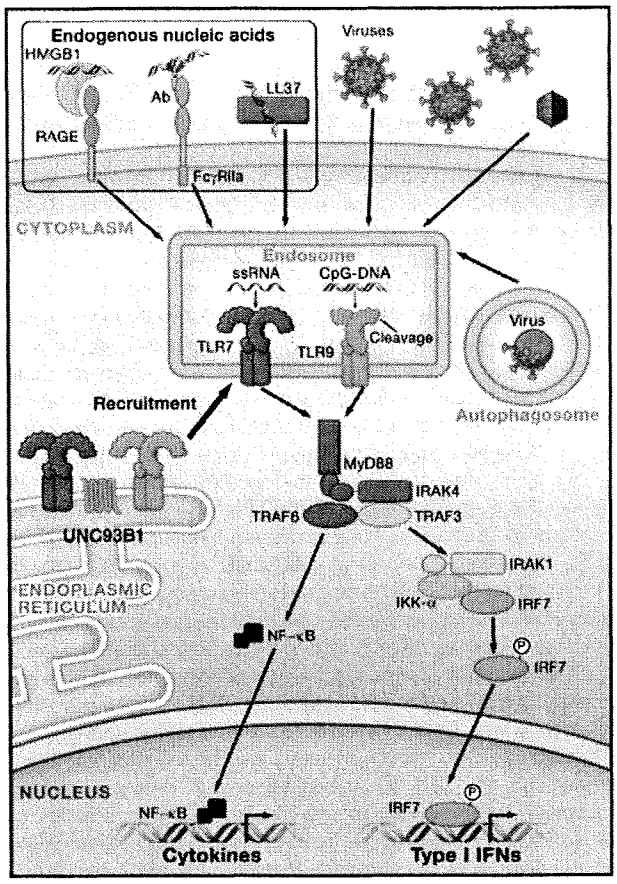

Figure 2. Nucleic Acid Sensing by TLR7 and TLR9

TLR7 and TLR9 recognize viral ssRNA and CpG DNA, respectively. Stimulation with ligands or infection by viruses induces trafficking of TLR7 and TLR9 from the ER to the endolysosome via. UNC93B1. TLR9 undergoes cleavage by proteases present in the endolysosome. A complex of MyD88, IRAK-4, TRAF6, TRAF3, IRAK-1, IKK-α, and IRF7 is recruited to the TLR. Phosphorylated IRF7 translocates into the nucleus and upregulates the expression of type I IFN genes. Viruses that have entered the cytoplasm are engulfed by autophagosomes and deliver viral nucleic acids to the endolysosome. An HMGB1-DNA complex released from damaged cells is captured by RAGE. Autoantibodies recognizing self-DNA or -RNA bind to FcγRIIa. LL37, an antimicrobial peptide, associates with endogenous DNA. These proteins are responsible for the delivery of endogenous nucleic acids to endolysosomes where they are recognized by TLR7 or TLR9.

revealed that UNC93B1 (an ER protein with 12 membrane-spanning domains) is responsible for TLR3, TLR7, and TLR9 signaling by governing the translocation of these TLRs from the ER to the endolysosome (Kim et al., 2008; Tabeta et al., 2006). When TLR9 is recruited from the ER to the endolysosome, it undergoes processing by proteases, such as cathepsins, in the endolysosome (Ewald et al., 2008; Park et al., 2008). The processed form of TLR9 is responsible for CpG-DNA recognition. It has been shown that cathepsins B, K, and L and asparagine endopeptidase are required for TLR9 responses (Asagiri et al., 2008; Matsumoto et al., 2008; Sepulveda et al., 2009). Currently, it remains unclear whether TLR7 is also cleaved in the endolysosome, although endosomal acidification is required for the sensing of TLR7 ligands.

TLR7 and TLR9 are essential for virus-induced type I IFN production by pDCs (Kato et al., 2005). Viral nucleotides can interact with TLR7 and TLR9 in pDCs after they have been endocytosed. Alternatively, once the viruses invade pDCs and virions are present in the cytoplasm, they can be delivered to the endolysosome where TLR7 and TLR9 are recruited for viral sensing. pDCs take advantage of a cellular process called autophagy in which self-proteins and damaged organelles are degraded in double-membraned vesicles called autophagosomes (Lee et al., 2007). In the absence of ATG5, a protein essential for autophagosome formation, pDCs fail to produce type I IFNs in response to virus infection, suggesting that the cytoplasmic virions are engulfed by autophagosomes and then fuse with lysosomes. However, ATG5 is also required for responses to CpG-DNA. Therefore, autophagy may control either the endosomal maturation required for CpG-DNA sensing or the TLR9 signaling pathways in pDCs, or both.

TLR-mediated microbial recognition is very important for host defense against pathogens. On the other hand, excess responses to TLR ligands induce lethal septic shock syndrome. These observations indicate that appropriate activation of TLRs is vital for eradicating invading pathogens without causing harmful damage to the host.

TLR Signaling Pathways

Recognition of PAMPs by TLRs leads to transcriptional upregulation of distinct genes, depending on the TLRs and cell types involved (Figure 1). The difference in the signaling cascades activated by the individual TLRs can be partly explained by the TIR domain-containing adaptor molecules recruited to TLRs (Akira et al., 2006). There are five TIR domain-containing adaptors including MyD88, TIR domain-containing adaptor inducing IFN-β (TRIF; also known as TICAM-1), TIRAP/Mal, TRIF-related adaptor molecule (TRAM), and Sterile-alpha and Armadillo motif-containing protein (SARM). TLR signaling is roughly divided into two distinct pathways depending on the usage of the distinct adaptor molecules, MyD88 and TRIF.

The MyD88-Dependent Signaling Pathway

MyD88 is composed of a death domain (DD) in addition to a TIR domain. MyD88 is essential for the downstream signaling of various TLRs, with the exception of TLR3. Children with MyD88 deficiency suffer from recurrent pyogenic bacterial infections. TLR2 and TLR4 signaling requires TIRAP/Mal for bridging between TLR and MyD88. MyD88 interacts with IL-1R-associated kinase (IRAK)-4, a serine/threonine kinase with an N-terminal death domain. IRAK-4 activates other IRAK family members, IRAK-1 and IRAK-2 (Kawagoe et al., 2008). The IRAKs then dissociate from MyD88 and interact with TNFR-associated factor 6 (TRAF6), which acts as an E3 ubiquitin protein ligase. Together with an E2 ubiquitin-conjugating enzyme complex comprising Ubc13 and Uev1A, TRAF6 catalyzes the formation of a lysine 63 (K63)-linked polyubiquitin chain on TRAF6 itself as well as the generation of an unconjugated free polyubiquitin chain (Xia et al., 2009). A complex of TGF-β-activated kinase 1 (TAK1), TAK1-binding protein 1 (TAB1), TAB2, and TAB3 is activated by the unconjugated free K63 polyubiquitin chain and

phosphorylates IκB kinase (IKK)-β and MAP kinase kinase 6. Subsequently, the IKK complex, composed of IKK-α, IKK-β, and NF-κB essential modulator (NEMO), phosphorylates IκBa, an NF-κB inhibitory protein. Phosphorylated IκB undergoes 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 the formation of another transcription factor complex, AP-1, that targets cytokine genes.

TLR7 and TLR9 signaling induces the production of type I IFNs in addition to other NF-κB-dependent cytokines in a MyD88-dependent manner. In pDCs, MyD88 forms a complex with IRAK-1, TRAF6, TRAF3, IKK-α, and IRF7, and phosphorylated IRF7 translocates to the nucleus to activate the expression of genes encoding type I IFNs (Figure 2). In cDCs, IRF1, but not IRF7, is activated downstream of TLR7 and TLR9, resulting in the activation of IFN-β gene expression (Negishi et al., 2006; Schmitz et al., 2007).

The TRIF-Dependent Signaling Pathway

In response to stimulation with dsRNA, TLR3 recruits another adaptor protein, TRIF. TLR4 triggers both MyD88-dependent and TRIF-dependent signaling. TLR4, but not TLR3, requires another adaptor, TRAM, for activating TRIF. A splice variant of TRAM called the TRAM adaptor with GOLD domain (TAG) acts as the negative regulator of TRIF-dependent signaling (Palsson-McDermott et al., 2009). TRIF associates with TRAF3 and TRAF6 through TRAF-binding motifs present in its N-terminal portion. TRIF also contains a C-terminal receptor-interacting protein (RIP) homotypic interaction motif (RHIM) and interacts with RIP1 and RIP3 via this motif. In humans, SARM functions as an inhibitor of TRIF-dependent signaling (Carty et al., 2006). The TNFR-associated death domain protein (TRADD), an essential adaptor for TNFR signaling, is involved in the TRIF-dependent signaling pathway (Ermolaeva et al., 2008; Pobezinskaya et al., 2008). TRADD forms a complex with FAS-associated death domain-containing protein (FADD) and RIP1, and TRADD mediates ubiquitination of RIP1, an event required for NF-κB activation. FADD activates caspase-8 or caspase-10 in response to poly I:C, and the cleaved form of caspases activates NF-κB (Takahashi et al., 2006).

