HHS Public Access

Author manuscript

Nat Immunol. Author manuscript; available in PMC 2010 July 26.

Published in final edited form as:

Nat Immunol. 2009 September ; 10(9): 965–972. doi:10.1038/ni.1771.

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

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Abstract

The intensity and duration of immune responses are controlled by multiple proteins that modulate Toll-like receptor (TLR) signaling. TRAF family member-associated NF-κB activator (TANK) has been implicated in positive regulation of interferon-regulatory factor-3 as well as NF-κB. 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 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 rescued by antibiotic treatment or the absence of interleukin (IL)-6 or MyD88. These results demonstrate that constitutive TLR signaling by intestinal commensal microflora is suppressed by TANK.

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Author contributions

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

Disclosure

The authors declare that they have no competing financial interests.

Introduction

Toll-like receptors (TLRs) recognize microbial components and evoke innate as well as adaptive immune responses. Stimulation with TLR ligands induces the production of proinflammatory cytokines and type I interferons (IFNs) in innate immune cells via intracellular signaling cascades1–3. Upon stimulation, TLRs trigger the recruitment of Toll/IL-1R homology (TIR) domain-containing adaptor molecules. One adaptor, MyD88, is essential for the downstream signaling of various TLRs, with the exception of TLR34–6. MyD88 interacts with IL-1R-associated kinase (IRAK)-4, which activates IRAK-1 and IRAK-27. In turn, the IRAKs dissociate from MyD88 and interact with TNFR-associated factor 6 (TRAF6) (http://www.signaling-gateway.org/molecule/query?afcsid=A002312), which acts as a ubiquitin protein ligase. Together with an E2 ubiquitin-conjugating enzyme complex comprised of Ubc13 and Uev1A, TRAF6 catalyzes formation of a lysine 63 (K63)-linked polyubiquitin chain on TRAF6 itself and on NF-κB essential modulator (NEMO). TGF-β-activated kinase 1 (TAK1) is also recruited to TRAF6, and phosphorylates IKK-β and MAP kinase kinase 68. Subsequently, the IκB kinase (IKK) complex, composed of IKK-α, IKK-β and NEMO, is formed. NF-κB binds to IκBα in resting cells and is sequestered in the cytoplasm. Phosphorylation of IκB by the IKK complex leads to its degradation by the ubiquitin-proteasome system, thereby freeing NF-κB to translocate into the nucleus and activate expression of proinflammatory cytokine genes. Activation of the MAP kinase cascade is responsible for AP-1-induced gene expression. In plasmacytoid dendritic cells (pDCs), MyD88-dependent signaling activates the production of type I IFNs via the transcription factor IFN-regulatory factor (IRF)-71, 9.

Multiple proteins control TLR signaling so as to ensure that the strength and duration of TLR signals is appropriate for any given immune response. TLRs have been implicated in the development of autoimmune diseases, and aberrant activation of innate immunity may contribute to rheumatoid arthritis, inflammatory bowel disease (IBD) and systemic lupus erythematosus (SLE)10, 11. Endogenous RNA molecules such as U1snRNP can activate autoreactive B cells and dendritic cells (DCs) via TLR712. Furthermore, duplication of the *Tlr7* gene accounts for the autoimmune phenotypes associated with Y chromosome-linked autoimmune accelerator (Yaa) mice13. TLR9 is also involved in the recognition of immune complexes of DNA and anti-double-stranded DNA (dsDNA) antibodies (Abs), together with B cell receptor (BCR)14. Signaling proteins that inhibit TLR signaling include IRAK-M, ST2, single immunoglobulin IL-1 receptor-related (SIGIRR), suppressor of cytokine signaling (SOCS)-1, the tumor suppressor cylindromatosis (CYLD) and A20 15–21. Cells lacking any one of these proteins show elevated production of proinflammatory cytokines in response to TLR stimulation. Furthermore, mice lacking SOCS-1 or A20 display immune disorders that lead to premature death. In addition, the immunosuppressive cytokine IL-10 suppresses colitis development by inhibiting TLR responses22–24. These studies indicate that negative regulation of TLR signaling is important for coordinated innate immune responses.

TRAF family member-associated NF-κB activator (TANK; also known as I-TRAF) was identified as a TRAF-binding protein25, 26. Among the 6 reported TRAF family members, TRAF1, 2, 3, 5 and 6 interact with TANK25–28. TANK has been implicated in positive

regulation of NF-κB activation. In addition to TRAF family members, inducible IKK (IKK-*i*) and TANK-binding kinase 1 (TBK1) are TANK binding partners29, 30. These proteins phosphorylate IRF-3 and IRF-7, which are transcription factors essential for the expression of type I IFN and IFN-inducible genes 31, 32. TBK1 and IKK-*i* are activated in response to recognition of viruses via TLRs and retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs)33, 34. TRAF3 is required for TBK1 and IKK-*i* activation downstream of TLRs and RLRs35, 36. It was reported that TANK functions as an adaptor that bridges TRAF3 and TBK1-IKK-*i* and TANK is required for type I IFN production in response to viral infection or TLR stimulation37. However, the functional roles of TANK *in vivo* have not yet been clarified.

In the present study, we use *Tank*-deficient ($Tank^{-/-}$) mice to demonstrate that TANK is not involved in IFN responses but is a negative regulator of TLR and BCR signaling. Macrophages and B cells from $Tank^{-/-}$ mice show elevated canonical NF-κB activation in response to stimulation of TLRs and BCR. TLR-induced polyubiquitination of TRAF6 was upregulated in $Tank^{-/-}$ macrophages, indicating 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 TNF. Furthermore, treatment of $Tank^{-/-}$ mice with antibiotics reduced autoantibody production, implying 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 roles of TANK *in vivo*, we generated $Tank^{-/-}$mice by homologous recombination in embryonic stem (ES) cells. We targeted exons 3 and 4 of the mouse *Tank* gene with a neo^{r} cassette in ES cells, and established $Tank^{-/-}$mice (Supplementary Fig. 1a). Homologous recombination of the *Tank* locus was confirmed by Southern blotting (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 in Mendelian ratios and grew normally.

$Tank^{-/-}$ mice displayed splenomegaly and lymphadenopathy (Fig. 1a,b). Flow cytometric analysis revealed a higher percentage of $CD19^{+}$ B cells in the spleen and lymph nodes (LNs) of $Tank^{-/-}$ mice (Fig. 1c,d). $IgM^{low}IgD^{high}$ mature B cells accumulated in the spleen of $Tank^{-/-}$ mice (Fig. 1e). The percentage of $CD19^{+}CD138^{+}$ plasma cells was also highly increased in the spleen and LNs 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 increased B cell populations, basal serum concentrations of IgM, IgG1, IgG2a, IgG2b and IgA were significantly elevated by 1.2–6.2-fold in $Tank^{-/-}$ mice compared with wild-type mice (Fig. 1h). Importantly, anti-nuclear Ab (ANA) and anti-dsDNA Abs were detected in the sera of $Tank^{-/-}$ mice (Fig. 1i,j)

Tank$^{-/-}$ mice spontaneously started to die at 3 months after birth and about 50% of *Tank*$^{-/-}$ mice had died by 12 months after birth (Fig. 2a). Histological studies revealed that 24 week old *Tank*$^{-/-}$ mice exhibited glomerulonephritis with mesangial cell proliferation and expansion of the mesangial matrix (Fig. 2b,c). The glomerular structure was devastated in terminally ill *Tank*$^{-/-}$ mice, suggesting that renal failure was the cause of death (data not shown). Although infiltration of lymphocytes was observed in the liver and lungs of *Tank*$^{-/-}$ mice, no histological changes were detected in their intestine, heart or joints (data not shown). In addition, deposition of IgG, IgM and complement components C3 and C1q was observed 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 is the cause of the glomerulonephritis in *Tank*$^{-/-}$ mice.

