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RIP1 is an essential mediator of Toll-like receptor 3–induced NF-κB activation

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Stimulation of Toll-like receptors (TLRs) initiates potent innate immune responses through Toll–interleukin 1 receptor (TIR) domain–containing adaptors such as MyD88 and Trif. Analysis of Trif-deficient mice has shown that TLR3-dependent activation of the transcription factor NF-κB by the TLR3 ligand double-stranded RNA is Trif dependent. Here we investigated the 'downstream' signaling events that regulate TLR3-dependent Trif-induced NF-κB activation. Trif recruited the kinases receptor interacting protein (RIP)-1 and RIP3 through its RIP homotypic interaction motif. In the absence of RIP1, TLR3-mediated signals activating NF-κB, but not the kinase JNK or interferon-β, were abolished, suggesting that RIP1 mediates Trif-induced NF-κB activation. In contrast, the presence of RIP3 negatively regulated the Trif-RIP1–induced NF-κB pathway. Therefore, in contrast to other TLRs, which use interleukin 1 receptor-associated kinase (IRAK) proteins to activate NF-κB, TLR 3-induced NF-κB activation is dependent on RIP kinases.

After recognizing conserved pathogen-associated molecular patterns, mammalian Toll-like receptors (TLRs) initiate an innate immune response. The ten known mammalian TLRs consist of an extracellular portion containing leucine-rich repeats, a transmembrane region and a cytoplasmic tail, called the Toll–interleukin 1 receptor (IL-1R) homology domain (TIR domain)[1–4]. Each TLR recognizes distinct ligands by means of its leucine-rich repeats and elicits different but often overlapping immune responses.

TLR3-induced signaling pathways are triggered by the binding of virus-derived double-stranded RNA, whereas TLR4 becomes activated after binding to lipopolysaccharide (LPS)[5–7]. Stimulation of either receptor induces a potent interferon-β (IFN-β) response, as well as activation of mitogen-activated protein kinases and the transcription factor NF-κB. It was originally proposed that the TIR-containing adaptor protein MyD88 was the crucial mediator of all TLR activities, including those of TLR3 and TLR4. However, analysis of MyD88-deficient mice showed the existence of parallel MyD88-independent pathways, because in these mice TLR3- and TLR4-induced NF-κB and JNK activation were not abolished but only delayed, whereas IFN-β responses were unaffected[8,9]. This second signaling pathway was found to be dependent on the TIR domain–containing adaptor protein Trif (also called TICAM-1)[10,11]. Trif associates with TLR3 and indirectly with TLR4 through another adaptor protein, TRAM[12,13].

Trif is an unexpectedly multifunctional adaptor protein, mediating activation of several transcription factors. Trif overexpression strongly activates IFN-β reporter genes, which correlates with the capacity of Trif to physically interact not only with interferon-regulatory factor 3 (IRF-3) but also with the IRF-3-activating kinase TBK-1 (refs. 14–16). Trif also triggers signals that result in activation of NF-κB and mitogen-activated protein kinases[10,11].

Analysis of Trif-deficient mice or mice carrying a frameshift mutation resulting in a C-terminal truncation of Trif (*Lps2* mutation)[17,18] showed that TLR3- and TLR4-induced IRF-3 activation was completely defective. TLR3-mediated NF-κB activation was also compromised, but TLR4-dependent NF-κB signals were still functional, most likely because of the MyD88-dependent pathway. Indeed, all TLR4 responses were abrogated in Trif-MyD88 double-deficient cells[18]. Thus, Trif seems to be an essential adaptor protein for the antiviral response triggered by TLR3, whereas two signaling pathways emanate from TLR4, one Trif dependent and the other MyD88 dependent.

