(Fig. 8 A and Fig. S5, available at http://www.jem.org/cgi/content/full/jem.20061523/DC1). In addition, production of IL-2 in response to TCR stimulation was not impaired in T cells from these mice (Fig. 8 B). Furthermore, wild-type, *IRAK-4*$^{-/-}$, and *IRAK-4*$^{KN/KN}$ T cells have equivalent ability to proliferate in response to allogenic DCs either untreated or treated with various TLR ligands, including MALP-2, LPS, and CpG-DNA (Fig. 8 C). Moreover, TCR-mediated activation of NF-κB as well as MAP kinases was also not altered between wild-type, *IRAK-4*$^{-/-}$, and *IRAK-4*$^{KN/KN}$ T cells (Fig. 8, D and E). We investigated whether IRAK-4 was involved in adaptive T cell responses in vivo. Wild-type and *IRAK-4*$^{-/-}$ mice were infected with lymphocytic choriomeningitis virus (LCMV). Splenocytes were prepared 8 d after infection, and induction of LCMV-specific CD8$^+$ T cells was analyzed by tetramer staining. As shown in Fig. 9 A, LCMV-specific CD8$^+$ T cells were induced both in wild-type and *IRAK-4*$^{-/-}$ mice in a similar manner after infection. Similarly, wild-type and *IRAK-4*$^{-/-}$ mice induced comparable ex vivo CTL responses as determined in a ^{51}Cr release assay (Fig. 9 B). These results indicate that IRAK-4 is not involved in the TCR signaling leading to the activation of NF-κB as well as T cell responses in vivo.

DISCUSSION

In the present study, we analyzed the role of IRAK-4 activity in vivo by generating mice with knockin mutation KK213AA and with null mutation. In agreement with previous papers, *IRAK-4*$^{-/-}$ macrophages showed severe defects in TLR-mediated cytokine responses (14, 15). Although the expression of IRAK-4 protein was slightly lower than wild-type cells, *IRAK-4*$^{KN/KN}$ macrophages also showed profound defects in the responses to various TLR ligands to the same extent as *IRAK-4*$^{-/-}$ cells. These results clearly indicate that the kinase activity of IRAK-4 is essential for the function of IRAK-4 in vivo. Previous in vitro studies implicated that the IRAK family members could activate NF-κB and inflammatory responses even in the absence of their kinase activity (19, 20). In the case of IRAK-4, one group has shown that the mutant IRAK-4 (KK213AA) restored IL-1β responsiveness (22), and the other group reported that the same mutation could restore the response only partially (23). It has been shown that expression of kinase-inactive IRAK-1 could also restore IL-1β–induced NF-κB activation. It may be possible that overexpressed IRAK-4 behaved differently compared with the physiological expression. In the physiological level of expression, the kinase activity of IRAK-4 is critical for its function. So far IRAK-4 substrates responsible for the signaling

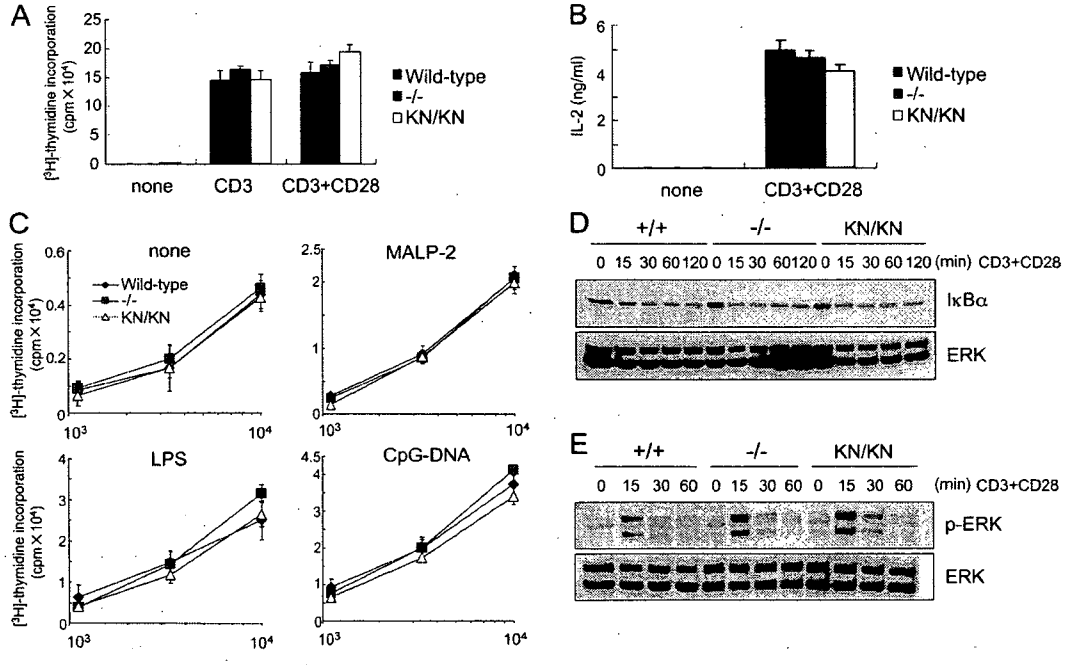

Figure 8. IRAK-4 is dispensable for TCR signaling. (A) Splenic T cells from wild-type, *IRAK-4*$^{-/-}$, and *IRAK-4*$^{KN/KN}$ mice were stimulated with immobilized (plate-bound) anti-CD3 (5 μg/ml) alone or anti-CD3 (5 μg/ml) plus anti-CD28 (2 μg/ml) for 48 h. The cells were pulsed with 1 μCi [^{3}H]thymidine for the last 16 h. [^{3}H]thymidine incorporation was measured by a β-scintillation counter. (B) Splenic T cells were stimulated with 5 μg/ml anti-CD3 plus 2 μg/ml anti-CD28 for 72 h. IL-2 concentrations in the culture supernatant were measured by ELISA. (C) Allogenic activity of wild-type, *IRAK-4*$^{-/-}$, and *IRAK-4*$^{KN/KN}$ T cells. T cells from indicated mice were mixed with bone-marrow DCs from BALB/c mice stimulated with MALP-2, LPS, and CpG-DNA. (D) Normal activation of NF-κB in response to TCR in *IRAK-4*$^{-/-}$ and *IRAK-4*$^{KN/KN}$ mice. The cell lysates from cells stimulated with plate-bound anti-CD3 plus anti-CD28 were immunoblotted with anti-IκBα. ERK1/2 levels are shown as loading control. (E) Normal activation of ERK in *IRAK-4*$^{-/-}$ and *IRAK-4*$^{KN/KN}$ mice. Wild-type, *IRAK-4*$^{-/-}$, and *IRAK-4*$^{KN/KN}$ T cells were stimulated with plate-bound anti-CD3 plus anti-CD28, and the cell lysates were immunoblotted with anti-phospho-ERK. ERK1/2 levels are shown as loading control.

