deposition of IgG, IgM and complement components C3 and C1q in the glomeruli of *Tank*^{-/-} mice (Fig. 2d). Such depositions are characteristic of lupus-like nephritis and suggest that deposition of immune complexes of autoantibodies was the cause of the glomerulonephritis in *Tank*^{-/-} mice.

TANK is a negative regulator of TLR responses

Next we examined the type I interferon responses of *Tank*^{-/-} cells to virus infection. In contrast to the results obtained by *in vitro* studies, interferon- β (IFN- β) production in response to infection with

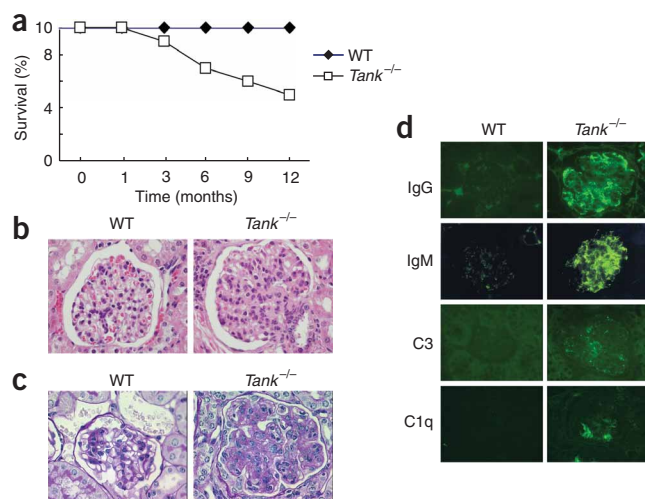


Figure 2 Development of lethal glomerulonephritis in *Tank*^{-/-} mice. (a) Survival of wild-type and *Tank*^{-/-} mice monitored for 1 year. (b,c) Kidney sections from 6-month-old wild-type and *Tank*^{-/-} mice, stained with hematoxylin and eosin (b) or periodic acid-Schiff (c). (d) Kidney sections from 6-month-old wild-type and *Tank*^{-/-} mice, stained with fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG, IgM, C3 and C1q. Original magnification, $\times 100$ (b-d). Data are representative of single experiments with a total of ten mice per genotype (a), or five (b,c) or two (d) experiments.

to LPS stimulation in *Tank*^{-/-} macrophages was less substantial than that induced by other TLR ligands. Conventional DCs from *Tank*^{-/-} mice also showed excessive cytokine production in response to these TLR ligands (data not shown).

We subsequently assessed the function of TANK in cytokine responses to stimulation with TLR ligands *in vivo*. We chose R-848, because the enhancement in cytokine production in *Tank*^{-/-} macrophages was most pronounced after stimulation with R-848. We injected R-848 into the peritoneum of 8-week-old mice and measured serum concentrations of IL-6 and IFN- α 1 and 3 h later. *Tank*^{-/-} mice had significantly more of these serum cytokines at both time points than did wild-type mice (Fig. 3g,h). Together these results indicate that TANK is a negative regulator of TLR-mediated responses but is not an essential positive regulator of type I interferon responses *in vivo*.

TANK controls TRAF6 ubiquitination

We examined whether the enhanced cytokine production in *Tank*^{-/-} macrophages was evident at the level of transcription. In response to R-848 stimulation, wild-type macrophages showed induction of the expression of *Il6*, *Tnf*, *Il12b*, *Ptgs2* (encoding cyclooxygenase 2), *Nfkbiz* (encoding I κ B ζ) and *Nos2* (encoding nitric oxide synthase 2). The expression of these genes was enhanced in *Tank*^{-/-} macrophages in response to R-848 stimulation (Fig. 4a), which indicated that initial TLR-induced gene expression was enhanced in *Tank*^{-/-} macrophages. Next we analyzed activation of the transcription factors NF- κ B and AP-1 by electrophoretic mobility-shift assay (EMSA). In response to R-848 stimulation, activation of NF- κ B and AP-1 was enhanced in *Tank*^{-/-} macrophages compared with that in wild-type macrophages (Fig. 4b,c).

Newcastle disease virus did not differ between wild-type and *Tank*^{-/-} conventional DCs derived from bone marrow cells (Fig. 3a). Wild-type and *Tank*^{-/-} conventional DCs also produced similar amounts of IL-6 (Fig. 3b). Newcastle disease virus is recognized by the RNA helicase RIG-I in conventional DCs, which indicates that TANK is not essential for the activation of signaling pathways by RLRs. TRAF3 has been shown to be activated downstream of TLR7 and TLR9 in plasmacytoid DCs. However, bone marrow plasmacytoid DCs induced by the cytokine Flt3L from *Tank*^{-/-} mice produced more rather than less IFN- α and IL-6 in response to A- or D-type CpG DNA (Fig. 3c,d). Collectively these results indicate that TANK is not essential for type I interferon responses.