TANK is a negative regulator of TLR responses

Then, we examined the type I IFN responses of *Tank*$^{-/-}$ cells to virus infection. In contrast to the results obtained by *in vitro* studies, IFN-β production in response to Newcastle disease virus (NDV) infection did not differ between wild-type and *Tank*$^{-/-}$ conventional DCs (cDCs) derived from bone marrow cells (Fig. 3a). Wild-type and *Tank*$^{-/-}$ cDCs also produced similar amounts of IL-6 (Fig. 3b). NDV is recognized by RIG-I in cDCs, indicating 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 pDCs. However, bone marrow pDCs induced by Flt3 ligand from *Tank*$^{-/-}$ mice produced increased, rather than decreased, amounts of IFN-α and IL-6 in response to A/D-type CpG-DNA (Fig. 3c,d). Collectively, these results indicate that TANK is not essential for type I IFN responses.

Next, we examined the production of proinflammatory cytokines in macrophages in response to a set of TLR ligands, including MALP-2 (TLR6-TLR2), poly I:C (TLR3), LPS (TLR4), R-848 (TLR7) and CpG-DNA (TLR9). The production of IL-6 and TNF in response to these TLR ligands, except poly I:C, were highly increased in *Tank*$^{-/-}$ peritoneal macrophages compared with wild-type cells (Fig. 3e,f). Of note, the enhanced cytokine production in response to LPS stimulation in *Tank*$^{-/-}$ macrophages was less severe compared to that induced by other TLR ligands. cDCs from *Tank*$^{-/-}$ mice also showed excessive cytokine production in response to these TLR ligands (data not shown).

We subsequently assessed the role 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 one and three hours later. *Tank*$^{-/-}$ mice contained significantly higher amounts of these serum cytokines at both time points compared with wild-type mice (Fig. 3g,h). Taken together, these results indicate that TANK is a negative regulator of TLR-mediated responses, but not an essential positive regulator of type I IFN responses *in vivo*.

TANK controls TRAF6 ubiquitination

We examined whether the increased cytokine production in *Tank*$^{-/-}$ macrophages was evident at the level of transcription. In response to R-848 stimulation, wild-type

macrophages showed induction of *Il6*, *Tnf*, *Il12b*, *Ptgs2*, *Nfkbiz* and *Nos2* gene expression. The expression of these genes was enhanced in *Tank*$^{-/-}$ macrophages in response to R-848 stimulation (Fig. 4a), indicating that initial TLR-induced gene expression is enhanced in *Tank*$^{-/-}$ macrophages. Next, we analyzed the activation of the transcription factors NF-κB and AP-1 by electrophoretic mobility shift assays (EMSA). In response to R-848 stimulation, activation of NF-κB and AP-1 was enhanced in *Tank*$^{-/-}$ macrophages compared to wild-type macrophages (Fig. 4b,c).

The above-described results indicate that TANK negatively regulates TLR-induced activation of NF-κB and AP-1. Activation of IRAK-1 in response to R-848 was not enhanced in *Tank*$^{-/-}$ macrophages (Fig. 5a). Furthermore, IRAK-1 was degraded after R-848 stimulation with similar kinetics in wild-type and *Tank*$^{-/-}$ macrophages (Fig. 5b), indicating that TANK regulates signaling downstream of IRAKs. TANK has been reported to interact with the TRAF family members TRAF1, 2, 3, 5 and 6. Among these, TRAF6 is needed for TLR signaling. Since TRAF6 is ubiquitinated in response to TLR stimulation, we examined whether TANK modifies the ubiquitination of TRAF6. As shown in Fig. 5c, induction of TRAF6 ubiquitination in response to R-848 stimulation was enhanced in *Tank*$^{-/-}$ macrophages compared with wild-type cells. Reciprocally, overexpression of TANK in HEK293 cells inhibited the ubiquitination of TRAF6 (Fig. 5d). Taken together, these results indicate that TANK inhibits TLR-induced NF-κB and AP-1 activation 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 BCR and CD40. After stimulation with R-848, CpG-DNA, anti-IgM or anti-CD40, *Tank*$^{-/-}$ B cells proliferated much more than wild-type B cells (Fig. 6a). On the other hand, the amounts of splenic B cell death following culture without a mitogen were comparable between wild-type and *Tank*$^{-/-}$ mice (Fig. 6b), indicating 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-κB. The non-canonical pathway is characterized by processing of the NF-κB2 precursor protein p100 to generate p52. As shown in Fig. 6c, activation of non-canonical NF-κB in response to CD40 stimulation was similar in wild-type and *Tank*$^{-/-}$ B cells. In contrast, NF-κB DNA-binding activity was enhanced in *Tank*$^{-/-}$ B cells compared with wild-type B cells (Fig. 6d), and the band was supershifted by anti-p65 and anti-p50 (data not shown). Ubiquitination of TRAF6 after anti-CD40 stimulation was also enhanced in *Tank*$^{-/-}$ B cells (Fig. 6e). Furthermore, BCR stimulation also induced enhanced activation of NF-κB and ubiquitination of TRAF6 in *Tank*$^{-/-}$ B cells (Supplementary Fig. 3a,b). Further, the expression of cyclin D2, an NF-κB-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 non-canonical, NF-κB activation pathways in B cells.

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 myristate acetate (PMA) and ionomycin *in vitro*, similar

proportions of wild-type and *Tank*$^{-/-}$ CD4^{+} T cells produced IFN-γ or IL-17 (Supplementary Fig. 2c), suggesting that TANK does not play a role in the development of T helper type 1 (T_H1) or T_H-17cells.

To explore the influence of TANK deficiency on Ab responses *in vivo*, wild-type and *Tank*$^{-/-}$ mice were immunized with a T cell-dependent antigen, NP-CGG, or a T cell-independent antigen, TNP-Ficoll. The NP-specific IgG1 and IgM titers were elevated in *Tank*$^{-/-}$ mice compared with wild-type mice (Fig. 6f). The TNP-specific IgG3 and IgM titers were also augmented in *Tank*$^{-/-}$ mice compared with wild-type mice (Fig. 6g). The difference between wild-type and *Tank*$^{-/-}$ mice was more severe in response to TNP-Ficoll immunization, suggesting that TANK may be more critical for T cell-independent than for T cell-dependent immune responses *in vivo*.