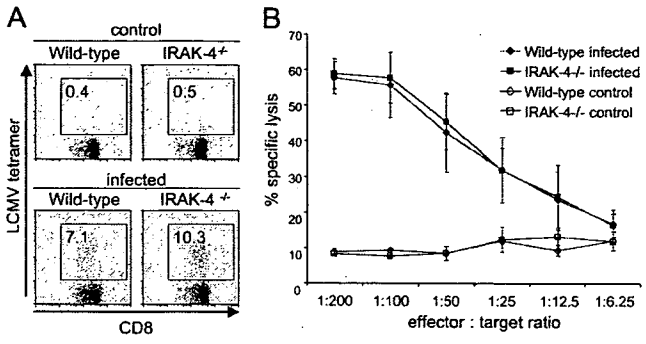

Figure 9. Responses of *IRAK-4*$^{-/-}$ T cells to LCMV infection. Wild-type and *IRAK-4*$^{-/-}$ mice were intravenously infected with 5×10^5 PFU of LCMV, and splenocytes were harvested at day 8 after infection. (A) The cells were stained with LCMV MHC class I tetramer and CD8a and were analyzed by flow cytometry. The data shown are representative of six different mice tested. (B) Ex vivo CTL activity in splenocytes was determined using 5-h ^{51}Cr release assay and GP33-loaded EL-4 cells as targets. Indicated values are mean ± SD of three mice. The data are representative of two separate experiments.

have not been well understood. Although it was shown that IRAK-4 phosphorylated IRAK-1 for activating TRAF6, TLR-mediated production of proinflammatory cytokines in *IRAK-1*$^{-/-}$ cells was not impaired in peritoneal macrophages. Further studies are required for identifying substrates other than IRAK-1 that are responsible for the TLR signaling pathway. Nevertheless, this is the first paper showing that the kinase activity of IRAK family members plays a critical role in their function in vivo.

The association between MyD88 and IRAK-4 was induced in response to IL-1β stimulation in both wild-type and *IRAK-4*$^{KN/KN}$ cells. A previous study showed that MyD88 interacted with kinase-negative, but not with wild-type, IRAK-4 when they were overexpressed in human embryonic kidney 293 cells (13). In contrast, it was reported that IL-1 stimulation induced an interaction between endogenous MyD88 and wild-type IRAK-4 (16). In that study, the kinase-truncated mutant of IRAK-4 was shown to constitutively interact with MyD88 even before IL-1 stimulation. Given that overexpression of wild-type IRAK-4 immediately activates NF-κB without further stimulation, the localization of overexpressed IRAK-4 may be different from endogenous protein. Based on our observation and that study (16), the endogenous IRAK-4 is probably recruited to MyD88 in response to stimulation, and IRAK-4 with KK213AA point mutation behaves similarly to wild-type IRAK-4 regarding association with MyD88.

Although IRAK-4 deficiency profoundly affected TLR2-mediated cytokine production, TNF-α gene induction was impaired, but not abrogated, as observed in MyD88 deficiency. TLR2-mediated expression of TNF-α and IκBζ genes was induced even in the absence of IRAK-4, though the expression in *IRAK-4*$^{-/-}$ and *IRAK-4*$^{KN/KN}$ cells was reduced and transient compared with wild-type cells. Furthermore, induction of NF-κB–DNA binding activity was also induced in *IRAK-4*$^{-/-}$ and *IRAK-4*$^{KN/KN}$ macrophages, although the activation was ~10 min delayed compared with wild-type cells. This finding is in contrast to the complete abrogation of TLR2 signaling in MyD88$^{-/-}$ macrophages and indicates the existence of an IRAK-4–independent signaling pathway. Stimulation with R-848 and CpG-DNA also induced NF-κB activation in an IRAK-4–independent manner without degrading IκBα, suggesting that the IRAK-4–independent pathway is not TLR2 specific. Given that the death domain of MyD88 is responsible for downstream signaling, other IRAK family members that contain an N-terminal death domain are candidates for mediating IRAK-4–independent signaling. Nevertheless, the activation of NF-κB in response to a TLR2 ligand was observed even in the absence of both IRAK-1 and -4. Because it was shown that IRAK-2 also positively regulated the IL-1β–signaling pathway, IRAK-2 may be responsible for the signaling pathway. Future studies will clarify if IRAK family members redundantly function in IL-1R/TLR responses in vivo. Although we clearly detected NF-κB–DNA binding activity, which is supershifted by anti-p50 and p65 Ab, we failed to detect activation of IKKs and phosphorylation of IκBα in the absence of IRAK-4 or its kinase activity in response to TLR2 stimulation. Induction of NF-κB activation without degradation of IκBα is quite unique, although the mechanism of activation is enigmatic. It has been reported that TLR2 stimulation leads to the recruitment of active Rac1 and phosphatidylinositol-3 kinase to the TLR2 cytosolic domain (27). Therefore, it is possible that the signaling is mediated through the small G protein–phosphatidylinositol-3 kinase pathway.

The TLR/IL-1R and antigen-receptor signaling share signaling molecules for activating NF-κB. In addition to IKK complex, TRAF6 was also reported to be involved in TCR-mediated NF-κB activation (28). TRAF6 can associate with MALT1, which forms a complex with BCL10 and CARMA1/CARD11. TRAF6 is oligomerized by the complex and activates IKKs by inducing polyubiquitination of IKK-γ/NF-κB essential modulator and activation of TGF-β–activated kinase 1. A recent paper showed that IRAK-4 was also involved in TCR responses via suppressing NF-κB activation by associating with ZAP-70 (18). However, the newly generated *IRAK-4*$^{-/-}$ mice did not show any defects in the T cell response as well as the TCR signaling pathway. Furthermore, IRAK-4 was not required for LCMV-induced CTL responses. *IRAK-4*$^{KN/KN}$ T cells also showed normal responses against TCR stimulation. We do not have a clear explanation for the discrepancy, and it may be due to the difference in the genetic background of the strains. However, it is unlikely that the critical TCR signaling components are different between mouse strains, suggesting that IRAK-4 is not critically involved in TCR signaling.

In summary, this study demonstrates that IRAK-4 activity plays a critical role in the physiological function of IRAK-4. Macrophages and DCs from *IRAK-4*$^{KN/KN}$ mice as well as *IRAK-4*$^{-/-}$ mice were profoundly defective in TLR-mediated

proinflammatory cytokine production. In addition, *IRAK-4*$^{KN/KN}$ mice were highly resistant to LPS-induced shock response. The exploration of small compounds targeting kinase activity of IRAKs has been challenged by the fact that expression of even kinase-inactive IRAK-4 mutant results in the activation of the intracellular signaling pathway. However, this study clearly indicates that the kinase activity of IRAK-4 is essential for the physiological functions, and the kinase activity of IRAK-4 is a good therapeutic target for inflammatory diseases and septic shock, without affecting acquired immune responses.

MATERIALS AND METHODS

Generation of *IRAK-4*$^{KN/KN}$ mice. The *IRAK-4* gene was isolated from genomic DNA extracted from ES cells (GSI) by PCR. A genomic fragment containing exon 2 of IRAK-4 was cloned into a pT7blue vector (Nugen), and point mutations resulting in the KK213AA conversion in the kinase domain were introduced by a site-directed mutagenesis. A targeting vector has a neomycin-resistance gene cassette (neo) flanked with two loxP sites, and a HSV thymidine kinase driven by PGK promoter was inserted into the genomic fragment for negative selection. The targeting vector was linearized and electroporated into ES cells (GSI). G418 and gancyclovir doubly resistant clones were selected and screened by PCR and further confirmed by Southern blotting. Three clones with homologous recombination were injected into blastocysts from C57BL/6 females, the obtained chimeric males were crossed with C57BL/6 females, and the obtained F1 generations with mutated IRAK-4 mice were crossed with CAG-Cre transgenic mice to excise the neo cassette. CAG-Cre transgene was removed from *IRAK-4*$^{KN/+}$ mice without a neo cassette by crossing the mice with C57BL/6 mice. *IRAK-4*$^{KN/+}$ mice were further intercrossed to obtain *IRAK-4*$^{KN/KN}$ mice. The *IRAK-4*$^{KN/KN}$ mice used were under 129Sv × C57BL/6 background. Mice were maintained in our animal facility and treated in accordance with the guidelines of Osaka University.