Next we examined the production of proinflammatory cytokines in macrophages in response to a set of TLR ligands, including MALP-2 (TLR6-TLR2), polyinosinic-polycytidylic acid (poly(I:C); TLR3), lipopolysaccharide (LPS; TLR4), the synthetic imidazoquinoline resiquimod (R-848; TLR7) and CpG DNA (TLR9). The production of IL-6 and TNF in response to these TLR ligands, except poly(I:C), was much higher in *Tank*^{-/-} peritoneal macrophages than in wild-type cells (Fig. 3e,f). Of note, the enhanced cytokine production in response

Figure 3 Enhanced proinflammatory cytokine production in response to TLR stimulation in *Tank*^{-/-} mice. (a,b) ELISA of IFN- α (a) and IL-6 (b) in culture supernatants of wild-type and *Tank*^{-/-} bone marrow-derived DCs infected for 24 h with Newcastle disease virus (NDV). (c,d) ELISA of IFN- α (c) and IL-6 (d) in culture supernatants of wild-type and *Tank*^{-/-} Flt3L-induced DCs stimulated for 24 h with 0.1 or 1 μ M CpG DNA. (e,f) ELISA of IL-6 (e) and TNF (f) in culture supernatants of wild-type and *Tank*^{-/-} peritoneal macrophages stimulated for 24 h with MALP-2 (10 ng/ml), poly(I:C) (100 μ g/ml), LPS (100 ng/ml), R-848 (10 nM) or CpG DNA (1 μ M). ND, not detectable. Data are representative of three (a-d) or five (e,f) experiments (error bars, s.d.). (g,h) ELISA of IL-6 (g) and IFN- α (h) in serum from wild-type mice ($n = 5$) and *Tank*^{-/-} mice ($n = 5$) injected intraperitoneally with 30 nmol R-848. Each symbol represents an individual mouse; small horizontal lines indicate the mean. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$, versus *Tank*^{-/-} mice (two-tailed Student's *t*-test). Data are representative of a single experiment.

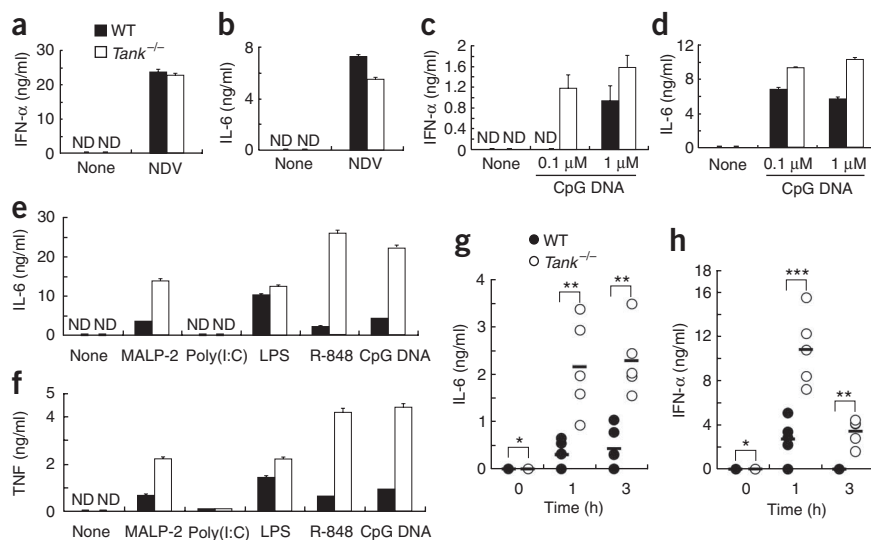
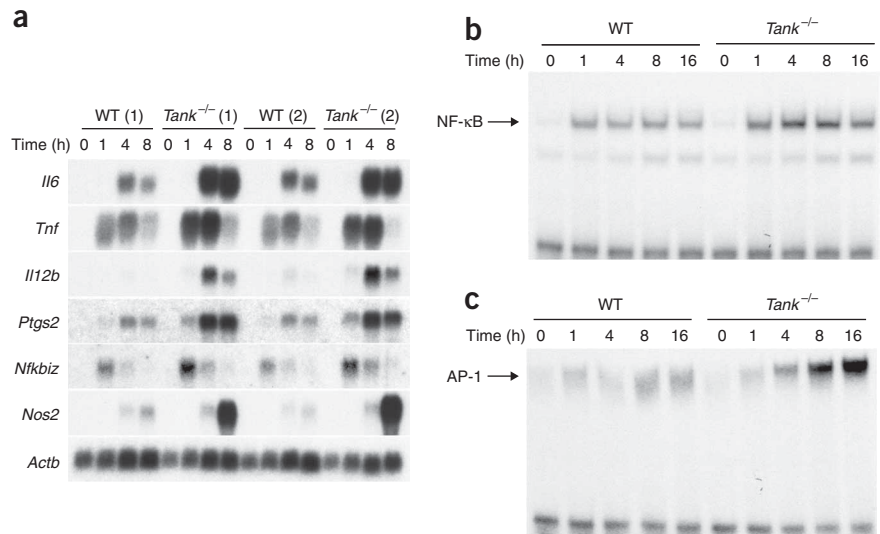


Figure 4 TANK negatively regulates the activation of NF- κ B and AP-1 as well as gene expression in response to TLR7 stimulation in macrophages.

(a) RNA blot analysis of the expression of *Il6*, *Tnf*, *Il12b*, *Ptgs2*, *Nfkbiz* and *Nos2* among total RNA extracted from wild-type and *Tank*^{-/-} peritoneal macrophages stimulated for various times (above lanes) with 10 nM R-848. Bottom, rehybridization of the same membrane with an *Actb* probe (encoding β -actin). Data are from two independent experiments (1 and 2). (b,c) EMSA of the DNA-binding activity of NF- κ B (b) and AP-1 (c) in nuclear extracts of wild-type and *Tank*^{-/-} macrophages stimulated for various times (above lanes) with 10 μ M R-848, assessed with NF- κ B- and AP-1-specific probes. Arrows indicate induced NF- κ B and AP-1 complexes. Data are representative of three independent experiments.



