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Sequential control of Toll-like receptor–dependent responses by IRAK1 and IRAK2

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Members of the IRAK family of kinases mediate Toll-like receptor (TLR) signaling. Here we show that IRAK2 was essential for sustaining TLR-induced expression of genes encoding cytokines and activation of the transcription factor NF-κB, despite the fact that IRAK2 was dispensable for activation of the initial signaling cascades. IRAK2 was activated 'downstream' of IRAK4, like IRAK1, and TLR-induced cytokine production was abrogated in the absence of both IRAK1 and IRAK2. Whereas the kinase activity of IRAK1 decreased within 1 h of TLR2 stimulation, coincident with IRAK1 degradation, the kinase activity of IRAK2 was sustained and peaked at 8 h after stimulation. Thus, IRAK2 is critical in late-phase TLR responses, and IRAK1 and IRAK2 are essential for the initial responses to TLR stimulation.

Toll-like receptors (TLRs) sense the invasion of microbes in the body by recognizing their structural components and activate intracellular signaling pathways leading to the expression of genes responsible for inflammatory and immune responses[1–3]. Studies have identified specific components detected by each TLR, such as diacyl lipoprotein (TLR6 and TLR2), triacyl lipoprotein (TLR1 and TLR2), double-stranded RNA (TLR3), lipopolysaccharide (LPS; TLR4), flagellin (TLR5), single-stranded RNA (TLR7 and TLR8) and CpG DNA (TLR9). Stimulation with TLR ligands induces the formation of homo- or heterodimers of TLRs for the recruitment of adaptor molecules containing the Toll–interleukin 1 receptor (IL-1R) homology domain (TIR domain). MyD88, an adaptor containing a TIR domain and a death domain, is essential for signaling 'downstream' of various TLRs, except TLR3 (refs. 4,5). MyD88 interacts with members of the IL-1R-associated kinase (IRAK) family, which dissociate from MyD88 and interact with tumor necrosis factor (TNF) receptor–associated factor 6 (TRAF6). TRAF6 acts as an ubiquitin protein ligase to catalyze the formation of a Lys63-linked polyubiquitin chain on TRAF6 itself and on the transcription factor NF-κB modulator NEMO. Transforming growth factor-β–activated kinase 1 (TAK1) is also recruited to TRAF6 and then phosphorylates inhibitor of NF-κB (IκB) kinase-β (IKKβ) and mitogen-activated protein (MAP) kinase kinase 6. Subsequently, the IKK complex, composed of IKKα, IKKβ and NEMO, is formed. NF-κB binds to IκB in resting cells, where it is sequestered in the cytoplasm. Phosphorylation of IκB by the IKK complex leads to degradation of IκB by the ubiquitin-proteasome system, freeing NF-κB to translocate into the nucleus, where it activates the expression of genes encoding proinflammatory cytokines. Activation of the MAP kinase cascade is responsible for gene expression induced by the transcription activator AP-1.

There are three additional TIR domain–containing adaptors involved in TLR signaling. TIRAP (also called Mal) has been shown to be critical for TLR2- and TLR4-induced NF-κB activation[6–9]. TLR3 and TLR4 trigger the signaling cascade leading to the production of type I interferons through another adaptor, TRIF[10–12]. The fourth adaptor, TRAM, bridges TLR4 and TRIF[13] and is critical for TLR4-induced interferon-inducible gene expression[14,15].

The IRAK family has four members: IRAK1 (A001277), IRAK2 (A001278), IRAKM and IRAK4 (A003450)[16]. IRAK family members are composed of an amino-terminal death domain and a serine-threonine kinase domain. IRAK4 is known to be essential for TLR–IL-1R-mediated cellular responses[17–19]. The kinase activity of IRAK4 is essential for its function in TLR-induced cytokine production[20–22]. IRAK4 deficiency in humans also leads to impaired TLR responses together with recurrent infections with pyrogenic bacteria, particularly *Streptococcus pneumoniae*[23–26]. In contrast, cells lacking IRAKM showed increased cytokine production in response to TLR stimuli, and IRAKM functions to inhibit formation of the IRAK1-TRAF6 complex[27]. IRAK1, the first member of the IRAK family to be discovered, is also involved in TLR–IL-1R signaling pathways[28]. IRAK1-deficient mice are more resistant to LPS-induced shock than are wild-type control mice[29,30], although *Irak4*$^{-/-}$ mice and *Myd88*$^{-/-}$ mice are more resistant to LPS inoculation[5,17,22]. IRAK1-deficient fibroblasts and macrophages reportedly show impaired cytokine production in response to IL-1β or LPS[4,17]. However, IRAK1-deficient cells are still able to produce cytokines after TLR or IL-1R stimulation, and it has been hypothesized that other IRAK family members or

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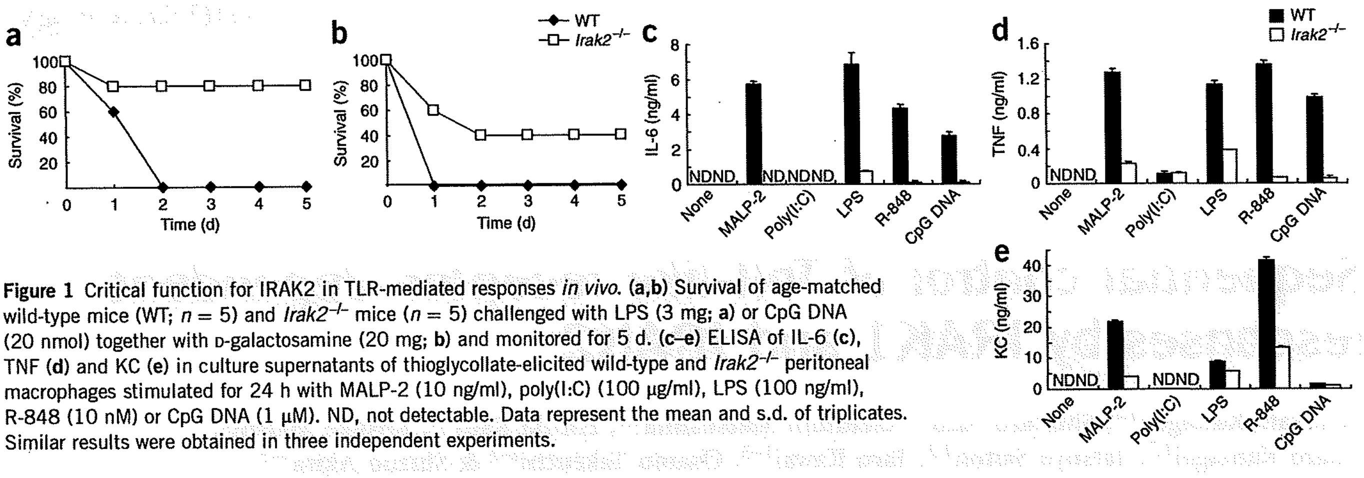


Figure 1 Critical function for IRAK2 in TLR-mediated responses *in vivo*. (**a**,**b**) Survival of age-matched wild-type mice (WT; $n = 5$) and *Irak2*$^{-/-}$ mice ($n = 5$) challenged with LPS (3 mg; **a**) or CpG DNA (20 nmol) together with D-galactosamine (20 mg; **b**) and monitored for 5 d. (**c**–**e**) ELISA of IL-6 (**c**), TNF (**d**) and KC (**e**) in culture supernatants of thioglycollate-elicited wild-type and *Irak2*$^{-/-}$ 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 represent the mean and s.d. of triplicates. Similar results were obtained in three independent experiments.

other proteins could take over the function of IRAK1. Instead, given that IRAK1 is critical for the actions of TLR9-mediated type I interferons but not for the actions of proinflammatory cytokines in plasmacytoid dendritic cells, it is most likely that the main contribution of IRAK1 to the TLR responses of plasmacytoid dendritic cells is in the regulation of interferon-regulatory factors but not of NF-κB[31].

IRAK2 is suggested to be involved in signaling through TIRAP, the adaptor protein responsible for TLR2 and TLR4 responses[6]. Overexpression of IRAK2 in cells results in NF-κB activation[32,33]. IRAK2 is also involved in TLR3 and TLR8 signaling[34]. However, the function of IRAK2 *in vivo* and its relation to other IRAK family members are not yet understood. It has remained unclear whether IRAK2 functions as an active kinase, as a critical aspartate residue in the IRAK catalytic domain is an asparagine residue in IRAK2, and IRAK2 fails to undergo autophosphorylation, unlike IRAK1 and IRAK4 (ref. 35).

Here we generated *Irak2*$^{-/-}$ mice and investigated the function of IRAK2 in TLR responses. *Irak2*$^{-/-}$ mice were resistant to TLR4- and TLR9-mediated shock responses and *Irak2*$^{-/-}$ cells showed impaired production of proinflammatory cytokines. IRAK2 was essential for sustaining TLR-mediated expression of genes encoding proinflammatory cytokines. Recruitment of NF-κB to the *Il6* promoter within 8 h of TLR2 stimulation was impaired in *Irak2*$^{-/-}$ macrophages, which suggested that IRAK2 is critical for sustaining IL-6 promoter activation. Macrophages lacking both IRAK1 and IRAK2 showed substantial defects in the induction of genes encoding proinflammatory cytokines in response to TLR ligands relative to that of cells lacking either IRAK alone, which indicated that IRAK1 and IRAK2 function redundantly in the TLR signaling pathway. An intact kinase domain of IRAK2 was essential for TLR-induced cytokine production, and the kinase activity of IRAK2 was sustained for longer than that of IRAK1 after TLR stimulation. Our results indicate that IRAK2 is critical in late-phase TLR responses and that IRAK1 and IRAK2 function redundantly in the initial response.

RESULTS

Essential function for IRAK2 in TLR-induced responses

To investigate the function of IRAK2 *in vivo*, we generated *Irak2*$^{-/-}$ mice by homologous recombination of embryonic stem cells. We targeted exons 4, 5 and 6 of mouse *Irak2* with a neomycin-resistance cassette in embryonic stem cells and established *Irak2*$^{-/-}$ mice (**Supplementary Fig. 1a** online). We confirmed homologous recombination of the *Irak2* locus by Southern blot analysis (**Supplementary Fig. 1b**). Expression of IRAK2 mRNA and protein was abrogated in *Irak2*$^{-/-}$ cells (**Supplementary Fig. 1c,d**). *Irak2*$^{-/-}$ mice grew normally and did not show any gross abnormalities until the age of 24 weeks. Flow cytometry showed that the spleens and lymph nodes of wild-type and *Irak2*$^{-/-}$ mice did not have a different composition of T cells, B cells, macrophages and dendritic cells (data not shown).

We first examined the function of IRAK2 in response to stimulation with TLR ligands *in vivo*. After challenge with LPS or CpG DNA together with D-galactosamine, all wild-type mice succumbed to shock and died. In contrast, *Irak2*$^{-/-}$ mice were more resistant to such challenge; 80% and 40% of mice survived after challenge with LPS and CpG DNA, respectively (**Fig. 1a,b**). These results indicate that IRAK2 is critical for TLR-induced shock *in vivo*. We next examined cytokine production by macrophages in response to TLR ligands, including MALP-2 (TLR6-TLR2), polyinosinic-polycytidylic acid (poly(I:C); TLR3), LPS (TLR4), resiquimod (R-848; TLR7) and CpG DNA (TLR9). We stimulated thioglycollate-elicited peritoneal macrophages with each TLR ligand and measured the production of proinflammatory cytokines by enzyme-linked immunoassay (ELISA). The production of IL-6, TNF and the inflammatory chemokine KC in response to these TLR ligands, except poly(I:C), was considerably impaired in *Irak2*$^{-/-}$ macrophages relative to that in wild-type cells (**Fig. 1c–e**). In contrast, TNF production in response to poly(I:C) was not different for wild-type versus *Irak2*$^{-/-}$ macrophages. Conventional dendritic cells from *Irak2*$^{-/-}$ mice also showed defective cytokine production in response to these TLR ligands (data not shown). Thus, IRAK2 is

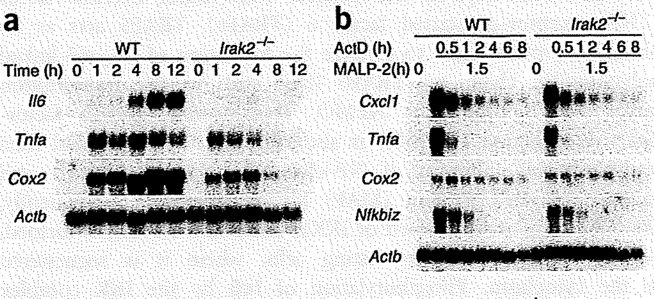


Figure 2 Impaired MALP-2-induced gene expression in *Irak2*$^{-/-}$ cells. (**a**) RNA blot analysis of the expression of *Il6*, *Tnfa* and *Cox2* in wild-type and *Irak2*$^{-/-}$ peritoneal macrophages stimulated for various times (above lanes) with MALP-2 (10 ng/ml). Bottom, rehybridization of same membrane with an *Actb* probe. (**b**) RNA blot analysis of the expression of *Cxcl1*, *Tnfa* and *Nfkbiz* in peritoneal macrophages left untreated (0) or treated for 1.5 h with MALP-2 (10 ng/ml) and then treated for various times (above lanes; ActD) with actinomycin D (5 μg/ml) and MALP-2 (10 ng/ml). Bottom, rehybridization of same membrane with an *Actb* probe. Similar results were obtained in three independent experiments.

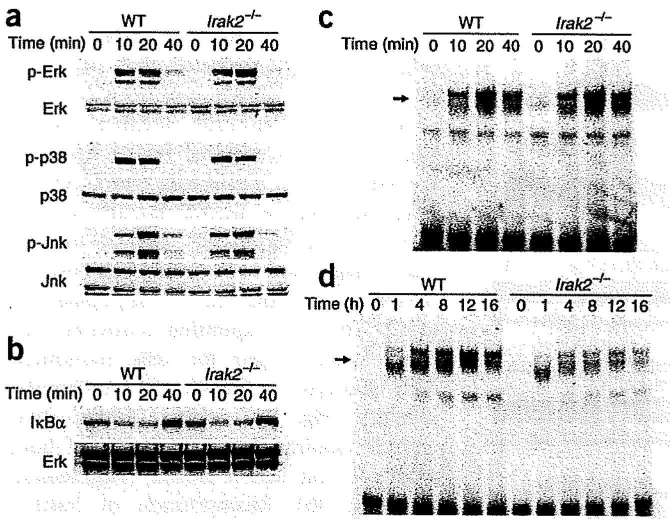


Figure 3 Activation of NF-κB and MAP kinases in *Irak2*$^{-/-}$ macrophages in response to MALP-2. (**a**,**b**) Immunoblot of whole-cell lysates of wild-type and *Irak2*$^{-/-}$ peritoneal macrophages stimulated for various times (above lanes) with MALP-2, analyzed with antibody to phosphorylated (p-) Erk, p38 or Jnk (**a**) or anti-IκBα (**b**). Total Erk, p38 and Jnk serve as a loading control. (**c**,**d**) EMSA of NF-κB DNA-binding activity in nuclear extracts of wild-type and *Irak2*$^{-/-}$ macrophages stimulated for various times (above lanes) with MALP-2 (10 ng/ml), assessed with an NF-κB-specific probe. Arrows indicate the induced NF-κB complex. Results are representative of three independent experiments.

important for eliciting cytokine production in response to various TLR ligands, except for a TLR3 ligand.