Generation of *IRAK-4*$^{-/-}$ mice. The *IRAK-4* gene was isolated from genomic DNA extracted from ES cells (GSI) by PCR. The targeting vector was constructed by replacing a 4.3-kb fragment encoding the *IRAK-4* ORF with a neo cassette, and a HSV thymidine kinase driven by PGK promoter was inserted into the genomic fragment for negative selection. After the targeting vector was transfected into ES cells, G418 and gancyclovir doubly resistant colonies were selected and screened by PCR and further confirmed by Southern blotting. Homologous recombinants were microinjected into blastocysts from C57BL/6 female mice, and heterozygous F1 progenies were intercrossed to obtain *IRAK-4*$^{-/-}$ mice. The *IRAK-4*$^{-/-}$ mice used were under 129Sv × C57BL/6 background.

Cells. Peritoneal exudate cells were isolated from the peritoneal cavity of mice 3 d after injection with 2 ml of 4.0% thioglycollate medium (Sigma-Aldrich) by washing with ice cold Hanks' balanced salt solution (Invitrogen). Bone-marrow DCs were prepared by cultivating either in the presence of 100 ng/ml human Flt3 ligand (PeproTech) or 10 ng/ml mouse GM-CSF (PeproTech) as described previously (29). Splenic T cells were isolated using MACS (Miltenyi Biotec).

Reagents. MALP-2 and PAM_3CSK_4 were synthesized as described previously (25, 26). LPS from *Salmonella minesota* Re-595 was purchased from Sigma-Aldrich. Poly I:C was purchased from GE Healthcare. R-848 was provided by the Pharmaceuticals and Biotechnology Laboratory of the Japan Energy Corporation. CpG oligonucleotide was synthesized as described previously (30). Polyclonal Ab to phosphorylated JNK (anti– phospho-JNK), anti–phospho-p38, anti–phospho-ERK, anti–phospho- IκBα (Ser32), and anti–phospho-NF-κB p65 (Ser536) were purchased from Cell Signaling. Polyclonal anti-JNK, anti-p38, anti-ERK, and anti–IκB-α were obtained from Santa Cruz Biotechnology, Inc. Abs to NF-κB p50 and p65 were purchased from Santa Cruz Biotechnology, Inc. Anti-MyD88 Ab was purchased from ProSci, and anti–IRAK-1 Ab was made as described previously (25). Rabbit anti–IRAK-4 polyclonal Ab was raised against a peptide corresponding to aa 436 to 459 of mouse IRAK-4. Specificity of this Ab was tested on overexpressed IRAK-4 (unpublished data) and on *IRAK-4*$^{-/-}$ cells (Fig. 1 D).

Measurement of cytokine production. Concentrations of cytokines in the culture supernatants were measured by ELISA. ELISA kits for mouse TNF-α, IL-6, IL-12 p40, and IL-2 were purchased from R&D Systems, and the kit for mouse IFN-α was purchased from PBL Biomedical Laboratories.

[^{3}H]thymidine uptake. Splenocytes were cultured with the indicated concentrations of MALP-2, poly I:C, LPS, CpG-DNA, anti-IgM (Jackson ImmunoResearch Laboratories), or anti-CD40 (BD Biosciences) for 48 h. For examining T cell responses, splenic T cells were activated with 10 μg/ml of plate-bound anti-CD3 (BD Biosciences) and 2 μg/ml of plate-bound anti-CD28 (BD Biosciences) for 48 h. Cells were pulsed with 1 μCi [^{3}H]thymidine for the last 16 h. [^{3}H]thymidine incorporation was measured by a scintillation counter (Packard Instrument Co.).

Synthesis of IRAK proteins and in vitro kinase assay. IRAK-4 cDNA was obtained by RT-PCR from mRNAs prepared from wild-type and *IRAK-4*$^{KN/KN}$ macrophages. The cDNAs were cloned into a pcDNA3 vector, which contains a T7 promoter and a Myc tag sequence. Recombinant Myc-tagged IRAK-4 proteins were expressed in the rabbit reticulocyte lysates using TNT T7 Quick coupled transcription/translation systems (Promega). A part of mouse IRAK-1 protein (aa 301–500), which contains the IRAK-1 activation loop, was also prepared the by same system. 10 μl of reticulocyte lysates, which contained recombinant kinase or exogenous substrate, was diluted with cell lysis buffer and combined as indicated. Kinase and substrate were immunoprecipitated with anti-Myc Ab (Cell Signaling), and then in vitro kinase assay was performed as described previously (31). For assessing autophosphorylation of endogenous IRAK proteins, peritoneal macrophages stimulated with 10 ng/ml MALP-2 were lysed and immunoprecipitated with anti–IRAK-1 and anti–IRAK-4 Ab. The kinase activity was then measured by in vitro kinase assay.

Northern blot analysis. Peritoneal macrophages were treated with 10 ng/ml MALP-2 for 0, 1, 2, 4, and 8 h, and total RNA was extracted using TRIzol reagent (Invitrogen). RNA was electrophoresed, transferred to nylon membranes, and hybridized with the indicated cDNA probes. To detect the expression of IRAK4 mRNA, a 394-bp fragment (707–1,101) was used as a probe. The same membrane was rehybridized with a β-actin probe.

Western blot analysis. Peritoneal macrophages were treated with 10 ng/ml MALP-2 for the indicated times. Cells were then lysed in a lysis buffer containing 1.0% NP-40, 150 mM NaCl, 20 mM Tris-Cl, pH 7.5, 1 mM EDTA, and protease inhibitor cocktail (Roche). Cell lysates were dissolved by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The membrane was blotted with the specific Ab to indicated proteins and visualized with an enhanced chemiluminescence system (NEN Life Science Products). For immunoprecipitation, 10^7 MEFs were treated with 10 ng/ml IL-1β for the indicated periods, and cell lysates were immunoprecipitated with anti-MyD88 or anti–IRAK-4 Ab, followed by immunoblot with the indicated Abs.

EMSA. The nuclear extracts were prepared from peritoneal macrophages (5×10^6) stimulated with MALP-2 as described previously (4). Nuclear extracts were incubated with or without Abs against NF-κB p65 or p50, and then further incubated with a specific probe for NF-κB DNA binding sites, electrophoresed, and visualized by autoradiography.

Allogenic T cell response assay. The allogenic T cell responses were analyzed as described previously (32). In brief, bone marrow–derived DCs stimulated with 10 ng/ml MALP-2, 1 μg/ml LPS, or 100 nM CpG-DNA

for 48 h from BALB/c mice were harvested at day 8, irradiated at a dose of 30 Gy, and plated at threefold serial dilutions in 96-well round-bottom plates. These DCs were incubated for 3 d with 5×10^4/well of splenic $CD4^+$ T cells from wild-type, *IRAK4*$^{-/-}$, and *IRAK-4*$^{KN/KN}$ mice isolated using MACS with CD4 microbeads (Miltenyi Biotec). [^{3}H]thymidine was added for the last 16 h. [^{3}H]thymidine incorporation was measured by a scintillation counter.

LCMV infection and analysis of T cell responses. LCMV-WE strain was obtained from T. Otheki (Akita University, Akita, Japan). Wild-type and *IRAK4*$^{-/-}$ mice were intravenously infected with 5×10^5 PFU of LCMV-WE and splenocytes were harvested at day 8 after infection. To investigate the induction of LCMV-specific T lymphocytes, splenocytes were incubated with T-select H-2Db LCMV tetramer-KAVYNFATC-PE (MBL International Corporation) and CD8a-APC Ab. Samples were acquired on a FACS Calibur (BD Biosciences) and analyzed with FlowJo software (TreeStar).