TRAF3 is important for activating two IKK-related kinases, TANK-binding kinase 1 (TBK1) and IKK-*i* (also known as IKK-ε) (Hacker et al., 2006; Oganesyan et al., 2006). TRAF3 undergoes K63-linked auto-ubiquitination in response to TLR3 and acts as an E3 ubiquitin ligase. TRAF3 activation is negatively regulated by a deubiquitination enzyme DUBA (Kayagaki et al., 2007), and MyD88-dependent signaling triggers K48-linked ubiquitination of TRAF3. Proteasome-mediated degradation of TRAF3 is important for the activation of MAP kinases and the production of proinflammatory cytokines (Tseng et al., 2009). A recent study identified an E2 ubiquitin ligase, Ubc5, as a molecule required for IRF3 activation by catalyzing K63-type polyubiquitin chain formation (Zeng et al., 2009). TBK1 and IKK-*i* phosphorylate IRF3 and IRF7; IRF3 and IRF7 dimers translocate to the nucleus, resulting in induction of type I IFNs and expression of IFN-inducible genes. IKK-*i* also phosphorylates STAT1 to facilitate the induction of a set of IFN-inducible genes including *Adar1*, *Ifit3*, and *Irf7* (Tenoever et al., 2007).

The activation of TBK1 and IKK-*i* is modulated by various proteins. TBK1 and IKK-*i* interact with TRAF family member-associated NF-κB activator (TANK) (also known as I-TRAF), NAK-associated protein 1 (NAP1), and the TBK1 adaptor (SINTBAD), which is similar to NAP1 (Guo and Cheng, 2007; Ryzhakov and Randow, 2007; Sasai et al., 2006). These molecules contain a TBK1-binding motif and show similarities in their coiled-coil domains. However, $TANK^{-/-}$ cells do not show impaired type I IFN production in response to dsRNA stimulation (Kawagoe et al., 2009). Although knockdown of either NAP1 or SINTBAD impairs TRIF signaling, the relationship between these molecules in TRIF signaling is not yet fully understood.

RLRs and Virus Recognition

The RIG-I-like receptor (RLR) family is composed of RIG-I, melanoma differentiation-associated gene 5 (MDA5), and LGP2 (Takeuchi and Akira, 2009; Yoneyama and Fujita, 2008). RLRs are composed of two N-terminal caspase recruitment domains (CARDs), a central DEAD box helicase/ATPase domain, and a C-terminal regulatory domain. They are localized in the cytoplasm and recognize the genomic RNA of dsRNA viruses and dsRNA generated as the replication intermediate of ssRNA viruses. The expression of RLRs is greatly enhanced in response to type I IFN stimulation or virus infection.

Mouse fibroblasts and cDCs lacking RIG-I are defective in the production of type I IFNs and inflammatory cytokines in response to various RNA viruses (Kato et al., 2006). These include the Paramyxoviridae such as Newcastle disease virus (NDV) and Sendai virus (SeV), vesicular stomatitis virus (VSV), influenza virus, and Japanese encephalitis virus (JEV). In contrast, cells lacking MDA5 respond normally to these viruses. Meanwhile, the IFN responses to several Picornaviridae, including encephalomyocarditis virus (EMCV), Mengo virus, and Theiler's virus, are abrogated in $MDA5^{-/-}$ cells, but not in cells lacking RIG-I. In addition to JEV, another flavivirus, hepatitis C virus (HCV), is also recognized by RIG-I, whereas both RIG-I and MDA5 redundantly recognize Dengue virus and West Nile virus (Loo et al., 2008; Sumpter et al., 2005). A double-stranded segmented RNA virus, reovirus, induces IFN production mainly through MDA5. However, the absence of both RIG-I and MDA5 completely abrogates this IFN production, suggesting that both RIG-I and MDA5 are involved in the recognition of reovirus (Kato et al., 2008; Loo et al., 2008). Mouse embryonic fibroblasts (MEFs) derived from $RIG\text{-}I^{-/-}MDA5^{-/-}$ mice failed to produce type I IFNs to any of the RNA viruses tested, indicating that RIG-I and MDA5 are essential and sufficient for evoking type I IFN production in response to RNA viruses.

RIG-I recognizes relatively short dsRNA (up to 1 kb), and the presence of a 5′ triphosphate end greatly enhances its type I IFN-inducing activity (Figure 3). It has been postulated that 5′ triphosphate ssRNA synthesized by in vitro transcription is a RIG-I ligand (Hornung et al., 2006; Pichlmair et al., 2006). However, recent studies have shown that T7 polymerase commonly produces extended byproducts generating dsRNA (Schlee et al., 2009; Schmidt et al., 2009). Chemically synthesized 5′ triphosphate ssRNA failed to stimulate RIG-I, indicating that RNA needs to be double-stranded for activation of RIG-I. Regarding a minimum length, the 19-mer or 21-mer dsRNA

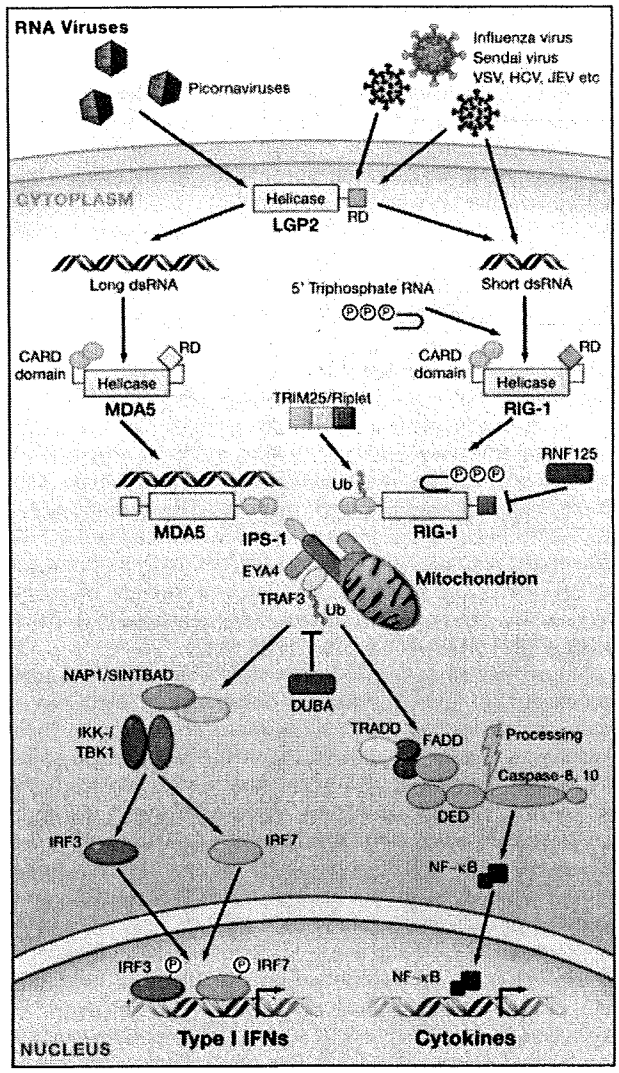

Figure 3. Recognition of RNA Viruses by RLRs

RIG-I and MDA5 recognize different RNA viruses by detecting short dsRNAs with 5′ triphosphate ends and long dsRNAs, respectively. LGP2 functions as a positive regulator in RIG-I-mediated and MDA5-mediated virus recognition. Activation of RIG-I is positively and negatively regulated by ubiquitin ligases TRIM25 and RNF125, respectively. RIG-I and MDA5 interact with IPS-1 through homophilic interactions between CARD domains. IPS-1 then activates signaling cascades leading to the expression of type I IFN genes via EYA4, TRAF3, NAP1/SINTBAD, TBK1/IKK-*i*, and IRF3/7. RLR signaling mediates polyubiquitination of TRAF3, which is removed by a deubiquitinase, DUBA. Simultaneously, IPS-1 signaling induces nuclear translocation of NF-κB via TRADD and FADD and caspase-8/-10. A cleaved fragment of caspase-8/-10 is responsible for the activation of NF-κB.

with a 5′ triphosphate end is able to potently induce type I IFNs. In contrast, a 5′ triphosphate end is not always necessary, as chemically synthesized dsRNAs with a 5′ monophosphate end or those without a 5′ phosphate can potently activate RIG-I (Takahasi et al., 2008; Kato et al., 2008). The amount of IFNs produced by these RNAs is low compared to dsRNA with a 5′ triphosphate end. HCV genomic RNA has been screened for RNA sequences that activate RIG-I. Poly(U)- or poly(A)-rich sequences from the HCV RNA 3′ untranslated region (UTR) are responsible for RIG-I-mediated IFN production, based on RNAs generated by in vitro transcription using T7 polymerase (Saito et al., 2008). However, in vitro-transcribed RNAs with low U/A content also potently activate RIG-I. Therefore, further studies are required to clarify whether particular RNA sequences are important for RIG-I-mediated recognition.