Little is known about the signaling components that regulate the Trif-dependent pathway triggered by TLR3 stimulation. Trif contains a TIR domain flanked by an N-terminal and C-terminal extension. Overexpression of a Trif mutant encompassing the TIR and the N-terminal region activates both IFN-β and NF-κB, whereas a Trif mutant consisting of the TIR and the C-terminal extension fails to activate IFN-β strongly but still induces NF-κB activation[10,11]. These results suggest that both the N-terminal and C-terminal regions of Trif have the capacity to activate NF-κB, probably through distinct mechanisms. TRAF6, which is involved in TLR4-mediated NF-κB signaling[19], binds the N-terminal region of Trif and activates NF-κB[16]. The effector protein(s) linking the

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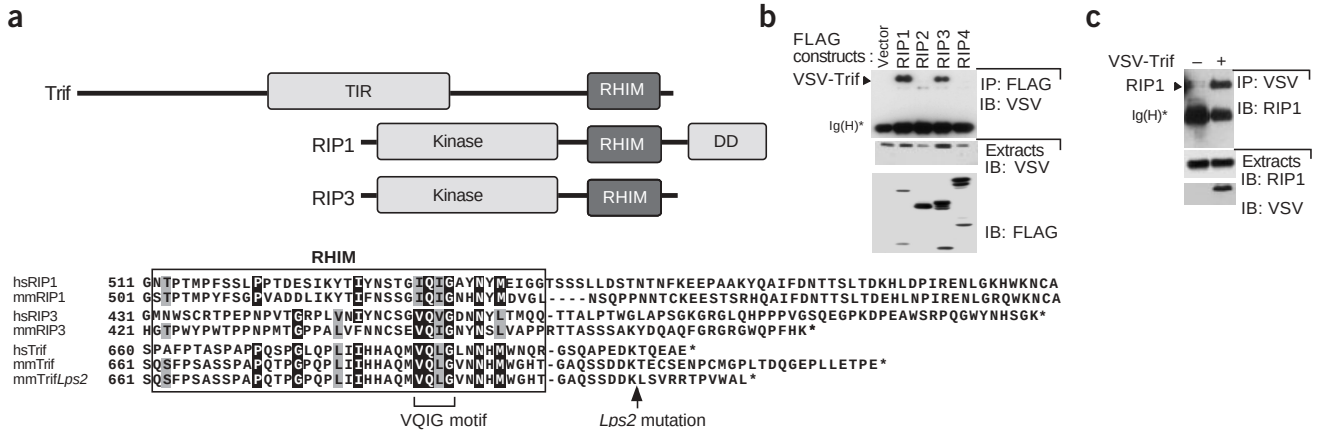

Figure 1 Trif interacts with RIP1 and RIP3. (**a**) Domain architecture of Trif and sequence alignment of the RHIM domain of human (hs) and mouse (mm) Trif, RIP1 and RIP3. Black and gray shading indicate ≥80% amino acid sequence identity and similarity, respectively. *, end of protein; DD, death domain; Ig(H)*, immunoglobulin heavy chain. The location of the *Lps2* frameshift mutation is shown. (**b**) 293T cells were transfected with the indicated FLAG-RIP constructs together with VSV-Trif, and anti-FLAG immunoprecipitates (IP) and cell extracts were analyzed by immunoblot (IB). (**c**) 293T cells were transfected with VSV-Trif, and anti-VSV immunoprecipitates (IP) and cell extracts were analyzed for the presence of endogenous RIP1 by immunoblot (IB).

C-terminal region of Trif with NF-κB activation remain(s) to be identified. Here we show that Trif contains a RIP homotypic interaction motif (RHIM) at the C terminus that is essential for binding of RIP1 and RIP3, two serine-threonine kinases linked to tumor necrosis factor (TNF)–mediated NF-κB activation[20–22]. TLR3-mediated NF-κB activation is impaired in mouse embryonic fibroblasts (MEFs) derived from RIP1-deficient mice, whereas the presence of RIP3 inhibits Trif-induced NF-κB activation.

RESULTS

Trif contains a RHIM and interacts with RIP1 and RIP3

Analysis of the predicted protein sequence of Trif showed notable similarity between a stretch of approximately 35 amino acids at the C terminus and a sequence present in the intermediary domains of RIP1 and RIP3. This stretch was previously identified as being responsible for mediating the interaction between RIP1 and RIP3 (ref. 23) and was designated RHIM (**Fig. 1a**). In particular, the four–amino acid (VQIG) motif that is most crucial for the RIP1-RIP3 interaction was highly conserved in Trif (VQLG), suggesting that Trif might interact with one or both of these kinases. Indeed, coexpression of Trif with all known RIP kinases (RIP1, RIP2, RIP3 and RIP4) in 293T HEK cells showed Trif associated with RIP1 and RIP3 but not with RIP2 and RIP4 (**Fig. 1b**), which lack the RHIM domain. Trif also interacted with endogenous RIP1 in 293T HEK cells (**Fig. 1c**). Thus, Trif is a RHIM-containing protein that interacts with the kinases RIP1 and RIP3.