For assessment of cytotoxicity of LCMV-specific T cells, splenocytes prepared from LCMV-infected mice were incubated for 5 h with EL-4 target cells that had been loaded with a peptide (GP33; KAVYNFATM; Peptide Institute) and labeled with ^{51}Cr. The percentage of specific lysis was calculated as [(experimental release − spontaneous release)/(maximal release − spontaneous release)] × 100%.

TNF bioassay. TNF activity was measured in macrophage culture supernatant after stimulation with MALP-2 for 1 and 2 h by cytotoxicity on L929 fibroblasts. L929 cells were plated on 96-well plates in RPMI 1640 medium supplemented with 2% FCS. Serial twofold dilutions of supernatants in 8 mg/ml actinomycin D were added to each well and incubated for 20 h. Viability of cells was determined using CellTiter-Glo (Promega) according to the manufacturer's instructions. Mouse recombinant TNF-α (R&D systems) was used to derive a standard curve, and the concentration of TNF-α was determined based on the standard curve.

Online supplemental material. Fig. S1 shows the generation of *IRAK-4*$^{-/-}$ mice. Fig. S2 shows the induction of TNF activity in response to MALP-2 stimulation. Fig. S3 shows that activation of NF-κB in response to MALP-2 was dependent on MyD88. Fig. S4 shows the activation of NF-κB in *IRAK-4*$^{-/-}$ and *IRAK-4*$^{KN/KN}$ macrophages in response to LPS, R-848, and CpG-DNA. Fig. S5 shows the proliferative responses of *IRAK-4*$^{-/-}$ and *IRAK-4*$^{KN/KN}$ T cells to soluble anti-CD3 plus anti-Ig Ab. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20061523/DC1.

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MDA5/RIG-I and virus recognition

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The innate immune system initially recognizes RNA virus infection and evokes antiviral responses by producing type I interferons (IFNs). Toll-like receptors (TLRs) and cytoplasmic retinoic acid-inducible gene I (RIG-I)-like helicases (RLHs) are the two major receptor systems for detecting RNA viruses. The RLH signaling pathways play essential roles in the recognition of RNA viruses in various cells, with the exception of plasmacytoid dendritic cells, which utilize TLRs for virus recognition. The route of infection determines the cell types responsible for type I IFN production. Recent studies have suggested that TLRs are critical for activation of adaptive immune responses against several virus infections, although it may be premature to draw such a conclusion for virus infections in general. In this review, we will discuss recent advances toward clarifying the signaling pathways activated by RLHs and TLRs.

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Introduction

Innate immunity is characterized by the use of germline-encoded pattern recognition receptors (PRRs) to sense components specific to micro-organisms [1–3]. Recent studies have identified three major classes of PRRs, designated Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like helicases (RLHs) and nucleotide-oligomerization domain (NOD)-like receptors (NLRs). In response to virus infections, viral components such as RNA and DNA are recognized by TLRs and RLHs, and cells are activated to produce type I interferons (IFNs) and proinflammatory cytokines. Type I IFNs, comprised of multiple IFN-α isoforms and a single IFN-β, and other members, such as IFN-ω, IFN-ε and IFN-κ, are pleiotropic cytokines that are essential for antiviral immune responses [4]. They induce apoptosis of virus-infected cells and cellular resistance to virus infection, as well as activating natural killer (NK) and T cells.

TLRs are comprised of leucine-rich repeats (LRRs), a transmembrane domain and a cytoplasmic Toll/interleukin 1 receptor (IL-1R) homology (TIR) domain [1]. Among the 13 TLRs identified in mammals, TLR3, TLR7, TLR8 and TLR9 are involved in the recognition of microbial nucleotides. These TLRs are expressed on the surface of cytoplasmic vesicles such as endosomes and the endoplasmic reticulum with the LRR domain in the vesicular space. TLR3 detects double-stranded (ds) RNA, while TLR7 and TLR8 recognize single-stranded (ss) RNA and TLR9 recognizes unmethylated DNA with CpG motifs. TLR-mediated signaling pathways recruit TIR domain-containing adaptors such as MyD88 and TIR domain-containing adaptor inducing IFN-β (TRIF), thereby leading to the activation of transcription factors such as nuclear factor-κB (NF-κB) and IFN-regulatory factors (IRFs), which regulate the expression of genes that encode proinflammatory cytokines and type I IFNs, respectively. Since TLRs are transmembrane proteins, they are not able to detect viral components present in the cytoplasm of a cell.

RIG-I (also known as DDX58) was identified as a candidate for a cytoplasmic viral RNA detector [5]. RIG-I is comprised of two N-terminal caspase-recruitment domains (CARDs) followed by a DExD/H box RNA helicase domain. RIG-I forms the RLH family together with melanoma differentiation-associated gene 5 (MDA5; also known as helicard or IFIH1) and LGP2 based on the high similarities among their helicase domains [6,7]. The helicase domains of RLH family members are highly similar to that of mammalian Dicer. The expression of genes encoding RLHs is strongly induced by IFNs. RLHs interact with dsRNAs through their helicase domain, and dsRNA stimulation induces their ATP catalytic activity. A C-terminal portion of RIG-I, designated the repressor domain (RD), was shown to inhibit the triggering of RIG-I signaling in the steady state [8]. The N-terminal CARDs are responsible for activating downstream signaling pathways that mediate dsRNA-induced type I IFN production.

In this review, we will describe the functions and signaling pathways of RLHs, and discuss the relationships among RLHs and TLRs in antiviral immune responses.

Recognition of RNA viruses by RLHs

RNA virus infections generate dsRNA for virus replication in infected cells. Initially, both RIG-I and MDA5 were implicated in the recognition of polyinosinic polycytidylic acid (poly I:C), a synthetic analogue of viral dsRNA. However, analysis of RIG-I$^{-/-}$ and MDA5$^{-/-}$

mice revealed that MDA5, but not RIG-I, was responsible for the response to poly I:C stimulation [9••,10]. On the other hand, RIG-I, but not MDA5, recognizes 5′-triphosphate ssRNA synthesized by T7 polymerase *in vitro* [11••,12••]. RNAs from some viruses are 5′-triphosphorylated and uncapped, whereas the 5′ ends of host mRNAs are capped. Thus, RIG-I discriminates virus and host RNAs based on the differences in the 5′ ends of their RNAs.

RNA viruses are also differentially recognized by RIG-I and MDA5. RIG-I$^{-/-}$ cells do not produce type I IFNs in response to various RNA viruses, including paramyxoviruses, vesicular stomatitis virus (VSV) and influenza virus [9••,13]. By contrast, MDA5$^{-/-}$ cells do not respond to infection with picornaviruses such as encephalomyocarditis virus (EMCV) and Theiler's virus. Cells infected with EMCV, but not influenza virus, generate dsRNA [12••]. Dephosphorylation of 5′-triphosphate RNA or the influenza genome results in a loss of their ability to induce IFNs, suggesting that recognition by RIG-I is mediated through 5′-triphosphate ssRNA. Consistent with defects in type I IFN production, RIG-I$^{-/-}$ and MDA5$^{-/-}$ mice are highly susceptible to inoculation with VSV and EMCV, respectively [9••]. Japanese encephalitis virus and hepatitis C virus, which both belong to the Flaviviridae family, are recognized by RIG-I. However, Dengue virus and West Nile virus, which also belong to the Flaviviridae family, were reported to induce type I IFN production even in the absence of either RIG-I or MDA5 [9••,14–16]. Furthermore, siRNA experiments suggested that Dengue virus was recognized by a combination of RIG-I and MDA5.