TLR-mediated gene expression in *Irak2*$^{-/-}$ macrophages

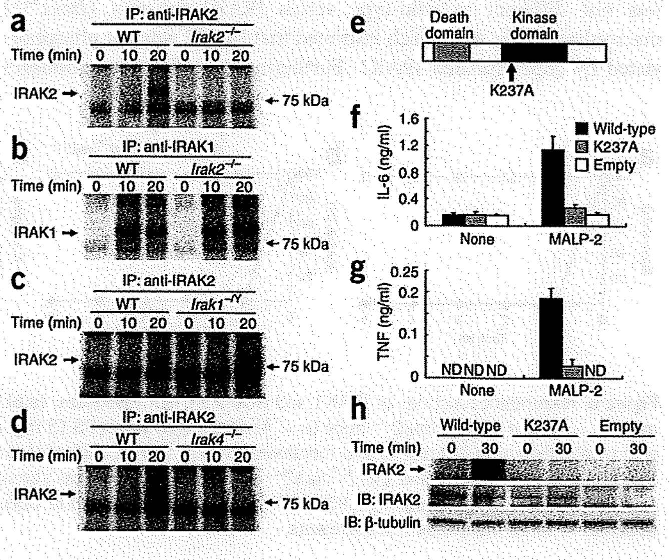
We next determined by RNA blot analysis whether the impaired cytokine production resulting from IRAK2 deficiency occurred at the 'message' level. We chose MALP-2, a TLR2 ligand, as the stimulant, because TLR2 signals only through the MyD88-dependent pathway. In response to stimulation with MALP-2, wild-type macrophages expressed *Il6*, *Tnf* (called '*Tnfa*' here) and *Ptgs2* (called '*Cox2*' here; **Fig. 2a**). In contrast, *Irak2*$^{-/-}$ macrophages failed to express *Il6* in response to stimulation with MALP-2. However, expression of *Tnfa* and *Cox2* was induced normally at 1 h after stimulation, even in the absence of IRAK2, and then was attenuated at 4 h after stimulation. Thus, we hypothesized that IRAK2 signaling was involved in regulating the mRNA stability of TLR-inducible genes. To examine the possible regulation of mRNA stability by IRAK2, we stimulated peritoneal macrophages from wild-type and *Irak2*$^{-/-}$ mice with MALP-2 for 1.5 h, then treated them with actinomycin D for an additional 0.5–8 h (**Fig. 2b**). The abundance of mRNA of TLR-inducible genes such as *Cxcl1*, *Tnfa*, *Cox2* and *Nfkbiz* gradually decreased after actinomycin D treatment with a similar time course in wild-type and *Irak2*$^{-/-}$ macrophages, which indicated that the impaired cytokine gene expression in *Irak2*$^{-/-}$ cells was not due to mRNA instability.

Next we examined the activation of signaling molecules, including MAP kinases and NF-κB, in *Irak2*$^{-/-}$ macrophages. Activation of the MAP kinases Jnk, p38 and Erk by MALP-2 was not impaired in *Irak2*$^{-/-}$ macrophages (**Fig. 3a**), which indicated that IRAK2 is dispensable for TLR-induced MAP kinase activation. We then analyzed NF-κB activation. In response to stimulation with MALP-2, IκBα was degraded within 10 min (**Fig. 3b**) and NF-κB DNA-binding activity was induced (**Fig. 3c**) in wild-type macrophages. It is well known that NF-κB induces the expression of IκB proteins, leading to the restoration of IκBα protein abundance to that seen before stimulation within 40 min. The degradation and recovery of IκBα protein, as well as NF-κB DNA-binding activity, were not impaired in *Irak2*$^{-/-}$ macrophages. Next we examined whether IRAK2 controls NF-κB activation later after stimulation with MALP-2. NF-κB DNA-binding activity was sustained until 16 h after stimulation in wild-type macrophages (**Fig. 3d**). In contrast, activation beyond 4 h after stimulation was impaired in *Irak2*$^{-/-}$ cells. Gel mobility 'supershift' assays showed that the p65 and p50 subunits of NF-κB were activated in both wild-type and *Irak2*$^{-/-}$ cells (**Supplementary Fig. 2** online). Antibodies to other NF-κB subunits such as c-Rel and p52 failed to produce 'supershifting' of NF-κB in wild-type or *Irak2*$^{-/-}$ macrophages, which suggested that the composition of NF-κB was not altered in wild-type versus *Irak2*$^{-/-}$ cells. These results indicate that IRAK2 is critical for sustaining NF-κB activation after TLR stimulation.

Requirement for IRAK2 kinase activity in the TLR signaling

It has been reported that IRAK2 overexpressed in IRAK1-deficient human embryonic kidney 293 (I1A) cells is unable to

Figure 4 Requirement for IRAK2 kinase activity in the response to stimulation with MALP-2. (**a**–**d**) *In vitro* kinase assay of the activation of IRAK1 and IRAK2 in wild-type and *Irak2*$^{-/-}$ (**a**,**b**), *Irak1*$^{-/Y}$ (**c**) or *Irak4*$^{-/-}$ (**d**) peritoneal macrophages stimulated for various times (above lanes) with MALP-2 (10 ng/ml); cell lysates were immunoprecipitated (IP) with anti-IRAK2 (**a**,**c**,**d**) or anti-IRAK1 (**b**). kDa, kilodaltons. Data are representative of three independent experiments. (**e**–**h**) Requirement for IRAK2's kinase activity in the response to TLR stimulation. (**e**) Mutant IRAK2 construct, containing a point substitution of the lysine at position 237 with an alanine residue (K237A). (**f**,**g**) ELISA of IL-6 (**f**) and TNF (**g**) in culture supernatants of macrophages generated from *Irak2*$^{-/-}$ bone marrow cells retrovirally transduced with wild-type or K237A IRAK2; macrophages were stimulated for 24 h with MALP-2 (10 ng/ml). Data represent the mean and s.d.; similar results were obtained in three independent experiments. (**h**) *In vitro* kinase assay of the kinase activity of IRAK2 (top) and immunoblot (IB) analysis of IRAK2 expression (middle) in retrovirally transduced bone marrow cells stimulated for 30 min with MALP-2 (10 ng/ml); lysates were immunoprecipitated with anti-IRAK2 for the kinase assay. Bottom, immunoblot analysis of β-tubulin (loading control). Data are representative of three independent experiments.



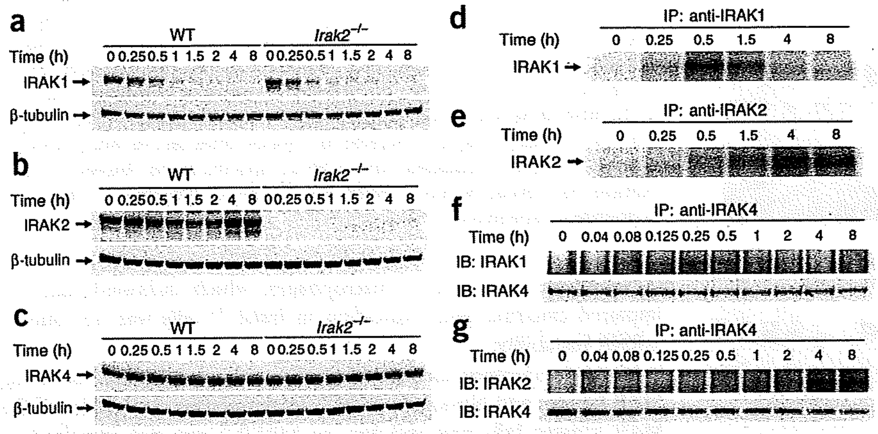


Figure 5 Sequential activation of IRAK1 and IRAK2 kinase activity after stimulation with MALP-2. (**a–c**) Immunoblot analysis of the expression of IRAK1 (**a**), IRAK2 (**b**) and IRAK4 (**c**) in response to stimulation with MALP-2. Bottom, immunoblot analysis of β-tubulin (loading control). Similar results were obtained in two independent experiments. (**d,e**) *In vitro* kinase assay of the activation of IRAK1 and IRAK2 in peritoneal macrophages stimulated for various times (above lanes) with MALP-2 (10 ng/ml); lysates were immunoprecipitated with anti-IRAK1 (**d**) or anti-IRAK2 (**e**). Data are representative of three independent experiments. (**f,g**) Immunoassay of wild-type macrophages stimulated for various times (above lanes) with MALP-2 (10 ng/ml); lysates were immunoprecipitated with anti-IRAK4, followed by immunoblot analysis with anti-IRAK1 (**f**) or anti-IRAK2 (**g**). Below, immunoblot analyses of IRAK4 serve as a loading control. Similar results were obtained in three independent experiments.

autophosphorylate because of the substitution in its kinase domain[35]. Nevertheless, all IRAK family members contain a functional ATP-binding pocket with an invariant lysine residue in the protein kinase subdomain[16]. Expression of IRAK1 and IRAK2 together leads to phosphorylation of IRAK2, whereas the expression of IRAK2 with a substitution (KK237AA) in its ATP-binding pocket fails to induce IRAK2 phosphorylation, which suggests that IRAK2 may function as an active kinase when phosphorylated by IRAK1 (ref. 35). To determine whether IRAK2 has intrinsic kinase activity in response to TLR stimulation *in vivo*, we immunoprecipitated IRAK2 from MALP-2-stimulated macrophages and did an *in vitro* kinase assay. IRAK2 phosphorylation was induced after 20 min in wild-type but not *Irak2*$^{-/-}$ macrophages in response to TLR2 stimulation (**Fig. 4a**). In contrast, IRAK2 deficiency did not affect MALP-2-induced IRAK1 autophosphorylation (**Fig. 4b**). The extent of IRAK2 phosphorylation was not different in wild-type versus IRAK1-deficient (*Irak1*$^{-/Y}$) macrophages (**Fig. 4c**), which indicated that IRAK2 was not phosphorylated by coprecipitated IRAK1. Furthermore, *Irak4*$^{-/-}$ macrophages failed to induce IRAK2 phosphorylation (**Fig. 4d**), which indicated that IRAK4 is essential for the activation of IRAK2.

We next determined whether IRAK2 kinase activity is required for its function. We retrovirally expressed wild-type IRAK2 or a kinase-defective IRAK2 mutant with a K237A substitution (**Fig. 4e**) in *Irak2*$^{-/-}$ macrophages and assessed the responses of the cells to TLR stimulation. The populations of CD11b$^+$ cells expressing retroviral constructs were similar for cells transduced with wild-type or K237A IRAK2 (**Supplementary Fig. 3a** online). Immunoblot analysis confirmed the expression of both wild-type and K237A IRAK2 (**Supplementary Fig. 3b**). Reconstitution of *Irak2*$^{-/-}$ macrophages with wild-type IRAK2 increased the production of IL-6 and TNF in response to stimulation with MALP-2. In contrast, K237A IRAK2 failed to restore MALP-2 responsiveness (**Fig. 4f,g**). Furthermore, reconstitution with wild-type IRAK2 restored the phosphorylation of IRAK2 in response to stimulation with MALP-2, but reconstitution with K237A IRAK2 did not (**Fig. 4h**), which confirmed that an intact kinase domain is essential for IRAK2 phosphorylation. These results indicate that IRAK2 kinase activity is indispensable for its function *in vivo*.

Sustained activation of IRAK2 after TLR2 stimulation

IRAK1 is known to be ubiquitinated and degraded after IL-1β stimulation[36,37]. However, it remains unknown how expression of IRAK2 and IRAK4 is regulated in response to TLR stimulation. In response to stimulation with MALP-2, there was a decrease in IRAK1 protein within 30 min of stimulation, and it remained suppressed for up to 8 h after stimulation (**Fig. 5a**). We noted a similar decrease in IRAK1 expression in *Irak2*$^{-/-}$ macrophages. In contrast, the abundance of IRAK2 or IRAK4 protein was not altered in response to stimulation with MALP-2 (**Fig. 5b,c**). The activation of IRAK1 autophosphorylation was induced rapidly and peaked at 0.5 h after stimulation with MALP-2 (**Fig. 5d**). Subsequently, this autophosphorylation decreased coincidentally with the diminished expression of IRAK1 protein. Notably, MALP-2-induced IRAK2 phosphorylation was sustained up to 8 h after stimulation (**Fig. 5e**). We further

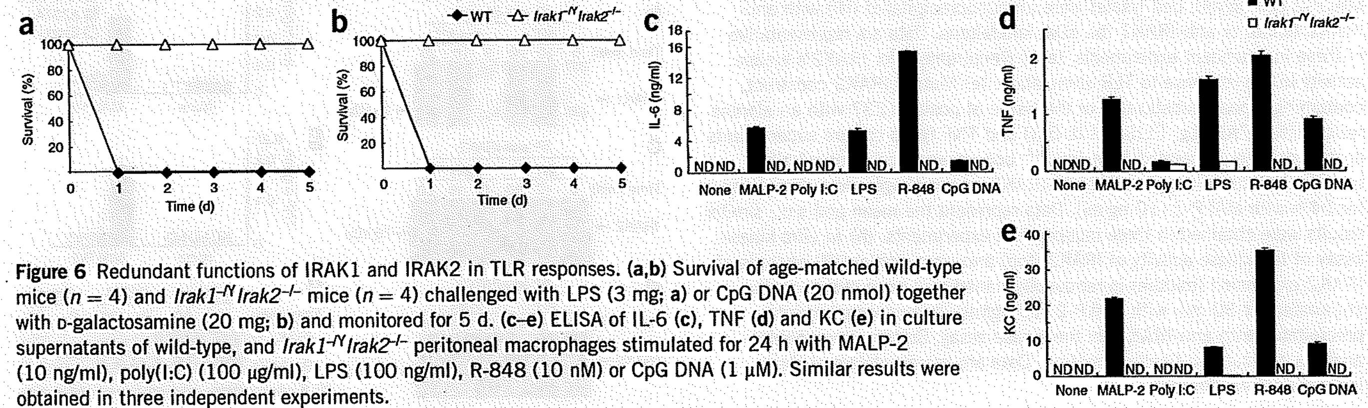


Figure 6 Redundant functions of IRAK1 and IRAK2 in TLR responses. (**a,b**) Survival of age-matched wild-type mice ($n = 4$) and *Irak1*$^{-/Y}$*Irak2*$^{-/-}$ mice ($n = 4$) challenged with LPS (3 mg; **a**) or CpG DNA (20 nmol) together with D-galactosamine (20 mg; **b**) and monitored for 5 d. (**c–e**) ELISA of IL-6 (**c**), TNF (**d**) and KC (**e**) in culture supernatants of wild-type, and *Irak1*$^{-/Y}$*Irak2*$^{-/-}$ 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). Similar results were obtained in three independent experiments.

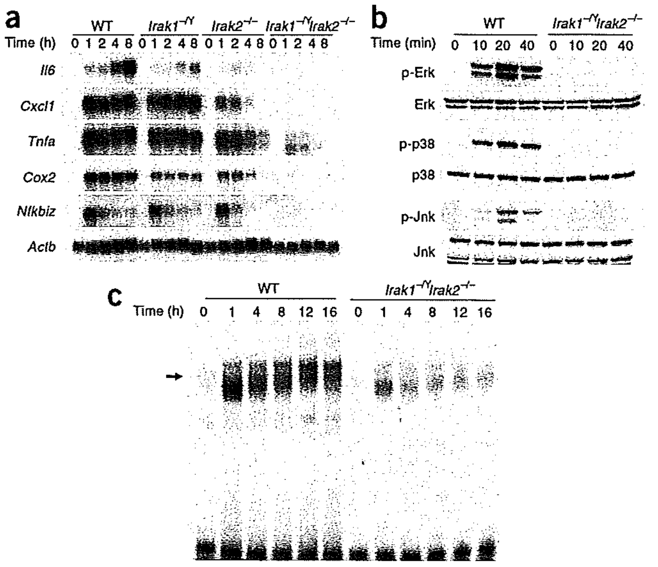


Figure 7 Double deficiency in IRAK1 and IRAK2 causes impaired signaling and is similar to IRAK4 deficiency. (**a**) RNA blot analysis of the expression of *Il6*, *Cxcl1*, *Tnfa*, *Cox2* and *Nfkbiz* in peritoneal macrophages stimulated for various times (above lanes) with MALP-2 (10 ng/ml). Bottom, rehybridization of same membrane with an *Actb* probe. (**b**) Immunoblot analysis of phosphorylated Erk, p38 and Jnk in whole-cell lysates of wild-type and *Irak1*$^{-/Y}$*Irak2*$^{-/-}$ macrophages stimulated with MALP-2. Total Erk, p38 and Jnk serve as a loading control. (**c**) EMSA of nuclear extracts of MALP-2-stimulated wild-type and *Irak1*$^{-/Y}$*Irak2*$^{-/-}$ macrophages, analyzed with an NF-κB-specific probe. Arrow indicates the induced NF-κB complex. Data are representative of three independent experiments.

examined association between IRAK4 and IRAK1 or IRAK2 in response to stimulation with MALP-2. The association between IRAK4 and IRAK1 was induced 5 min after stimulation and decreased at 1 h after stimulation (**Fig. 5f**). In contrast, IRAK2 was precipitated together with IRAK4 at later time points and this was sustained until 8 h after stimulation (**Fig. 5g**). These results suggest that IRAK1 and IRAK2 are activated in response to TLR stimulation at early and late time points, respectively.