VSV produces dsRNA in infected cells (Kato et al., 2008). Disruption of dsRNA among RNAs from VSV-infected cells reduces the IFN-β-inducing activity, suggesting that the presence of dsRNA in VSV-infected cells is important for recognition by RIG-I. Interestingly, the dsRNA fragments produced by VSV infection are about 2.0–2.5 kb, and much shorter than the size of the VSV genomic RNA. It has been reported that defective interfering (DI) particles are generated in VSV-infected cells, and that the sizes of DI particle dsRNAs are about 2.2 kb (Pattnaik et al., 1995). Thus, the dsRNA generated during the course of VSV replication may be derived from DI particles, although further studies are needed to clarify the source of this dsRNA. As DI particles are known to strongly induce type I IFNs, RIG-I may play a role in detecting the presence of dsRNA in DI particles. In contrast, it is difficult to detect dsRNA in cells infected with influenza virus. Genomic RNA from influenza virus lost its IFN-inducing activity after a phosphatase treatment that removed the 5′ triphosphate end. The sequences of the 5′ and 3′ ends of the viral RNA are partially complementary to each other, and it has been suggested that a panhandle structure can form (Hsu et al., 1987). Thus, it is tempting to speculate that short panhandle dsRNA with a 5′ triphosphate is responsible for recognition by RIG-I.

In contrast to RIG-I, MDA5 detects long dsRNA (more than 2 kb) such as poly I:C. *MDA5*$^{-/-}$ mice show severely reduced production of type I IFNs in response to poly I:C inoculation in vivo, whereas their production of IL-12p40 is not impaired (Kato et al., 2006). Shortening the length of the poly I:C by treatment with a dsRNA-specific nuclease converts poly I:C from an MDA5 ligand to a RIG-I ligand, indicating that long, but not short, dsRNA is recognized by MDA5. EMCV produces high levels of dsRNA in infected cells, and the 5′ end of its genomic RNA is covalently linked to a small protein, VPg. Investigation of RNA from EMCV-stimulated cells has revealed that higher-order structure RNA containing both dsRNA and ssRNA, but not simple dsRNA as the replication intermediate, has MDA5-stimulating activity (Pichlmair et al., 2009).

LGP2, the third member of the RLR family, lacks a CARD, and in vitro studies have suggested that LGP2 functions as a negative regulator of RIG-I and MDA5 responses by sequestering dsRNA or inhibiting RIG-I conformational changes (Rothenfusser et al., 2005; Saito et al., 2007; Yoneyama et al., 2005). However, the generation of *LGP2*$^{-/-}$ mice and mice with a point mutation, D30A, that disrupts the ATPase activity of LGP2 revealed that LGP2 positively regulates production of type I IFNs in response to RNA viruses recognized by both RIG-I and MDA5 (Satoh et al., 2010). Nevertheless, LGP2 is dispensable for type I IFN production following transfection by synthetic RNAs. These results suggest that LPS2 may modify viral RNA by removing

proteins from viral ribonucleoprotein (RNP) complexes or unwinding complex RNA structures to facilitate MDA5-mediated and RIG-I-mediated recognition of dsRNA.

RLRs contain a C-terminal regulatory domain, which is responsible for the binding to dsRNAs. The recent solution of RLR C-terminal regulatory domain structures revealed that the C-terminal domains of LGP2 and RIG-I have a large basic surface forming an RNA-binding loop (Cui et al., 2008; Takahasi et al., 2008, 2009). The RIG-I and LGP2 C-terminal domains bind to the termini of dsRNA. Although the MDA5 C-terminal domain also has a large basic surface, it is extensively flat because of the open conformation of the RNA-binding loop. Therefore, the RNA-binding activity of MDA5 is much weaker than that of RIG-I and LGP2. RLRs catalyze ATP via the DExD/H helicase domain, and the ATPase activity is essential for RLRs to induce type I IFN production. Although RIG-I has helicase activity, it is not clear if the unwinding of dsRNA by RIG-I is required for triggering the signaling pathway. RIG-I might change its conformation to expose the CARDs and dimerize by catalyzing ATP, or alternatively, RIG-I may act as a translocase for dsRNA.

The RLR Signaling Pathways

The RIG-I conformation is known to be modulated by ubiquitination. TRIM25 and Riplet (also known as RNF135) act as E3 ubiquitin ligases that mediate the K63-linked polyubiquitination of RIG-I (Gack et al., 2007; Pichlmair et al., 2009). This modification is required for the activation of RLR signaling. On the other hand, K48-type polyubiquitination of RIG-I by RNF125 leads to RIG-I degradation by the proteasome and inhibition of RIG-I signaling (Arimoto et al., 2007).

The CARDs of RLRs are responsible for triggering signaling cascades by interacting with the N-terminal CARD-containing adaptor IFN-β-promoter stimulator 1 (IPS-1) (also known as MAVS, CARDIF, or VISA) (Kawai and Akira, 2006) (Figure 3). IPS-1 is localized on the mitochondrial membrane, and cleavage of IPS-1 by an HCV NS3/4A protease, which dislodges it from the mitochondrial membrane, results in abrogation of RLR signaling. NLRX1 (also known as NOD9), an NLR family member, is localized on the mitochondrial membrane and acts as an inhibitor of IPS-1 signaling (Moore et al., 2008). However, another report showed that NLRX1 is a mitochondrial matrix protein responsible for the generation of reactive oxygen species. Therefore, further studies are required to clarify the role of NLRX1 in RLR signaling (Arnoult et al., 2009). IPS-1 activates TRAF3 and TRADD (Michallet et al., 2008). The downstream signaling molecules for the expression of IFN-inducible genes are shared between the IPS-1 and TRIF signaling pathways. The recently identified protein Eyes absent 4 (EYA4) associates with IPS-1 and NLRX1 and stimulates the expression of IFN-inducible genes in response to DNA stimulation (Okabe et al., 2009). EYA4 is a phosphatase for both phosphotyrosine and phosphothreonine, and the threonine phosphatase activity is essential for enhancing IFN-β gene activation. Identification of molecules that are dephosphorylated by EYA4 will be important for understanding the mechanism for how EYA4 regulates IFN responses.

In contrast to pDCs, autophagy negatively regulates IFN responses in fibroblasts and cDCs by suppressing RLR signaling (Jounai et al., 2007; Tal et al., 2009). In the absence of autophagy-related proteins, such as ATG5 or ATG16L1, damaged mitochondria accumulate together with IPS-1, and reactive oxygen species associated with dysfunctional mitochondria are responsible for the overproduction of type I IFNs.

Recognition of Cytoplasmic DNA

Although DNA with a CpG motif is sensed by TLR9, introduction of dsDNA into cells evokes type I IFN responses in a TLR9-independent manner. Right-handed spiral B-DNA, but not left-handed Z-DNA, activates these responses (Ishii et al., 2006). Although the sequence specificity has not been clearly observed, synthetic poly (dA:dT) is more potent for induction of IFN responses than poly (dI:dC) or poly (dG:dC). Transfection of dsDNA leads to the activation of IRF3 via TBK1 and IKK-*i*, but activation of NF-κB is barely detected in response to DNA. It is believed that cytoplasmic DNA recognition is important for the production of type I IFNs to infection with DNA viruses. Infection with intracellular bacteria such as *Listeria monocytogenes* and *Legionella pneumophila* induces type I IFN production in response to bacterial DNA in the cytoplasm (Stetson and Medzhitov, 2006). In addition, the adjuvant effect of DNA vaccines can be explained by TLR9-independent cytoplasmic recognition of DNAs that activates the TBK1-IRF3-dependent pathway (Ishii et al., 2008).

Two studies have shown that poly (dA:dT) can be transcribed into dsRNA by polymerase III and that dsRNA is recognized in a RIG-I-IPS-1-dependent manner in human cells or transformed mouse MEFs (Figure 4) (Ablasser et al., 2009; Chiu et al., 2009; Choi et al., 2009). However, primary DCs prepared from RIG-I-deficient mice do not show a defect in IFN production, suggesting that cytoplasmic DNAs are sensed in both a polymerase III-RIG-I-dependent and -independent manner depending on the species or cell types involved. A cytoplasmic DNA-binding protein, DNA-dependent activator of IRF (DAI, also known as Zbp1 or DLM1), interacts with TBK1 and activates type I IFN responses (Takaoka et al., 2007). Nevertheless, *Zbp1*$^{-/-}$ MEFs are still capable of producing type I IFNs in response to cytoplasmic DNA, suggesting that cytoplasmic DNA is redundantly recognized by as-yet unidentified receptors (Ishii et al., 2008).

