Pellino1 Is Required for Interferon Production by Viral Double-stranded RNA

Karine Enesa, Alban Ordueau, Hilary Smith, David Barford, Peter C. F. Cheung, Janet Patterson-Kane, J. Simon C. Arthur, and Philip Cohen

Background: Signaling networks activated by viral double-stranded (ds) RNA stimulate interferonβ (IFNβ) production.

Results: Myeloid cells and fibroblasts from mice expressing an E3 ligase-deficient mutant of Pellino1 produce less IFNβ in response to viral dsRNA due to impaired interaction of IRF3 with the IFNβ promoter.

Conclusion: Pellino1 is needed to stimulate IFNβ gene transcription.

Significance: Pellino1 is a new component required to combat viral infection.

Viral double-stranded RNA, a ligand for Toll-like Receptor 3 (TLR3) and the cytoplasmic RNA receptors RIG1 and MDA5, activate a signaling network in which the IKK-related protein kinase TBK1 phosphorylates the transcription factor Interferon Regulatory Factor 3 (IRF3) and the E3 ubiquitin ligase Pellino1. IRF3 then translocates to the nucleus where it stimulates transcription of the interferonβ (IFNβ) gene, but the function of Pellino1 in this pathway is unknown. Here, we report that myeloid cells and embryonic fibroblasts from knock-in mice expressing an E3 ligase-deficient mutant of Pellino1 produce reduced levels of IFNβ mRNA and secrete much less IFNβ in response to viral double-stranded RNA because the interaction of IRF3 with the IFNβ promoter is impaired. These results identify Pellino1 as a novel component of the signal transduction network by which viral double-stranded RNA stimulates IFNβ gene transcription.

Following infection by bacteria or viruses, components of these pathogens bind to Pattern Recognition Receptors (PRRs) that are associated with membranes (e.g. Toll-like receptors (TLRs)) or are cytoplasmic (e.g. the double-stranded RNA (dsRNA) sensors, retinoic acid-inducible gene 1 (RIG1) and melanoma-differentiation-associated gene 5 (MDA5)). The activation of these receptors switches on signaling networks that stimulate the production of inflammatory mediators, such as pro-inflammatory cytokines, chemokines and interferons (IFNs). Most TLRs signal via myeloid differentiation primary response gene 88 (MyD88) (1, 2) and the interleukin-1 receptor-associated kinases (IRAKs) but TLR3, a receptor for dsRNA, signals via TIR-domain-containing adaptor-inducing interferon β (TRIF), while TLR4, which responds to lipopolysaccharide (LPS), signals via both TRIF and MyD88 (3–5).

The TLR3/4-TRIF and RIGI/MDA5 signaling networks both induce the phosphorylation of the transcription factor interferon regulatory factor 3 (IRF3), which dimerizes, translocates to the nucleus, and stimulates transcription of the IFNβ gene. In these pathways, the activation of IRF3 is catalyzed by TANK-binding kinase 1 (TBK1), although the related IKKε may also participate in this pathway (6–8) to ensure that it leads to the production of Type 1 rather than Type 2 interferons (9).

Pellino was originally identified as a protein in Drosophila melanogaster that interacts with Pelle, the Drosophila orthologue of mammalian IRAKs (10) where it is required for the secretion of anti-microbial peptides, such as drosomycin, and innate immunity (11). In mammalian cells, there are three peli genes, peli 1, -2, and -3, which encode three isoforms of Pellino, termed Pellino1, -2, and -3. The Pellino isoforms are E3 ubiquitin ligases, but only display this catalytic activity when they are phosphorylated at particular serine and threonine residues. The activation of the Pellino isoforms can be catalyzed in vitro by IRAK1, IRAK4 (12–14), TBK1, or IKKε (15), but the protein kinases that activate Pellino1 in vivo depend on the ligand and cell type. We found that IRAK1 is the major protein kinase that activates Pellino1 when mouse embryonic fibroblasts (MEFs) or HEK293 cells that stably express the interleukin 1 (IL-1) receptor are stimulated with IL-1, a ligand that signals via MyD88. In contrast, TBK1/IKKε are the major kinases that activate Pellino1 when MEFs are stimulated with TNFα or RAW264.7 macrophages are stimulated with PRRs that activate TLRs (15, 16).
Pellino1 Regulates the Production of IFNβ