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

Proinflammatory cytokines play critical roles in the development of autoimmune diseases. 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 Abs were significantly lower in *Tank*$^{-/-}$*IL-6*$^{-/-}$ than in *Tank*$^{-/-}$ 5 month old mice (Fig. 7a). Moreover, IL-6 deficiency rescued the glomerulonephritis that developed in *Tank*$^{-/-}$ mice (Fig. 7b). On the other hand, TNF deficiency did not significantly alter the amount of anti-dsDNA Ab production in *Tank*$^{-/-}$ mice (Fig. 7c). To examine whether MyD88 deficiency protects against the disease progress, we crossed *Tank*$^{-/-}$ mice with *MyD88*$^{-/-}$ mice. The anti-dsDNA Ab titers in 5-month-old *Tank*$^{-/-}$*MyD88*$^{-/-}$ mice were significantly lower than in *Tank*$^{-/-}$ mice (Fig. 7d), indicating 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 in the pathogenesis of autoimmune diseases, such as colitis in IL-10-deficient mice. Therefore, we orally treated *Tank*$^{-/-}$ mice with a combination of antibiotics to clear the intestinal microflora. As shown in Fig. 7e, the antibiotic treatment significantly ameliorated the production of anti-dsDNA Abs, suggesting that continuous stimulation of TLRs by intestinal microflora contributes to the generation of autoantibodies in the absence of TANK.

Discussion

In the present study, we generated *Tank*$^{-/-}$ mice and showed that TANK is essential for negative regulation of canonical NF-κB signaling. *Tank*$^{-/-}$ mice displayed enhanced activation of macrophages and B cells in response to TLR ligands and antigens, culminating in the development of fatal immune complex-mediated renal failure. Although TANK has been shown to positively regulate TBK1 and IKK-*i*-mediated type I IFN production by *in vitro* studies, analyses of *Tank*$^{-/-}$ mice revealed that TANK was not needed for activation of the type I IFN pathway downstream of RLRs or TRIF. TANK forms a family with NAK-associated protein 1 (NAP1) and similar to NAP1 TBK1 adaptor (SINTBAD)38, 39, which

are comprised of an N-terminal coiled-coil domain and a TBK1-binding domain. NAP1 and SINTBAD have also been implicated in the activation of TBK1 and IKK-*i* downstream of virus sensors. Knockdown of NAP1, SINTBAD or TANK by siRNA has been linked to impaired IFN responses. Hence, it is possible that these three proteins function redundantly in the activation of TBK1 and IKK-*i*.

Although previous studies showed that TANK is a positive regulator of NF-κB, our results clearly demonstrate that TANK is critical for the negative regulation of canonical NF-κB via suppression of TRAF6 ubiquitination. K63-type ubiquitination is important for the activation of TAK1 via 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 deubiquitinases40–42, TANK does not harbor a deubiquitination enzyme domain. Immunoprecipitation experiments revealed that overexpressed A20 or CYLD failed to co-immunoprecipitate with overexpressed TANK, suggesting that TANK may suppress ubiquitination of TRAF6 independently of A20 or CYLD (data not shown). Further studies are required to assess the precise mechanism through which TANK modifies TRAF6. In addition, canonical NF-κB activation in response to BCR and CD40 stimulation was augmented in *Tank*$^{-/-}$ B cells. Consistently, proliferation of B cells in response to TLR and BCR stimulation was highly elevated in *Tank*$^{-/-}$ mice. In TCR signaling, TRAF2 and TRAF6 were reported to participate in NF-κB activation downstream of Bcl10 and MALT143. 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. On the other hand, activation of non-canonical NF-κB activation was not enhanced in *Tank*$^{-/-}$ B cells, and it was reported that TRAF3 mainly controls non-canonical NF-κB activation in B cells44. Hence, these observations suggest that TANK is not involved in signaling downstream of TRAF3. Further, TRAF2 can control non-canonical NF-κB as well as marginal zone B cell development. The relationship between TANK and TRAF2 needs to be further explored in future.

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 Abs and ANA 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 phenotypes of *Tank*$^{-/-}$ mice are reminiscent of mice overexpressing IL-6 in B cells45, which are 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 T_H-17 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 via aberrant activation of B cells in response to antigen stimulation.

The generation of anti-dsDNA Abs in *Tank*$^{-/-}$ mice was significantly decreased in response to oral treatment with antibiotics or in the absence of MyD88, suggesting that TLR signaling is critical for the development of autoimmune diseases in *Tank*$^{-/-}$ mice. Although various proteins have been identified as negative regulators of TLR signaling, few mice lacking any single one of these proteins spontaneously develop autoimmune diseases spontaneously, with the exception of mice lacking A20. *A20*$^{-/-}$ mice spontaneously develop multiorgan inflammation and premature lethality, which can be rescued by MyD88 deficiency46, 47. Unlike *Tank*$^{-/-}$ mice, *A20*$^{-/-}$ mice do not develop immune complex-mediated glomerulonephritis. A20 controls TNFR in addition to TLR signaling, and 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.

Since 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 revealed that hematopoietic cells were responsible for the lethality in *Tank*$^{-/-}$ mice (data not shown). Intestinal microflora contribute to the pathogenesis of IBD48, 49, and the colitis observed in IL-10-deficient mice was rescued by the absence of MyD8824, suggesting that TLR signaling is involved in the pathogenesis of IBD. Since 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 required 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 be due to the enhanced DC and B cell activation in response to antigens and the adjuvant in *Tank*$^{-/-}$ mice. It will be interesting 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 useful in vaccinations when administered together with an adjuvant.

In summary, the results of the present study clearly demonstrate that TANK is a negative regulator of TLR and BCR responses. Future studies involving cell-type specific deletion of TANK will clarify the complex interplay between immune cells needed to prevent the development of autoimmune diseases.

Methods

Generation of *Tank*$^{-/-}$ mice

The *Tank* gene was isolated from genomic DNA extracted from ES cells (GSI-I) by PCR. The targeting vector was constructed by replacing a 2.0-kb fragment encoding the *Tank* ORF with a *neomycin-resistance* gene cassette (*neo*^{r}), and a herpes simplex virus thymidine

kinase (HSV-TK) driven by the PGK promoter was inserted into the genomic fragment to facilitate negative selection. After transfection of the targeting vector into ES cells, G418 and gancyclovir doubly-resistant colonies were selected, screened by PCR and further confirmed by Southern blotting. Homologous recombinants were microinjected into blastocysts from C57BL/6 female mice, and heterozygous F1 progenies were intercrossed to obtain *Tank*$^{-/-}$ mice. *Tank*$^{-/-}$ mice under the 129Sv × C57BL/6 background and their littermate controls were used for the experiments.

Mice and cells

MyD88$^{-/-}$, and *Tnf*$^{-/-}$ mice were described previously4, 23. *Il6*$^{-/-}$ mice were provided by T. Yasui (Osaka University, Osaka, Japan). All animal experiments were carried out with the approval of the Animal Research Committee of the Research Institute for Microbial Diseases (Osaka University, Osaka, Japan). At 3 days after injection of 2 ml of 4.0% thioglycolate 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-Thy1.2 magnetic beads (Miltenyi Biotec). The cell purities were confirmed to be >90% by flow cytometric analysis.

Reagents

MALP-2 was provided as described previously7. LPS from *Salmonella minnesota* Re-595 was purchased from Sigma-Aldrich. Poly I:C was purchased from Amersham Biosciences. R-848 was provided by the Pharmaceuticals and Biotechnology Laboratory of the Japan Energy Corporation. The CpG oligonucleotide was synthesized as described previously7. Polyclonal anti-IRAK1 was described previously7.