Relationship between IRAK1 and IRAK2 in TLR responses

The differential activation of IRAK1 and IRAK2 suggested that these kinases function redundantly in TLR signaling and responses. The production of TNF, IL-6 and KC in response to TLR ligands was modestly impaired in *Irak1*$^{-/Y}$ macrophages (**Supplementary Fig. 4** online). Therefore, we generated mice doubly deficient in IRAK1 and IRAK2 (*Irak1*$^{-/Y}$*Irak2*$^{-/-}$ mice) and examined their responses to TLR simulation. When we inoculated wild-type and *Irak1*$^{-/Y}$*Irak2*$^{-/-}$ mice with LPS or CpG DNA, *Irak1*$^{-/Y}$*Irak2*$^{-/-}$ mice were completely resistant to septic shock induced by LPS or CpG DNA (**Fig. 6a,b**). The production of IL-6, TNF and KC in response to these TLR ligands, except poly(I:C), was abrogated in *Irak1*$^{-/Y}$*Irak2*$^{-/-}$ macrophages (**Fig. 6c–e**).

We next analyzed MALP-2-induced mRNA expression in peritoneal macrophages (**Fig. 7a**). In response to stimulation with MALP-2, *Irak1*$^{-/Y}$*Irak2*$^{-/-}$ macrophages failed to express *Il6*, *Cxcl1*, *Cox2* or *Nfkbiz*, even at early time points. Although *Tnfa* was expressed in response to MALP-2 even in the absence of IRAK1 and IRAK2, its expression was lower in *Irak1*$^{-/Y}$*Irak2*$^{-/-}$ macrophages. Analysis of signaling molecules showed that IRAK1 and IRAK2 were essential for the activation of MAP kinases, including Jnk, p38 and Erk (**Fig. 7b**). Nevertheless, NF-κB DNA-binding activity was induced after stimulation with MALP-2, even in *Irak1*$^{-/Y}$*Irak2*$^{-/-}$ macrophages, although the activity was lower (**Fig. 7c**).

IRAK1 and IRAK2 have been linked to the response to IL-1β. The production of IL-6 in response to IL-1β in *Irak2*$^{-/-}$ and *Irak1*$^{-/Y}$ mouse embryonic fibroblasts was impaired relative to that in wild-type mouse embryonic fibroblasts and was abrogated in *Irak1*$^{-/Y}$*Irak2*$^{-/-}$ mouse embryonic fibroblasts (**Supplementary Fig. 5** online), which indicated that IRAK1 and IRAK2 are involved in the IL-1R signaling.

These findings collectively suggest that IRAK1 and IRAK2 function redundantly in TLR and IL-1R signaling downstream of IRAK4. The defects in the MALP-2 responsiveness of *Irak1*$^{-/Y}$*Irak2*$^{-/-}$ macrophages are reminiscent of those of *Irak4*$^{-/-}$ macrophages[22]. Therefore, these results indicate that either IRAK1 or IRAK2 is required for IRAK4 signaling.

Gene expression in the absence of IRAK1 and IRAK2

Finally, we examined the expression of MALP-2-inducible genes in wild-type, *Irak2*$^{-/-}$ and *Irak1*$^{-/Y}$*Irak2*$^{-/-}$ macrophages by microarray analysis. In wild-type macrophages, in response to stimulation with MALP-2, 171 genes were upregulated more than tenfold at 2, 4 or 8 h after stimulation; we defined these as 'MALP-2-inducible genes' (**Supplementary Table 1** online). As shown by a 'heat map' of MALP-2-inducible genes in wild-type, *Irak2*$^{-/-}$ and *Irak1*$^{-/Y}$*Irak2*$^{-/-}$ macrophages, the expression of various MALP-2-inducible genes at 2 h after stimulation was similar in wild-type and *Irak2*$^{-/-}$ macrophages (**Fig. 8**). Nevertheless, the expression of most genes in *Irak2*$^{-/-}$ cells was lower by 8 h after stimulation. This finding is consistent with impaired activation of NF-κB at later time points. In the absence of both IRAK1 and IRAK2, the expression of MALP-2-inducible genes was impaired much more substantially. However, their expression was not completely abrogated, which suggests that signaling pathways independent of the IRAK family are involved in the regulation of TLR2-inducible genes.

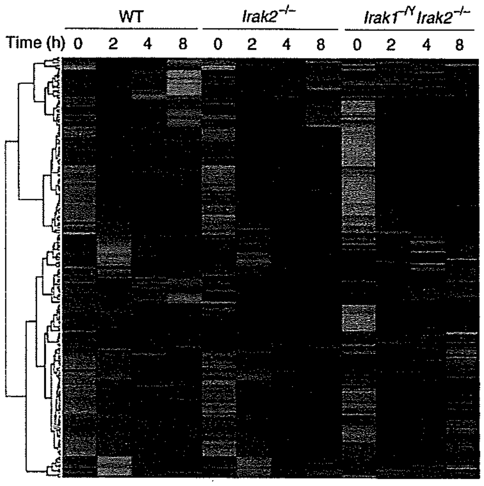


Figure 8 Expression of MALP-2-inducible genes in IRAK1- and IRAK2-deficient macrophages. Cluster images of microarray analysis (heat map and dendrogram) of MALP-2-inducible genes in wild-type, *Irak2*$^{-/-}$ and *Irak1*$^{-/Y}$*Irak2*$^{-/-}$ macrophages stimulated for various times (above columns) with MALP-2 (10 ng/ml). Genes upregulated more than tenfold in wild-type macrophages at 2, 4 or 8 h after stimulation were defined as 'MALP-2-inducible genes'.

DISCUSSION

In this study we generated *Irak2*$^{-/-}$ mice and examined the function of IRAK2 in TLR signaling, relative to that of IRAK1. *Irak2*$^{-/-}$ mice were resistant to shock responses mediated by LPS and CpG DNA. Although *Irak1*$^{-/Y}$ mice are also reported to be resistant to LPS shock, the difference in the mortality rates of wild-type and *Irak1*$^{-/Y}$ mice is subtle[38]. Thus, IRAK2 seems to be more involved in the mortality caused by TLR stimulation *in vivo* than is IRAK1. IRAK2 deficiency attenuated responses to various TLR ligands, including MALP-2, LPS, R-848 and CpG DNA, with the lone exception of poly(I:C). IL-1β-induced cytokine production was also impaired in *Irak2*$^{-/-}$ cells. Although previous results have suggested that IRAK2 acts downstream of TIRAP, an adaptor responsible for TLR2 and TLR4 signaling, our findings here have indicated that IRAK2 is critical not only for the TLR2 and TLR4 signaling pathways but also for the signaling pathways downstream of other TLRs with MyD88.

IRAK2 is critical for sustaining the expression of genes encoding proinflammatory cytokines in response to TLR stimulation. Although the initial upregulation of *Tnfa* and *Cxcl1* in response to TLR2 stimulation was not different control versus *Irak2*$^{-/-}$ macrophages, their expression was lower in *Irak2*$^{-/-}$ macrophages 4 h after stimulation. Thus, the amount of TNF protein produced during the initial 24 h was much lower in *Irak2*$^{-/-}$ macrophages. Although we hypothesized that IRAK2 was essential for ensuring the stability of mRNA encoding various cytokines, there was no difference in the degradation of cytokine transcripts in wild-type versus *Irak2*$^{-/-}$ macrophages. Thus, it seems that IRAK2 is important for sustaining the transcription of genes encoding proinflammatory cytokines involved in TLR signaling. Indeed, NF-κB DNA-binding activity was lower at later time points after TLR2 stimulation, which indicated that IRAK2-dependent signaling is critical for sustaining the activation of transcription factors such as NF-κB.

It has been reported that IRAK2 is involved in the activation of a MyD88-independent signaling pathway by activating TIRAP[6]. Another report has shown that IRAK2 is involved in TLR3 signaling 'upstream' of TRIF[34]. However, we found that TLR4-induced interferon-inducible gene expression was not impaired in *Irak2*$^{-/-}$ macrophages (data not shown). In addition, cytokine production in response to poly(I:C) stimulation was not impaired in *Irak2*$^{-/-}$ or *Irak1*$^{-/Y}$*Irak2*$^{-/-}$ macrophages. Although the reason for discrepancy with previous reports is not clear, we believe that IRAK2 is involved in MyD88-dependent signaling pathways but not in the TRIF-dependent pathway emanating from TLR4.

Among IRAK family members, IRAK1 and IRAK4 have been shown to have intrinsic kinase activity[39,40]. However, a critical aspartate residue in the IRAK catalytic domain is an asparagine or serine in IRAK2 or IRAKM, and their kinase domains are supposedly inactive. However, it has also been shown that coexpression of IRAK1 and IRAK2 leads to phosphorylation of IRAK2, whereas expression of IRAK2 with a substitution in its ATP-binding pocket (KK237AA) together with IRAK1 fails to induce IRAK2 phosphorylation[35]. That report points out the possibility that IRAK2 is phosphorylated by IRAK1, thereby activating the intrinsic kinase activity of IRAK2. We have also shown here, by an *in vitro* kinase assay, that TLR2 stimulation induced phosphorylation of IRAK2 when cell lysates were immunoprecipitated with an IRAK2-specific antibody. Although it has been hypothesized that IRAK1 activates IRAK2 (ref. 35), there was TLR2-induced IRAK2 phosphorylation even in *Irak1*$^{-/Y}$ macrophages. These results indicate that IRAK1 is not responsible for the phosphorylation of IRAK2 in response to TLR stimulation. However, IRAK4 deficiency abrogated the phosphorylation of IRAK2, which suggests that activated IRAK4 phosphorylates IRAK1 and IRAK2, thereby inducing their autophosphorylation activity. Further, reconstitution of *Irak2*$^{-/-}$ macrophages with wild-type IRAK2 restored the phosphorylation of IRAK2 in response to MALP-2, but reconstitution with K237A IRAK2 did not, which indicates that the kinase activity of IRAK2 is critical for IRAK2 phosphorylation. It has also been reported that IRAK2 expression promotes the ubiquitination of TRAF6 (ref. 34). Thus, both IRAK1 and IRAK2 activate intracellular signaling cascades through TRAF6.

The requirement for the kinase activity of IRAK family members is still not well understood. Although we have shown here that IRAK4's kinase activity was critical for its function, IRAK1's kinase activity is reportedly dispensable for IL-1β-mediated signaling[37,40–43]. Furthermore, a kinase-defective IRAK1 mutant potently induced NF-κB activation. Reconstitution of *Irak2*$^{-/-}$ macrophages with wild-type IRAK2 restored IL-6 production in response to stimulation with MALP-2, but reconstitution with K237A IRAK2 did not, which suggests that IRAK2's kinase activity is essential for its function in regulating cytokine production.

It has been shown that IRAK1 is rapidly ubiquitinated and degraded by the ubiquitin-proteasome system after TLR stimulation[36]. IRAK1 is also reported to have 'PEST' sequences, which are involved in regulating proteolysis[16,44]. IRAK1 protein abundance and autophosphorylation were lower after TLR2 stimulation. In contrast, IRAK2 expression was not altered in response to TLR2 stimulation. Notably, IRAK2 autophosphorylation began 20 min after TLR2 stimulation, peaked at 8 h after stimulation and was sustained as late as 16 h after stimulation. Thus, IRAK2 may function exclusively to sustain TLR-mediated signaling and cause robust proinflammatory cytokine production.

The initial TLR2-induced expression of *Cxcl1* and *Tnfa* mRNA was lower in *Irak1*$^{-/Y}$ macrophages, although their expression was similar to the expression in wild-type cells at later time points. Those observations suggest that the presence of two kinases is beneficial for inducing strong initial cytokine responses to the invasion of pathogens. We speculate that the degradation of IRAK1 might be a prerequisite for preventing the overproduction of proinflammatory cytokines, which may cause harmful septic shock.

Among the IRAK family members, IRAK4 has been shown to be essential for the responses to TLR and IL-1R stimulation in *Irak4*$^{-/-}$ mice[17,19]. In response to TLR stimulation, IRAK4 phosphorylates IRAK1, inducing IRAK1 activation and an association between IRAK1 and TRAF6. Although IRAK1 is critical for IL-1R-mediated signaling in human embryonic kidney 293 cells lacking IRAK1 expression[35], analysis of *Irak1*$^{-/Y}$ mice shows that IRAK1 contributes modestly to TLR-induced cytokine production in macrophages[29,30], and the presence of IRAK1-independent signaling pathways has been predicted. We have shown here that TLR-mediated cytokine production was abrogated in the absence of both IRAK1 and IRAK2. Activation of MAP kinases was also abrogated when both IRAK1 and IRAK2 were absent. These results indicate that IRAK1 and IRAK2 function redundantly in the production of cytokines and MAP kinase activation. The phenotype of *Irak1*$^{-/Y}$*Irak2*$^{-/-}$ macrophages is reminiscent of that of *Irak4*$^{-/-}$ macrophages. It has been shown that IRAK4 phosphorylates IRAK1, thereby activating its kinase activity. Thus, it is plausible that IRAK2 is also phosphorylated by IRAK4, leading to its activation. Indeed, phosphorylation of IRAK2 has not been reported in *Irak4*$^{-/-}$ macrophages[22]. These results indicate that IRAK4 acts upstream of both IRAK1 and IRAK2 to activate downstream signaling cascades.

Our microarray analysis showed that IRAK1 and IRAK2 were critical for the regulation of only some MALP-2-inducible genes, although cytokines and chemokines are regulated by IRAK1 and

IRAK2. That result is consistent with the induction of NF-κB even in the absence of IRAK1 and IRAK2. A reported MyD88-dependent IRAK4 independent pathway[22] may be responsible for the expression of these genes. Identifying the signaling molecules responsible for the IRAK-independent signaling pathways will be essential for understanding complex TLR-induced gene expression mechanisms. However, the gene expression profiles of wild-type and *Irak2*$^{-/-}$ macrophages 2 h after stimulation with MALP-2 were similar. The expression of genes encoding cytokines was not sustained in *Irak2*$^{-/-}$ macrophages, and the expression of 38 genes was impaired 50% in *Irak2*$^{-/-}$ cells 8 h after stimulation. That result supports the idea that IRAK2 is involved in the expression of genes encoding cytokines later in response to the TLR stimulation. Further studies are needed to understand the mechanism underlying this signaling pathway.

Finally, our data have shown that IRAK1 and IRAK2 acted redundantly at early time points after TLR stimulation, whereas IRAK2 was critical for sustaining the responses at later time points. Both IRAK1 and IRAK2 seemed to be activated downstream of IRAK4. Moreover, the kinase activity of IRAK2 was essential for cytokine production in response to TLR stimulation in macrophages. Given that IRAK2 deficiency minimally affected TLR-induced gene expression, except for that of genes encoding proinflammatory cytokines, the development of a small molecule targeting IRAK2 kinase activity will be beneficial to therapies for septic shock by preventing a wide spectrum of immune suppression.