RIG-I-mediated signaling is positively and negatively controlled by ubiquitination of RIG-I. First, the CARDs of RIG-I undergo Lys 63-linked ubiquitination by tripartite motif (TRIM) 25, a ubiquitin E3 ligase composed of a RING finger domain, a B box/coiled-coil domain and a SPRY domain [17•]. This ubiquitination is necessary for efficient activation of the RIG-I signaling pathway, and TRIM25$^{-/-}$ cells display impaired production of type I IFNs against viral infection. On the other hand, RIG-I also undergoes ubiquitination by the ubiquitin ligase RNF125, which leads to its proteasomal degradation [18]. Thus, RIG-1 ubiquitination by RNF125 is considered to inhibit aberrant activation of RIG-I signaling.

RNase L, an endonuclease originally thought to cleave viral ssRNA, was reported to be involved in the production of IFN-β in response to RNA virus infection or dsRNA stimulation [19]. Briefly, 2′,5′-linked oligoadenylate generated by virus infection activates RNase L to cleave self RNA, resulting in the generation of small RNA products. These small RNAs are responsible for RIG-I/MDA5-mediated recognition and subsequent production of type I IFNs. However, the precise structures of the RNAs generated by RNase L that are recognized by RIG-I/MDA5 need to be further investigated.

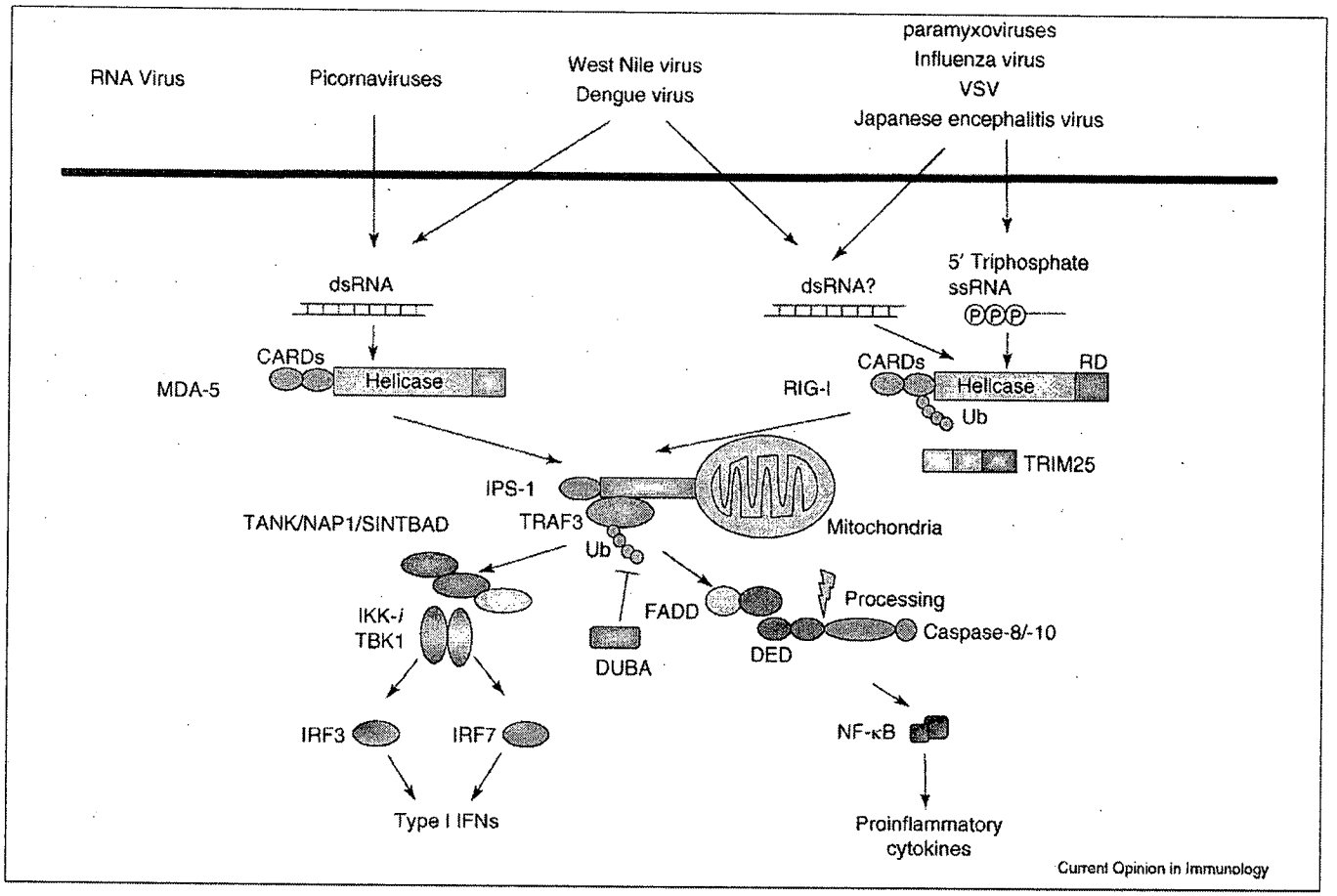
Since LGP2 lacks a CARD, it is suggested to function as a negative regulator of RIG-I/MDA5 signaling. Overexpression of LGP2 inhibits Sendai virus and Newcastle disease virus (NDV) signaling [6–8]. LGP2 also contains an RD, and this RD was found to interact with the RD of RIG-I and suppress its self-association. Recently, Lgp2$^{-/-}$ mice were generated and analyzed by Barber and coworkers [20]. Lgp2$^{-/-}$ mice show highly elevated induction of type I IFNs in response to poly I:C stimulation, and modestly increased IFN production in response to VSV infection. On the other hand, Lgp2$^{-/-}$ mice exhibit partially impaired type I IFN production in response to EMCV infection. The authors proposed that LGP2 is a negative regulator of RIG-I, but not of MDA5. However, given that both poly I:C and EMCV are recognized by MDA5, the difference cannot be simply explained by differential usage of LGP2 for RIG-I and MDA5 signaling.

The RLH signaling pathway

The CARDs of RIG-I and MDA5 are responsible for initiating signaling cascades (Figure 1). RIG-I and MDA5 associate with an adaptor protein, IFN-β promoter stimulator 1 (IPS-1; also known as MAVS, VISA or CARDIF), which also contains a CARD [21–24]. Overexpression of IPS-1 induces the activation of IFN gene promoters as well as NF-κB. IPS-1$^{-/-}$ mice are defective in producing type I IFNs and proinflammatory cytokines in response to all RNA viruses recognized by either RIG-I or MDA5 [25,26]. These findings indicate that IPS-1 plays an essential role in RIG-I/MDA5 signaling. Interestingly, this protein is present in the outer mitochondrial membrane, suggesting that mitochondria may be important for IFN responses, in addition to their roles in metabolism and cell death [22]. Recently, IPS-1 and RIG-I were found to associate with Atg5–Atg12 conjugates, which are essential components for autophagy [27]. Atg5$^{-/-}$ mouse embryonic fibroblasts show increased type I IFN production in response to RNA virus infection, suggesting that the autophagic machinery directly affects the RIG-I signaling pathway in addition to autophagosome formation.

IPS-1 was found to associate with TNF-receptor-associated factor (TRAF) 3, an E3 ubiquitin ligase assembling lysine 63-linked polyubiquitin chains, through its C-terminal TRAF domain [28–30]. TRAF3$^{-/-}$ cells show severely impaired production of type I IFNs in response to virus infection. Recently, a deubiquitinase named DUBA was shown to deubiquitinate TRAF3 and suppress RLH signaling [31•]. TRAF3 recruits and activates two IKK-related kinases, designated TANK-binding kinase 1 (TBK1) and inducible IκB kinase (IKK-*i*; also known as IKKε), which phosphorylate IRF-3 and IRF-7

Figure 1



RLH-mediated recognition of RNA viruses. RIG-I and MDA5 recognize 5′-triphosphate RNA and dsRNA from RNA viruses, and interact with IPS-1. TRAF3 is required for activation of IPS-1 signaling pathways, and K63-type polyubiquitination, which is controlled by the presence of DUBA. Subsequently, TRAF3 recruits TANK/NAP1/SINTBAD and TBK1/IKK-*i*, which phosphorylate IRF-3 and IRF-7. Next, nuclear translocation of IRFs takes place to induce the expression of type I IFN genes. NF-κB is also activated by IPS-1 via a FADD and caspase-8/caspase-10-dependent pathway.