Expression cloning of a gene inducing IFN-β promoter activation identified STING (also known as MITA, ERIS, or TMEM173), a transmembrane protein expressed in the ER (Ishikawa and Barber, 2008; Sun et al., 2009; Zhong et al., 2008). Cells and mice lacking *STING* show impaired IFN production in response to both RNA and DNA stimulation. STING associates with a member of the translocon-associated protein (TRAP) complex that is required for protein translocation across the ER in resting cells. In response to transfection of dsDNA, STING translocates from the ER to the Golgi apparatus and subsequently to cytoplasmic punctate structures where it colocalizes with TBK1 (Ishikawa et al., 2009). The exocyst complex is involved in secretory vesicle sorting and is especially required for post-Golgi transport to the plasma membrane. Sec5, a component of the exocyst complex, colocalizes with STING. Activation of the RalB GTPase promotes the assembly of TBK1 and Sec5, leading to phosphorylation of Sec5 by TBK1. *STING*$^{-/-}$ mice are highly susceptible to HSV-1 or VSV infection, indicating a role for STING in host defense against virus infection. Interestingly,

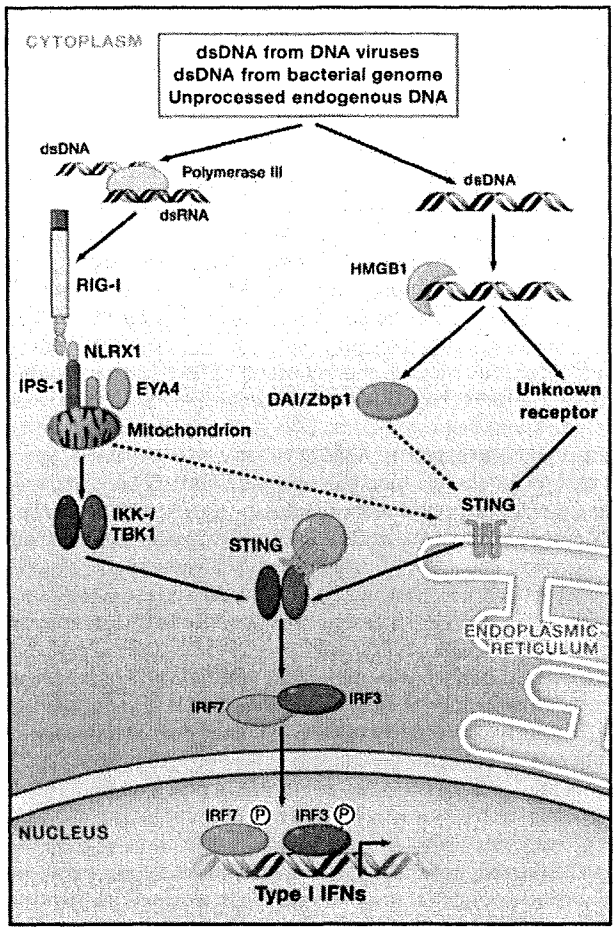

Figure 4. Recognition of Cytoplasmic DNA and IFN Production
Double-stranded DNA (dsDNA) accumulates in the cytoplasm after infection by viruses or bacteria or through failure to process endogenous DNA. The intracellular DNA is captured by HMGB1 and recognized by an unidentified cytoplasmic receptor or DAI. Alternatively, dsDNA is transcribed into dsRNA by polymerase III in a species- and cell type-specific manner. Generated dsRNA is recognized by RIG-I and production of type I IFNs is induced. Following DNA stimulation, an ER protein STING translocates from the ER to the cytoplasmic punctate structure, where STING colocalizes with TBK1.

autophagy-related gene 9a (ATG9a) colocalizes with STING (Saitoh et al., 2009). Loss of ATG9a, but not another autophagy-related gene (ATG7), greatly enhances the assembly of STING with TBK1 in response to dsDNA, followed by increased production of type I IFNs. Notably, the introduction of dsRNA did not induce the translocation of STING; it will be interesting to explore how STING regulates the IFN response to dsRNA. It has been shown that TBK1 activation by RalB is involved in cell transformation and tumor progression (Chien et al., 2006). Furthermore, a recent study revealed that TBK1 is required for KRAS-driven cancer formation, probably by activating the NF-κB-induced antiapoptotic gene *BCL-xL* (Barbie et al., 2009). Thus, TBK1 is a pleiotropic kinase involved in both innate immunity and tumorigenesis.

High-mobility group box (HMGB) proteins were originally identified as nuclear proteins with DNA-binding capacity. HMGB1 is also known to be secreted in response to cell damage and evokes inflammatory responses. Recently, HMGB proteins present in the cytoplasm were found to act as the initial sensors for cytoplasmic nucleic acids that lead to the activation of downstream receptors such as RLRs, TLRs, and unknown DNA receptors (Yanai et al., 2009).

Although stimulation with TLR ligands alone does not lead to activation of the inflammasome, the introduction of dsDNA induces not only the production of pro-IL-β but also its cleavage via caspase-1. Sensing of cytoplasmic DNA by absent-in-melanoma 2 (AIM2) activates the inflammasome via ASC and caspase-1, leading to the production of IL-1β (see Review by K. Schroder and J. Tschopp on page 821).

NLR- and CLR-Mediated Pathogen Recognition

The NLR family consists of cytoplasmic pathogen sensors that are composed of a central nucleotide-binding domain and C-terminal leucine-rich repeats (Inohara et al., 2005). The N-terminal portions of most NLRs harbor protein-binding motifs, such as CARDs, a pyrin domain, and a baculovirus inhibitor of apoptosis protein repeat (BIR) domain. NLRs harboring a pyrin domain or a BIR domain in their N terminus are not involved in the transcriptional activation of inflammatory mediators and are components of the inflammasome that regulates caspase-1 activation. NOD1 and NOD2, which harbor CARDs in addition to NOD and LRR domains, activate NF-κB via an adaptor, RIP2/RICK. NOD1 and NOD2 induce transcriptional upregulation of proinflammatory cytokine genes. NOD1 and NOD2 recognize the structures of bacterial peptidoglycans, g-D-glutamyl-meso-diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP), respectively. As TLRs also recognize bacterial peptidoglycan components, TLRs and NODs synergistically activate proinflammatory cytokine production. In addition to bacteria, a recent study found that the expression of NOD2 is involved in 5′-triphosphate RNA-induced type I IFN production and host defense against respiratory syncytial virus infection (Sabbah et al., 2009).

CLRs comprise a transmembrane receptor family characterized by the presence of a carbohydrate-binding domain. CLRs recognize carbohydrates on microorganisms such as viruses, bacteria, and fungi. CLRs either stimulate the production of proinflammatory cytokines or inhibit TLR-mediated immune complexes. The functions of CLRs are described in detail elsewhere (Geijtenbeek and Gringhuis, 2009), and here we give just a few examples of CLR-mediated microbial recognition. Dectin-1 and dectin-2 are immunoreceptor tyrosine-based activation motif (ITAM)-coupled CLRs responsible for sensing β-glucans from fungi. DCs activated by dectin-1 or dectin-2 are able to instruct T cells to confer protective immunity against *Candida albicans* (Robinson et al., 2009). The macrophage C-type lectin MINCLE (also known as Clec4e and Clecsf9) senses infection by fungi such as Malassezia and Candida (Yamasaki et al., 2009). In addition, MINCLE is responsible for the detection of an endogenous protein, spliceosome-associated protein 130 (SAP130), which is a component of U2 snRNP from necrotic host cells (Yamasaki et al., 2008). CLEC9A is

expressed on $CD8^+$ DCs in the spleen and also recognizes necrotic cells for cross-priming. CLRs activate intracellular signaling either via the ITAM domain of CLRs or via adaptors harboring an ITAM domain, such as FcRγ, DAP10, or DAP12. The Syk tyrosine kinase is activated by ITAM-containing proteins either directly or indirectly via ITAM-containing adaptors. Engagement of Syk with CLRs activates MAP kinases, the transcription factor NF-AT, and NF-κB through CARD9. As a result, proinflammatory cytokines are produced. However, the signaling mechanism for the production of proinflammatory cytokines is not well understood.

Transcriptional Regulation of Inflammatory Mediators

Activation of PRR signaling pathways leads to the nuclear translocation of a set of transcription factors, including NF-κB, AP-1, IRFs, and C/EBPβ. These factors cooperatively regulate the transcription of their target genes. Furthermore, remodeling of chromatin is important for controlling the transcriptional regulation of a set of TLR-inducible genes (Ramirez-Carrozzi et al., 2006). In addition, epigenetic controls of a set of TLR-inducible genes are critical for induction of tolerance to LPS, a well-known phenomenon rendering cells refractory to a second exposure to LPS (Foster et al., 2007) (see Review by S.T. Smale on page 833 of this issue). Here, we focus on the TLR-inducible proteins that modulate inflammatory responses. Besides cytokines, chemokines, and IFNs, TLR stimulation upregulates the expression of hundreds of genes in macrophages. These genes are potentially involved in antimicrobial defense (e.g., defensins, lipocaline), metabolic changes, and tissue repair (e.g., secretory leukocyte peptidase inhibitor). Nevertheless, the functions of various TLR-inducible genes remain largely unexplored. Some gene products are associated with the positive and negative regulation of inflammatory responses by controlling TLR-signaling pathways. The genes immediately induced by TLR ligands potentially regulate TLR signaling or responses after their expression.