The results described above indicated that TANK negatively regulates the TLR-induced activation of NF- κ B and AP-1. Activation of IRAK1 in response to R-848 was not enhanced in *Tank*^{-/-} macrophages (Fig. 5a). Furthermore, IRAK1 was degraded after R-848 stimulation with similar kinetics in wild-type and *Tank*^{-/-} macrophages (Fig. 5b), which indicated that TANK regulates signaling downstream of IRAKs. TANK has been reported to interact with the TRAF family members TRAF1, TRAF2, TRAF3, TRAF5 and TRAF6. Among these, TRAF6 is needed for TLR signaling. As TRAF6 is ubiquitinated in response to TLR stimulation, we examined whether TANK modifies the ubiquitination of TRAF6. We found that induction of TRAF6 ubiquitination in response to R-848 stimulation was enhanced in *Tank*^{-/-} macrophages compared with that in wild-type cells (Fig. 5c). Reciprocally, overexpression of TANK in human embryonic kidney (HEK293) cells inhibited the ubiquitination of TRAF6 (Fig. 5d). Together these results indicate that TANK inhibits TLR-induced activation of NF- κ B and AP-1 by suppressing TRAF6 ubiquitination.

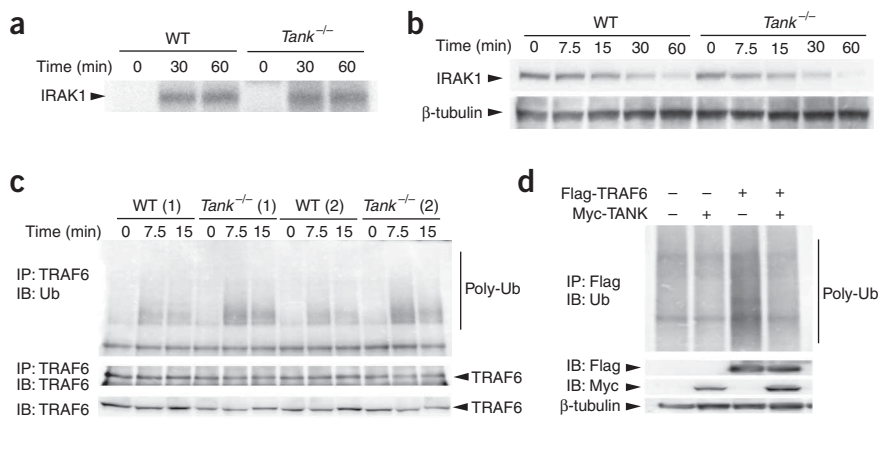
TANK is involved in BCR and CD40 signaling

Next we investigated the responses of *Tank*^{-/-} B cells to mitogens such as TLR ligands and crosslinking of the BCR and CD40. After stimulation with R-848, CpG DNA, antibody to IgM (anti-IgM) or

anti-CD40, *Tank*^{-/-} B cells proliferated much more than did wild-type B cells (Fig. 6a). In contrast, splenic B cell death after culture without mitogen was similar in wild-type and *Tank*^{-/-} mice (Fig. 6b), which indicates that TANK is not involved in the control of B cell apoptosis. In response to anti-CD40, B cells activate both canonical and non-canonical NF- κ B. The noncanonical pathway is characterized by processing of the NF- κ B2 precursor protein p100 to generate p52. We found that activation of noncanonical NF- κ B in response to CD40 stimulation was similar in wild-type and *Tank*^{-/-} B cells (Fig. 6c). In contrast, NF- κ B DNA-binding activity was enhanced in *Tank*^{-/-} B cells compared with that in wild-type B cells (Fig. 6d), and the band was supershifted by anti-p65 and anti-p50 (data not shown). Ubiquitination of TRAF6 after stimulation with anti-CD40 was also enhanced in *Tank*^{-/-} B cells (Fig. 6e). Furthermore, BCR stimulation also induced enhanced activation of NF- κ B and ubiquitination of TRAF6 in *Tank*^{-/-} B cells (Supplementary Fig. 3a,b). Furthermore, expression of cyclin D2, an NF- κ B-inducible protein, was higher in *Tank*^{-/-} B cells than in wild-type B cells after stimulation with anti-CD40 or anti-IgM (Supplementary Fig. 4). These data suggest that TANK is involved in canonical but not noncanonical NF- κ B activation pathways in B cells.

Figure 5 TANK controls TRAF6 ubiquitination in response to TLR7 stimulation in macrophages.

(a) *In vitro* kinase assay of anti-IRAK1 immunoprecipitates from lysates of wild-type and *Tank*^{-/-} peritoneal macrophages stimulated for various times (above lanes) with 10 μ M R-848. Data are representative of two experiments. (b) Immunoblot analysis of whole-cell lysates of wild-type and *Tank*^{-/-} macrophages stimulated for various times (above lanes) with 10 μ M R-848, probed with anti-IRAK1. Below, immunoblot analysis of β -tubulin (loading control). Data are representative of two experiments. (c) Immunoblot (IB) analysis of anti-TRAF6 immunoprecipitates (IP) from lysates of macrophages treated for various times (above lanes) with R-848, probed with antibody to ubiquitin (Ub). Poly-Ub, polyubiquitin. Below, immunoblot analysis of TRAF6 (loading control), with (middle) and without (bottom) anti-TRAF6 immunoprecipitation. Data are from two independent experiments (1 and 2). (d) Immunoblot analysis of anti-Flag immunoprecipitates from lysates of HEK293 cells cotransfected with Flag-tagged TRAF6 and Myc-tagged TANK, probed with anti-ubiquitin. Below, immunoblot analysis of lysates without immunoprecipitation; β -tubulin, loading control. Data are representative of three independent experiments.