Measurement of cytokines and autoantibodies

The concentrations of cytokines in culture supernatants and sera were measured by ELISA. The ELISA kits for mouse TNF and IL-6 were purchased from R&D Systems. The ELISA kit for mouse IFN-α was purchased from PBL Biomedical Laboratories. The ELISA kits for mouse anti-dsDNA Abs and ANA were purchased from Alpha Diagnostic International. Serum Ig concentrations were determined as described previously50.

Histological analysis

Formalin-fixed tissues were stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS). For detection of renal IgG deposits, kidneys were rapidly frozen in liquid nitrogen and 2-μm cryostat sections were fixed in 100% acetone for 15 min. The sections were incubated with FITC-conjugated goat anti-mouse IgG (ICN Biomedicals), FITC-conjugated donkey anti-mouse IgM (Jackson ImmunoResearch), FITC-conjugated sheep anti-human C3c complement (Thermo Electron Corporation) or FITC-conjugated anti-mouse C1q (RmC7H8) (Cedarlane Laboratories) at 10 mg/ml overnight at 4°C.

RNA hybridization

Peritoneal macrophages were treated with 10 nM R-848 for 0, 1, 4 and 8 h, and total RNA was extracted using the TRIzol reagent (Invitrogen). The extracted RNA was electrophoresed, transferred to nylon membranes and hybridized with various cDNA probes. To detect the expression of *Tank* mRNA, a 319-bp fragment (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-IRAK-1 antibody. Then, IRAK-1 activity was measured by an *in vitro* kinase assay, as described previously7.

Immunoblot analysis

Peritoneal macrophages were treated with 10 nM R-848 for various times. The cells were then lysed in a lysis buffer comprising 1.0% Nonidet-P40, 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1 mM EDTA and a protease inhibitor cocktail (Roche). The cell lysates were separated by SDS-PAGE and analyzed by immunoblots. Polyclonal anti-TANK (2141) was purchased 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 obtained from Santa Cruz Biotechnology.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from peritoneal macrophages (4×10^6) stimulated with 10 nM R-848 as described previously7. The nuclear extracts were then incubated with or without Abs against NF-κB p65 or p50 (Santa Cruz), and further incubated with a specific probe for NF-κB DNA-binding sites, before being electrophoresed and visualized by autoradiography.

Immunoblot, immunoprecipitation and *in vivo* ubiquitination assays

Peritoneal macrophages (4×10^6) were stimulated with 10 nM R-848 for various times. Immunoblotting and immunoprecipitation were carried out as described previously7. For detection of *in vivo* ubiquitination of TRAF6, cell lysates were boiled in 1% SDS at 90°C for 10 min to remove noncovalently attached proteins, followed by immunoprecipitation with anti-TRAF6 in 0.1% SDS lysis buffer in the presence of protease inhibitors. Ubiquitin was detected by immunoblot analysis.

B and T cell proliferation assays

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

In vivo immunization and ELISA

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

Cell viability

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

Construction of TANK expression plasmids

A murine full-length TANK cDNA was obtained by PCR from a murine cDNA library, and cloned into the Myc-pcDNA3 vector.

Statistical analysis

Statistical significance was calculated with the two-tailed Student's *t*-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to T. Yasui (Research Institute for Microbial Diseases, Osaka University) for providing *Il6*$^{-/-}$ mice and plasmids. We thank all the colleagues in our laboratory, E. Kamada for secretarial assistance, and Y. Fujiwara, M. Kumagai and R. Abe for technical assistance. We thank S. Sato for helpful discussions. This work was 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 NIH (P01 AI070167).

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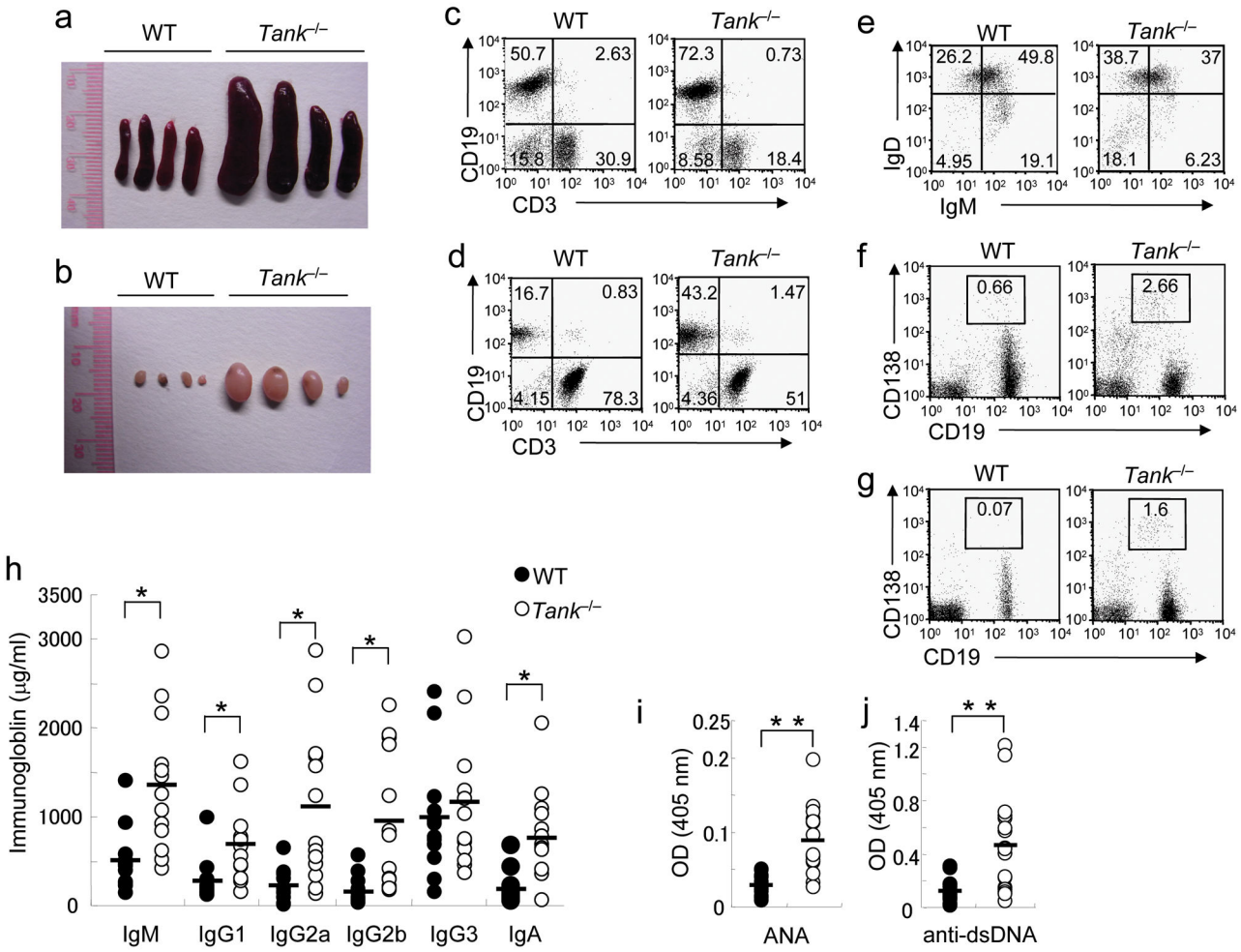