[32,33]. However, IKK-*i* is also activated by IFN-β to directly phosphorylate STAT1, thereby controlling a set of IFN-inducible genes such as the dsRNA-activated adenosine deaminase gene (*Adar1*) [34].

TBK1 and IKK-i interact with TRAF family member-associated NF-κB activator (TANK), NAK-associated protein 1 (NAP1) and similar to NAP1 TBK1 adaptor (SINTBAD). These molecules show similarities with each other, and knockdown of any of them was reported to impair RLH signaling [35–37].

Phosphorylation of IRF-3 and IRF-7 by these kinases induces the formation of homodimers and/or heterodimers [38], which translocate into the nucleus and bind to IFN-stimulated response elements (ISREs), thereby resulting in the expression of type I IFN genes and a set of IFN-inducible genes. In addition, FAS-associated death domain-containing protein (FADD) interacts with caspase-8, caspase-10 and IPS-1, and the FADD-dependent pathway is responsible for the activation of NF-κB downstream of IPS-1 [39].

Type I IFN-producing cells in response to viral infection

Although RLHs play essential roles in the production of type I IFNs and cytokines in various cell types, such as fibroblasts and conventional dendritic cells (cDCs), plasmacytoid DCs (pDCs) produce cytokines in the absence of RLH signaling [13]. pDCs produce huge amounts of type I IFNs in response to virus infections, and TLR signaling is essential for type I IFN production by pDCs. TLR7 and TLR9 are highly expressed on pDCs, and stimulation with viral RNA and DNA induces the recruitment of a complex of MyD88, IRAK-1, IRAK-4, IKK-α and IRF-7 to the receptor, thereby leading to the phosphorylation and nuclear translocation of IRF-7 to activate the expression of IFN-inducible genes [40,41]. Although the importance of pDCs as a source of type I IFNs *in vivo* has been emphasized, direct identification of IFN-producing cells *in vivo* has not been achieved. Recently, a reporter mouse strain monitoring IFN-α6 (*Ifna6*$^{GFP/+}$) has been established. Although pDCs are highly potent in expressing GFP upon systemic NDV infection, lung infection of *Ifna6*$^{GFP/+}$ mice with NDV results in increases in GFP$^+$ alveolar macrophages and cDCs, but not pDCs [42•]. These observations indicate that cells other than pDCs can act as sources of type I IFNs depending on the route of infection. Interestingly, pDCs start to produce IFN-α when alveolar macrophages are depleted, suggesting that pDCs function when the first line of defense is broken.

Roles of RLHs and TLRs in the activation of adaptive immune responses to viruses

Immediate innate responses are important for mounting acquired immune responses to viral infections. However, it was not clear how the innate PRRs are involved in the activation of acquired immunity. Recently, two different virus infection models have been tested to examine the roles of RLHs and TLRs in the activation of acquired immune responses. The first model virus is lymphocytoid choriomeningitis virus (LCMV), an ambisense ssRNA virus belonging to the *Arenaviridae* family, which induces cytotoxic T lymphocyte (CTL) responses in a type I IFN-dependent manner [43]. Using MyD88$^{-/-}$ and IPS-1$^{-/-}$ mice, the serum levels of type I IFNs and pro-inflammatory cytokines were found to mainly depend on the presence of MyD88, but not IPS-1. The generation of virus-specific CTLs was critically dependent on MyD88, but not IPS-1. Analyses of *Ifna6*$^{GFP/+}$ reporter mice revealed that pDCs are the major sources of IFN-α in LCMV infection. These results suggest that recognition of LCMV by plasmacytoid DCs via TLRs is responsible for the production of type I IFNs *in vivo*. Furthermore, TLRs, but not RLHs, appear to be important for mounting CTL responses to LCMV infection.

Influenza virus has also been used to study the activation of adaptive immune responses [44]. Induction of type I IFNs in response to intranasal influenza A virus infection is abrogated in the absence of both MyD88 and IPS-1, although mice lacking either of these molecules are capable of producing type I IFNs. Induction of B cells or CD4 T cells specific for viral proteins is dependent on the presence of MyD88, but not IPS-1, whereas induction of nuclear protein Ag-specific CD8 T cells is not impaired in the absence of either MyD88 or IPS-1. These results suggest that adaptive immune responses to influenza A virus infection are governed by a TLR pathway.

The virus infection models tested to date support roles for TLRs, rather than RLHs, in instructing the adaptive immune system. However, further studies are required, since these two PRR systems provide different contributions depending on the viruses involved and also may depend on the route of infection.

Conclusions

In this review, we have described the roles of RLHs, their signaling pathways and the relationships among RLHs and TLRs in responses against RNA viruses. Although recent studies have clarified the functions and signaling pathways of RLHs, the molecular structures of the RNAs recognized by MDA5 are not understood. In addition, it remains unclear whether RIG-I can recognize dsRNA in addition to 5′-triphosphate ssRNA. Although a previous report showed that small dsRNAs without 3′ overhangs induced IFN-inducible genes via RIG-I in certain cell types [45], chemically synthesized small dsRNAs (20-27 nucleotides) were only found to activate mouse fibroblasts and cDCs (OT and SA: unpublished observations). Thus, further investigations are required to clarify the structures of RIG-I and MDA5 ligands.

Furthermore, the mechanisms of DNA virus recognition are not well understood. Recently, the presence of a cytoplasmic DNA sensor was predicted [46,47], and a protein named DAI was proposed as a candidate for this sensor [48]. However, loss-of-function studies are still required to prove the importance of this protein. Although we focused on the mechanisms of innate responses and T cell activation toward RNA viruses in this review, many other cell types such as NK cells and NK T cells are involved in antiviral responses *in vivo*. Further studies are required to fully elucidate the complex regulation of antiviral responses *in vivo*.

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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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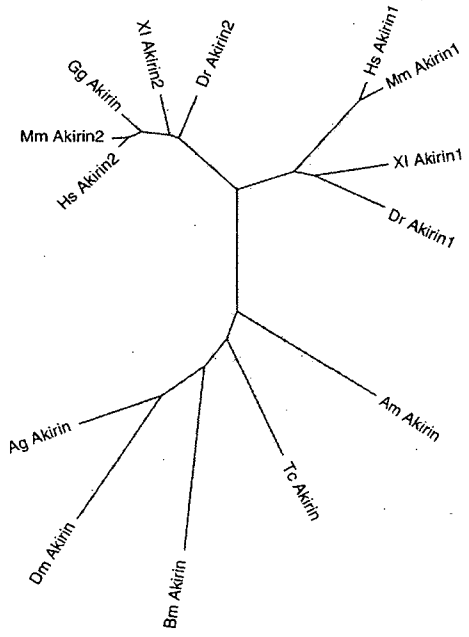

Figure 1 Unrooted evolutionary tree of Akirin homologs: Akirins are highly conserved. Dm, *Drosophila melanogaster*; Ag, *Anopheles gambiae*; Bm, *Bombyx mori*; Am, *Apis mellifera*; Tc, *Tribolium castaneum*; Gg, *Gallus gallus*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Xl, *Xenopus laevis*; Dr, *Danio 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