Prolonged stimulation of RAW264.7 or primary BMDM with LPS or the synthetic dsRNA mimic poly(I:C) or bone marrow-derived dendritic cells (BMDC) with lipid A greatly increases the level of Pellino1 mRNA (15, 17) and protein (15). This induction of the Pellino1 protein did not occur in BMDM from TRIF−/− mice or IRF3−/− mice, was prevented by pharmacological inhibition of TBK1/IKKe, but only reduced modestly in BMDM from mice that do not express IFNAR (15). These findings established that the induction of Pellino1 is regulated by IRF3 “downstream” of TRIF.

To investigate the physiological roles of Pellino1, we generated mice in which the wild type protein was replaced by Pellino1[F397A], a mutant devoid of E3 ligase activity. Here, we have exploited these mice to demonstrate that Pellino1 is required to induce the interaction of IRF3 with the IFNβ promoter in poly(I:C)-stimulated myeloid cells or in MEFs infected with Sendai virus. These findings identify Pellino1 as a new player in the signal transduction network triggered by viral dsRNA.

**EXPERIMENTAL PROCEDURES**

**Materials**—Poly(I:C) was purchased from Invivogen, LPS (Escherichia coli strain O5:B55) from Alexis Biochemicals, IFNβ and IFNα from PBL Interferon Source. The JAK kinase inhibitors CP 690550 (Tofacitinib) and INCB18424 (Ruxolitinib) were purchased from Selleck Chemicals and ChemieTek, respectively. The pFN18A HaloTag® T7 Flexi® Vector was purchased from Promega and vectors expressing Halo-tagged-NFκB-essential modifier (NEMO) and Halo-tagged-NEMO[Δ311N] were made by Dr. Mark Peggie, MRC Protein Phosphorylation Unit, Dundee.

**Antibodies and Other Proteins**—Phosphospecific antibodies that recognize IRF3 phosphorylated at Ser-396 (Catalogue #4947), p38 MAP kinase (p38 MAPK) phosphorylated at Thr-180 and Tyr-185 (#44682) and an antibody was from BD Transduction (#610459) and an anti-ubiquitin antibody was from Dako (#Z0458). Rabbit and mouse secondary antibodies conjugated to horseradish peroxidase were from Pierce. The Pellino1 antibody has been described previously (15). The Pellino1, UBE1, Ubc13-Uev1a proteins were expressed and purified in the Division of Signal Transduction Therapy, MRC Protein Phosphorylation Unit, University of Dundee (12, 14). Ubiquitin was purchased from Sigma. The polyubiquitin–binding protein NEMO and the polyubiquitin binding-defective mutant NEMO[Δ311N] were expressed in Escherichia coli as Halo-tagged proteins, and attached covalently to HaloLink™ resin in the bacterial extracts as described by the manufacturer (Promega). The resin was then washed extensively with 50 mM Tris/HCl pH 7.5, 1 M NaCl, 0.1 mM EDTA, 270 mM sucrose, 0.03% (w/v) Brij 35, 0.1% (v/v) 2-mercaptoethanol, 0.2 mM phenylmethylsulphonyl fluoride, and 1 mM benzamidine.

**Generation of Pellino1[F397A] Knock-in Mice**—Pellino1 knock-in mice were generated by Taconic-Artemis. Briefly, a targeting vector was designed to create a knock-in mutation in which Phe-397 of the endogenous peli 1 gene was mutated to Ala (supplemental Fig. S1A). ES cells derived from C57/B16N mice were targeted using standard techniques, and ES cells that were targeted using correctly were identified by Southern Blotting. Knock-in ES cells were microinjected into mouse blastocysts to obtain chimeric mice. Germline chimeras were crossed with C57/B16 Flpe transgenic mice to delete the puromycin resistance gene. Following this deletion, the Pellino1[F397A] knock-in mice were bred away from the Flpe transgene and maintained on a C57/B16 background. Routine genotyping was carried out by PCR from ear biopsies using primers across the 3′ loxP/Frt sites, which generated a band of 384 bp from the knock-in allele or 229 bp from the wild-type allele. Sequences of the primers used are 5′-GACAAGCGTTAGTGGCACCAC-3′ and 5′-GGCGAGAACGCGCTTATCG-3′ that generate a 343 bp fragment in Flpe-positive samples. Animals were maintained under specific pathogen-free conditions in line with EU and UK regulations. Experiments were carried out under a UK project license that was subject to local ethical review.