It is well known that the components of NF-κB are TLR-induced genes that undergo positive feedback regulation by NF-κB (Figure 5). In addition, IκBζ and activating transcription factor 3 (ATF3) are nuclear factors that are rapidly induced by the TLRs (Gilchrist et al., 2006; Yamamoto et al., 2004). IκBζ harbors C-terminal ankyrin repeat motifs that bind to the NF-κBp50 subunit, and expressed IκBζ positively regulates the induction of a set of genes including IL-6 and IL-12p40, which are transcribed later. In contrast, ATF3 negatively regulates the transcription of genes encoding IL-6 and IL-12p40 by increasing histone deacetylase activity. Epigenetic changes can be regulated by TLR-inducible proteins. Trimethyl-histone 3 lysine 27 demethylase, Jmjd3, is expressed in response to TLR stimulation via NF-κB and is recruited to the transcription start sites of LPS-inducible genes (De Santa et al., 2007). Given that Jmjd3 is recruited to the transcription start site of various LPS-inducible genes, it may be responsible for the fine-tuning of LPS-induced gene expression in macrophages (De Santa et al., 2009).

In response to TLR ligand stimulation, genes encoding RNA-binding proteins with a CCCH-type zinc finger motif (Zf) are rapidly induced. These proteins include Zc3h12a, Zc3h12c, Zc3hav1, and tristetraprolin (TTP; also known as Zfp36)

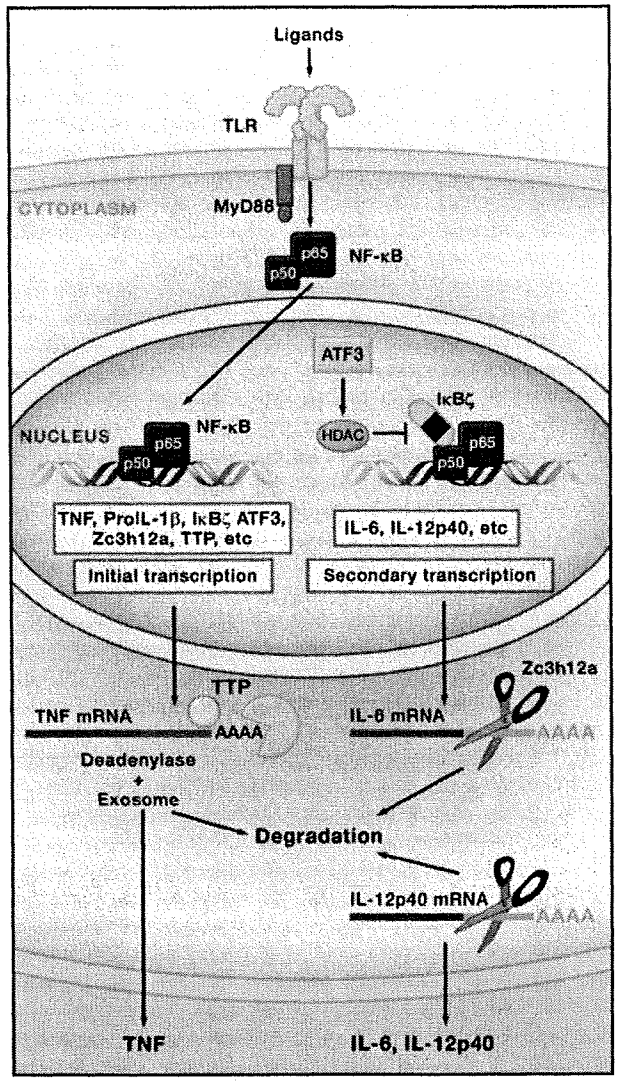

Figure 5. TLR-Inducible Proteins Regulate Inflammation

TLR signaling initially induces genes encoding TNF, Pro-IL-1β, IκBζ, ATF3, Zc3h12a, TTP, and so on. Proteins generated from these genes modulate inflammatory protein expression by various mechanisms. IκBζ positively regulates transcription of genes including IL-6 and IL-12p40 by associating with NF-κBp50. In contrast, ATF3 negatively regulates transcription of these genes by activating HDAC, which suppresses gene expression by epigenetic regulation. Zc3h12a acts as an RNase degrading IL-6 and IL-12p40 mRNA, whereas TTP binds to TNF mRNA and recruits a deadenylase, which induces its degradation through an exosome.

(Matsushita et al., 2009). TTP harbors two CCCH-type Zf domains and associates with mRNA via AU-rich elements present in the 3'UTR, leading to removal of the poly(A) tail by recruitment of a deadenylase (Carrick et al., 2004). The deadenylated mRNAs traffic to exosomes where the RNA is degraded. TTP is required to prevent the generation of autoimmune arthritis in mice through controlling mRNAs encoding TNF. Zc3h12a and Zc3h12c are composed of a CCCH-type Zf domain and an

RNase domain (Matsushita et al., 2009). Zc3h12a is responsible for degrading mRNAs for TLR-inducible proteins such as IL-6 and IL-12p40 in macrophages. Zc3h12a controls its target mRNAs via the 3′UTR independently of AU-rich elements; the RNase activity is essential for mRNA degradation. *Zc3h12a*$^{-/-}$ mice spontaneously develop a fatal inflammatory disease characterized by highly elevated immunoglobulin levels and autoantibody production. RLR signaling induces another gene encoding a CCCH-type Zf protein, ZAP (also known as Zc3hav1). ZAP contains four clusters of CCCH-type Zf domains and directly binds to viral RNAs as well as exosomes where viral RNA is degraded (Gao et al., 2002). ZAP overexpression is reported to confer resistance against various viruses such as retroviruses and alphaviruses. Zcchc11 is a CCHC-type Zf domain-containing RNA-binding protein with a nucleotidyltransferase domain. A recent study found that Zcchc11 adds terminal uridines to miR-26 family microRNAs, which target IL-6 mRNA (Jones et al., 2009). Uridylated miR-26 fails to repress IL-6 mRNA, and Zcchc11 thereby potentiates IL-6 production in response to TNF stimulation. It was reported that a set of mRNAs rapidly expressed in response to TNF stimulation are critically controlled through mRNA stability. These mRNAs tend to have abundant AU-rich elements in their 3′UTRs compared with mRNAs expressed at later time points (Hao and Baltimore, 2009). Therefore, control of mRNA decay may be as important as control of transcription in terms of the regulation of innate immune responses.

TLR signaling induces the expression not only of protein-coding mRNAs but also of noncoding RNAs (Guttman et al., 2009). Some of the noncoding RNAs produce microRNAs including miR-146a/b, miR-147, and miR-155 (Taganov et al., 2006). These microRNAs interact with their target mRNAs and fine-tune their expression to modulate the inflammatory response. One of the best characterized microRNAs is miR-155, which is rapidly induced in response to TLR ligands and modulates innate and adaptive immune responses by modulating the expression of multiple target mRNAs encoding PU.1, IKK-*i*, SHIP1, and so on (Faraoni et al., 2009). A recent report shows that miR-21 negatively regulates TLR4 signaling by the tumor suppressor PDCD4, a protein required for NF-κB activation (Sheedy et al., 2009).

PRR-Dependent Recognition of Self-Nucleotides in Autoimmunity

Under physiological conditions, PRRs strictly discriminate self and microbial components to prevent the development of autoimmune disease. A hallmark of autoimmune disease is the production of antibodies recognizing self-antigens. In human autoimmune diseases such as systemic lupus erythematosus (SLE), immune complexes are formed by autoantibodies and deposited in the kidneys, causing glomerular nephritis. It has been shown that serum type I IFN levels in SLE patients positively correlate with disease severity.

TLRs, especially TLR7 and TLR9, have been implicated in the production of autoantibodies in various mouse models. Self-nucleic acids released from damaged cells are rapidly degraded by serum nucleases and do not encounter TLR7 or TLR9. However, when self-nucleic acids interact with cellular proteins such as HMGB1, RNPs, antimicrobial peptides, and autoantibodies, endocytosis of the resulting nucleic acid-protein complexes is facilitated and induces TLR7- and TLR9-mediated type I IFN production (Figure 2). For instance, HMGB1 interacts with the receptor for advanced glycation end-products (RAGE) on the cell surface, and HMGB1-DNA-containing immune complexes released from damaged cells stimulate TLR9 and RAGE, which cooperate to stimulate pDCs and B cells (Tian et al., 2007). Endogenous RNA-protein complexes, such as U1 snRNP, can activate autoreactive B cells and DCs via TLR7 (Vollmer et al., 2005). Furthermore, nucleic acids bound to autoantibodies are endocytosed through B cell receptors and FcγRIIA in B cells and DCs (Means et al., 2005; Viglianti et al., 2003). Recognition of immune complexes by these receptors leads to the activation of autoreactive B cells as well as the production of type I IFNs, thus exacerbating the causes of autoimmune diseases. Similarly, the cationic antimicrobial peptide LL37 forms a complex with self-DNA or self-RNA, thereby facilitating endocytosis by pDCs and stimulating TLR-mediated IFN responses (Ganguly et al., 2009; Lande et al., 2007). LL37 is highly expressed in psoriasis skin lesions, and the production of IFNs contributes to the pathogenesis of this disease. Taken together these data show that the responses of nucleic acid-sensing TLRs are positively regulated by endogenous proteins that facilitate endocytosis of nucleic acids. Type I IFNs and cytokines produced by the TLRs cause inflammation, and this positive feedback mechanism is involved in the exacerbation of autoimmune diseases.