METHODS

Generation of *Irak2*$^{-/-}$ mice. *Irak2* was isolated from genomic DNA extracted from embryonic stem cells by PCR. The targeting vector was constructed by replacement of a 2.9-kilobase fragment encoding the *Irak2* open reading frame with a neomycin-resistance cassette and a herpes simplex virus thymidine kinase cassette driven by the promoter of the gene encoding phosphoglycerate kinase, which had been inserted into the genomic fragment for negative selection. After the targeting vector was transfected into embryonic stem cells, colonies doubly resistant to the aminoglycoside G418 and gancyclovir were selected and screened by PCR and their identities were further confirmed by Southern blot analysis. Homologous recombinants were microinjected into blastocysts from C57BL/6 female mice, and heterozygous F_1 progeny were intercrossed to obtain *Irak2*$^{-/-}$ mice. *Irak2*$^{-/-}$ mice on the 129/Sv × C57BL/6 background and their littermates (controls) were used. All animal experiments were with the approval of the Animal Research Committee of the Research Institute for Microbial Diseases (Osaka University).

Cells. Peritoneal exudate cells were isolated from the peritoneal cavities of mice 3 d after mice were injected with 2 ml of 4.0% (wt/vol) thioglycollate medium (Sigma) by washing with ice-cold Hank's buffered-salt solution (Invitrogen).

Reagents. MALP-2 was provided as described[45]. LPS from *Salmonella minnesota* Re-595 was from Sigma-Aldrich; poly(I:C) was from Amersham Biosciences; and R-848 was provided by the Pharmaceuticals and Biotechnology Laboratory of Japan Energy. CpG oligonucleotide was synthesized as described[46]. Polyclonal antibodies to phosphorylated Jnk (9251), p38 (9211) and Erk (9101) were from Cell Signaling. Polyclonal antibody to β-tubulin (anti-β-tubulin; sc-5274), anti-IκBα (sc-371), anti–NF-κB p50 (sc-1192) and anti–NF-κB p65 (sc-109) were from Santa Cruz. Rabbit polyclonal anti-IRAK1 and anti-IRAK4 were made as described[22,47]. Rabbit polyclonal anti-IRAK2 (3595) was from ProSci.

Measurement of cytokine production. Concentrations of cytokines in culture supernatants were measured by ELISA. ELISA kits for mouse TNF, IL-6 and KC were from R&D Systems.

***In vitro* kinase assay.** Peritoneal macrophages stimulated with MALP-2 (10 ng/ml) were lysed and immunoprecipitated with anti-IRAK1, anti-IRAK2 or anti-IRAK4, then the activity of IRAK1, IRAK2 and IRAK4 was measured by an *in vitro* kinase assay as described[22].

RNA hybridization. Peritoneal macrophages were treated for 0, 1, 2, 4 or 8 h with MALP-2 (10 ng/ml) and total RNA was extracted with TRIzol reagent (Invitrogen). RNA was separated by electrophoresis and transferred to nylon membranes and then hybridized with the appropriate cDNA probe. For the detection of *Irak2* mRNA expression, a 314–base pair fragment (nucleotides 894–1208) was used as a probe. The same membrane was rehybridized with an *Actb* probe (encoding β-actin) as a loading control.

Immunoblot analysis. Peritoneal macrophages were treated for various times with MALP-2 (10 ng/ml). Cells were then lysed in lysis buffer containing 1.0% (vol/vol) Nonidet-P40, 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA and a protease inhibitor 'cocktail' (Roche). Cell lysates were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blotted with the appropriate specific antibodies and were visualized with an enhanced chemiluminescence system (NEN Life Science Products).

Electrophoretic mobility-shift assay. Nuclear extracts prepared from 4×10^6 peritoneal macrophages stimulated with MALP-2 (10 ng/ml) as described[12] were incubated with or without anti–NF-κB p65 or anti–NF-κB p50 and then were further incubated with a specific probe for NF-κB DNA-binding sites, separated by electrophoresis and visualized by autoradiography.

Construction of IRAK2 expression plasmids. Full-length IRAK2 cDNA was obtained by RT-PCR from a human cDNA library, and a point mutation resulting in a K237A substitution in the kinase domain was introduced by site-directed mutagenesis. Full-length or mutated IRAK2 cDNA was cloned into the 'LZR' vector.

Retroviral transduction. Highly efficient retroviral transduction of macrophages was achieved by transduction of hematopoietic stem cells before differentiation into macrophages as described[48]. Bone marrow was isolated from *Irak2*$^{-/-}$mice that had been injected intraperitoneally 4 d earlier with 5 mg of 5-fluorouracil (Nacalai Tesque). Cells were cultured in stem cell media (DMEM supplemented with 15% (vol/vol) FCS, sodium pyruvate (10 mM), L-glutamine (2 mM), β-mercaptoethanol (50 μM), penicillin (100 U/ml), streptomycin (100 g/ml), stem cell factor (100 ng/ml), IL-6 (10 ng/ml) and IL-3 (10 ng/ml)). Then, 48 h later, cells were transduced with retroviral supernatant (supplemented with stem cell factor, IL-6, IL-3 and 10 ng/ml of polybrene) on 2 successive days. Virus was produced with the PlatE retrovirus packaging cell line. After the second transduction, cells were washed and resuspended in macrophage growth media (RPMI 1640 medium supplemented with 10% (vol/vol) FCS, HEPES (10 mM), pH 7.0, sodium pyruvate (10 mM), L-glutamine (2 mM), β-mercaptoethanol (50 μM), penicillin (100 U/ml), streptomycin (100 g/ml) and macrophage colony–stimulating factor (40 ng/ml)). Cells were cultivated for 7 d and then analyzed.

Microarray. Peritoneal macrophages were stimulated for 0, 2, 4 or 8 h with MALP-2 (10 ng/ml), then total RNA was extracted with TRIzol (Invitrogen Life Technologies) and further purified with an RNeasy kit (Qiagen). Biotin-labeled cDNA was synthesized from 100 ng total RNA with the Ovation Biotin RNA Amplification and Labeling System (Nugen) according to the manufacturer's protocol. Affymetrix mouse Genome 430 2.0 microarray chips were hybridized, stained, washed and scanned according to the manufacturer's instructions. Data were analyzed MicroArray Suite software (Affymetrix) and ArrayAssist software (Stratagene).

Accession codes. UCSD-Nature Signaling Gateway (http://www.signaling-gateway.org): IRAK1 (A001277), IRAK2 (A001278), IRAKM and IRAK4 (A003450); GEO: microarray data, GSE10765.

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

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AUTHOR CONTRIBUTIONS

T. Kawagoe, O.T. and S.A. designed the research and analyzed data; T. Kawagoe did most of the experiments; S.S., K. Matsushita., H.K., K. Matsui., Y.K., T.S. and T.K. provided advice; and T. Kawagoe, O.T. and S.A. prepared the manuscript.

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Akirins are highly conserved nuclear proteins required for NF-κB-dependent gene expression in drosophila and mice

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During a genome-wide screen with RNA-mediated interference, we isolated *CG8580* as a gene involved in the innate immune response of *Drosophila melanogaster*. *CG8580*, which we called *Akirin*, encoded a protein that acted in parallel with the NF-κB transcription factor downstream of the Imd pathway and was required for defense against Gram-negative bacteria. *Akirin* is highly conserved, and the human genome contains two homologs, one of which was able to rescue the loss-of-function phenotype in drosophila cells. Akirins were strictly localized to the nucleus. Knockout of both *Akirin* homologs in mice showed that one had an essential function downstream of the Toll-like receptor, tumor necrosis factor and interleukin (IL)-1β signaling pathways leading to the production of IL-6. Thus, Akirin is a conserved nuclear factor required for innate immune responses.

The innate immune system shields all metazoans against invading microorganisms. This well conserved defense mechanism relies on host-pathogen interactions between nonclonally distributed pattern recognition receptors in the host and pathogen-associated molecular patterns in microbes[1–4]. In contrast, the acquired immune system, based on selection of lymphocytes and their antigen-specific receptors, is specific to vertebrates. Drosophila has become an attractive model organism for the study of the innate immune system due to its well established genetics, the absence of an acquired immune system and the striking conservation between its immune system and many mammalian innate immune defenses.

One of the hallmarks of the drosophila defense is the systemic response, which involves the synthesis of small cationic antimicrobial peptides by the fat body, a functional equivalent of the mammalian liver. Two distinct signaling pathways, namely the immune deficiency (Imd) and the Toll pathways, control the transcription of the antimicrobial peptide genes[2,4,5]. Fungal or Gram-positive bacterial infections activate the Toll pathway[6]. The cytokine-like peptide Spaetzle is cleaved in response to microbial challenge in the open circulatory system of the fly and binds to the transmembrane receptor Toll[7]. The subsequent intracellular cascade leads to the dissociation of the NF-κB family member Dorsal-related immunity factor (Dif)[8,9] from its inhibitor, the IκB-like protein Cactus, through the recruitment of the myeloid differentiation factor 88 homolog (MyD88)[10], the adaptor molecule Tube, and the IL-1R-associated kinase (IRAK)-like serine-threonine kinase Pelle[2]. Dif nuclear translocation then activates many genes, including the gene encoding the antifungal peptide Drosomycin (*Drs*)[4,6,9].

In contrast, Gram-negative bacterial infection activates the Imd pathway, resulting in the expression of genes encoding antimicrobial peptides such as Attacin, Cecropin and Diptericin[3–5]. Expression of these effector genes requires the signal-dependent cleavage and subsequent nuclear translocation of Relish, another member of the NF-κB family of transcription factors[11–13]. Several genetic screens have identified many players in the Imd pathway and shown striking similarities with components of the mammalian tumor necrosis factor (TNF) pathway[14]. Gram-negative bacterial peptidoglycan (PGN) binds to peptidoglycan recognition protein LC (PGRP-LC) and PGRP-LE, which are the most upstream components of the Imd pathway[15–21]. Imd itself encodes a protein with a death domain (DD) similar to that of the mammalian receptor-interacting protein (RIP) that is important in both NF-κB activation and apoptosis[22,23]. Yeast two-hybrid experiments and genetic analysis have demonstrated that Imd forms a complex with the death domain–containing adaptor Fadd and the caspase Dredd[24,25]. This upstream protein complex then activates, through a TAK1-binding protein called dTAB2 (ref. 26) and inhibitor of apoptosis protein 2 (IAP2)[27], the drosophila TGF-β–activated kinase-1 (dTAK1), a member of the MAPKKK family of kinases[28]. Both IκB kinase (IKK)-β (IKKβ) and IKKγ are also required downstream of Imd and dTAK1 for Relish activation[29,30].

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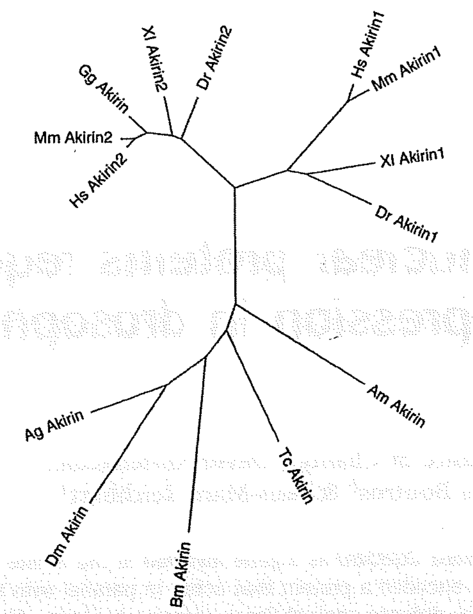

Figure 1 Unrooted evolutionary tree of Akirin homologs: Akirins are highly conserved. Dm, *Drosophila melanogaster*; Ag, *Anopheles gambiae*; Bm, *Bombyx mori*; Am, *Apis mellifera*; Tc, *Tribolium castaneum*; Gg, *Gallus gallus*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Xl, *Xenopus laevis*; Dr, *Danio rerio*. In vertebrates, similarity splits *Akirins* into two groups that we have numbered 1 and 2, with *Akirin2s* more closely related to invertebrate *Akirins*.

In mammals, Gram-negative bacteria are sensed by Toll-like receptors (TLRs) that activate, similarly to the drosophila Imd pathway, an IKK complex and NF-κB. In response to TLR or IL-1R stimulation, MyD88 and IRAKs are recruited to the receptor, and then interact with TNF receptor–associated factor 6 (TRAF6), which acts as an ubiquitin protein ligase (E3). Subsequently, TRAF6, together with a ubiquitination E2 enzyme complex consisting of UBC13 and UEV1A, catalyzes the formation of a K63-linked polyubiquitin chain on TRAF6 and on IKK-γ–NF-κB essential modulator (NEMO)[31]. A complex comprising TAK1 and the TAK1-binding proteins, TAB1, TAB2 and TAB3, is also recruited to TRAF6 (ref. 32). After stimulation by TLR ligands, IκBα is phosphorylated on two serine residues by an IKK complex activated by TAK1. Phosphorylated IκBα is then ubiquitinated and degraded by the proteasome. Liberated NF-κB translocates into the nucleus, where it activates the transcription of its target genes.

Despite more than ten years of research since the initial discovery of the Imd mutation, the pathway bearing its name is still not fully understood. We undertook a functional genome-wide RNA-mediated interference (RNAi) screen in drosophila cell culture to isolate new components in the Imd pathway. We report here the isolation of *CG8580* (that we renamed *Akirin*) encoding a nuclear protein with no recognizable domain and required for NF-κB-dependent transcription. RNAi-mediated knock down of *Akirin* led to impaired Imd pathway signaling and enhanced sensitivity of flies to Gram-negative bacterial infection. Moreover, epistatic analysis allowed us to place the *Akirin* function at the level of the transcription factor itself. As *Akirin* shows striking evolutionary conservation, we generated mice deficient for *Akirin* homologs and demonstrated that one of these mouse *Akirin* homologs was required for NF-κB dependent IL-6 production after TLR agonist, IL-1β or TNF stimulation of embryonic fibroblasts. A drosophila loss of function phenotype could also be restored by expression of the human homolog of *Akirin*. We therefore propose that *Akirin* is an ancient conserved nuclear factor regulating NF-κB dependent transcription.

RESULTS

Identification of drosophila and mouse Akirin homologs

To identify new components of the Imd pathway, we performed a high-throughput RNAi screen with cultured drosophila S2 hemocyte-like cells[27,33]. Of 21,306 RNAi probes, several induced a moderate to marked effect on the expression of the Imd pathway-dependent *Attacin* gene activated by an *Escherichia coli* infection. We selected *CG8580* for further study, as the corresponding RNAi reduced the induction of Attacin expression by 90%. *CG8580* encoded a putative 201–amino acid protein with no recognizable domains. Two homologs of the *CG8580* sequence were present in zebrafish (*Danio rerio*), African clawed frog (*Xenopus laevis*), human (*Homo sapiens*) and mouse (*Mus musculus*) databases. Only one copy was present in insects (*Apis mellifera, Tenebrio molitor, Anopheles gambiae, D. melanogaster*) and in birds (*Gallus gallus*); none was found in plants, yeast or bacteria. The similarities allowed the sequences to be split into discrete groups, one in insects and two in vertebrates (**Fig. 1**). The conservation was highest for the putative C- and N-terminal domains. All sequences showed a clear nuclear localization signal (NLS) located between residues 24 and 29 near the N terminus (**Supplementary Fig. 1** online). We renamed the gene *Akirin* (*Akirin1* and *Akirin2* in the case of vertebrates) from the Japanese 'akiraka ni suru', which means 'making things clear'.

Akirins are ubiquitously expressed nuclear proteins

Microarray data in Flybase[34] indicate that *D. melanogaster Akirin* expression is ubiquitous. Similarly, an analysis based on a blot with human RNA points to almost ubiquitous expression of human *Akirins* (**Supplementary Fig. 2** online). To monitor the cellular localization of drosophila Akirin, we fused the *D. melanogaster Akirin* coding sequence to a V5 tag and transfected S2 cells. Immunoblot analysis with antibody to V5 (anti-V5) showed that drosophila *Akirin* was expressed as a single ~27-kDa protein that was not modified after *E. coli* stimulation (**Supplementary Fig. 3** online). Antibody staining of the S2 cells established that drosophila Akirin had a strict nuclear localization, which was dependent on the presence of the N-terminal NLS (**Fig. 2a**) and did not change after *E. coli* treatment (data not shown). Similarly, we fused the *H. sapiens Akirin1* and *Akirin2* sequences to a Flag tag and transfected HeLa cells. Antibody staining of the human cells clearly showed the nuclear localization of human Akirin1 and human Akirin2, which was again dependent on the NLS (**Fig. 2b**).