Figure 1. Increased Ig and autoantibody production due to B cell abnormalities in *Tank*$^{-/-}$ mice

(**a,b**) Images of representative spleens (**a**) and inguinal LNs (**b**) from 6-month-old wild-type (WT) and *Tank*$^{-/-}$ mice. (**c**–**g**) Expanded plasma cell populations in spleens and LNs of *Tank*$^{-/-}$ mice. The percentages of B cells and T cells in spleens (**c**) and LNs (**d**), expression of IgM and IgD on splenic B cells (**e**) and expression of CD138 and CD19 on cells in spleens (**f**) and LNs (**g**), from WT and *Tank*$^{-/-}$ mice were analyzed by FACS. (**h**) Basal titers of Ig isotypes in sera from nonimmunized 3-month-old WT (n = 13) and *Tank*$^{-/-}$ (n = 13) mice were measured by ELISA. (**i**, **j**) ANA (**i**) and anti-dsDNA Abs (**j**) in sera from 12-month-old WT (n = 12) and *Tank*$^{-/-}$ (n = 12) mice were measured by ELISA. *, $P<0.005$, **, $P<0.001$, versus *Tank*$^{-/-}$ cells.

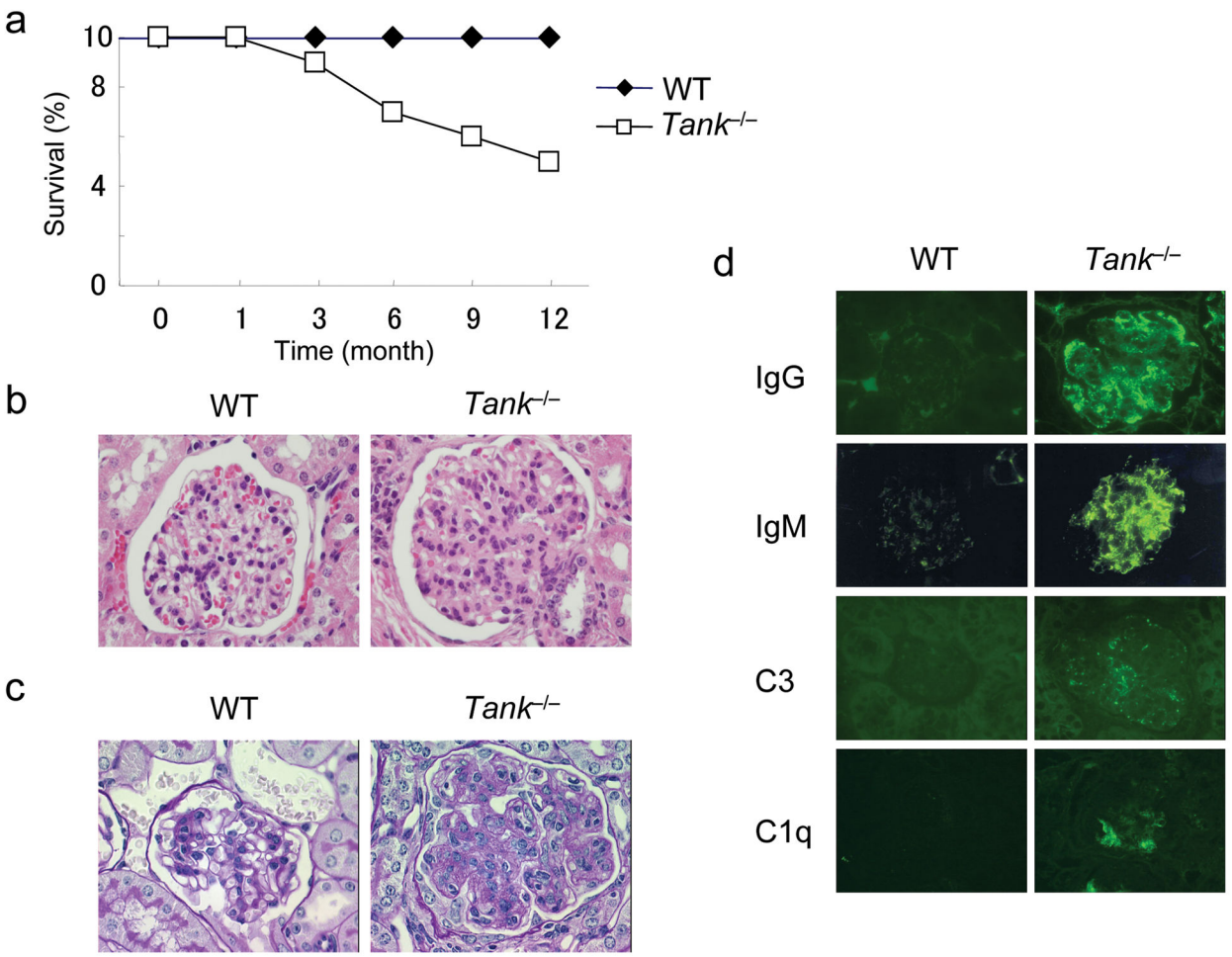

Figure 2. Development of lethal glomerulonephritis in *Tank*$^{-/-}$ mice

(**a**) The survival of WT (n = 10) and *Tank*$^{-/-}$ (n = 10) mice was monitored for 1 year. (**b**,**c**) Kidney sections from 6-month-old WT and *Tank*$^{-/-}$ mice were stained with hematoxylin and eosin (H&E) (**b**) or Periodic acid-Schiff (PAS) (**c**). (**d**) Kidney sections from 6-month-old WT and *Tank*$^{-/-}$ mice were stained with FITC-labeled anti-mouse IgG, IgM, C3 and C1q.