The contributions of nucleic acid-sensing TLRs to autoimmunity have been examined using mouse models that develop autoimmune disease spontaneously. Duplication of the *Tlr7* gene accounts for the autoimmune phenotypes associated with Y chromosome-linked autoimmune accelerator (Yaa) mice (Pisitkun et al., 2006; Subramanian et al., 2006). Reciprocally, TLR7 deficiency in lupus-prone MRL *lpr/lpr* mice results in reduced levels of autoantibodies recognizing RNA-containing antigens and abrogation of disease (Christensen et al., 2006). But the relationship between TLR9 and autoimmune disease mouse models is complicated. Although CpG-DNA has been used as an adjuvant to generate organ-specific autoimmune disease in mouse models, the lack of TLR9 exacerbated the severity of disease in the MRL *lpr/lpr* murine lupus model. Although the TLR7 and TLR9 signaling pathways are indistinguishable, there is a clear difference in the roles of TLR7 and TLR9 in the establishment of systemic autoimmune disease. A mutation of amino acid D34 in UNC93B1 that changes its ability to bind to TLR7 and TLR9 has been identified (Fukui et al., 2009). The mutant UNC93B1 increases TLR7 responses but causes hyporesponsiveness to TLR9. Given that TLR7 is important for the generation of autoimmune disease models in mice, it is possible that UNC93B1 suppresses RNA sensing by skewing the response toward DNA.

Appropriate clearance of nucleic acids is essential for preventing autoimmune disease. DNase II is located in the lysosomes of macrophages and is required for the degradation of DNA from apoptotic cells. In the absence of DNase II, undigested DNA accumulates in macrophages, and the resulting production of IFN-β and TNF causes embryonic lethality (Kawane et al.,

2003). The type I IFNs are produced independently of TLR9, suggesting that recognition of DNA by intracellular receptors is responsible for the embryonic lethality of DNase II-deficient mice (Okabe et al., 2005). Similarly, deficiency in DNase I, a major DNase present in serum, results in the generation of SLE-like autoimmune disease in mice; a mutation in DNase I has been identified in human SLE patients (Lee-Kirsch et al., 2007). Aicardi-Goutières syndrome (AGS) is a human disorder characterized by fatal encephalopathy owing to overproduction of IFN-α. Recent studies have revealed that AGS is caused by a mutation in 3′ repair exonuclease 1 (Trex1) (Crow et al., 2006). It has been shown that DNA derived from endogenous retroelements accumulates in *Trex1*$^{-/-}$ cells and stimulates the induction of IFN-inducible genes (Stetson et al., 2008). In addition, mutations in components of RNase H2 are also involved in AGS (Rice et al., 2009). Nucleic acid accumulation because of a deficiency in these nucleases probably leads to recognition of the nucleic acids by cytoplasmic PRRs resulting in the production of type I IFNs. Interestingly, mutations in MDA5 that disrupt its signaling ability correlate with resistance to type I diabetes, although the mechanism for the contribution of MDA5 to this disease remains unclear (Nejentsev et al., 2009). Future studies will clarify how cytoplasmic PRRs contribute to the generation of autoimmune disease.

PRR Activation in Acute and Chronic Inflammation

PRRs recognizing non-nucleic acid ligands are also involved in various inflammatory and autoimmune diseases. It is well known that activation of TLR signaling by bacterial components leads to acute inflammation, culminating in septic shock in some cases. TLR ligands also are commonly used as adjuvants to generate organ-specific autoimmune disease models in mice for arthritis or encephalitis, for example.

A mutation in NOD2 that generates a truncated form of the protein is frequently found in patients with Crohn's disease, an inflammatory bowel disorder (Cho, 2008). This disease may arise through impairment of proper responses to intestinal bacteria and aberrant bacterial growth causing increased inflammatory responses in the intestine. Mutations in the autophagy-related gene ATG16L1 are also implicated in susceptibility to Crohn's disease. In the absence of ATG16L1 in mice, overactivation of the inflammasome occurs, and increased production of IL-1β and IL-18 contributes to inflammation in the intestine (Saitoh et al., 2008). Furthermore, ATG16L1 is critical for the normal function of intestinal Paneth cells (Cadwell et al., 2008).

Lack of negative regulators for TLRs can cause autoimmune disease based on various mouse models. Loss of the protein tyrosine phosphatase SHP1 in mice induced by N-ethyl-N-nitrosourea (ENU) mutagenesis leads to the generation of inflammatory lesions together with overactivation of macrophages in response to TLR stimulation (Croker et al., 2008). The inflammation is suppressed by loss of MyD88, suggesting that microbe-induced hyperactivation of TLR signaling is responsible for the generation of this inflammatory disease. A20 is a protein with dual enzyme domains, comprising an E3 ubiquitin ligase and a deubiquitinase that removes K63-linked polyubiquitin chains. A20 negatively regulates NF-κB activation downstream of TLR2 and NOD1/NOD2 (Boone et al., 2004; Hitotsumatsu et al., 2008). In the absence of A20, mice develop multiorgan inflammatory disorders that lead to premature death. Another example of a TLR negative regulator is TANK. Generation of mice lacking TANK revealed that TANK is critical for controlling TLR signaling in macrophages as well as antigen receptor signaling in B cells, by inhibiting the activation of TRAF6 (Kawagoe et al., 2009). *TANK*$^{-/-}$ mice spontaneously develop autoimmune glomerular nephritis depending on the presence of MyD88 or IL-6. These results suggest that aberrant activation of innate immune cells and B cells is responsible for the generation of autoimmune diseases resembling human SLE. These studies indicate that negative regulation of PRRs, especially TLR signaling, is important for coordinated innate immune responses.

It is well known that TLR-dependent inflammation contributes significantly to the pathogenesis of ischemia-reperfusion myocardial injury. Mice lacking TLR2, TLR4, or MyD88 show reduced infarcted regions in response to cardiac as well as cerebral ischemic-reperfusion injury (Arumugam et al., 2009). In contrast, pretreatment of mice with TLR ligands such as LPS induces tolerance and reduces the infarct size after ischemic-reperfusion injury. The effects of TLR agonists may be explained by tolerance induction through initial stimulation of TLRs. TLRs are similarly involved in the pathogenesis of cerebral ischemia-reperfusion injury models. Other extensive studies have shown that TLR2 and TLR4 are required for the production of atherosclerotic lesions in mice with hyperlipidemia.

Given that TLR2 and TLR4 are clearly responsible for the severity of inflammation induced by nonmicrobial agents, it has been postulated that endogenous molecules directly activate these TLRs. It has been shown that HMGB1 released from necrotic cells stimulates TLR2 and TLR4 to induce the production of proinflammatory cytokines (Lotze and Tracey, 2005). Furthermore, heat shock proteins (HSPs) such as HSP60, HSP70, gp96, and HSP22 are also reported to be recognized by TLR2 and TLR4 (Asea et al., 2002; Ohashi et al., 2000; Roelofs et al., 2006; Warger et al., 2006). However, most studies describing endogenous protein ligands for TLR2 and TLR4 used recombinant proteins generated in an *Escherichia coli* expression system. We need to interpret the data carefully as it is very difficult to completely eliminate the possibility of contamination by *E. coli* components that stimulate TLRs. Nevertheless, it is apparent that these TLRs contribute to inflammatory diseases caused by nonmicrobial agents. Further studies will uncover how these agents activate TLR-induced inflammation.

Future Perspectives

In this Review, we have discussed how the sensing of pathogens and cellular components by PRRs triggers inflammation and its consequences. The cellular mechanisms for individual PRRs to induce pleiotropic outcomes are complex, and we are far from predicting the entire immune response, which is mediated by crosstalk among the various PRRs. Furthermore, we do not know much about the dynamic regulation of immune cell activation or behavior. Investigations into how inflammation is physiologically controlled to cause appropriate outcomes are in their infancy. We believe that one direction for future research is to define the dynamics of immune cell activation and behavior

in vivo by using imaging techniques, as immune cell activation is differentially regulated depending on the cell type involved. A second important approach is to integrate accumulating knowledge using a systems biology strategy. Indeed, there have been several attempts to incorporate systems biology into immunology research. Using a systems biology approach, Aderem and colleagues identified ATF3 and C/EBPγ as a suppressor and amplifier of TLR-mediated gene expression, respectively (Gilchrist et al., 2006; Litvak et al., 2009). There have also been attempts to comprehensively understand the transcription networks activated in response to PRR stimulation in DCs. Recently, Regev and coauthors selected 125 transcription regulators involved in TLR-mediated gene expression based on gene expression profiles and constructed a network model by knocking down each of them in turn (Amit et al., 2009). However, multiple PRRs are activated simultaneously in the course of microbial infection. Thus, the dynamic changes in transcriptional networks activated in response to inflammatory stimuli are likely to be highly complex. In the future, the merging of imaging, systems biology, and immunology will uncover the dynamics of PRR-mediated inflammatory responses and their role in autoimmune disease.