Akirin function in drosophila

To analyze the effects of drosophila *Akirin* on the Imd pathway, we used an RNAi-mediated knock down strategy in S2 cells. A truncated form of PGRP-LCa (containing only the transmembrane and intracellular segment) can induce a robust expression of an Attacin-luciferase (Att-Luc) reporter (refs. 10,15,16,27 and A.G., unpublished data). Compared with GFP RNAi controls, the induction of the Att-Luc reporter was strongly suppressed by double-stranded RNA (dsRNA) against *Akirin* (**Fig. 3a,b**), in keeping with reduced *Akirin* mRNA abundance (**Supplementary Fig. 4** online). The degree of reduction was similar to that obtained with dsRNA against *Imd* (**Fig. 3b**). In further experiments we confirmed the specificity of the suppression with two different, nonoverlapping dsRNAs directed against *Akirin*, which both produced a considerable reduction in

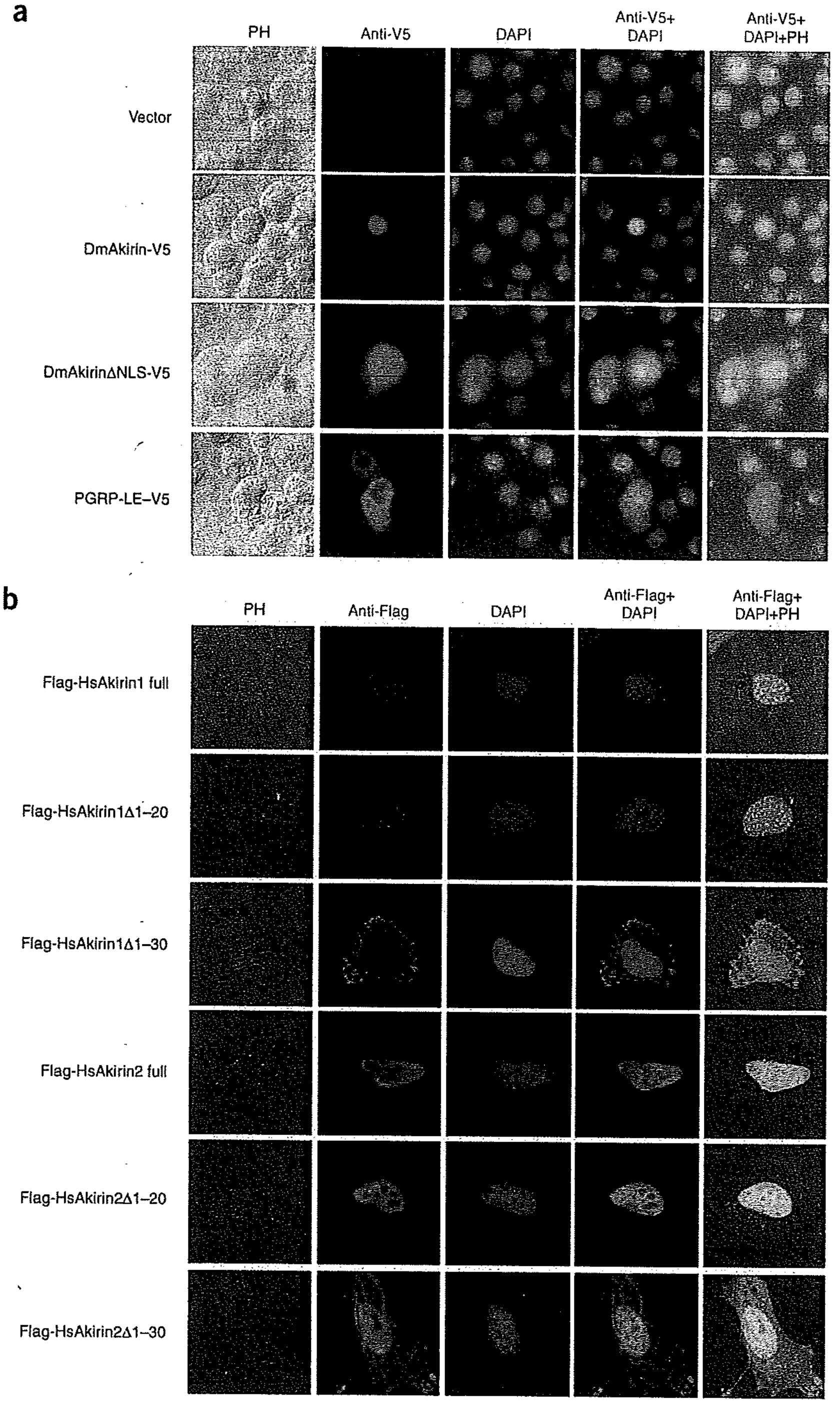

Figure 2 Nuclear localization of Akirins. **(a)** S2 cells transfected with constructs encoding V5-tagged drosophila Akirin, NLS-deleted drosophila Akirin or PGRP-LE. Cells transfected with an empty vector were used as control. Nuclei were visualized with DAPI (blue). Akirin, NLS-deleted Akirin and PGRP-LE were visualized by V5 antibody (green). The merged fields including phase contrast (PH) showed nuclear localization of drosophila Akirin (anti–V5+DAPI+PH), in contrast to the cytoplasmic localization of PGRP-LE. This nuclear localization was abrogated when the NLS was deleted from Akirin. Results are representative of three independent experiments. **(b)** HeLa cells transfected with Flag-tagged full-length or N-terminally deleted (amino acids 1–20 or 1–30) *H. sapiens Akirin1* or *H. sapiens Akirin2*. Nuclei were visualized with DAPI (blue) and human Akirins were visualized with an anti-Flag antibody (green). The merged fields (anti–Flag+DAPI+PH) showed NLS-dependent (amino acids 20–30; **Supplementary Fig. 1**) nuclear localization for both human Akirin1 and human Akirin2.

Att-Luc, similar to that of the original dsRNA, demonstrating that the suppression is gene specific (**Fig. 3a,c**).

The Imd pathway responds to Gram-negative bacteria, but the Toll pathway is predominantly activated by Gram-positive bacteria or fungi and culminates in the expression of many genes, including the antifungal peptide Drs[5]. To address whether *Akirin* is also involved in the Toll pathway, we transfected an expression construct encoding *D. melanogaster* TollΔLRR, a constitutively active form of Toll lacking its extracellular leucine-rich repeat (LRR) domain, into S2 cells together with a Drs-luciferase reporter. As expected, transfection of this constitutively active TollΔLRR resulted in a marked luciferase expression[10], which was reduced by dsRNA targeting *Pelle*, a gene encoding a serine-threonine kinase required in the Toll pathway (**Fig. 3d** and ref. 10). However, dsRNA against either *Akirin* or *Imd* did not affect Drs-Luciferase expression, demonstrating that drosophila *Akirin* is not involved in the Toll pathway and eliminating the possibility that dsRNA against drosophila *Akirin* might affect luciferase expression itself.

We next undertook epistatic experiments to analyze the position of drosophila *Akirin* within the Imd pathway. For this, we transfected S2 cells with expression constructs encoding several genes of the Imd pathway—*PGRP-LE, Imd, Fadd, Dredd* and *Relish*—and monitored Att-Luc expression. Transfection of *PGRP-LE, Imd* and *Relish* constructs led to abundant Att-Luc expression (**Fig. 4a–c**). *Fadd* transfection led to a dominant-negative effect on *E. coli*–induced Att-Luc expression, whereas *Dredd* expression resulted in lower cell viability (data not shown). Notably, in *PGRP-LE*-transfected S2 cells, the enhanced Att-Luc expression was significantly decreased by transfection of dsRNA against either *Imd* or *Akirin* (~60% ($P = 0.001$)) and ~80% ($P = 0.007$), respectively; **Fig. 4a**). Expression of *Imd* also resulted in a robust Att-Luc expression that could be suppressed by both dsRNAs against *Akirin*, indicating that *Akirin* acts downstream of *Imd* (**Fig. 4b**).

As expression of Fadd and Dredd in S2 cells did not cause any Att-Luc expression, we decided to transfect the cells with a construct encoding the NF-κB family member Relish, which acts downstream in the Imd pathway. As shown earlier, transfection of a construct encoding full-length Relish only moderately activated the Imd

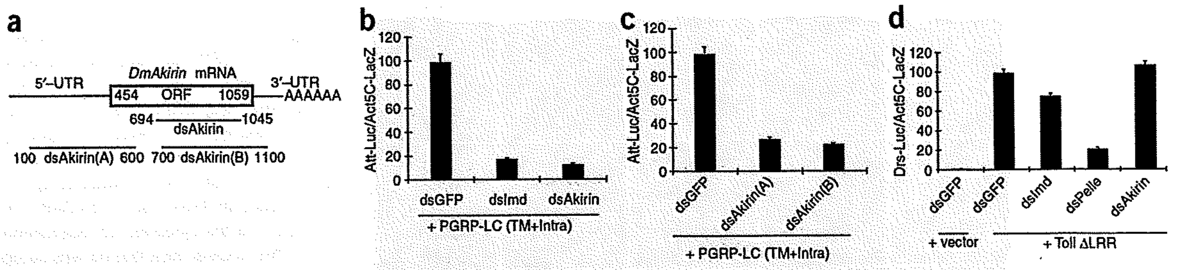

Figure 3 Effect of RNAi knock-down of drosophila *Akirin* on the activation of the Imd and Toll pathways in drosophila S2 cells. (**a**) Structure of *D. melanogaster Akirin* mRNA and of the dsRNAs against drosophila *Akirin*. An original dsRNA (dsAkirin) covering nucleotides 694–1045 was used for the screen. We synthesized two more dsRNAs, (dsAkirins(A) and (B)) covering nucleotides 100–600 and 700–1100, respectively. ORF, open reading frame. (**b**) S2 cells transfected with PGRP-LC (TM+Intra) constitutively express the Attacin-Luciferase (Att-Luc) reporter gene as an indicator of activation of the Imd pathway; this expression is lower in cells treated with dsAkirin than in control cells treated with dsGFP and is similar to that in cells treated with dsImd. (**c**) Both dsAkirin(A) and (B) suppressed the Att–Luc induction in the same way as the original dsAkirin. (**d**) S2 cells transfected with TollDLRR constitutively express the Drosomycin-Luciferase (Drs-Luc) reporter gene as an indicator of activation of the Toll pathway. In contrast to the expression in cells treated with dsPelle, this expression is unchanged in cells treated with dsImd and dsAkirin relative to that of control cells treated with dsGFP. Each bar represents the mean of three independent experiments. Error bars are s.d.

pathway, but a Relish construct deleted for the nucleotides encoding a serine-rich region (ΔS29–S45) led to a strong Att-Luc expression[11]. We confirmed this result (**Fig. 4c**) and further noted that the strong RelishΔS29–S45-dependent reporter gene induction was significantly suppressed by both dsRNAs against *Akirin* ($P = 0.0003$). This result indicated that *Akirin* acts downstream of or at the level of *Relish* (**Fig. 4c**), which is in agreement with the nuclear localization of Akirin.

Drosophila *Akirin* expression in S2 cells by itself did not activate the Imd pathway, as monitored by expression of Att-Luc, nor result in lower cell viability. Further, it did not show any dominant-negative effect against *E. coli* treatment (data not shown). To ascertain that the expressed *Akirin* construct was functional, we set up a rescue experiment. dsRNA against the *Akirin* 5′ untranslated region (UTR) was synthesized and shown to suppress activation of the Imd pathway in PGRP-LC transfected cells that actively expressed the reporter gene. However, when the coding sequence of *Akirin* devoid of its 5′ UTR—that is, the target of the dsRNA sequence—was coexpressed in the same cells, Att-Luc expression was rescued such that it was equivalent to wild-type expression. We could also rescue this phenotype with the human ortholog of *D. melanogaster Akirin*, *H. sapiens Akirin2*, clearly indicating that Akirin is functionally and evolutionary conserved (**Fig. 4d**).

To analyze the *in vivo* function of drosophila *Akirin*, we first generated null mutants by imprecise excision of EY08097, a P element located in the first intron of *CG8580*. Out of 430 lines, we isolated seven representing a deletion removing the *Akirin* gene. However, all deletion lines were homozygous embryonic lethal, indicating that *Akirin* is critically required during drosophila embryonic development (see Discussion). We next tried to knock down *Akirin* through a transgenic RNA interference (RNAi) approach[35]. We generated UAS-Akirin RNAi transgenic flies and crossed them with different GAL4 drivers (**Fig. 5**). *Akirin* knock down with heat-shock–GAL4 and yolk-GAL4 resulted in reduction of Imd pathway–dependent *Diptericin* gene expression after infection with a mix of Gram-positive and Gram-negative bacteria (**Fig. 5a,b**). Consistent with cell culture data (**Fig. 2d**), *Drs* expression was unchanged in these experiments (**Fig. 5a,c**), indicating that Toll pathway activation does not require *Akirin* function. Finally, RNAi-mediated knock-down of *Akirin* in whole flies led to enhanced sensitivity to Gram-negative bacterial infection (**Fig. 5d**).

Akirin loss-of-function mouse embryonic fibroblasts

To investigate whether the function of *Akirins* is conserved in the immune response between drosophila and mammals, we generated mice deficient in either the mouse *Akirin1* or the mouse *Akirin2* gene. A gene-targeting vector was constructed by placing two *loxP* sites flanking the first coding exon of the *Akirin1* gene and inserting a *loxP* site–flanked ('floxed') *neo*^{r} gene into intron 1 of the *Akirin1* gene

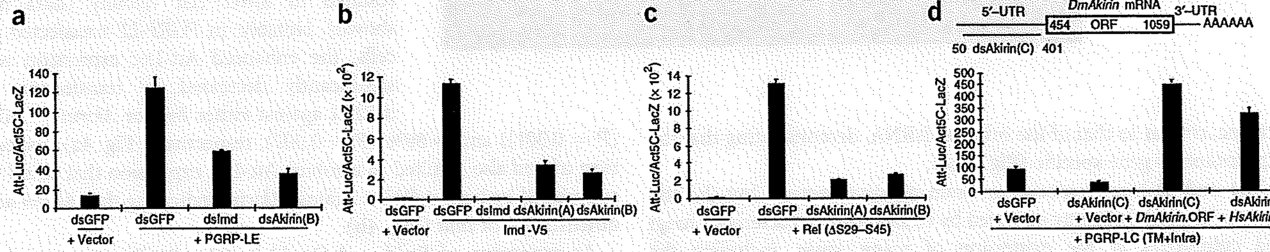

Figure 4 Epistatic analysis of *D. melanogaster* Akirin position within the Imd pathway. Constitutive activation of the Imd pathway induced by the transfection of S2 cells with PGRP-LE-V5 (**a**), Imd-V5 (**b**), Rel (ΔS29-S45; **c**) or PGRP-LC (TM+Intra; **d**) is highly compromised when cells are also treated with dsAkirin, as demonstrated by expression of the Att-Luc reporter gene ($P < 0.05$). (**d**) The compromised expression is restored by the coexpression in the same cells of the coding sequence of *D. melanogaster Akirin or of H. sapiens Akirin2*. Cells treated with vector alone serve as a control. Each bar represents the mean of three independent experiments (error bars, s.d.).