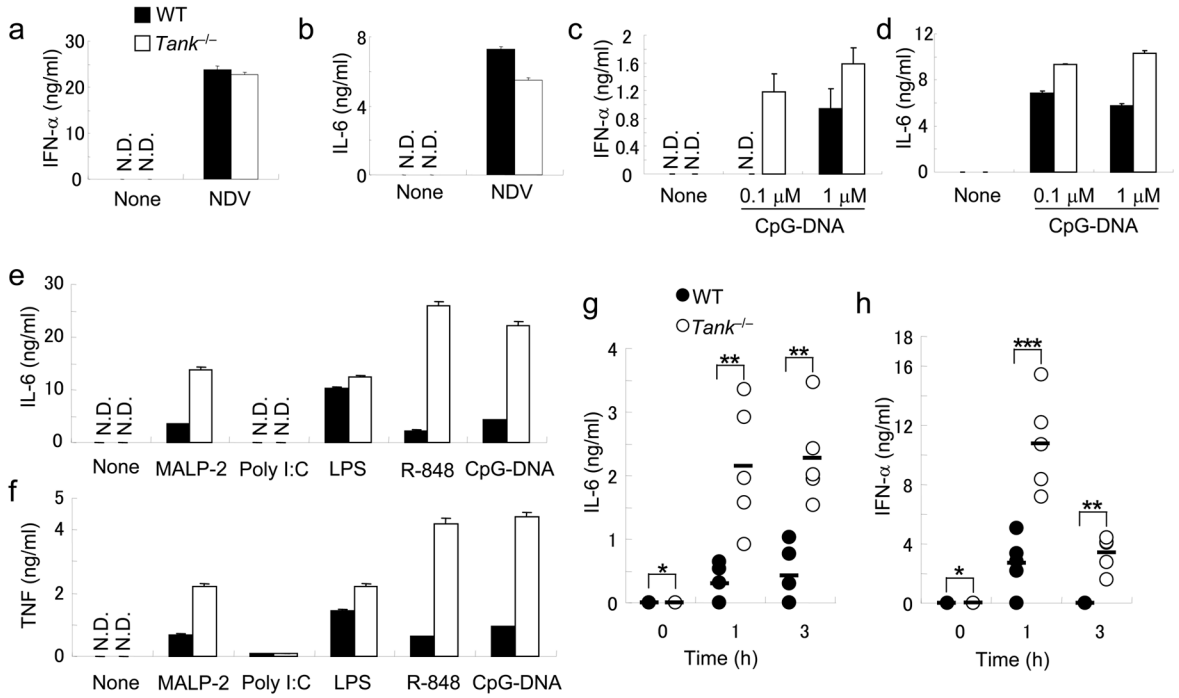

Figure 3. Enhanced proinflammatory cytokine production in response to TLR stimulation in *Tank*$^{-/-}$ mice

(**a**,**b**) BM-DCs from wild-type and *Tank*$^{-/-}$ mice were infected with NDV for 24 h. The concentrations of IFN-α (**a**) and IL-6 (**b**) in the culture supernatants were measured by ELISA. (**c**,**d**) Flt3L-DCs from wild-type and *Tank*$^{-/-}$ mice were stimulated with 0.1 or 1μM CpG-DNA for 24 h. The concentrations of IFN-α (**c**) and IL-6 (**d**) in the culture supernatants were measured by ELISA. (**e**,**f**) Peritoneal macrophages from wild-type and *Tank*$^{-/-}$ mice were stimulated with MALP-2 (10 ng/ml), poly I:C (100 μg/ml), LPS (100 ng/ml), R-848 (10 nM) or CpG-DNA (1 μM) for 24 h. The concentrations of IL-6 (e) and TNF (f) in the culture supernatants were measured by ELISA. (g, h) Wild-type (n = 5) and *Tank*$^{-/-}$ (n = 5) mice were intraperitoneally injected with 30 nmol of R-848. Sera were collected and the concentrations of IL-6 (g) and IFN-α (h) were determined by ELISA. Data represent the means ± s.d. of triplicate assays. Similar results were obtained in three independent experiments. *, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.005$, versus *Tank*$^{-/-}$ mice.

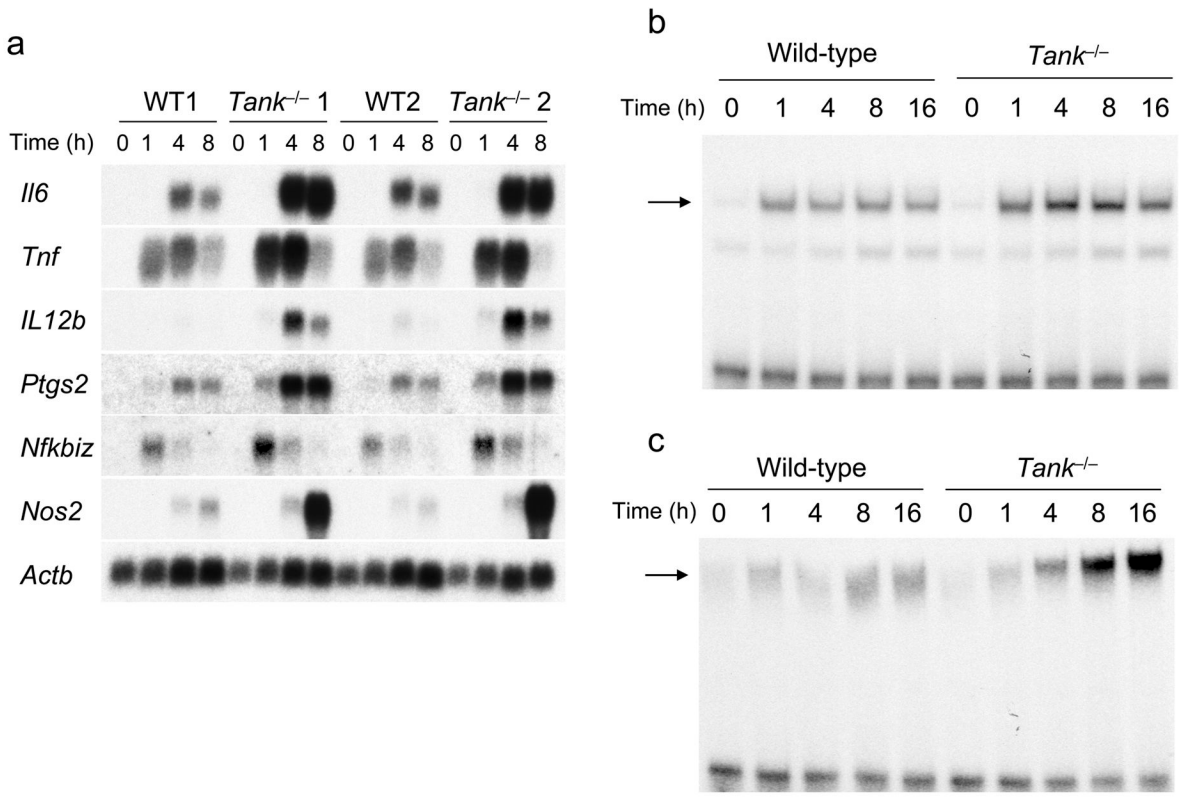

Figure 4. TANK negatively regulates the activation of NF-κB and AP-1 as well as gene expression in response to TLR7 stimulation in macrophages

(**a**) Peritoneal macrophages from wild-type (WT) and *Tank*$^{-/-}$ mice were stimulated with 10 nM R-848 for the indicated periods. Total RNA was extracted and subjected to Northern blot analyses for the expression of *Il6*, *Tnf*, *Il12b*, *Ptgs2*, *Nfkbiz* and *Nos2*. The same membranes were rehybridized with an *Actb* probe. Data of two independent experiments (lanes marked 1 and 2 represent distinct experiments) are shown. (**b**,**c**) Wild-type and *Tank*$^{-/-}$ macrophages were stimulated with R-848 (10 μM) for the indicated periods. Nuclear extracts were prepared, and the NF-κB (**b**) and AP-1 (**c**) DNA-binding activities were determined by EMSA using NF-κB- and AP-1-specific probes. The arrows indicate the induced NF-κB and AP-1 complexes. The results are representative of three independent experiments.