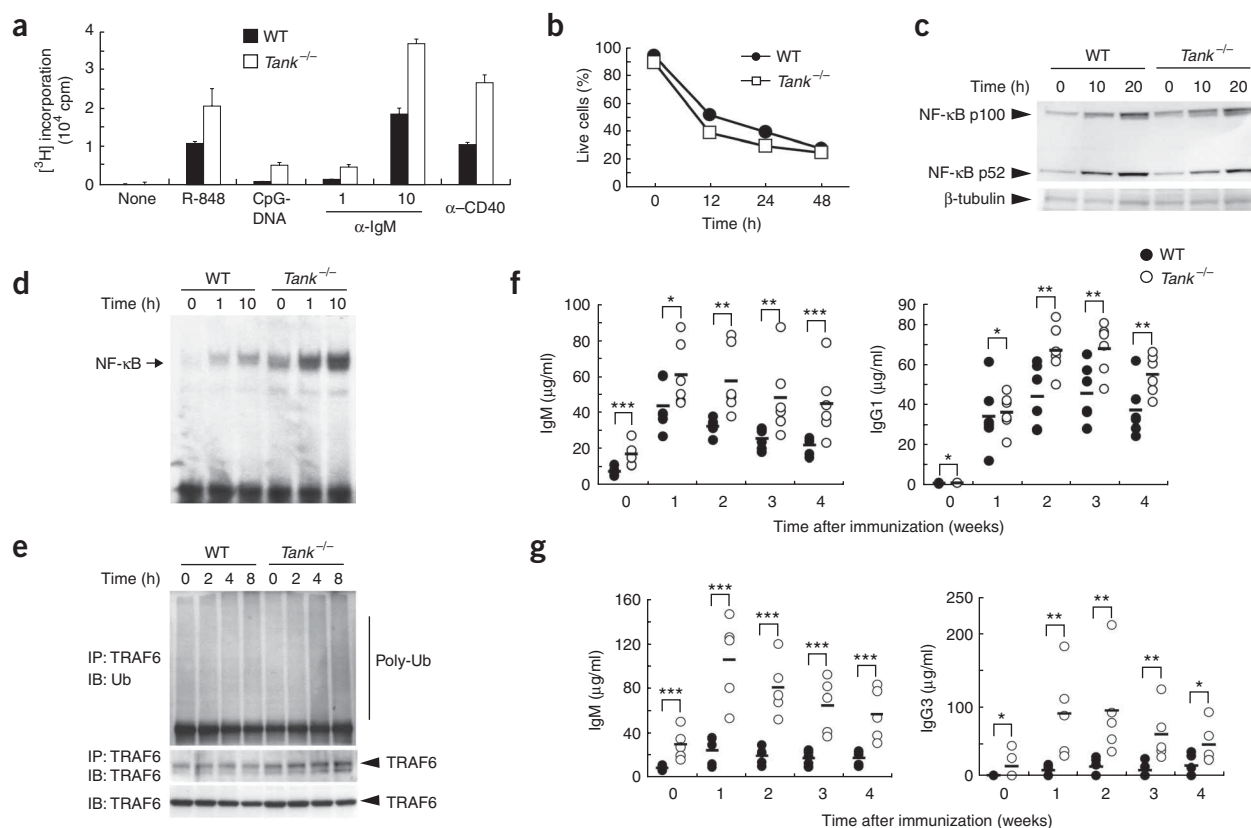


Figure 6 Enhanced activation of B cells in *Tank*^{-/-} mice. **(a)** [³H]thymidine incorporation by purified splenic B cells cultured for 48 h with R-848 (10 nM), CpG DNA (10 nM), anti-IgM (1 or 10 μg/ml) or anti-CD40 (1 μg/ml) and then pulsed with [³H]thymidine (1 μCi) for the final 16 h, measured in a β-scintillation counter. **(b)** Viability of splenic B cells cultured for various times (horizontal axis) in the absence of cytokines, assessed by annexin V staining followed by flow cytometry. **(c)** Immunoblot analysis of the processing of p100 to p52 in whole-cell lysates of wild-type and *Tank*^{-/-} B cells stimulated for various times (above lanes) with anti-CD40 (5 μg/ml). Bottom, immunoblot analysis of β-tubulin (loading control). **(d)** EMSA of NF-κB DNA-binding activity in nuclear extracts of wild-type and *Tank*^{-/-} B cells stimulated for various times (above lanes) with anti-CD40 (5 μg/ml). Arrow indicates the induced NF-κB complex. **(e)** Immunoblot analysis of anti-TRAF6 immunoprecipitates from lysates of splenic B cells treated various times (above lanes) with anti-CD40 (5 μg/ml), probed with anti-ubiquitin. Bottom, immunoblot analysis of TRAF6 (loading control). **(f)** ELISA of the production of NP-specific IgM and IgG1 by mice immunized with NP-CGG, measured 1, 2, 3 and 4 weeks after immunization. **(g)** ELISA of the production of TNP-specific IgM and IgG3 by mice immunized with TNP-Ficoll, measured at 1, 2, 3 and 4 weeks after immunization. Each symbol represents an individual mouse; small horizontal lines indicate the mean (**f,g**). **P* > 0.05, ***P* < 0.05 and ****P* < 0.01, versus *Tank*^{-/-} mice (two-tailed Student's *t*-test). Data are representative of three (**a** (error bars, s.d.) and **c–e**) or two (**b**) independent experiments, or single experiments with a total of five mice per genotype (**f,g**).