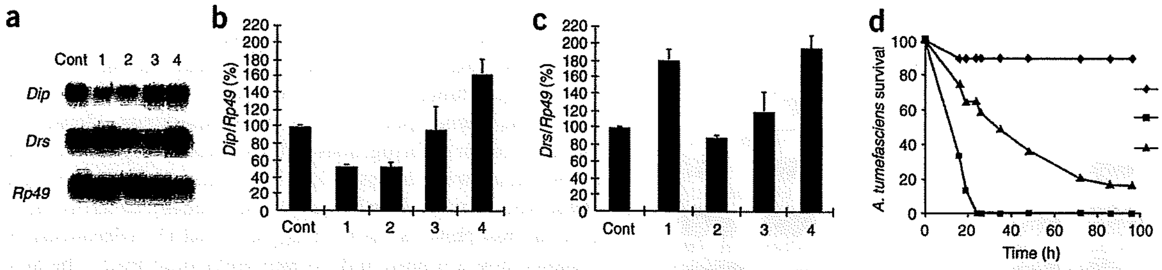

Figure 5 *In vivo* function of *D. melanogaster* Akirin. **(a)** The Imd and Toll pathway activations were monitored by RNA blot analysis of the *Diptericin* (*Dip*) and *Drosomycin* (*Drs*) messengers expression 6 h and 24 h, respectively, after infection with a mixture of Gram-positive and Gram-negative bacteria. The *Rp49* messenger was used as loading control. **(b,c)** Quantification of *Dip* **(b)** and *Drs* **(c)** normalized with *Rp49*. 1: *hs–GAL4/+;UAS–dsDmAkirin/+*, 2: *yolk–GAL4/+; UAS–dsDmAkirin/+* (females), 3: *CyO/+; UAS–dsDmAkirin/+*, 4: *yolk–GAL4/+; UAS–dsDmAkirin/+* (males). Homozygous *white*^{1118} flies were used as a control (cont). Each bar represents the mean of three independent experiments. Error bars are s.d. **(d)** Survival of adult flies infected with a Gram-negative bacterium (*Agrobacterium tumefasciens*). The Imd pathway mutant flies, *Relish*^{E20} (*Rel*), are highly sensitive to this bacterial infection. Compared with control (*white*^{1118}) flies, flies in which drosophila *Akirin* was knocked down showed an increased sensitivity to infection. Results are representative of three independent experiments.

(**Supplementary Fig. 5** online). We transiently transfected the targeted embryonic stem cells with a plasmid encoding the Cre protein to excise the *neo*^{r} gene. We then crossed *Akirin1*$^{flox/+}$ mice with a transgenic mouse line expressing Cre in germ cells (CAG-Cre mice). The deletion of the *Akirin1* gene was confirmed by Southern blot analysis (**Supplementary Fig. 5**). *Akirin1*$^{-/-}$ mice were born in a mendelian ratio, grew healthily and did not show gross developmental abnormalities. *Akirin1* mRNA was not expressed in mouse embryonic fibroblasts (MEFs) obtained from *Akirin1*$^{-/-}$ mice (**Supplementary Fig. 5**).

To generate an *Akirin2* flox allele, we constructed a targeting vector inserting two *lox*P sites flanking the first coding exon of the mouse *Akirin2* gene, with a *lox*P site–flanked *neo*^{r} gene (**Supplementary Fig. 6** online). The targeted embryonic stem cells were transiently transfected with a plasmid encoding Cre to eliminate *neo*^{r}. *Akirin2*$^{+/-}$ mice were obtained by mating *Akirin2*$^{flox/+}$ mice with CAG-Cre mice. In contrast to *Akirin1*$^{-/-}$, *Akirin2*$^{-/-}$ was embryonic lethal, and we did not find *Akirin2*$^{-/-}$ embryos even on embryonic day 9.5, indicating that the *Akirin2* gene is essential for normal embryonic development in mice (**Supplementary Table 1** online). Thus, we generated MEFs from *Akirin2*$^{flox/+}$ and *Akirin2*$^{flox/-}$ embryos and excised the *lox*P-flanked genomic fragment by retroviral expression of the Cre protein together with the puromycin resistance gene (*Puro*). We examined puromycin-resistant cells for the expression of *Akirin2* by RT-PCR. The expression of *Akirin2* was suppressed in Cre-transduced *Akirin2*$^{flox/-}$ (*Akirin2*$^{-/-}$) MEFs (**Supplementary Fig. 6**). This enabled us to analyze MEFs specifically lacking *Akirin1* or *Akirin2*.

Mouse *Akirin2* in IL-1β- and TLR-mediated responses

As drosophila Akirin was critical for the Imd pathway, which activates NF-κB through the IKK complex similarly to the mammalian TNF signaling pathway, we hypothesized that mouse Akirins could likewise be involved in TLR-, IL-1β- and TNF-mediated responses. We first examined the production of cytokines in *Akirin1*$^{-/-}$ MEFs in response to TLR ligands, IL-1β and TNF. The production of IL-6 was similar in wild-type and *Akirin1*$^{-/-}$ MEFs in response to all stimuli tested (**Fig. 6a**). However, when *Akirin2*$^{-/-}$ MEFs were stimulated with TLR ligands (MALP-2 and lipopolysaccharide (LPS)), IL-1β and TNF, production of IL-6 was much less than in control *Akirin2*$^{+/-}$ MEFs (**Fig. 6b**). Thus, Akirin2, but not Akirin1, was responsible for the production of IL-6 in response to TLR or IL-1R activation.

Next we examined whether Akirin2 regulated IL-6 production at the level of gene expression. LPS-induced expression of genes encoding IL-6, IP-10, RANTES and BCL3 two hours after challenge was severely impaired in *Akirin2*$^{-/-}$ MEFs relative to that in control cells, indicating that Akirin2 is critical for the expression of several LPS-inducible genes (**Fig. 7a**). However, the induction of genes encoding IκBα, IκBζ and the CXCL1 chemokine KC was similar in *Akirin2*$^{-/-}$ and control MEFs. The gene induction in response to IL-1β stimulation was similarly impaired in *Akirin2*$^{-/-}$ MEFs (**Fig. 7b**). Thus, mouse Akirin2 regulates the expression of a set of LPS- and IL-1β-inducible genes.

As drosophila Akirin acts together with or downstream of Relish, we next examined the IL-1β- and LPS-dependent activation of NF-κB

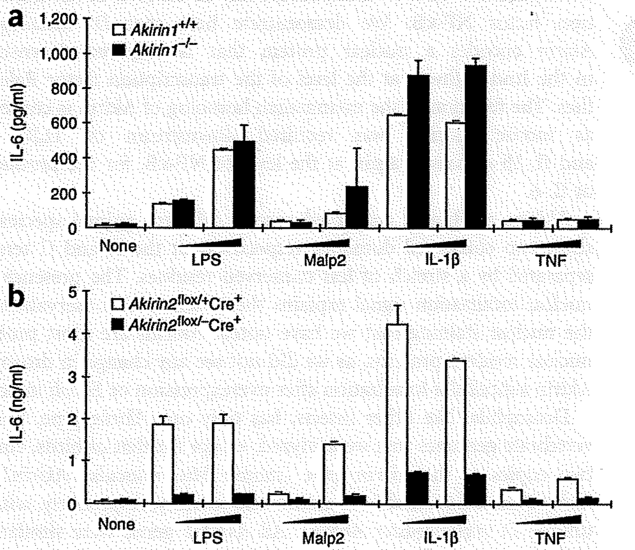

Figure 6 TLR-, IL-1β- and TNF-induced IL-6 production in *Akirin1*$^{-/-}$ and *Akirin2*$^{-/-}$ mouse embryonic fibroblasts (MEFs). **(a,b)** IL-6 concentrations in *Akirin1*$^{+/+}$ and *Akirin1*$^{-/-}$ MEFs **(a)** and Cre-transduced *Akirin2*$^{flox/+}$ and *Akirin2*$^{flox/-}$ MEFs **(b)** stimulated with increasing concentrations of LPS (1, 10μg/ml), MALP-2 (1, 10 nM), IL-1β (1, 10 ng/ml) and TNF (1, 10 ng/ml) for 24 h. Unlike IL-6-induced production in *Akirin*$^{-/-}$ MEFs, that in *Akirin2*$^{-/-}$ MEFs is reduced compared with corresponding wild-type control cells. Each bar represents the mean of three independent experiments. Error bars are s.d.

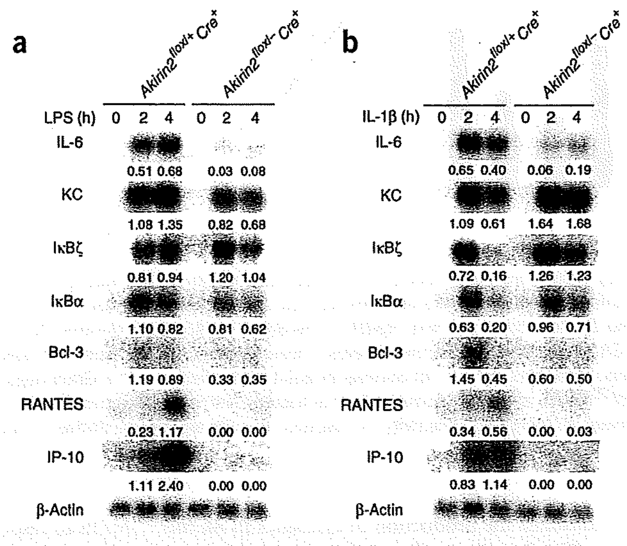

Figure 7 LPS- and IL-1β-induced gene expression in *Akirin2*$^{-/-}$ MEFs. **(a,b)** Total RNA blot analysis of the expression of IL-6, KC, IκBζ, IκBα, BCL3, RANTES and IP-10 in Cre-transduced *Akirin2*$^{flox/+}$ and *Akirin2*$^{flox/-}$ MEFs stimulated with LPS (10 μg/ml) **(a)** or IL-1β (10 ng/ml) **(b)** for 2 and 4 h. The β-actin messenger is used as loading control. Signals were quantified, and values indicate relative density compared with the corresponding loading control. The expression of several LPS- and IL-1β-inducible genes is reduced in *Akirin2* deficient MEFs compared with wild-type control cells. Results are representative of three independent experiments.

in *Akirin2*$^{-/-}$ MEFs. In response to these stimuli, neither degradation of IκBα (**Fig. 8a,b**) nor induction of NF-κB DNA binding (**Fig. 8c,d**) was impaired in *Akirin2*$^{-/-}$ MEFs. These data indicated that mouse Akirin2 acts together with or downstream of NF-κB in the control of TLR- and IL-1β-inducible gene expression.

DISCUSSION

Akirins represent previously unknown, extremely conserved, nuclear factors that are involved in the metazoan innate immune system. Akirins function during immune and inflammatory responses in drosophila as well as in mice, most likely at the level of the transcription factor NF-κB. We demonstrate here that *D. melanogaster Akirin* encodes a nuclear protein that is required downstream in the Imd pathway at the level of the transcription factor Relish in flies. The function of the mammalian homolog of Akirin is conserved, as mouse Akirin2 was required downstream of TLR, TNF and IL-1β signaling, again at the level of NF-κB, for the production of IL-6.

Akirins are highly conserved among different animal species and show two conserved domains, respectively at the N and C termini, separated by a stretch of less conserved residues. The presence of a nuclear localization signal explains the N-terminal conservation and the nuclear staining that we have noted. Akirins are most probably nuclear resident proteins, as we did not see any change in drosophila Akirin subcellular localization after overexpression or *E. coli* infection.

Drosophila, like other insects, has only one *Akirin* gene, but the vertebrate genomes that we analyzed, except for that of birds, contain two copies of the *Akirin* gene (mouse *Mus musculus Akirin1* and *Akirin2* show 34% and 39% amino acid identity, respectively, with the unique *D. melanogaster Akirin*). All *Akirin1* genes were similar and segregated from the group containing the *Akirin2* genes, indicating an early duplication event followed by divergence in the evolution of vertebrates. Birds would then have secondarily lost the *Akirin1* gene. The diverging function between Akirin1 and 2 was attested to by the contrasting phenotypes of mouse *Akirin* knockouts. Mouse *Akirin2* was essential for embryogenesis and the cytokine response to TLR and IL-1R stimulation, whereas *Akirin1* knockout mice showed no obvious phenotype. Mouse *Akirin2* would be functionally closer to the single gene in drosophila, as the homozygously null *D. melanogaster Akirin* mutants show a similar, mid- to early embryonic death. The function of mouse Akirin1, which is clearly an Akirin on the basis of its sequence conservation, is unknown. It is possible that mouse Akirin1 and Akirin2 work redundantly in the regulation of target gene expression in MEFs. Generation of cells lacking both *Akirin1* and *Akirin2* will help to elucidate the function of Akirin1 *in vivo*.

Both drosophila Akirin and mammalian Akirin2 regulate the expression of a set of genes together with or downstream of NF-κB. These results imply that both drosophila and mammalian Akirins associate with similar protein(s) for controlling gene expression in the nucleus. Transcription by RNA polymerase II involves the cooperative assembly of an initiation complex, which is restrained by the incorporation of promoter DNA into nucleosomes and other chromatin structures. Transcription is then modulated by chromatin remodeling cofactors targeting the nucleosomes or by general cofactors that associate with the basal transcription machinery. It is unlikely that Akirins regulate transcription by binding directly to DNA, as Akirin sequences show no obvious DNA- or RNA-binding motifs. According to Occam's razor principle, the prediction would be that Akirins act as cofactors to regulate or fine-tune NF-κB transcriptional activity by interacting with components of the chromatin or the transcriptional engine. We tested the hypothesis of a direct interaction of drosophila Akirin with DNA or Relish, but we could not precipitate DNA in chromatin immunoprecipitation assays with tagged Akirin or Akirin with a tagged Relish (data not shown), which means that the postulated associations are either weak or most probably require intermediary components. The notion that Akirins could function to modulate transcriptional factors in several other immune-related processes is strengthened by the report that drosophila *Akirin* was found as interacting genetically with *pannier*, one of the GATA factors involved in heart and blood cell development[36]. Along the same line, after another genome-wide RNAi screen, drosophila *Akirin* appeared in a list of putative modulators of the Wingless pathway[37], which was recently shown to be involved in the inflammatory response[38]. Taken

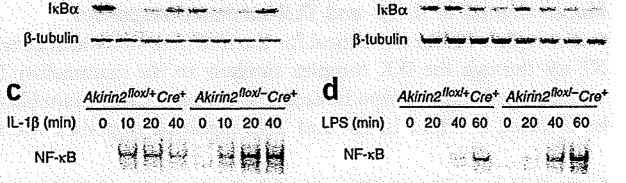

Figure 8 LPS- and IL-1β-induced activation of NF-κB in *Akirin2*$^{-/-}$ MEFs. **(a,b)** IκBα expression in the whole cell lysates, analyzed by immunoblotting. **(c,d)** NF-κB-DNA binding activity in the nuclear extracts, determined by electrophoretic mobility-shift assay. Cre-transduced *Akirin2*$^{flox/+}$ and *Akirin2*$^{flox/-}$ MEFs were stimulated with IL-1β (10 ng/ml) **(a,c)** or LPS (10 μg/ml) **(b,d)** for the indicated periods. IκBα degradation and NF-κB-DNA binding activity were similar in wild-type and *Akirin2*-deficient cells after IL-1β and LPS stimulations. Results are representative of three independent experiments.

together, these results indicate that Akirins are previously unknown, important nuclear cofactors regulating the transcriptional activities of main transactivators. However, further studies are needed to clarify how Akirins control gene expression in the Imd and the TLR–IL-1R pathways.