To map the Trif-RIP interaction site, we generated a series of Trif and RIP1 deletion constructs (**Fig. 2**). We overexpressed these constructs in 293T cells and looked for an interaction by coimmunoprecipitation. The RHIM domain of Trif alone was sufficient for RIP1 interaction and it interacted with the RHIM-containing intermediary domain of RIP1. However, there was no interaction between the kinase domain of RIP1, which lacks the RHIM, and Trif (**Fig. 2b**). When four amino acids (QLGL) in the RHIM domain of Trif were replaced by four alanine residues (AAAA), the Trif-RIP1 interaction was impaired (**Fig. 2a**, right). The corresponding four amino acids in RIP3 are known to be essential for RIP1 binding[23]. These results therefore show that the RHIM domain of Trif can bind the RHIM domains of RIP1 and RIP3.

Because RIP1 is essential for TNF-induced NF-κB activation[24], it was likely that the previously noted activation of NF-κB by overexpression of the C-terminal region of Trif was RIP1 dependent. We measured Trif-dependent

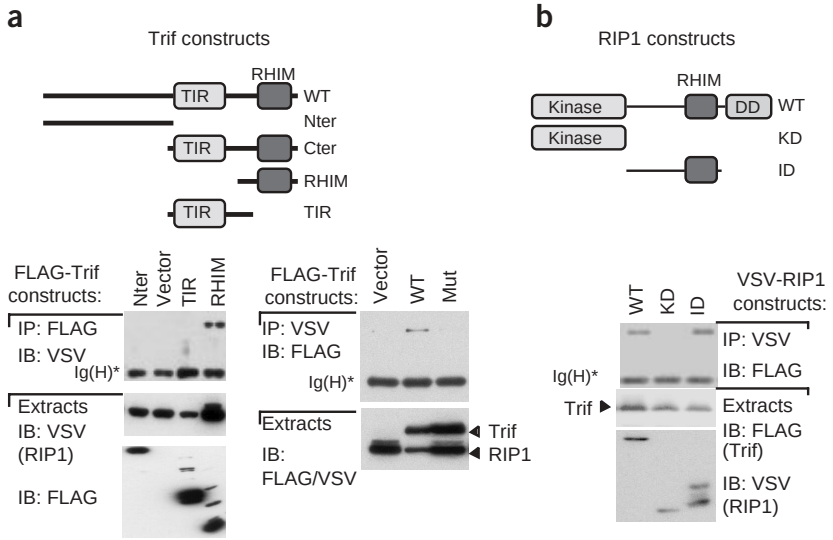

Figure 2 Trif and RIP1 interact through their RHIM. (**a**,**b**) 293T cells were transfected with different Trif and RIP1 constructs and the immunoprecipitates (IP) and cell extracts were analyzed by immunoblot (IB) as described in **Figure 1**. In the mutant (Mut) construct, the four amino acids QLGL within the RHIM domain of wild-type (WT) Trif were replaced with AAAA. KD, kinase domain; ID, intermediate domain; Ig(H)*, immunoglobulin heavy chain.

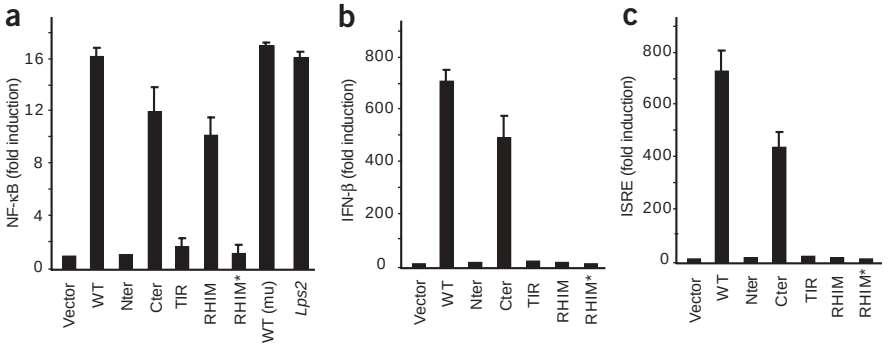

Figure 3 Trif-induced NF-κB activation is RHIM dependent. (**a**–**c**) 293T cells were transfected with an NF-κB, IFN-β or ISRE reporter plasmid, together with a Trif construct (horizontal axes) or an empty plasmid (Vector), and were analyzed for NF-κB-, IFN-β- or ISRE-dependent luciferase activity, respectively. Data are mean values ± s.d. from one experiment representative of at least three independent experiments. RHIM*, mutant RHIM domain (**Fig. 2**); WT (mu), mouse wild-type Trif; *Lps2*, *Lps2* mouse mutation (**Fig. 1a**).