In contrast to B cells, wild-type and *Tank*^{-/-} T cells proliferated to a similar degree after stimulation with anti-CD3 or anti-CD3 together with anti-CD28 (**Supplementary Fig. 2b**). When stimulated with phorbol 12-myristate 13-acetate and ionomycin *in vitro*, similar proportions of wild-type and *Tank*^{-/-} CD4⁺ T cells produced IFN-γ or IL-17 (**Supplementary Fig. 2c**). This suggests that TANK is not involved in the development of T helper type 1 or IL-17-producing T helper cells.

To explore the influence of TANK deficiency on antibody responses *in vivo*, we immunized wild-type and *Tank*^{-/-} mice with the T cell-dependent antigen nitrophenol-chicken γ-globulin (NP-CGG) or the T cell-independent antigen trinitrophenyl-Ficoll (TNP-Ficoll). NP-specific IgG1 and IgM titers were higher in *Tank*^{-/-} mice than in wild-type mice (**Fig. 6f**). TNP-specific IgG3 and IgM titers were also higher in *Tank*^{-/-} mice than in wild-type mice (**Fig. 6g**). The difference between wild-type and *Tank*^{-/-} mice was greater in response to immunization with TNP-Ficoll, which suggests that TANK may be more critical for T cell-independent immune responses than for T cell-dependent immune responses *in vivo*.

Intestinal microflora in the autoimmunity of *Tank*^{-/-} mice

Proinflammatory cytokines are critical in the development of autoimmune disease. Overproduction of IL-6 and TNF in mice results in the development of mesangioproliferative glomerulonephritis and chronic polyarthritis, respectively. To investigate whether IL-6 or TNF is involved in disease pathogenesis in *Tank*^{-/-} mice, we generated mice lacking IL-6 or TNF on the *Tank*^{-/-} genetic background. The titers of anti-dsDNA antibodies were significantly lower in 5-month-old *Tank*^{-/-}*Il6*^{-/-} mice than in 5-month-old *Tank*^{-/-} mice (**Fig. 7a**). Moreover, IL-6 deficiency 'rescued' the glomerulonephritis that developed in *Tank*^{-/-} mice (**Fig. 7b**). In contrast, TNF deficiency did not significantly alter the amount of anti-dsDNA antibody production in *Tank*^{-/-} mice (**Fig. 7c**). To determine whether MyD88 deficiency protects against the disease progress, we crossed *Tank*^{-/-} mice with *MyD88*^{-/-} mice. Anti-dsDNA antibody titers were significantly lower in 5-month-old *Tank*^{-/-}*MyD88*^{-/-} mice than in *Tank*^{-/-} mice (**Fig. 7d**), which indicates that TLR and/or IL-1R family members are critical for the autoimmunity caused by TANK deficiency. The next question we addressed was how TLR and/or IL-1R signaling was activated to cause IL-6 production. Intestinal microflora has been shown to be involved

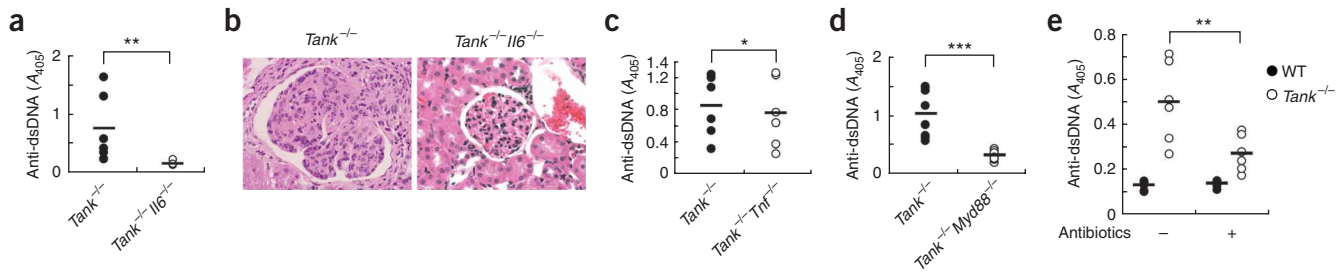


Figure 7 Antibiotic treatment, as well as deficiency of MyD88 or IL-6, ameliorates autoantibody production in *Tank*^{-/-} mice. **(a)** Anti-dsDNA antibodies in serum from 5-month-old *Tank*^{-/-} and *Tank*^{-/-}*Il6*^{-/-} mice. **(b)** Hematoxylin and eosin staining of kidney sections from *Tank*^{-/-} mice and *Tank*^{-/-}*Il6*^{-/-} mice. Original magnification, $\times 100$. **(c,d)** ELISA of anti-dsDNA antibodies in *Tank*^{-/-} and *Tank*^{-/-}*Tnf*^{-/-} mice **(c)** or *Tank*^{-/-} and *Tank*^{-/-}*Myd88*^{-/-} mice **(d)**. **(e)** ELISA of serum anti-dsDNA antibodies in 16-week-old wild-type and *Tank*^{-/-} mice given drinking water (from birth onward) containing ampicillin (1 g/l), neomycin (1 g/l), vancomycin (0.5 g/l) and metronidazole (1 g/l); control wild-type and *Tank*^{-/-} mice received untreated drinking water (-). Each symbol represents an individual mouse; small horizontal lines indicate the mean **(a,c-e)**. * $P > 0.05$, ** $P < 0.05$ and *** $P < 0.01$, versus *Tank*^{-/-} mice (two-tailed Student's *t*-test). Data are representative of single experiments with a total of six mice per genotype **(a,c-e)** or are representative of three experiments **(b)**.