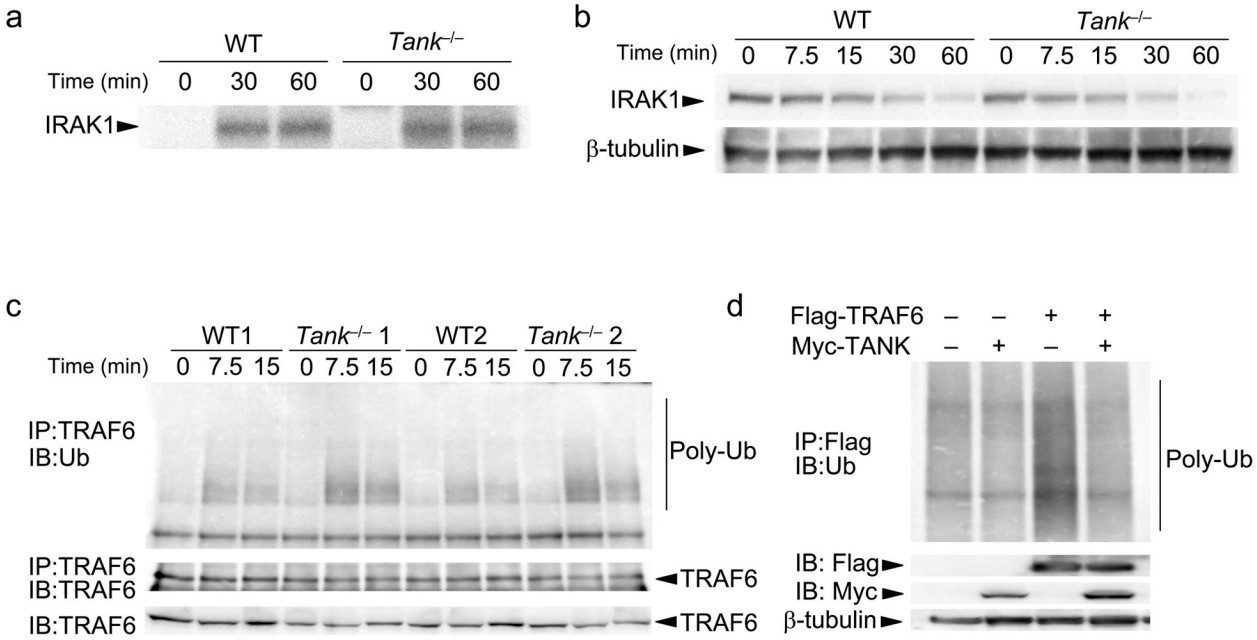

Figure 5. TANK controls TRAF6 ubiquitination in response to TLR7 stimulation in macrophages

(**a**) Peritoneal macrophages from wild-type (WT) and *Tank*$^{-/-}$ mice were stimulated with 10μM R-848 for the indicated periods. Cell lysates were prepared and immunoprecipitated with anti-IRAK1. The kinase activities in the immunoprecipitates were measured using an *in vitro* kinase assay. (**b**) Macrophages from wild-type and *Tank*$^{-/-}$ mice were stimulated with 10 μM R-848 for the indicated periods. Whole cell lysates were subjected to immunoblot analysis with anti-IRAK1. Immunoblots ofβ-tubulin are shown as a loading control. (**c**) Cell lysates of macrophages treated with R-848 for the indicated periods were immunoprecipitated with anti-TRAF6, followed by immunoblot analysis with anti-Ub. Immunoblots of TRAF6 are shown as a loading control. Data of two independent experiments are shown. (**d**) HEK293 cells were cotransfected with Flag-TRAF6 and Myc-TANK. Cell lysates were immunoprecipitated with anti-Flag, followed by immunoblot analysis with anti-Ub. Immunoblots of β-tubulin are shown as a loading control. The data shown are representative of three independent experiments.