METHODS

DNA constructs. The expressed sequence tag clone of *PGRP-LCa* (LP06704) was obtained from MRC geneservice. We subcloned all constructs into the *Bam*HI–*Kpn*I sites of the expression vector pPAC (ref. 39). A PCR fragment of *PGRP-LCa* was amplified with forward 5′-CCCCGGATCCGATAATTCCCGCCATGCCTTTTAGCAATGAAACG-3′ and reverse 5′-GGGGGGTACCTCAGTTCAACGTCTTTCCGAAGAG-3′ primers. The *PGRP-LE-V5* fragment was obtained from *UAS-PGRP-LE* transgenic flies[20] with forward 5′-CCCCGGATCCGATAATTCCCGCCATGTCCGAATCGGGAATC-3′ and reverse 5′-GGGGGGTACCTCAGGTGGAATCCAGGCCCAGCAGGGGGTTGGGGATGGCTTGCCTTGTTCCTCCTCCTCGATATTG -3′ primers. The V5-tagged *D. melanogaster Akirin* vector was constructed with forward 5′-CCCCGGATCCGATAATTCCCGCCATGGCCTGTGCAACCCTGAAAC-3′ and reverse 5′-GGGGGGTACCTCAGGTGGAATCCAGGCCCAGCAGGGGGTTGGGGATGGGCTTGCCCGACAGGTAGCTAGGCGCTG-3′ primers. The NLS was deleted from V5-tagged drosophila *Akirin* with the 5′-CTAGACTGGGAGTCGATCAACCGTTGCAATCCCTTTGGCCAG-3′ primer. The Imd-V5 construct was obtained by exchanging the tag in an Imd-hemagglutinin construct[25]. *H. sapiens Akirin2* was amplified with forward 5′-CCCCGGATCCGATAATTCCCGCCATGGCGTGCGGAGCCACTCTG-3′ and reverse 5′-GGGGGGTACCTCATGAAACATAGCTAGCAGGC-3′ primers. *Relish* constructs were from ref. 11 and *TollΔLRR* construct from ref. 10. *UAS-dsDmAkirin* fly stocks were established as in ref. 35 with 5′-GGGGCCGGATCCATGGCCTGTGCAACCC-3′ and 5′-GGGGCCGCTAGCTTACGACAGGTAGC-3′ primers. N-terminal deletions from *H. sapiens Akirin1* and *H. sapiens Akirin2* were constructed by PCR with the following primers: 5′-AGCTTGGCTCCCCGAAGCGGCGGCGCTGC-3′ (Δ20), 5′-AAGCTTCTGCCCGGCCCCACTCCGGGCCTC-3′ (Δ30), 5′-AAGCTTTCCCCGAAGCGCAGGCGATGTGCG3′ (Δ20) and 5′-AAGCTTTCGGCGCCCACCTCGGCCGCTGCC-3′ (Δ30), respectively.

Sequence analysis. We retrieved sequences by homology search with BLAST with the *D. melanogaster CG8580* from the US National Center for Biotechnology Information (NCBI) database, except for *Bombyx mori*, for which we used SilkBase (http://morus.ab.a.u-tokyo.ac.jp/). The sequences were as follows: *D. melanogaster* Akirin, NP_648113; *Anopheles gambiae* Akirin, XP_308938, modified; Akirin for *Bombyx mori* Akirin, wdS20131; *Apis mellifera* Akirin, XP_395252; *Tribolium castaneum* Akirin, XP_971340; *Gallus gallus* Akirin, XP_419845; *H. sapiens* Akirin1, NP_078871; *Mus musculus* Akirin1, NP_075912; *Xenopus laevis* Akirin1, AAH72831; *Danio rerio* Akirin1, NP_001007187; *H. sapiens* Akirin2, NP_060534; *Mus musculus* Akirin2, NP_001007590; *Xenopus laevis* Akirin2, AAH72831; and *Danio rerio* Akirin2, NP_998707. Sequences were aligned with MULTALIN[40] (**Supplementary Fig. 1**). Subsequent assembly into a majority consensus minimum evolution bootstrap tree was made with the MEGA3 software[41].

Cell culture and transfection assays. Akirin was identified in a large-scale RNAi screen as previously described[27,33]. In brief, 384-well screening plates were prespotted with approximately 75 nM dsRNA in 5 μl of 1 mM Tris at pH 7. Hemocyte-like Kc167 cells were batch-transfected with an IMD-specific *mtk*-luciferase reporter[27], a truncated form of PGRP-LC and a constitutive expressed *Renilla* luciferase and transferred to dsRNA-containing screening plates. Then 15,000 cells in 20 μl were dispensed per well and incubated for 1 h before the addition of serum-containing medium. After 5 d, medium was removed, cells were lysed and both firefly and *Renilla* luciferase activities were determined.

Akirin was also identified in IMD-pathway experiments in S2 cells (as described in ref. 27). S2 cells (Invitrogen and DGRC) were grown at 23 °C in Schneider's medium (Biowest) supplemented with 10% FCS. Cells (1.2×10^6/ml) were transfected in 24-wells plates by calcium phosphate precipitation with 10 μg of *Attacin (Att)*-luciferase or *Drosomycin (Drs)*-luciferase reporter vector, 10 μg of an *Actin5C-lacZ* transfection control vector and dsRNAs (1.0 μg/well). After 12–16 h, the cells were washed with PBS and incubated in fresh medium. Cells were stimulated by heat-killed *E. coli* (~20–30 bacteria per cell) the next day. After 12–16 h of *E. coli* stimulation, cells were lysed and luciferase activity was measured in a luminometer (BCL Book, Promega) immediately after addition of the substrate (luciferin, Promega). β-Galactosidase activity was measured with *O*-nitrophenyl-β-D-galactoside as a substrate, and the values were used to normalize variability in transfection efficiency. For epistatic analysis various amounts (0.001, 0.002, 0.01, 0.02, 0.2 or 0.5 μg per well) of expression vectors were used. For rescue experiments, 0.75 μg of Akirin, 0.025 μg of *PGRP-LC* and 0.25 μg of dsRNAs were transfected. All experiments were done more than twice independently with duplicate wells.

dsRNA preparation. Templates for dsRNA preparation were PCR-derived fragments between two T7 promoter sequences. Fragments for each gene were as follows: *GFP* (nucleotides 35–736, GenBank accession L29345), *Key* (nucleotides 222–744, NCBI accession NM_079132), *Imd* (nucleotides 331–1015, NCBI accession NM_133166) *Akirin* (nucleotides 50–401, 100–600, 694–1045, 700–1100; GenBank accession number AY095189) and *PGRP-LCa*: LP06704 (nucleotides 318–1028, NCBI accession AY119048). Single-stranded RNAs were synthesized with the MEGAscript T7 transcription kit (Ambion). Annealed dsRNAs were ethanol precipitated and dissolved in injection buffer (0.1 mM sodium phosphate, pH 6.8; 5 mM KCl).

Cell staining. S2 cells were fixed 3 d after transfection with 2% paraformaldehyde in PBS for 15 min. Cells were then permeabilized with 0.1% Triton X-100, 1% BSA, PBS for 1 h, incubated overnight with monoclonal antibody to V5 (Invitrogen; 500-fold dilution in PBT: PBS containing 0.1% Tween 20), washed and incubated with fluorescein isothiocyanate–conjugated anti-mouse IgG (500-fold dilution in PBS, Jackson ImmunoResearch). Cells were stained with DAPI in PBS to visualize nuclei and examined with a Zeiss Axioskop 2 microscope.

Microbial infection, survival experiments and RNA blot analysis. We used the following bacterial strains: *E. coli* (1106), *Micrococcus luteus* (CIP A270) and *Agrobacterium tumefasciens*. Survival experiments were carried out as previously described[42]. For RNA blot analysis, flies were challenged with a thin tungsten needle previously dipped into a concentrated culture of mixed Gram-positive (*M. luteus*) and Gram-negative (*E. coli*) bacteria. After 6 h (for *Dip*) or 24 h (for *Drs*), flies were collected. MEFs (1×10^6) were stimulated with 10 ng/ml of IL-1β or 10 mg/ml of LPS for 2 or 4 h. Total RNA was extracted with TRIzol (Invitrogen). RNA (20 μg for flies; 10 μg for MEFs) was electrophoresed, transferred to nylon membrane (Hybond N+; Amersham Pharmacia Biotech) and hybridized with specific cDNA probes for *Dip, Drs, Il6, Nfkbia, Nfkbiz, Bcl3, Ccl5, Cxcl1* and *Cxcl10*. The same membrane was stripped and rehybridized with an *Rp49* (flies) or an *Actb* cDNA probe as internal control. Signals were quantified with BAS 2000 Image Analyzer (Fuji) for fly RNA data and with NIH Image software (US National Institutes of Health) for mouse RNA data.

Fly strains and crosses. Flies were grown on standard medium at 25 °C. Drosophila Gal4 driver stocks are described in ref. 43. We used *Relish*^{E20} and *white*^{1118} as Imd pathway mutant and wild-type control, respectively. Transgenic *w*^{1118}; +/+; *UAS-dsDmAkirin*/*TM3* males were crossed with either *w*^{1118}; *heat-shock (hs)-GAL4*/*CyO*; +/+, or *w*^{1118}; +/+; *yolk-GAL4* females and the progeny kept at 29 °C.

Establishment of *Akirin2*$^{-/-}$ MEFs. We obtained MEFs from embryonic day 13.5 *Akirn2*$^{flox/+}$ or *Akirin2*$^{flox/-}$ embryos. To excise the floxed genomic fragment containing exon 1, we infected the MEFs with retrovirus expressing Cre protein together with puromycin-resistance gene product. At 24 h after infection, we added 3 mg/ml of puromycin (Invivogen) and grew the cells under this selection for 72 h. Then the MEFs were used for analysis. All animal experiments were done with the approval of the Animal Research Committee of the Research Institute for Microbial Diseases (Osaka University, Osaka, Japan).

Measurement of IL-6 production. MEFs (2×10^4) were stimulated with 0.1 and 1 μg/ml of recombinant mouse IL-1β (R&D Systems), 10 μg/ml of LPS

(Sigma), 1 and 10 nM of MALP-2 or 1 and 10 ng/ml of recombinant mouse TNF (R&D Systems) for 24 h. We collected culture supernatants and measured IL-6 concentrations with the ELISA kit (R&D Systems).

Immunoblot analysis. MEFs (2×10^6) preincubated in FBS-free medium for 1 h were stimulated with 10 ng/ml of IL-1β in FBS-free medium or 10 mg/ml of LPS in medium containing 0.3% FBS for various periods. MEFs were then lysed in a lysis buffer containing 1.0% Nonidet-P40, 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA and a protease inhibitor cocktail (Roche). Lysates were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (BioRad). Membranes were probed with antibodies and visualized with an enhanced chemiluminescence system (Perkin-Elmer). Polyclonal antibody to IκBα (anti-IκBα and HRP-conjugated monoclonal anti-β-tubulin (clone D-10) were purchased from Santa Cruz. Monoclonal anti–phosho-p65 (Ser536) (clone 7F1) was purchased from Cell Signaling.

Electrophoretic mobility-shift assay. MEFs (2×10^6) preincubated in FBS-free medium for 1 h were stimulated with 10 ng/ml of IL-1β in FBS-free medium or 10 mg/ml of LPS in medium containing 0.3% FBS for various periods. Nuclear extracts were purified from cells, incubated with a probe specific for NF-κB DNA-binding sites, separated by electrophoresis and visualized by autoradiography.

Additional methods. Information on multiple-tissue RNA blot analysis and the generation of *Akirin*$^{-/-}$ and *Akirin*$^{\mathrm{flox/flox}}$ mice is available in the **Supplementary Methods** online.

Statistical analysis. Mean values and s.d. were calculated with Excel software (Microsoft).

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

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AUTHOR CONTRIBUTIONS

A.G., V.G., L.E.C. and D.K. did the drosophila experiments. K.M. and O.T. did the mouse experiments. S.A., M.B., O.T. and J.-M.R. conceived and directed the experiments. A.G., O.T., J.A.H. and J.-M.R. wrote the paper. All authors contributed to manuscript criticism.

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Corrigendum: Akirins are highly conserved nuclear proteins required for NF-κB-dependent gene expression in drosophila and mice

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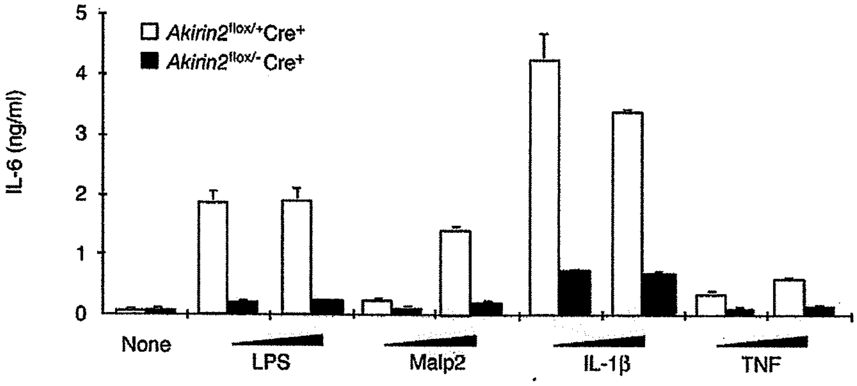
In the version of this article initially published, the bars for the LPS samples in Figure 6b are incorrect. The correct data are presented here. The error has been corrected in the HTML and PDF versions of the article.



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LETTERS

TANK-binding kinase-1 delineates innate and adaptive immune responses to DNA vaccines

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Successful vaccines contain not only protective antigen(s) but also an adjuvant component that triggers innate immune activation and is necessary for their optimal immunogenicity[1,2]. In the case of DNA vaccines[3], this consists of plasmid DNA; however, the adjuvant element(s) as well as its intra- and inter-cellular innate immune signalling pathway(s) leading to the encoded antigen-specific T- and B-cell responses remain unclear. Here we demonstrate *in vivo* that TANK-binding kinase 1 (TBK1), a non-canonical IκB kinase, mediates the adjuvant effect of DNA vaccines and is essential for its immunogenicity in mice. Plasmid-DNA-activated, TBK1-dependent signalling and the resultant type-I interferon receptor-mediated signalling was required for induction of antigen-specific B and T cells, which occurred even in the absence of innate immune signalling through a well known CpG DNA sensor—Toll-like receptor 9 (TLR9) or Z-DNA binding protein 1 (ZBP1, also known as DAI, which was recently reported as a potential B-form DNA sensor[4]). Moreover, bone-marrow-transfer experiments revealed that TBK1-mediated signalling in haematopoietic cells was critical for the induction of antigen-specific B and CD4$^+$ T cells, whereas in non-haematopoietic cells TBK1 was required for CD8$^+$ T-cell induction. These data suggest that TBK1 is a key signalling molecule for DNA-vaccine-induced immunogenicity, by differentially controlling DNA-activated innate immune signalling through haematopoietic and non-haematopoietic cells.

To develop optimal vaccines for clinical applications, it is important to understand the mechanisms of their actions on immune systems in terms of efficacy as well as safety. In particular, the innate immune recognition of the adjuvant element of vaccine formulations had been shown to be critical for its immunogenicity[2]. Many adjuvants, such as monophosphoryl lipid A and CpG DNA, seem to be ligands for TLRs[5,6]. In contrast, some conventional adjuvants, including aluminium hydroxide (alum) and incomplete Freund's adjuvant, as well as unconventional adjuvant-containing vehicles, such as apoptotic cells, are free of TLR ligand[7,8], suggesting that multiple innate immune recognition and signalling pathways are required for an adjuvant to function.

In the case of DNA vaccines, which have been shown to elicit humoral[9] and cellular[10] immune responses, unmethylated CpG motifs expressed within a plasmid backbone have been considered to be 'built-in' adjuvants, owing to their ability to activate the innate immune system by means of TLR9 (ref. 11). TLR9-deficient mice, however, mounted humoral and cellular immune responses to the encoded antigen comparable to those of wild-type mice[12,13]. Although another report showed a partial reduction of immune responses to a DNA vaccine in TLR9-deficient mice[14], the molecular and/or cellular mechanisms underlying the adjuvant effect and element(s) of DNA vaccines have not been fully clarified[15]. To address this issue, we used an optimized immunization protocol for DNA vaccination by electroporation as described previously[16,17]. After DNA vaccination, mice lacking TLR9 or its essential adaptor, MyD88, mounted both humoral and cellular immune responses to DNA vaccines comparable to those of wild-type mice (Supplementary Figs 1a–d and 2). Moreover, plasmid DNA electroporation activated dendritic cells to produce type-I interferons (IFNs) and inflammatory cytokines in a TLR9-independent manner (Supplementary Fig. 1e and data not shown). Although the immunogenicity of DNA vaccines may vary due to many factors such as the quality of plasmid DNA, injected sites, injection methods or modification of CpG motifs within plasmid DNA[11], our results support previous findings[12,13] indicating that TLR9-mediated recognition of plasmid DNA and subsequent signalling are not essential for optimal DNA vaccination.