in the pathogenesis of autoimmune diseases, such as colitis in IL-10-deficient mice. Therefore, we treated *Tank*^{-/-} mice orally with a combination of antibiotics to clear the intestinal microflora. The antibiotic treatment significantly ameliorated the production of anti-dsDNA antibodies (**Fig. 7e**), which suggests that continuous stimulation of TLRs by intestinal microflora contributes to the generation of autoantibodies in the absence of TANK.

DISCUSSION

Here we generated *Tank*^{-/-} mice and have shown that TANK is essential for the negative regulation of canonical NF- κ B signaling. *Tank*^{-/-} mice had enhanced activation of macrophages and B cells in response to TLR ligands and antigens, which culminated in the development of fatal immune complex-mediated renal failure. Although TANK has been shown to positively regulate TBK1- and Ikki-mediated production of type I interferon by *in vitro* studies, analysis of *Tank*^{-/-} mice showed that TANK was not needed for activation of the type I interferon pathway downstream of RLRs or TRIF. TANK forms a family with the adaptor proteins NAP1 and SINTBAD^{38,39}, which are composed of an amino-terminal coiled-coil domain and a TBK1-binding domain. NAP1 and SINTBAD have also been linked to the activation of TBK1 and Ikki downstream of virus sensors. Knockdown of NAP1, SINTBAD or TANK by small interfering RNA has been associated with impaired interferon responses. Hence, it is possible that these three proteins function redundantly in the activation of TBK1 and Ikki.

Although published studies have shown that TANK is a positive regulator of NF- κ B, our results have shown that TANK is critical for the negative regulation of canonical NF- κ B through suppression of TRAF6 ubiquitination. Lysine 63-type ubiquitination is important for the activation of TAK1 with the binding partners TAB2 and TAB3 in TLR signaling, and TANK may inhibit TRAF6 ubiquitination by directly binding to TRAF6 in response to TLR stimulation. Although A20 and CYLD have been identified as deubiquitinases^{40–42}, TANK does not contain a deubiquitination enzyme domain. Immunoprecipitation experiments showed that overexpressed A20 or CYLD failed to immunoprecipitate together with overexpressed TANK, which suggests that TANK may suppress ubiquitination of TRAF6 independently of A20 or CYLD (data not shown). Further studies are needed to assess the precise mechanism through which TANK modifies TRAF6. In addition, activation of canonical NF- κ B in response to BCR and CD40 stimulation was augmented in *Tank*^{-/-} B cells. Consistent with that, proliferation of B cells in response

to TLR and BCR stimulation was much higher in *Tank*^{-/-} mice. In TCR signaling, TRAF2 and TRAF6 are reported to participate in NF- κ B activation downstream of the adaptors Bcl-10 and MALT1 (ref. 43). Given that TANK suppresses the polyubiquitination of TRAF6 in response to TLR stimulation in macrophages, it is possible that TANK suppresses BCR and CD40 signaling by regulating the activation of TRAF proteins in B cells. However, activation of non-canonical NF- κ B was not enhanced in *Tank*^{-/-} B cells, and it has been reported that TRAF3 controls mainly that activation in B cells⁴⁴. Hence, these observations suggest that TANK is not involved in signaling downstream of TRAF3. Furthermore, TRAF2 can control noncanonical NF- κ B as well as the development of marginal zone B cells. The relationship between TANK and TRAF2 needs to be explored further.

The disease caused by the absence of TANK was characterized by glomerulonephritis due to deposition of immune complexes in the glomeruli. In addition, anti-dsDNA antibodies and antinuclear antibodies were present in high concentrations in *Tank*^{-/-} mice. These observations indicate that *Tank*^{-/-} mice may represent a mouse model of lupus-like immune diseases. The phenotype of *Tank*^{-/-} mice is reminiscent of that of mice that overexpress IL-6 in B cells⁴⁵, which is characterized by lymphadenopathy and plasmacytosis culminating in the development of severe glomerular nephritis. IL-6 is a pleiotropic cytokine responsible for fever, acute-phase protein expression, osteoclast activation and the development of IL-17-producing T helper cells and plasma cells. Indeed, *Tank*^{-/-} macrophages showed enhanced production of proinflammatory cytokines, including IL-6 and TNF, in response to TLR stimulation. Furthermore, *Tank*^{-/-} mice failed to produce autoantibodies and did not develop glomerulonephritis in the absence of IL-6. These results indicate that IL-6 is essential for the development of the *Tank*^{-/-} B cells that are responsible for the production of autoantibodies. In contrast, *Tank*^{-/-} T cells responded normally to TCR stimulation. Given that TANK is critical for inhibiting BCR-induced B cell activation, it is possible that the lack of TANK in B cells is important for the generation of autoimmune nephritis through aberrant activation of B cells in response to antigen stimulation.