NF-κB activation using a luciferase reporter gene under control of an NF-κB–responsive promoter. All Trif constructs containing the RHIM domain were active, including the *Lps2* construct bearing a C-terminal mutation after the RHIM (**Fig. 3a**). In contrast, the N terminus alone (lacking the TIR domain) and the RHIM mutant were inactive. The N-terminal fragment of Trif, also containing the TIR domain, activates NF-κB when overexpressed[16]. The TIR domain spontaneously oligomerizes after overexpression[25] and thus is important in transmitting signals when Trif is overexpressed in the absence of TLR3.

Overexpression of the RHIM domain, however, did not suffice for the activation of an IFN-β luciferase reporter gene (**Fig. 3b**). The transcription enhancer of the IFN-β promoter contains four positive regulatory domains, which function cooperatively to activate the expression of IFN-β in response to viral infection[26]. The transcription factors that bind to these elements include NF-κB, IRF-3 and the heterodimeric transcription factor ATF-2–c-Jun. The RHIM domain alone was unable to activate the IRF-3-dependent interferon-stimulated regulatory element (ISRE; **Fig. 3c**). For stimulation of the ISRE and the IFN-β promoters, both the TIR domain and the RHIM domain were required. Thus, different regions of the Trif molecule are involved in regulating different transcription factors, and the RHIM domain is crucial for NF-κB activation.

RIP3 downregulates Trif-mediated NF-κB activation

These results suggest that RIP1 and possibly RIP3 could be essential for TLR3-mediated NF-κB activation. RIP3-dependent phosphorylation of RIP1 inhibits NF-κB activation by RIP1 and TNF[23]. We confirmed these findings (**Fig. 4a**) and also showed that RIP3 inhibited Trif-induced NF-κB activation in a dose-dependent way when overexpressed in 293T cells. RIP3-induced inhibition was specific for Trif, as NF-κB was not substantially inhibited when triggered by overexpression of MyD88 (**Fig. 4a**). Competition for binding explained this inhibition (**Fig. 4b**). Binding of RIP1 to Trif was greatly reduced in the presence of RIP3, suggesting that the RHIM-mediated interaction of RIP1 with RIP3 was of higher affinity than the interaction of RIP1 with Trif or RIP3 with Trif. Thus, RIP3 acts as an inhibitor of Trif-induced NF-κB activation by competing with the binding of Trif to RIP1.

RIP1 mediates the TLR3-induced NF-κB activation

Because Trif can bind TLR3 (refs. 10,11), we used coimmunoprecipitation to determine whether a trimolecular complex, TLR3-Trif-RIP1, was formed. When Trif, Rip1 and TLR3 were overexpressed in 293T cells, formation of the trimolecular complex was possible and was dependent on the presence of Trif (**Fig. 5a**). Thus, Trif serves as an adaptor that links RIP1 to TLR3.

To investigate the importance of RIP1 in a physiological setting, we stimulated MEFs derived from Rip1-deficient (*Ripk1*$^{-/-}$) and *Ripk1*$^{+/-}$ mice with poly(I:C). We then assessed NF-κB and JNK activation by immunoblot with antibodies specifically recognizing the phosphorylated forms of the inhibitor of NF-κB (IκB) and JNK, respectively. Although NF-κB and JNK activation in *Ripk1*$^{+/-}$ MEFs was intact (**Fig. 5b**), NF-κB activation was ablated in MEFs from *Ripk1*$^{-/-}$ mice. However, JNK activation was not affected by the absence of RIP1. This suggests that RIP3 or another binding partner of Trif may transmit signals that lead to JNK activation. The absence of RIP1 also had no effect on the signaling pathways emanating from IL-1R (**Fig. 5b**) and TLR4 (data not shown), which are completely or mostly MyD88, IRAK1 and IRAK4 dependent[27].