Recent accumulating evidence, in contrast, suggests that the double-stranded structure of DNA, independently of CpG motifs, possesses immunomodulatory effects when introduced into the cytosol or its homeostatic clearance is hampered[18]. Intracellular administration of double-stranded B-form DNA (B-DNA) triggers TLR-independent, TBK1- and interferon regulatory factor 3 (IRF3)-dependent innate activation of both immune and non-immune cells to produce type-I IFNs and their inducible genes[19–21]. On the basis of these results, we hypothesized that the immunogenicity of DNA vaccines may be controlled by these TLR9-independent immunostimulatory activities of B-DNA as 'built-in' adjuvant(s), thereby prompting us to investigate whether TBK1-mediated innate immune activation contributes to DNA vaccine immunogenicity.

Because induction of type-I IFNs is a hallmark of TLR9-independent innate immune activation by B-DNA[19–21], which has been shown to have an important role in the following adaptive immune responses[22,23], we initially examined the effect of type-1 IFNs on the immunogenicity of DNA vaccines. IFN-αβR-deficient mice that lack type-I IFN-mediated signalling (*Ifnar2*$^{-/-}$) and wild-type mice were immunized with plasmid DNA encoding LacZ or the influenza NP protein. After immunization, wild-type but not *Ifnar2*$^{-/-}$ mice elicited strong T- and B-cell responses to LacZ, including serum LacZ-specific immunoglobulin (Ig)Gs (Fig. 1a, b), spleen CD8$^+$ T-cell frequency and IFN-γ secretion (Fig. 1c, d). Similarly, NP-specific IFN-γ production by CD4$^+$ or

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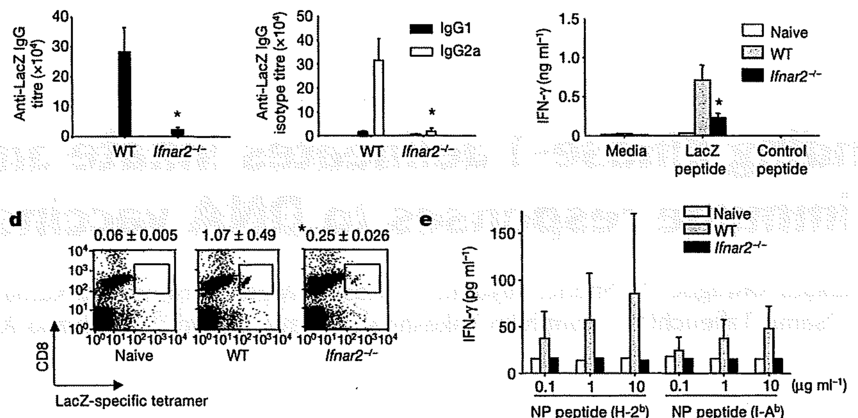

Figure 1 | Optimal DNA vaccine immunogenicity requires type-I interferons. Mice lacking type-I IFN signalling (*Ifnar2*$^{-/-}$) or wild-type (WT) mice were immunized twice by i.m. electroporation with a DNA vaccine encoding LacZ (**a–d**) or influenza A virus NP (**e**) protein at intervals of 4 weeks. The serum titres of anti-LacZ IgG (**a**), of IgG1 or IgG2a (**b**), and of spleen LacZ-antigen-specific IFN-γ production (**c**), as well as the frequency of CD8$^+$ T cells (**d**; average % of the gated population ± s.d.), were measured two weeks after the second immunization. Similarly, NP-specific IFN-γ production in the immunized spleen in response to NP peptides (I-A^b or H-2^b) for CD4$^+$ or CD8$^+$ T cells, respectively, was also measured (**e**). Data are the averages ± s.d. of 3–5 mice per group; *$P < 0.01$ against wild-type mice.

CD8$^+$ T-cell-specific peptide was dependent on IFN-αβR (Fig. 1e). These results indicated that signalling by type-I IFNs is indispensable among the critical factors for DNA-vaccine-induced immunogenicity, although minimal immune responses were still observed in *Ifnar2*$^{-/-}$ mice.

We confirmed *in vitro* that plasmid DNA electroporation activated bone-marrow-derived dendritic cells to produce type-I IFNs in a TBK1-dependent manner (Supplementary Fig. 3a, b and data not shown), consistent with our previous findings with cationic liposomes[20]. To examine the role of TBK1 in DNA vaccination *in vivo*, we used TBK1-deficient mice on a *Tnf*$^{-/-}$ background[24]. Although *Tbk1*$^{-/-}$ mice die *in utero*, this lethal effect can be resuscitated in the absence of tumour necrosis factor (TNF)[24]. An advantage of using these mice is that deficiency of TBK1 or of TNF does not influence TLR9-dependent innate immune activation and vice versa[20]. Wild-type and *Tnf*$^{-/-}$ mice elicited comparable antigen-specific immune responses to the encoded antigen after DNA vaccination (Figs 2a and 3a, and data not shown). In contrast, however, *Tbk1*$^{-/-}$ mice on a *Tnf*$^{-/-}$ background failed to increase the frequency and cytotoxicity of antigen-specific CD8$^+$ T cells and spleen IFN-γ production

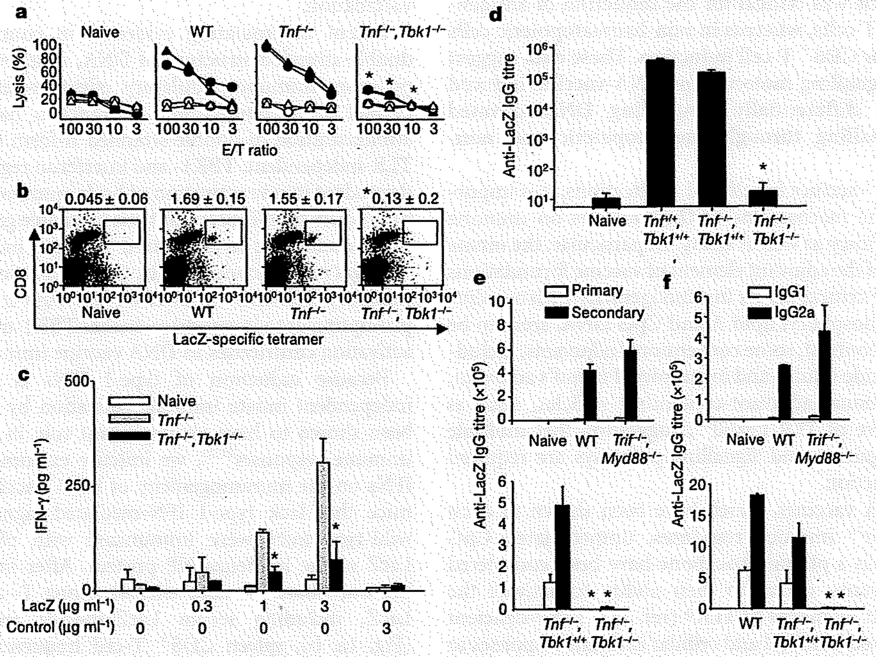

Figure 2 | *Tbk1*$^{-/-}$ mice failed to elicit antigen-specific T- and B-cell responses after DNA vaccination. Mice (five per group) deficient for TNF, TNF and TBK1 or MyD88 and TRIF were immunized with a DNA vaccine encoding LacZ protein on days 0 and 28 by intramuscular electroporation, as described in the Methods. Fourteen days after the second immunization, the antigen-specific CTL activity (**a**, representative individual data with (filled symbol) or without (open symbol) LacZ peptide), the frequency of CTL (**b**, positive for LacZ-specific tetramer, in which the number is the average percentage of the gated population ± s.d.) and the LacZ-protein-specific IFN-γ production (**c**) by spleen cells were analysed. LacZ-specific IgG titre (**d–f**), including their isotypes, were analysed by ELISA. Data are the averages ± s.d. of five mice per group; *$P < 0.01$ against control (*Tbk1*$^{+/+}$) mice. E/T ratio, effector to target ratio.

(Fig. 2a–c). Moreover, *Tbk1*$^{-/-}$, but not control (*Tnf*$^{-/-}$) or *Myd88*$^{-/-}$,*Trif*$^{-/-}$ (deficient for any known TLR signalling), mice immunized with a different DNA vaccine encoding influenza A virus NP protein also failed to induce IFN-γ-producing spleen cells in response to NP peptides specific to both CD4^{+} and CD8^{+} T cells (Supplementary Fig. 4a). To examine whether *Tbk1*$^{-/-}$ T cells are able to induce TLR9-adjuvanted immune responses or not, we immunized control (*Tnf*$^{-/-}$) and *Tbk1*$^{-/-}$ mice with a vaccine consisting of a NP peptide and CpG ODN, the adjuvant effect of which is totally dependent on TLR9 (ref. 25). Both control (*Tnf*$^{-/-}$) and *Tbk1*$^{-/-}$ immunized mice showed antigen-specific IFN-γ production and cytotoxicity of spleen cells (Supplementary Fig. 4b, c), suggesting that the TLR9-mediated adjuvant effects of CpG ODN are intact in the absence of TBK1 and TNF. The normal functions of *Tbk1*$^{-/-}$ T cells and dendritic cells were further confirmed by several assays, because *Tbk1*$^{-/-}$ T cells respond normally to anti-CD3 and anti-CD28 (Supplementary Fig. 5a and b), and *Tbk1*$^{-/-}$ dendritic cells had intact antigen-presenting functions (Supplementary Fig. 5c). These data clearly demonstrate that TBK1-dependent signalling, but not TLR signalling, is essential for DNA-vaccine-induced T-cell responses to the encoded antigen.

We next examined the role of TBK1 in the humoral responses elicited by DNA vaccination. When wild-type and control (*Tnf*$^{-/-}$) mice were immunized with DNA vaccine, their IgG titres against the encoded LacZ protein were significantly augmented in serum; however, titres in *Tbk1*$^{-/-}$ mice were reduced to the level observed in naive mice, nearly 4 log lower than those in control (*Tnf*$^{-/-}$) or wild-type mice (Fig. 2d). This was the case for either primary or secondary immune responses including isotypes (Fig. 2e, f), whereas those in *Myd88*$^{-/-}$ or *Trif*(also called Ticam 1)$^{-/-}$ mice were comparable to those in wild-type mice (Fig. 2e, f). This was not due to malfunction of *Tbk1*$^{-/-}$ B cells, because the levels of total serum IgG, including IgG1 and IgG2a, were at comparable levels to those of wild-type mice (Supplementary Fig. 5d). Taken together, these results strongly suggest that TBK1 is required for the induction of both humoral and cellular immune responses by DNA vaccination *in vivo*.

To elucidate further the intercellular mechanism(s) by which the TBK1-mediated signalling contributes to DNA-vaccine immunogenicity, we next examined the role of TBK1 signalling in haematopoietic and non-haematopoietic cells by transferring the bone marrows of *Tbk1*$^{+/+}$ or *Tbk1*$^{-/-}$ mice into *Tbk1*$^{-/-}$ or *Tbk1*$^{+/+}$ mice on a *Tnf*$^{-/-}$ background, respectively. When *Tbk1*$^{+/+}$ or *Tbk1*$^{-/-}$ chimaeric mice with *Tbk1*$^{-/-}$ bone marrow were immunized with DNA vaccines, the antigen-specific IgG and IFN-γ production were significantly impaired compared with those in *Tbk1*$^{+/+}$ or *Tbk1*$^{-/-}$ chimaeric mice with *Tbk1*$^{+/+}$ bone marrow (Fig. 3a–d). It has been shown that direct transfection of dendritic cells with DNA vaccines can prime both humoral and cellular immune responses to the encoded antigen[26]. To examine whether TBK1-mediated signalling of dendritic cells directly transfected with DNA vaccine is involved in DNA-vaccine-induced immune responses, splenic dendritic cells from wild-type, control (*Tnf*$^{-/-}$) or *Tbk1*$^{-/-}$ mice were transfected with DNA vaccine by electroporation *in vitro*, and were then transferred to naive, control (*Tnf*$^{-/-}$) mice. Serum IgG and IgG2a titers were significantly increased in mice that received wild-type or control (*Tnf*$^{-/-}$) dendritic cells, but not in those that received *Tbk1*$^{-/-}$ (*Tnf*$^{-/-}$) dendritic cells (Fig. 3e), suggesting that TBK1 signalling in dendritic cells is sufficient to prime antigen-specific antibody responses. Taken together, TBK1 signalling in bone-marrow-derived cells, probably dendritic cells, and to a lesser extent that in non-haematopoietic cells, is critical for the optimal humoral response as well as for helper T (Th)1 cytokine production after DNA vaccination.

We also examined the roles of TBK1 in haematopoietic and non-haematopoietic cells, in the induction of antigen-specific CD4^{+} and

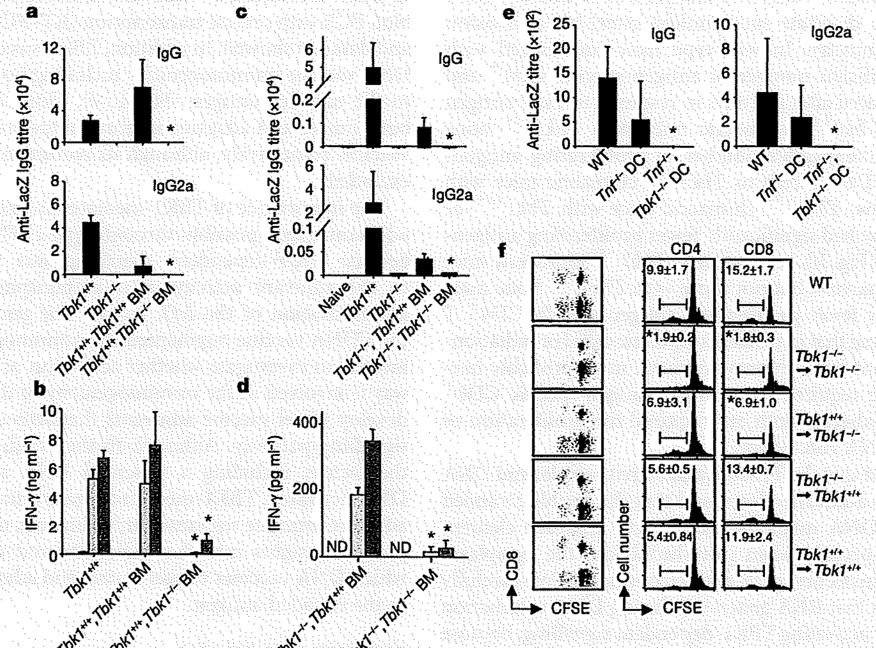

Figure 3 | Contribution of haematopoietic and non-haematopoietic cells to DNA-vaccine-induced immunogenicity. Bone marrow (BM) chimaeric mice with TNF-deficient or TNF and TBK1-double-deficient bone marrow were immunized with a DNA vaccine encoding LacZ, as described in the Methods. Fourteen days after the second immunization, sera from the chimaeric mice (TNF-deficient (**a**, **b**) or TNF and TBK1-double-deficient mice (**c**, **d**)) were analysed for LacZ-specific IgG titre (**a**, **c**), and spleen cells were analysed for their antigen-specific IFN-γ production (**b**, **d**) as well as for their antigen-specific CD4^{+} or CD8^{+} T-cell proliferation (**f**) in response to LacZ antigen. Splenic dendritic cells from wild-type, control (*Tnf*$^{-/-}$) or *Tbk1*$^{-/-}$ mice were transfected with DNA vaccine by electroporation *in vitro*, and were then transferred to naive, control (*Tnf*$^{-/-}$) mice (**e**). Serum LacZ-specific IgG titre was analysed 3 weeks after dendritic-cell transfer (**e**). Data are the averages ± s.d. of three mice per group (except **f**, which is representative of two experiments); $^{*}P < 0.01$ against wild-type mice. DC, dendritic cell. N.D., not detected.