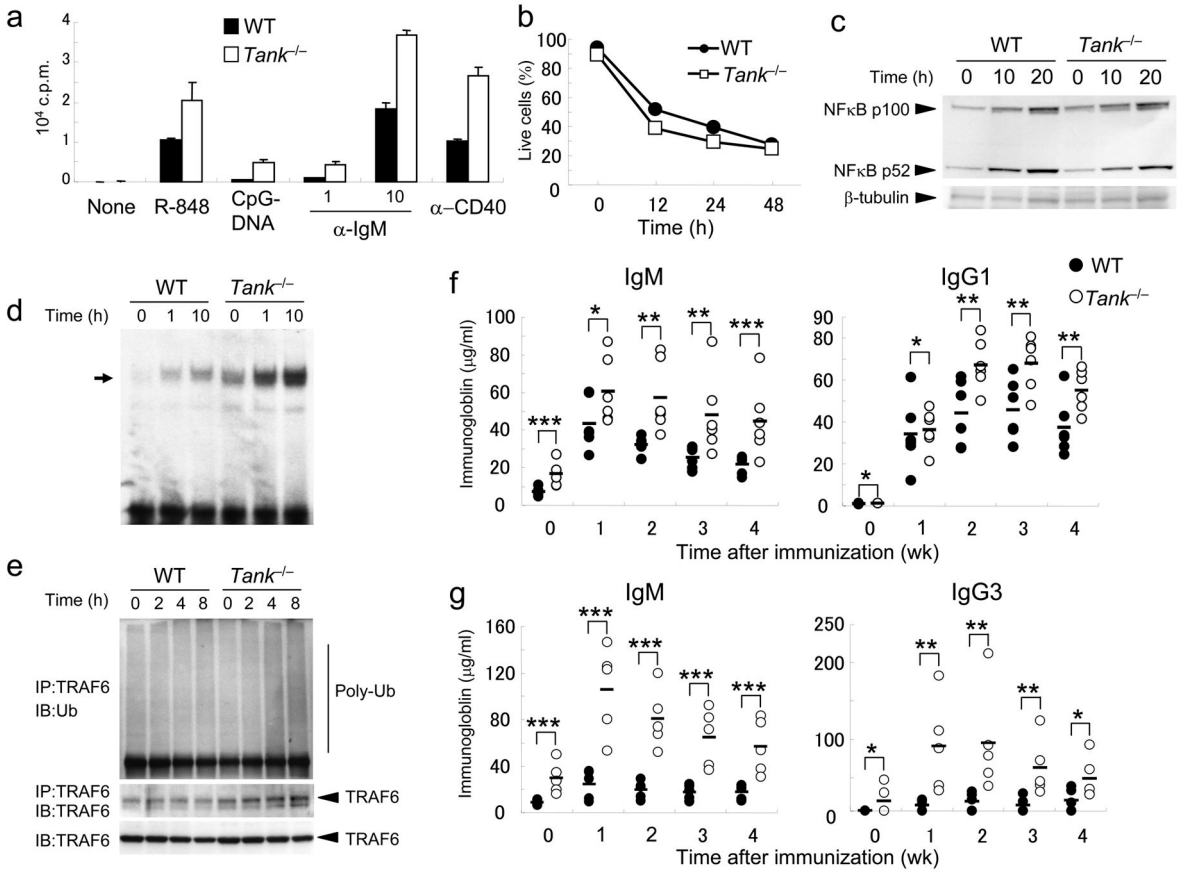

Figure 6. Enhanced activation of B cells in *Tank*$^{-/-}$ mice

(**a**) Purified splenic B cells were cultured with R-848 (10 nM), CpG-DNA (10 nM), anti-IgM (1, 10μg/ml) or anti-CD40 (1 μg/ml) for 48 h. The samples were pulsed with [^{3}H]-thymidine (1 μCi) for the last 16 h. [^{3}H]-thymidine incorporation was measured using a β-scintillation counter. (**b**) Splenic B cells were cultured in the absence of cytokines for the indicated periods. The viability of the cells was determined by annexin V staining followed by flow cytometric analysis. (**c**) B cells from wild-type and *Tank*$^{-/-}$ mice were stimulated with 5μg/ml anti-CD40 for the indicated periods, and the processing of p100 to p52 in whole cell lysates was detected by immunoblot analysis. Immunoblots of β-tubulin are shown as a loading control. (**d**) B cells from wild-type and *Tank*$^{-/-}$ mice were stimulated with 5μg/ml anti-CD40 for the indicated periods. Nuclear extracts were prepared and the NFκB DNA-binding activity was determined by EMSA. The arrow indicates the induced NF-κB complex. (**e**) Cell lysates of splenic B cells treated with 5μg/ml anti-CD40 for the indicated periods were immunoprecipitated with anti-TRAF6, followed by immunoblot analysis with anti-Ub. Immunoblots of TRAF6 are shown as a loading control. The data shown are representative of three independent experiments. (**f**) Mice were immunized with nitrophenol-chicken γ-globulin, and nitrophenol (NP)-specific IgM and IgG1 production was measured by ELISA at 1, 2, 3 and 4 weeks after immunization. The data for 5 representative mice per genotype are shown. (**g**) Mice were immunized with trinitrophenol-Ficoll, and trinitrophenol (TNP)-specific IgM and IgG3 production was measured at 1, 2, 3 and 4 weeks after

immunization. The data for 5 representative mice per genotype are shown. *, $P > 0.05$, **, $P < 0.05$ and ***, $P < 0.01$, versus $Tank^{-/-}$ mice.

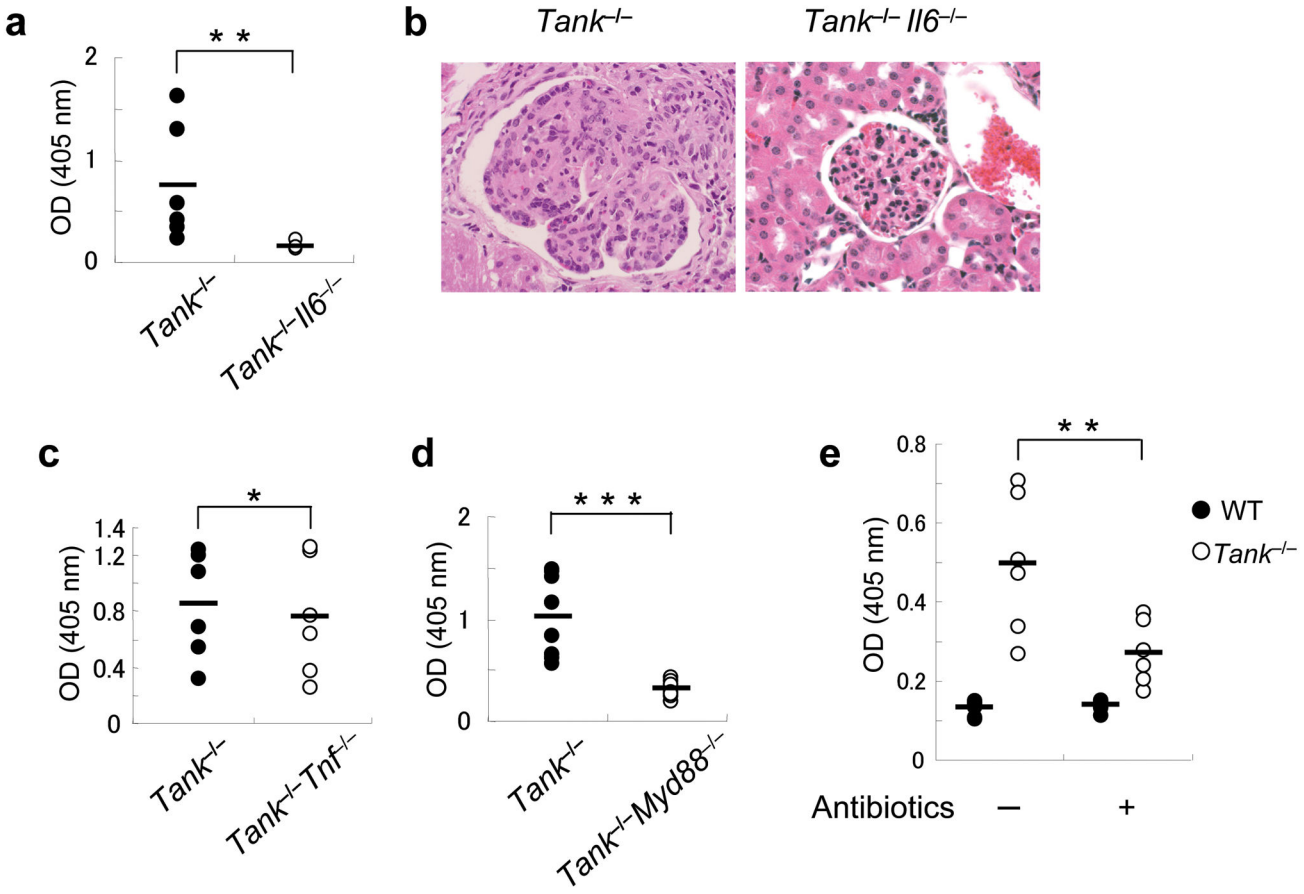

Figure 7. Antibiotic treatment as well as deficiency of MyD88 or IL-6 ameliorates autoantibody production in *Tank*$^{-/-}$ mice

(**a**) Anti-dsDNA Abs in sera from 5-month-old *Tank*$^{-/-}$ (*n* = 6) and *Tank*$^{-/-}$*IL-6*$^{-/-}$ (*n* = 6) mice. (**b**) H&E staining of kidney sections from *Tank*$^{-/-}$ and *Tank*$^{-/-}$*IL-6*$^{-/-}$ mice. (**c**,**d**) Anti-dsDNA Abs in *Tank*$^{-/-}$ (*n* = 6) and *Tank*$^{-/-}$*Tnf*$^{-/-}$ (*n* = 6) mice (**c**), *Tank*$^{-/-}$ (*n* = 6) and *Tank*$^{-/-}$*MyD88*$^{-/-}$ (*n* = 6) mice (**d**) were measured by ELISA. (**e**) Oral treatment with antibiotics reduces the serum anti-dsDNA Ab concentrations in *Tank*$^{-/-}$ mice. WT (*n* = 6) and *Tank*$^{-/-}$ (*n* = 6) mice were given drinking water containing ampicillin (1 g/L), neomycin (1 g/L), vancomycin (0.5 g/L) and metronidazole (1 g/L) after birth. Control wild-type (*n* = 6) and *Tank*$^{-/-}$ (*n* = 6) mice received untreated drinking water. The serum anti-dsDNA Ab concentrations were measured by ELISA at 16 weeks of age.