RT-PCR analysis of different genes consistent with these results showed that transcription of the NF-κB-dependent *Icam1* was abolished after poly(I:C) treatment in RIP1-deficient cells (**Fig. 5c**). In contrast, transcription of *Ifnb* was not affected in the absence of RIP1, indicating that RIP1-mediated NF-κB activation is not essential for the IFN-β response to poly(I:C). To prove that RIP1 functions downstream of Trif, we overexpressed Trif in MEFs and showed that Trif-induced NF-κB activation was greatly reduced in RIP1-deficient cells (**Fig. 5d**). Hence, RIP1 mediates the TLR3-induced NF-κB activation 'downstream' of the adaptor Trif protein.

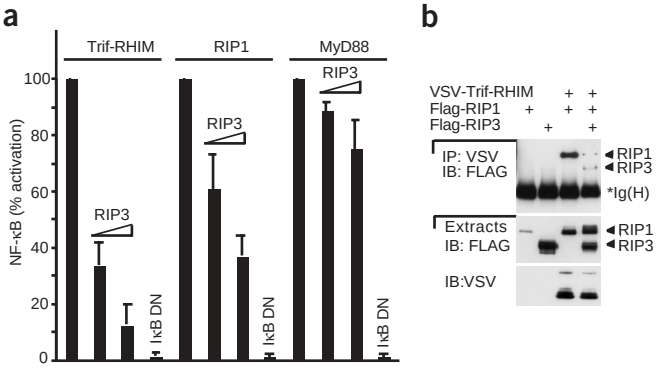

Figure 4 RIP3 inhibits Trif-dependent activation of NF-κB. (**a**) 293T cells were transfected with an NF-κB reporter plasmid, together with Trif-RHIM, RIP1 or MyD88, in combination with increasing concentrations (wedges) of RIP3 or an IκBα dominant negative (DN) or an empty plasmid, and were analyzed for NF-κB-dependent luciferase activity. Data are mean values ± s.d. from one experiment representative of at least three independent experiments. (**b**) 293T cells were transfected with VSV-Trif-RHIM, FLAG-RIP1 or FLAG-RIP3 as indicated, and anti-VSV immunoprecipitates (IP) and cell extracts were analyzed by immunoblot (IB). *Ig(H), immunoglobulin heavy chain.