The generation of anti-dsDNA antibodies in *Tank*^{-/-} mice was significantly lower in response to oral treatment with antibiotics or in the absence of MyD88, which suggests that TLR signaling is critical for the development of autoimmune disease in *Tank*^{-/-} mice. Although various proteins have been identified as negative regulators of TLR signaling, few mice lacking any a single one of these proteins

spontaneously develop autoimmune disease, with the exception of mice lacking A20. A20-deficient mice spontaneously develop multiorgan inflammation and premature death, which can be 'rescued' by MyD88 deficiency^{46,47}. Unlike *Tank*^{-/-} mice, A20-deficient mice do not develop immune complex-mediated glomerulonephritis. A20 controls TNF receptor signaling in addition to TLR signaling, yet the responses to TNF were not altered in *Tank*^{-/-} cells. TNF is involved in the pathogenesis of organ-specific autoimmune diseases, such as rheumatoid arthritis and Crohn's disease⁴⁸. Hence, the differences in the signaling pathways regulated by A20 and TANK may explain the differences in the types of autoimmune disease caused by A20 or TANK deficiency.

As oral treatment with antibiotics ameliorated autoantibody production in *Tank*^{-/-} mice, constitutive stimulation of TLRs by intestinal microflora seems to be responsible for the generation of autoimmunity in the absence of TANK. Bone marrow-transfer experiments showed that hematopoietic cells were responsible for the death of *Tank*^{-/-} mice (data not shown). Intestinal microflora contribute to the pathogenesis of inflammatory bowel disease^{48,49}, and the colitis observed in IL-10-deficient mice was 'rescued' by the absence of MyD88 (ref. 24), which suggests that TLR signaling is involved in the pathogenesis of inflammatory bowel disease. As TLRs are expressed on intestinal DCs and are responsible for sensing microbes in the intestine, it is possible that TANK controls the production of certain cytokines in intestinal tissues. Further studies are needed to understand why TANK deficiency causes autoimmune nephritis but not colitis.

In addition, the antigen-specific humoral immune responses to haptens were enhanced in *Tank*^{-/-} mice. This may have been due to the enhanced DC and B cell activation in response to antigens and the adjuvant in *Tank*^{-/-} mice. It will be useful to explore whether inhibition of TANK expression in certain cell types is beneficial for inducing antigen-specific immune responses *in vivo*. Modification of TANK may be helpful in vaccines administered together with an adjuvant. In summary, our results here have shown that TANK is a negative regulator of TLR and BCR responses. Future studies involving cell type-specific deletion of TANK will clarify the complex interaction between cells of the immune system needed to prevent the development of autoimmune disease.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/natureimmunology/>.

Accession code. UCSD-Nature Signaling Gateway (<http://www.signaling-gateway.org>): A002312.

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

ACKNOWLEDGMENTS

We thank T. Yasui (Osaka University) for *Il6*^{-/-} mice and plasmids; colleagues in our laboratories; E. Kamada for secretarial assistance; Y. Fujiwara, M. Kumagai and R. Abe for technical assistance; and S. Sato for discussions. Supported by the Special Coordination Funds of the Japanese Ministry of Education, Culture, Sports, Science and Technology, and grants from the Ministry of Health, Labour and Welfare in Japan, the Global Center of Excellence Program of Japan, and the US National Institutes of Health (P01 AI070167).

AUTHOR CONTRIBUTIONS

T.K., O.T. and S.A. designed the research and analyzed data; T.K. generated *Tank*^{-/-} mice and did most of the experiments; Y.T., Y.I. and T.T. did histological examination of kidneys; H.K. provided advice; and T.K., O.T. and S.A. prepared the manuscript.

Published online at <http://www.nature.com/natureimmunology/>.

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ONLINE METHODS

Generation of *Tank*^{-/-} mice. *Tank* was isolated from genomic DNA extracted from embryonic stem cells (GSI-1) by PCR. The targeting vector was constructed by replacement of a 2.0-kilobase fragment encoding the *Tank* open reading frame with a neomycin-resistance gene cassette; the gene encoding herpes simplex virus thymidine kinase driven by the promoter of the gene encoding phosphoglycerate kinase was inserted into the genomic fragment to facilitate negative selection. After transfection of the targeting vector into embryonic stem cells, colonies doubly resistant to the aminoglycoside G418 and gancyclovir were selected, screened by PCR and further confirmed by Southern blot analysis. Homologous recombinants were microinjected into blastocysts from C57BL/6 female mice, and heterozygous F₁ progenies were intercrossed to obtain *Tank*^{-/-} mice. *Tank*^{-/-} mice on the 129Sv × C57BL/6 background and their littermate controls were used.

Mice and cells. *MyD88*^{-/-} and *Tnf*^{-/-} mice have been described^{4,23}. *Il6*^{-/-} mice were provided by T. Yasui. All animal experiments were carried out with the approval of the Animal Research Committee of the Research Institute for Microbial Diseases (Osaka University). At 3 d after injection of 2 ml of 4.0% (wt/vol) thioglycollate medium (Sigma), peritoneal exudate cells were isolated from the peritoneal cavities of mice by washing with ice-cold Hank's buffered-salt solution (Invitrogen). Resting B cells were isolated from splenocyte single-cell suspensions by positive selection with anti-B220 magnetic beads (Miltenyi Biotec). T cells were isolated from splenocyte single-cell suspensions by positive selection with anti-Thy-1.2 magnetic beads (Miltenyi Biotec). Cell purity was confirmed to be above 90% by flow cytometry.