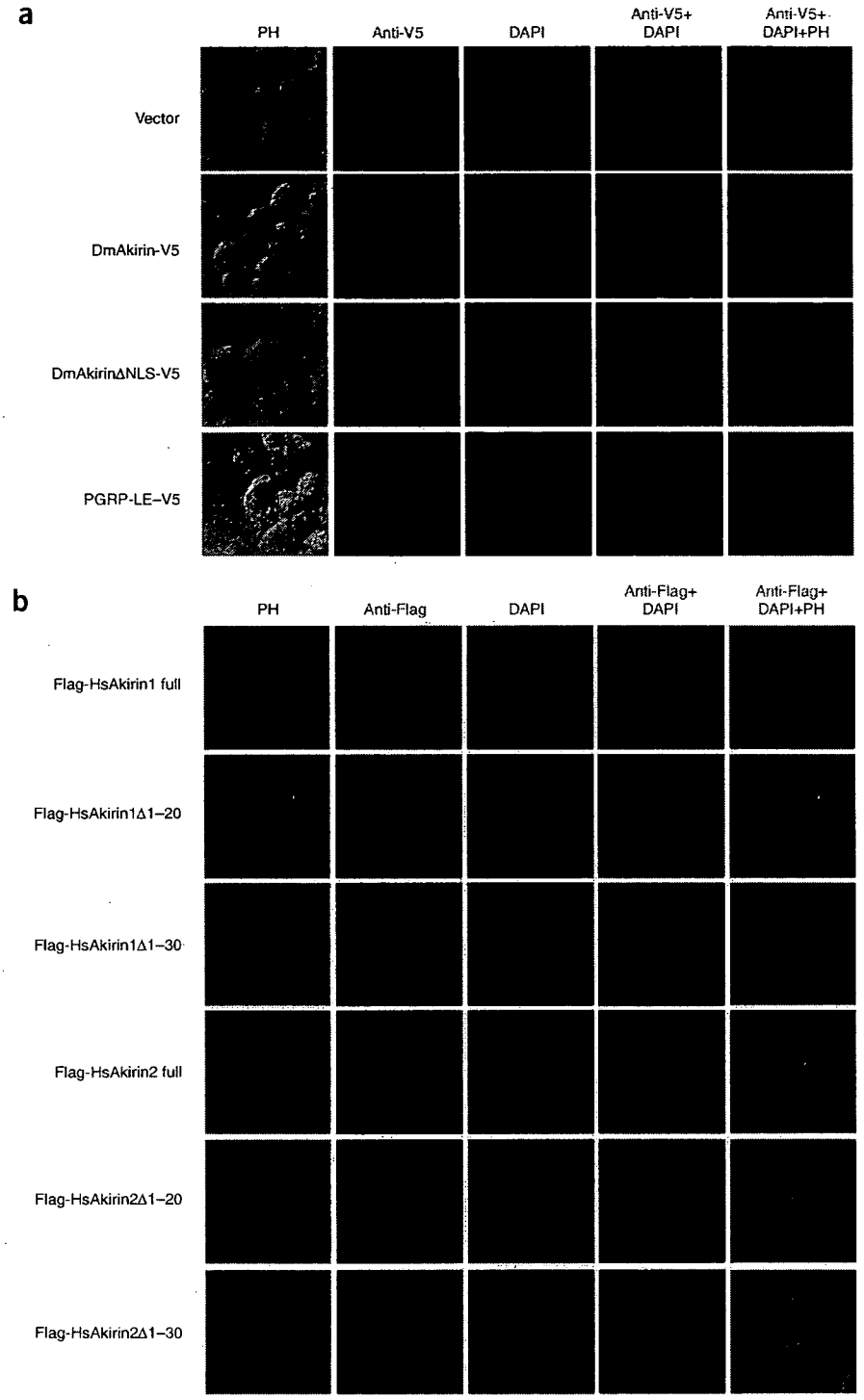

Figure 2 Nuclear localization of Akirins. (**a**) S2 cells transfected with constructs encoding V5-tagged drosophila Akirin, NLS-deleted drosophila Akirin or PGRP-LE. Cells transfected with an empty vector were used as control. Nuclei were visualized with DAPI (blue). Akirin, NLS-deleted Akirin and PGRP-LE 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

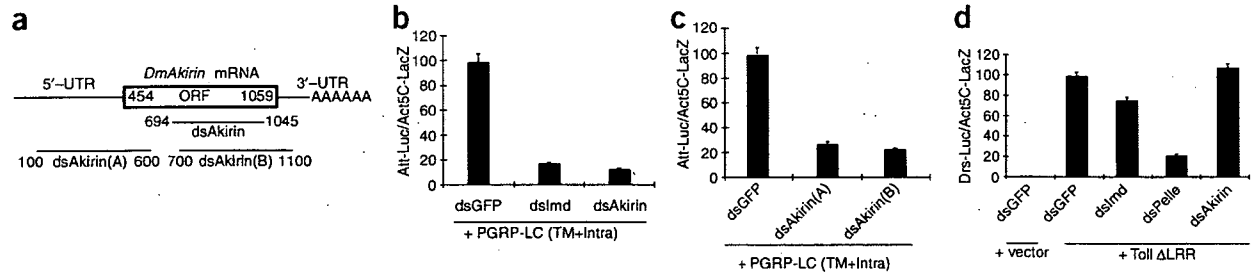

Figure 3 Effect of RNAi knock-down of drosophila *Akirin* on the activation of the Imd and Toll pathways in drosophila S2 cells. (**a**) Structure of *D. melanogaster Akirin* mRNA and of the dsRNAs against drosophila *Akirin*. An original dsRNA (dsAkirin) covering nucleotides 694–1045 was used for the 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 *lox*P sites flanking the first coding exon of the *Akirin1* gene and inserting a *lox*P site–flanked ('floxed') *neo*^{r} gene into intron 1 of the *Akirin1* gene

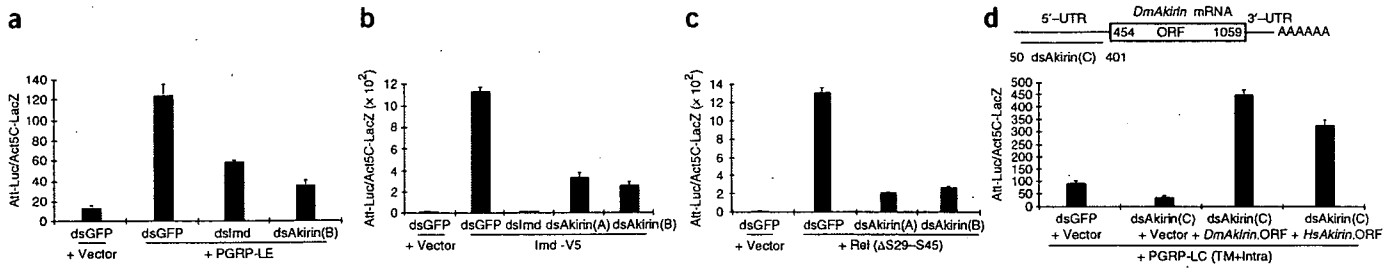

Figure 4 Epistatic analysis of *D. melanogaster* Akirin position within the Imd pathway. Constitutive activation of the Imd pathway induced by the transfection of S2 cells with PGRP-LE-V5 (**a**), Imd-V5 (**b**), Rel (ΔS29-S45; **c**) or PGRP-LC (TM+Intra; **d**) is highly compromised when cells are 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.).