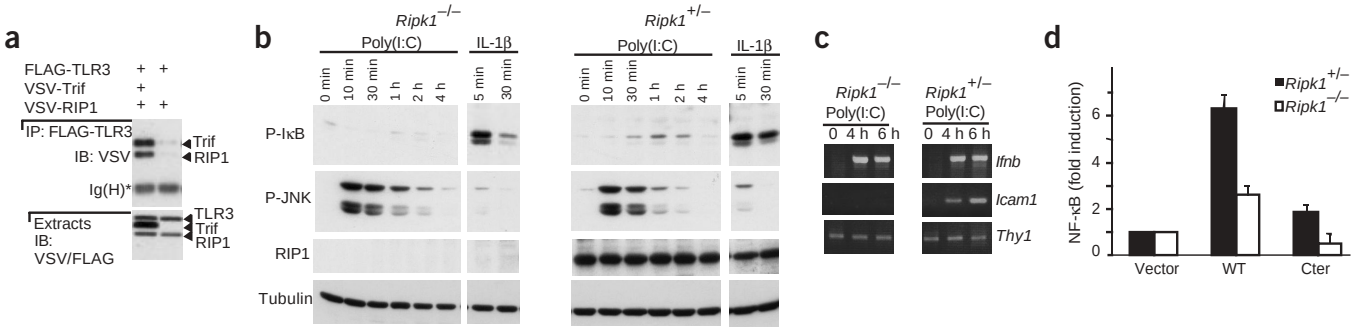

Figure 5 TLR3-induced NF-κB activation is RIP1 dependent. (**a**) 293T cells were transfected with TLR3, Trif or RIP1, as indicated, and anti-FLAG immunoprecipitates (IP) and cell extracts were analyzed by immunoblot (IB). Ig(H)*, immunoglobulin heavy chain. (**b**) *Ripk1*$^{-/-}$*Tnfrsf1a*$^{-/-}$ and *Ripk1*$^{+/-}$*Tnfrsf1a*$^{-/-}$ mouse embryonic fibroblasts were stimulated with poly(I:C) (100 μg/ml) to activate TLR3 or with IL-1β (20 ng/ml) to activate IL-1R. Activation of NF-κB and JNK was measured with phospho-specific (P-) antibodies to IκB and JNK, respectively. Equal loading was assessed by immunoblot for tubulin. (**c**) MEFs were stimulated with 100 μg/ml poly(I:C) for various periods of time (above lanes). Total RNA was isolated and assessed for expression of IFN-β (*Ifnb*), ICAM-1 (*Icam1*) and Thy1 (*Thy1*) by RT-PCR. (**d**) MEFs were transfected with an NF-κB reporter plasmid, together with a Trif construct (horizontal axis) or an empty plasmid (Vector), and NF-κB-dependent luciferase activity was analyzed. Data are mean values ± s.d. from one experiment representative of at least three independent experiments.

DISCUSSION

To combat viruses, the immune system has evolved several distinct viral recognition systems that converge on similar pathways to induce NF-κB and IFN-β activation. TLR3 is such a viral recognition system for which a specific adaptor protein, Trif, has been characterized[17,18]. Although Trif is essential for NF-κB and IFN-β responses triggered by TLR3, NF-κB can be activated independently of Trif in the TLR4 pathway[18], with MyD88 as an adaptor. MyD88 links TLR4 (and IL-1R) to the IRAK kinases and TRAF6, which in turn activate mitogen-activated protein kinases and, through IκB kinase-α (IKKα) and IKKβ, the transcription factor NF-κB.

Trif is a multipurpose adaptor protein that is able to recruit several proteins essential for the activation of the IFN-β response, such as IRF-3 and TBK1 (refs. 14–16). We have shown here that the RHIM domain of Trif recruits RIP1 to induce NF-κB activation. Trif also contains TRAF6-binding sites in the N-terminal region that are important for NF-κB signaling[16]. Therefore, Trif acts as a 'starting platform' for the recruitment of both TRAF6 and RIP1. This mode of activation of the NF-κB pathway is very reminiscent of MyD88-induced signals, of which TRAF6, IRAK1 and IRAK4 are essential components. The sequences of the kinase domains of IRAKs and RIPs are very similar[28], suggesting comparable functions. IRAKs are mainly associated with signaling pathways that are triggered by TLRs and IL-1Rs (containing TIR domains)[29], whereas RIPs are involved in pathways emanating from receptors of the TNF family[24]. Thus, Trif acts as an adaptor protein that links TLRs to RIP.

Trif also binds RIP3 through its RHIM domain. The physiological function of RIP3 is unknown, because RIP3-deficient mice are not yet available. It is likely, however, that the function of RIP3 is the down-regulation of the NF-κB response, as previously demonstrated for TNF signaling[23] and as supported by our results. The function of RIP3 may therefore be comparable to the function of IRAK3 (also known as IRAK-M) in the negative regulation of TLR-MyD88–mediated NF-κB activation. In that case, IRAK3 prevents binding of IRAK to TRAF6 (ref. 30). Like IRAK3, RIP3 has a restricted expression pattern, which contrasts with the ubiquitous expression of the other RIP or IRAK members. However, unlike IRAK3, RIP3 expression does not seem to be induced by proinflammatory signals (data not shown).

The *Lps2* mutation was identified in mice that had lost responses to double-stranded RNA after treatment with *N*-ethyl-*N*-nitrosourea[17]. The C-terminal 24 amino acids of Trif are replaced by 11 unrelated amino acids in this mutant Trif. The replacement starts immediately after the C-terminal region of the RHIM domain[23]. We therefore investigated whether this mutation could compromise the capacity of the RHIM domain to activate NF-κB. This, however, was not the case, in line with the fact that the C-terminal extension affected by the mouse *Lps2* mutation is not conserved in human Trif, a potent activator of NF-κB.

In summary, RIP1 is an essential component of the TLR3-Trif–mediated NF-κB signaling pathway. RIP1 is therefore not only important for NF-κB activation in later phases of the immune response when TNF is active but also at the very beginning, when an antiviral immune response is engaged via TLR3.