ACKNOWLEDGMENTS

We thank D.M. Standley for critical reading of this manuscript and E. Kamada for secretarial assistance. This work was supported by the Special Coordination Funds of the Japanese Ministry of Education, Culture, Sports, Science and Technology, the 21st Century Center of Excellence Program of Japan, and the NIH (P01 AI070167).

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

Tatsukata Kawagoe[1,2,6], Osamu Takeuchi[1,2,6], Yoshitsugu Takabatake[3], Hiroki Kato[1,2], Yoshitaka Isaka[4], Tohru Tsujimura[5] & Shizuo Akira[1,2]

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-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 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-κB modulator NEMO (also called IKKγ). The kinase TAK1 is also recruited to TRAF6 and phosphorylates the kinases IKKβ and MEKK6 (ref. 8). Subsequently, the inhibitor of NF-κB (IκB) kinase (IKK) complex, composed of IKKα, IKKβ 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 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].

[1]Laboratory of Host Defense, World Premier International Immunology Frontier Research Center, and [2]Research Institute for Microbial Diseases, Osaka University, Osaka, Japan. [3]Department of Nephrology and [4]Department of Advanced Technology for Transplantation, Osaka University Graduate School of Medicine, Osaka, Japan. [5]Department of Pathology, Hyogo College of Medicine, Hyogo, Japan. [6]These authors contributed equally to this work. Correspondence should be addressed to S.A. (sakira@biken.osaka-u.ac.jp).

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

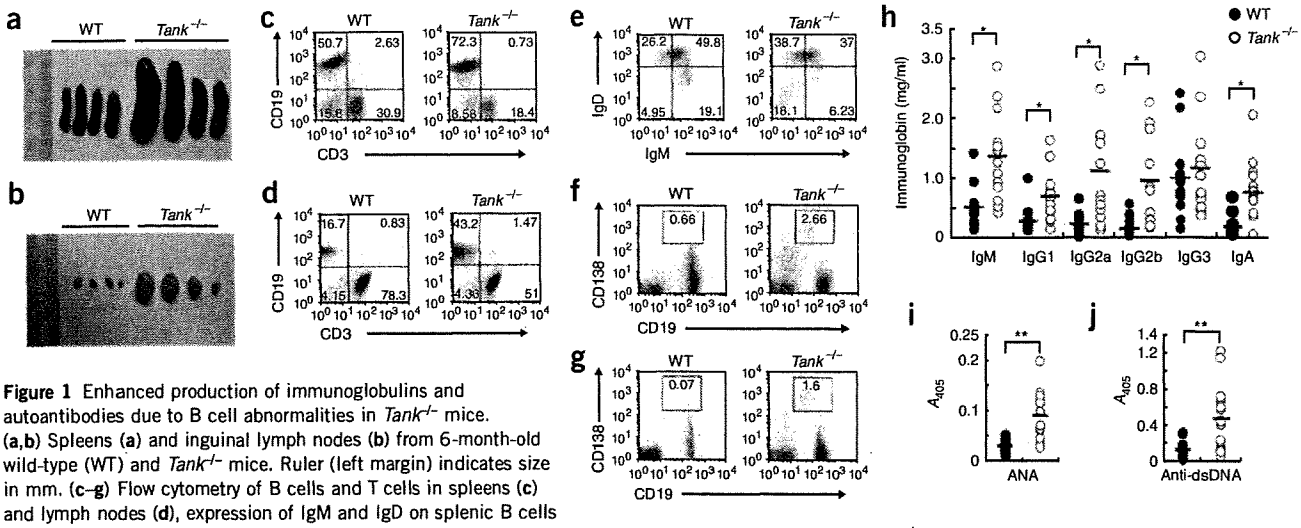

Figure 1 Enhanced production of immunoglobulins and autoantibodies due to B cell abnormalities in *Tank*$^{-/-}$ mice. (**a**,**b**) Spleens (**a**) and inguinal lymph nodes (**b**) from 6-month-old wild-type (WT) and *Tank*$^{-/-}$ mice. Ruler (left margin) indicates size in mm. (**c**–**g**) Flow cytometry 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. A_{405}, 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-κB 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 stimulation[37]. 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-κB 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

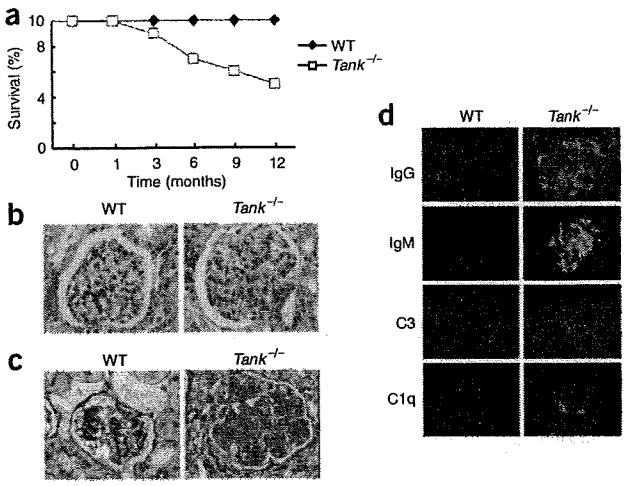

Figure 2 Development of lethal glomerulonephritis in *Tank*$^{-/-}$ mice. (**a**) Survival of wild-type and *Tank*$^{-/-}$ mice monitored for 1 year. (**b,c**) Kidney sections from 6-month-old wild-type and *Tank*$^{-/-}$ mice, stained with hematoxylin and eosin (**b**) or periodic acid–Schiff (**c**). (**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, ×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.

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 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 IκBζ) 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-κB and AP-1 by electrophoretic mobility-shift assay (EMSA). In response to R-848 stimulation, activation of NF-κB and AP-1 was enhanced in *Tank*$^{-/-}$ macrophages compared with that in wild-type macrophages (**Fig. 4b,c**).

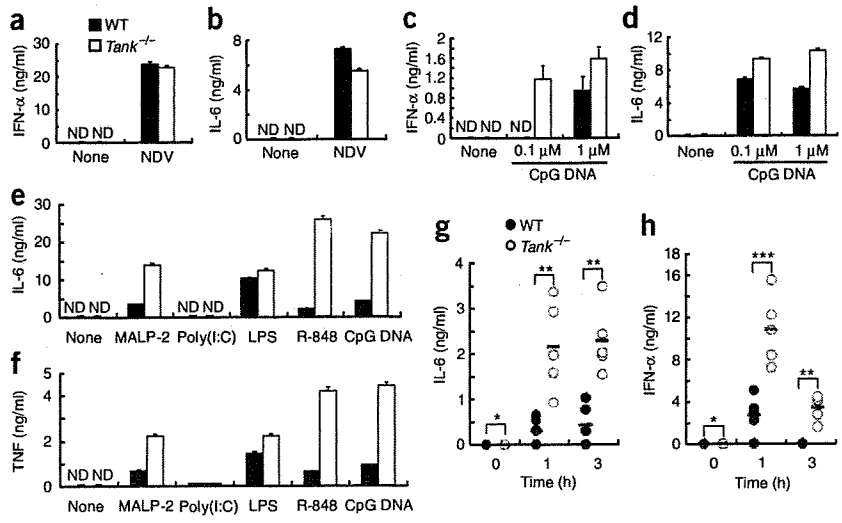

Figure 3 Enhanced proinflammatory cytokine production in response to TLR stimulation in *Tank*$^{-/-}$ mice. (**a,b**) ELISA of IFN-α (**a**) and IL-6 (**b**) in culture supernatants of wild-type and *Tank*$^{-/-}$ bone marrow–derived DCs infected for 24 h with Newcastle disease virus (NDV). (**c,d**) 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 μM 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 μg/ml), LPS (100 ng/ml), R-848 (10 nM) or CpG DNA (1 μM). 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.