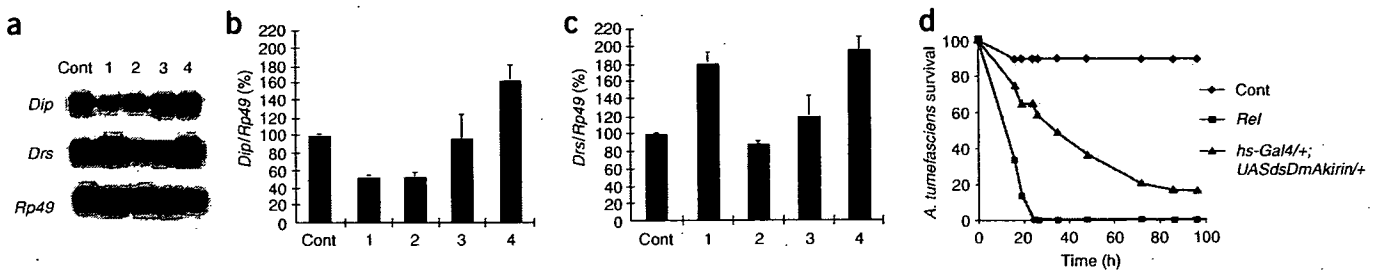

Figure 5 *In vivo* function of *D. melanogaster* Akirin. (**a**) The Imd and Toll pathway activations were monitored by RNA blot analysis of the *Diptericin* (*Dip*) and *Drosomycin* (*Drs*) messengers expression 6 h and 24 h, respectively, after infection with a mixture of Gram-positive and Gram-negative 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

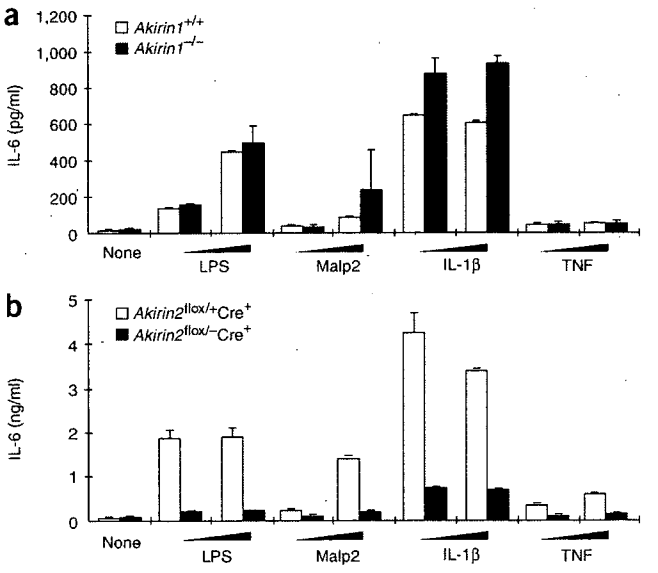

Figure 6 TLR-, IL-1β- and TNF-induced IL-6 production in *Akirin1*$^{-/-}$ and *Akirin2*$^{-/-}$ mouse embryonic fibroblasts (MEFs). (**a,b**) IL-6 concentrations in *Akirin1*$^{+/+}$ and *Akirin1*$^{-/-}$ MEFs (**a**) and Cre-transduced *Akirin2*$^{flox/+}$ and *Akirin2*$^{flox/-}$ MEFs (**b**) stimulated 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.

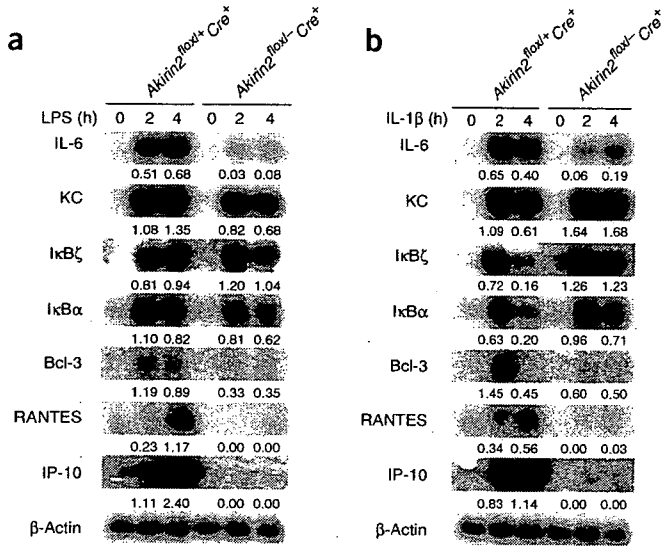

Figure 7 LPS- and IL-1β-induced gene expression in *Akirin2*$^{-/-}$ MEFs. (**a**,**b**) Total RNA blot analysis of the expression of IL-6, KC, IκBζ, IκBα, BCL3, RANTES and IP-10 in Cre-transduced *Akirin2*$^{floxl+}$ and *Akirin2*$^{floxl-}$ MEFs stimulated with LPS (10 μg/ml) (**a**) or IL-1β (10 ng/ml) (**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

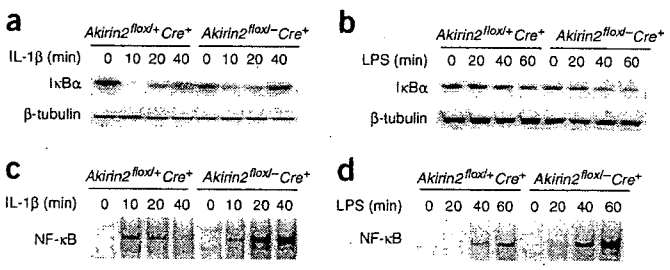

Figure 8 LPS- and IL-1β-induced activation of NF-κB in *Akirin2*$^{-/-}$ MEFs. (**a**,**b**) IκBα expression in the whole cell lysates, analyzed by immunoblotting. (**c**,**d**) NF-κB-DNA binding activity in the nuclear extracts, determined by electrophoretic mobility-shift assay. Cre-transduced *Akirin2*$^{floxl+}$ and *Akirin2*$^{floxl-}$ 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′-CCCCGGATCCGATAATTCCC GCCATGCCTTTTAGCAATGAAACG-3′ and reverse 5′-GGGGGGTACCTCA GTTCAACGTCTTTCCGAAGAG-3′ primers. The *PGRP-LE-V5* fragment was obtained from *UAS-PGRP-LE* transgenic flies[20] with forward 5′-CCCCGG ATCCGATAATTCCCGCCATGTCCGAATCGGGAATC-3′ and reverse 5′-GGGG GGTACCTCAGGTGGAATCCAGGCCCAGCAGGGGGTTGGGGATGGCTTG CCTTGTTCCTCCTCCTCGATATTG -3′ primers. The V5-tagged *D. melanogaster Akirin* vector was constructed with forward 5′-CCCCGGATCCGATAAT TCCCGCCATGGCCTGTGCAACCCTGAAAC-3′ and reverse 5′-GGGGGGTA CCTCAGGTGGAATCCAGGCCCAGCAGGGGGTTGGGGATGGGCTTGCCC GACAGGTAGCTAGGCGCTG-3′ primers. The NLS was deleted from V5-tagged drosophila *Akirin* with the 5′-CTAGACTGGGAGTCGATCAACCGTTG CAATCCCTTTGGCCAG-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′-CCCCGGATCCGATAATTCCCGCCATGGCGTGC GGAGCCACTCTG-3′ and reverse 5′-GGGGGGTACCTCATGAAACATAGCTA GCAGGC-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′-GGGG CCGCTAGCTTACGACAGGTAGC-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′-AAGCTTTCC CCGAAGCGCAGGCGATGTGCG3′ (Δ20) and 5′-AAGCTTTCGGCGCCCACC TCGGCCGCTGCC-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*$^{flox/flox}$ 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.