METHODS

Expression vectors. Full-length Trif cDNA was amplified from an expressed sequence tag clone by standard PCR with Pwo polymerase (Roche). Trif was subsequently cloned into a derivative of pCR3 (Invitrogen), in-frame with an N-terminal VSV or FLAG tag. Trif Nter (N-terminal; amino acids 1–385), Cter (C-terminal; amino acids 381–712), RHIM (amino acids 534–712) and TIR (amino acids 381–660) were amplified by PCR. Mutation of QLGL (amino acids 688–691) to AAAA (Mut) to generate a mutated RHIM in the gene encoding Trif was achieved by single PCR with a 3′ oligonucleotide containing the desired mutation. The RIP1 kinase domain (amino acids 1–303) and intermediate domain (amino acids 304–588) were amplified by PCR. The fidelity of the PCR amplifications was confirmed by sequencing. The luciferase reporter plasmid NF-κBLuc was provided by V. Jongeneel (University of Lausanne, Switzerland) and IFN-βLuc was provided by T. Taniguchi (University of Tokyo, Japan). TLR3 cDNA was a gift from T. Seya (Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan). pISRE-Luc was from Stratagene. The Renilla luciferase transfection efficiency vector (phRLTK) was purchased from Promega.

RT-PCR. Total RNA from *Ripk1*$^{+/-}$ TNF receptor–deficient (*Tnfrsf1a*$^{-/-}$) and *Ripk1*$^{-/-}$*Tnfrsf1a*$^{-/-}$ fibroblasts[31] was isolated with TRIzol reagent (Gibco BRL). Cells from six-well plates were lysed and collected in 500 μl TRIzol and 6 μl glycogen (Roche). Chloroform (100 μl) was then added with vortexing. After 5 min of incubation at room temperature (20° C) followed by 15 min of centrifugation at 4 °C, 3,000*g*, the aqueous phase was transferred to a new tube to which 250 μl isopropanol was added. An additional step of centrifugation

was done for 10 min. The pellet was then washed with 80% ethanol in diethyl pyrocarbonate–treated water and was resuspended in 50 μl diethyl pyrocarbonate–treated water for measurement of the concentration of RNA. The RNA (5 μg) was reverse transcribed with the ReadyToGo TPrimed FirstStrand Kit (Amersham Biosciences), according to the manufacturer's instructions. A portion of the cDNA obtained (5%) was used as a template for PCR amplification with Taq DNA polymerase (Sigma). The following primers were used: 5′-TTCCTGCTGTGCTTCTCCAC-3′ and 5′-GATTCACTACCAG TCCCAGAGTC-3′ for IFN-β; 5′-TGTCAGCCACTGCCTTGGTA-3′ and 5′-C AGGATCTGGTCCGCTAGCT-3′ for ICAM-1; and 5′-CCATCCAGCATGAGT TCAGCC-3′ and 5′-GCATCCAGGATGTGTTCTGA-3′ for Thy1.

Cell culture conditions. *Ripk1*$^{+/-}$*Tnfrsf1a*$^{-/-}$ and *Ripk1*$^{-/-}$*Tnfrsf1a*$^{-/-}$ mouse embryonic fibroblasts[31] and human embryonic kidney (HEK) 293T cells were grown in DMEM (Invitrogen) supplemented with 10% heat-inactivated FCS and penicillin plus streptomycin (100 μg/ml of each). Fibroblasts of *Ripk1*$^{+/-}$*Tnfrsf1a*$^{-/-}$ and *Ripk1*$^{-/-}$*Tnfrsf1a*$^{-/-}$ double-deficient mice were used, as breeding of these mice is easier than breeding of RIP1-deficient mice[31]. Where indicated, the MEFs were treated with 20 ng/ml mouse IL-1β or 100 μg/ml poly(I:C) (Sigma).

Transfection, immunoprecipitation and immunoblot. These assays were done in 293T cells as described[32]. MEFs were transfected in 24-well plates with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Antibody to FLAG M2 (anti-FLAG M2) and anti-VSV P5D4 were from Sigma. Anti-RIP1 38 was from Transduction Laboratories. Anti-phospho-JNK and anti-phospho-IκB were from Cell signaling (catalog numbers 9251 and 9241, respectively). Anti-tubulin was a gift from S. Lens (Netherlands Cancer Institute, Amsterdam, The Netherlands).

Luciferase reporter assays. These assays were done as described[28].

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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