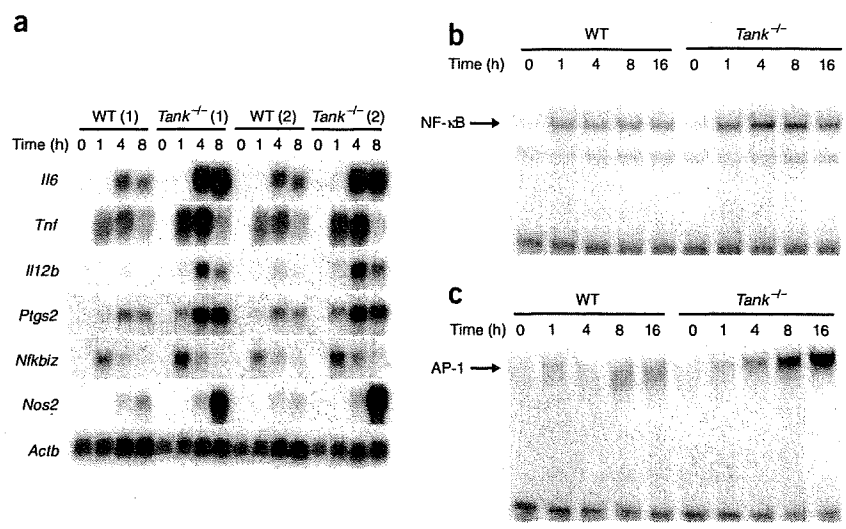

Figure 4 TANK negatively regulates the activation of NF-κB and AP-1 as well as gene expression in response to TLR7 stimulation in macrophages. (**a**) RNA blot analysis of the expression of *Il6*, *Tnf*, *Il12b*, *Ptgs2*, *Nfkbiz* and *Nos2* among total RNA extracted from wild-type and *Tank*$^{-/-}$ 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-κB (**b**) and AP-1 (**c**) in nuclear extracts of wild-type and *Tank*$^{-/-}$ macrophages stimulated for various times (above lanes) with 10 μM R-848, assessed with NF-κB- and AP-1-specific probes. Arrows indicate induced NF-κB 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-κB 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-κB 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 noncanonical NF-κB. The noncanonical pathway is characterized by processing of the NF-κB2 precursor protein p100 to generate p52. We found that activation of noncanonical NF-κB in response to CD40 stimulation was similar in wild-type and *Tank*$^{-/-}$ B cells (**Fig. 6c**). In contrast, NF-κB 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-κB and ubiquitination of TRAF6 in *Tank*$^{-/-}$ B cells (**Supplementary Fig. 3a,b**). Furthermore, 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 noncanonical NF-κB-activation pathways in B cells.

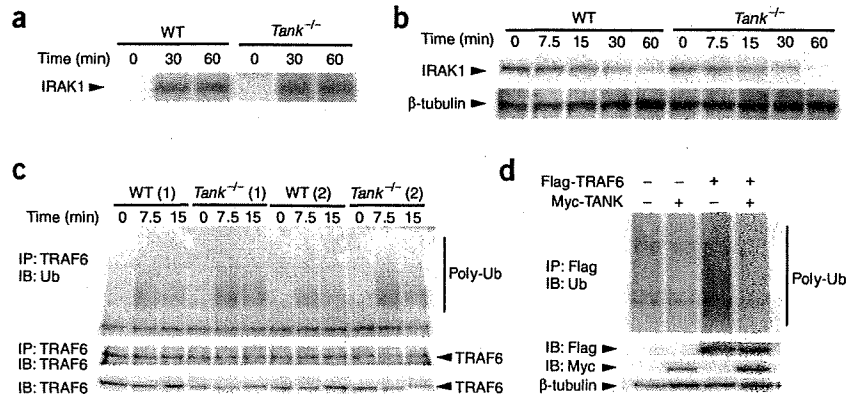

Figure 5 TANK controls TRAF6 ubiquitination in response to TLR7 stimulation in macrophages. (**a**) *In vitro* kinase assay of anti-IRAK1 immunoprecipitates from lysates of wild-type and *Tank*$^{-/-}$ peritoneal macrophages stimulated for various times (above lanes) with 10 μM R-848. Data are representative of two experiments. (**b**) Immunoblot analysis of whole-cell lysates of wild-type and *Tank*$^{-/-}$ macrophages stimulated for various times (above lanes) with 10 μM 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.

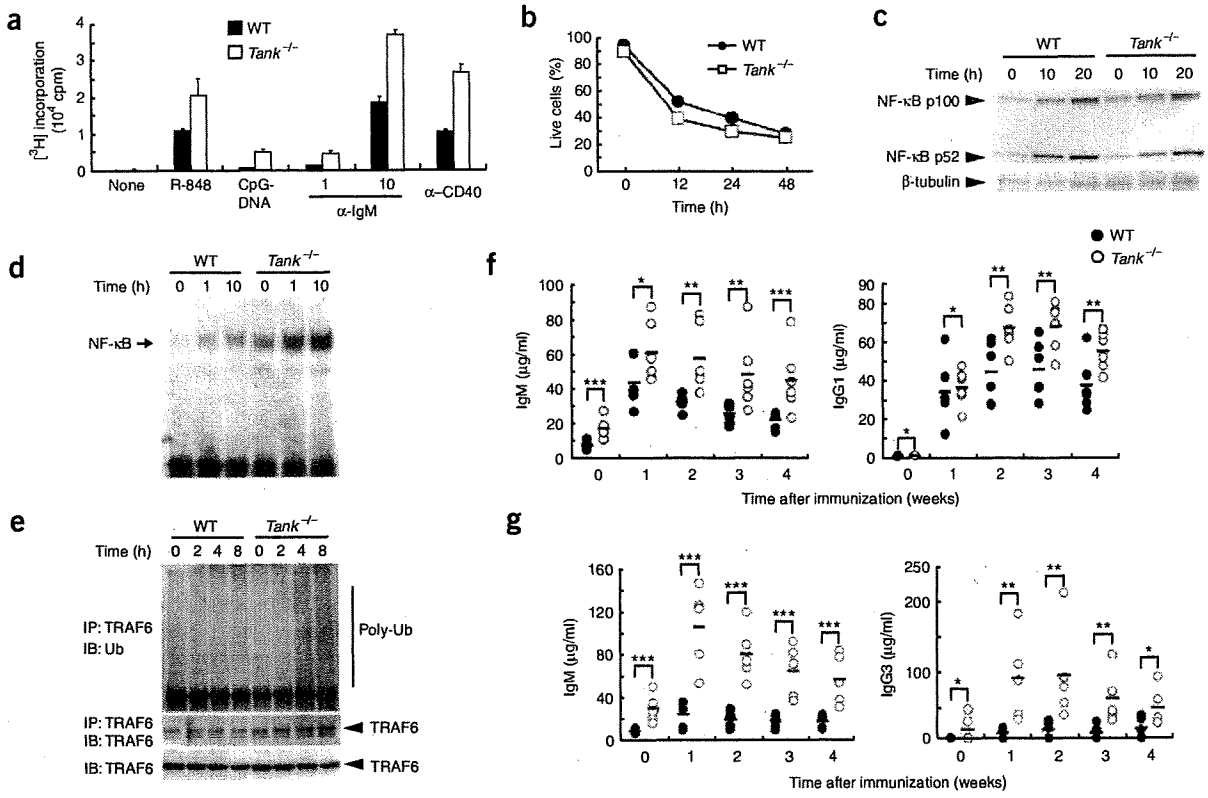

Figure 6 Enhanced activation of B cells in *Tank*$^{-/-}$ mice. (**a**) [^{3}H]thymidine incorporation by purified splenic B cells cultured for 48 h with R-848 (10 nM), CpG DNA (10 nM), anti-IgM (1 or 10 μg/ml) or anti-CD40 (1 μg/ml) and then pulsed with [^{3}H]thymidine (1 μCi) for the final 16 h, measured 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

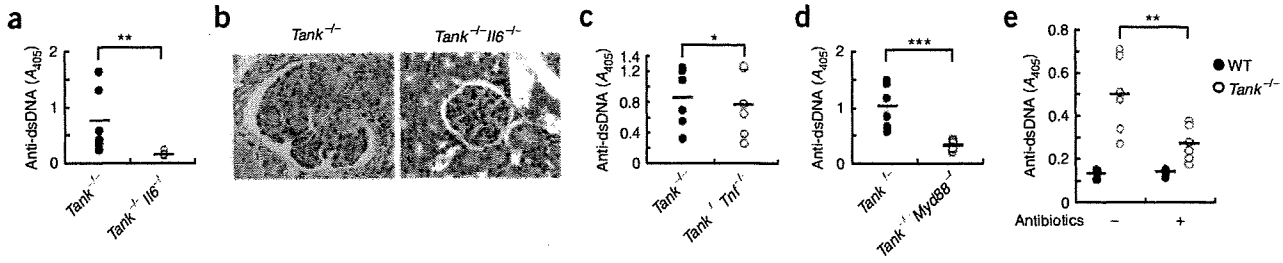

Figure 7 Antibiotic treatment, as well as deficiency of MyD88 or IL-6, ameliorates autoantibody production in *Tank*$^{-/-}$ mice. (**a**) Anti-dsDNA antibodies in serum from 5-month-old *Tank*$^{-/-}$and *Tank*$^{-/-}$*Il6*$^{-/-}$ mice. (**b**) Hematoxylin and eosin staining of kidney sections from *Tank*$^{-/-}$ mice and *Tank*$^{-/-}$*Il6*$^{-/-}$ mice. Original magnification, ×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-κB 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-κB, our results have shown that TANK is critical for the negative regulation of canonical NF-κB 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-κB 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-κB 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-κB was not enhanced in *Tank*$^{-/-}$ B cells, and it has been reported that TRAF3 controls mainly that activation in B cells[44]. Hence, these observations suggest that TANK is not involved in signaling downstream of TRAF3. Furthermore, TRAF2 can control noncanonical NF-κB 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 cells[45], 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 disease[48]. 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.signalinggateway.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.

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