Reagents. MALP-2 was provided as described⁷. LPS from *Salmonella minnesota* strain Re-595 was from Sigma-Aldrich. Poly(I:C) was from Amersham Biosciences. R-848 was provided by the Pharmaceuticals and Biotechnology Laboratory of the Japan Energy Corporation. The CpG oligonucleotide was synthesized as described⁷. Polyclonal anti-IRAK1 has been described⁷.

Measurement of cytokines and autoantibodies. Concentrations of cytokines in culture supernatants and serum were measured by ELISA. ELISA kits for mouse TNF and IL-6 were from R&D Systems. The ELISA kit for mouse IFN- α was from PBL Biomedical Laboratories. ELISA kits for mouse anti-dsDNA antibodies and antinuclear antibody were from Alpha Diagnostic International. Serum immunoglobulin concentrations were measured as described⁵⁰.

Histological analysis. Formalin-fixed tissues were stained with hematoxylin and eosin or periodic acid-Schiff reagent. For detection of renal IgG deposits, kidneys were rapidly frozen in liquid nitrogen and cryostat sections 2 μ m in thickness were fixed for 15 min in 100% (vol/vol) acetone. Sections were incubated overnight at 4 °C with FITC-conjugated goat anti-mouse IgG (67228; ICN Biomedicals), FITC-conjugated donkey anti-mouse IgM (715-095-050; Jackson ImmunoResearch), FITC-conjugated sheep anti-human C3c complement (433004; Thermo Electron) or FITC-conjugated anti-mouse C1q (RmC7H8; Cedarlane Laboratories), each at a concentration of 10 μ g/ml.

RNA hybridization. Peritoneal macrophages were treated for 0, 1, 4 and 8 h with 10 nM R-848, and total RNA was extracted with the TRIzol reagent (Invitrogen). The extracted RNA was separated by electrophoresis, transferred to nylon membranes and hybridized with various cDNA probes. For detection of the expression of *Tank* mRNA, a 319-base pair fragment (nucleotides 350–669) of *Tank* cDNA was used as a probe. The same membranes were rehybridized with an *Actb* probe.

In vitro kinase assay. Peritoneal macrophages stimulated with 10 nM R-848 were lysed and immunoprecipitated with anti-IRAK1. Then, IRAK1 activity was measured by *in vitro* kinase assay as described⁷.

Immunoblot analysis. Peritoneal macrophages were treated for various times with 10 nM R-848, then were lysed in a lysis buffer composed of 1.0% (vol/vol) Nonidet P-40, 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 analyzed by immunoblot. Polyclonal anti-TANK (2141) was from Cell Signaling. Polyclonal anti-TRAF6 (sc-7221), monoclonal anti-Ub (F-7), monoclonal anti- β -tubulin (D-10) and anti-cyclin D2 (34B1-3) were from Santa Cruz Biotechnology.

EMSA. Nuclear extracts were prepared from peritoneal macrophages (4×10^6) stimulated with 10 nM R-848 as described⁷, then were incubated with or without antibodies to NF- κ B p65 (C-20) or p50 (D-17; Santa Cruz) and were further incubated with a probe specific for NF- κ B DNA-binding sites, before being separated by electrophoresis and visualized by autoradiography.

Immunoblot, immunoprecipitation and in vivo ubiquitination assays. Peritoneal macrophages (4×10^6) were stimulated for various times with 10 nM R-848. Immunoblot analysis and immunoprecipitation were done as described⁷. For detection of *in vivo* ubiquitination of TRAF6, cell lysates were boiled for 10 min at 90 °C in 1% (wt/vol) SDS for removal of noncovalently attached proteins, followed by immunoprecipitation with anti-TRAF6 in 0.1% (wt/vol) SDS lysis buffer in the presence of protease inhibitors. Ubiquitin was detected by immunoblot analysis.

B cell and T cell proliferation assays. Purified splenic B cells (5×10^4) were cultured for 48 h in 96-well plates with various concentrations of R-848, CpG DNA, anti-IgM (Jackson ImmunoResearch) or anti-CD40 (HM40-3, Pharmingen). Purified splenic T cells were stimulated for 48 h with plate-bound anti-CD3 alone (1 or 5 μ g/ml; 2C11; Pharmingen) or with anti-CD3 (1 μ g/ml) plus anti-CD28 (1 μ g/ml; 37.51; Pharmingen). Samples were pulsed with 1 μ Ci [³H]thymidine for the final 16 h and ³H uptake was measured with a β -scintillation counter (Packard).

In vivo immunization and ELISA. Mice were immunized intraperitoneally with 50 μ g NP-CGG (Biosearch Technologies) precipitated with Imject alum (Pierce) or with 25 μ g of TNP-Ficoll (Biosearch Technologies). Antigen- and isotype-specific antibodies in serum collected from peripheral blood at various time points were measured by ELISA on plates coated with NP-BSA or TNP-BSA (Biosearch Technologies). Alkaline phosphatase-conjugated antibodies to mouse IgM (1020-04), IgG1 (1070-04) and IgG3 (1100-04) were from Southern Biotechnology.

Cell viability. Purified splenic B cells (1×10^6) were cultured for various periods in RPMI medium containing 10% (vol/vol) FCS. Cell viability was assessed with annexin V-indocarbocyanine (BioVision) and a FACSCalibur (Becton Dickinson).

Construction of TANK expression plasmids. Full-length mouse TANK cDNA was obtained by PCR from a mouse cDNA library and was cloned into the Myc-pcDNA3 vector.

Statistical analysis. Statistical significance was calculated with the two-tailed Student's *t*-test. *P* values of less than 0.05 were considered significant.

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