**Real-time PCR**—Total RNA was extracted from 1.2 × 10⁶ BMDM or BMDC or 1.0 × 10⁵ primary MEFs using a Qiagen RNeasy kit following the manufacturer’s instructions and quantitated by measuring the absorbance at 260 nm. 1 μg of total RNA was reverse trancribed into cDNA for 30 min at 42°C using the qScript cDNA SuperMix from Quanta Bioncisces. 50 ng of cDNA was incubated with primers (100 nm) in a total volume of 20 μl using the PerfeCT Syber Green Fast mix from Quanta Biosciences and the cDNA corresponding to the mRNA amplified was measured using the ΔΔ Cycle Threshold (CT) method and the constitutively expressed gene hypoxanthine phospho-ribosyl transferase as an internal control. The following primers were used in this study: Pellino1-F-5′-CTGCGTGAACACAGATCGTACAGAG-3′; Pellino1-R-5′-CCGAGCTGATTGATCTCCTGTCTTAAAGC-3′; IL-12p40-F-5′-TCATCAAGGAGACATCAAACC-3′; IL-12p40-R-5′-TGAGGGAGAAGTAGGAATGGG-3′; IL-6-R-5′-TTCCATCCAGTGCTTCTT-3′; IL-6-R-5′-AGGCTTCGTTGGGAGTGTATC-3′; TNFα-F-5′-CAGACCTCAACTCAGATCTAC-3′; TNFα-R-5′-GCTACAGGCGTGTCTCTGCT-3′; IFNβ-F-5′-GGAAAAGCAAGAGGAAAGATGG-3′; IFNβ-R-5′-CATCTCAGTGGTAGCTAGCG-3′; ISG15-F-5′-CAGGACGGTCTTACCCTTTCC-3′; ISG15-R-5′-GCTGATCGGAGAAGAGTTCG-3′; Cxcl10-F-5′-CAGGACGGTCTTACCCTTTCC-3′; Cxcl10-R-5′-GCTGATCGGAGAAGAGTTCG-3′; SendaivirusPgene-R-5′-CATCCTCAGGTAGTTGTTTG-3′.

**Cells**—Both Splenic and bone marrow-derived dendritic cells (BMDC) and macrophages (BMDM) were generated in-house from 6- to 12-week-old C57/Bl6 mice obtained under specific pathogen-free conditions following guidelines established in the Supplemental Information.

**Elution of Halo-tagged Proteins**—The pFN18A HaloTag® T7 Flexi® Vector was purchased from Promega. The resin was then washed extensively with 50 mM Tris/HCl pH 7.5, 1 M NaCl, 0.1 mM EDTA, 270 mM sucrose, 0.03% (w/v) Brij 35, 0.1% (v/v) 2-mercaptoethanol, 0.2 mM phenylmethylsulphonyl fluoride, and 1 mM benzamidine.
5′-GGGCTCCAAACCCCAAGCCC-3′; IRF7-R-5′-ctgcgtctcggtagagctcgg-3′.

ELISA—The concentration of IFNβ and other cytokines released into the cell culture medium was determined by ELISA using a Verikine mouse IFNβ and mouse IFNα ELISA kit from PBL Interferon Source and murine IL-12, TNFα, and IL-6 ELISA kit from Peprotech. The concentration of cytokines in the cell culture medium was calculated using a standard curve established with known amounts of purified recombinant murine proteins.

Virus Infection—Sendai virus (Cantell Strain) was supplied by Charles River Laboratories with a minimum HA titer of 2000 HA units/ml. MEFs from wild type and Pellino1[F397A] mice were seeded at 1 × 10^5 in a 12-well plate. The following day, cells were infected using 100 HA units/ml in a serum-free medium. Cells were incubated for 60 min at 37 °C with the virus in serum-free media. The serum-free media were then replaced by complete media, and the cells incubated at 37 °C for times indicated in figure legends.

Chromatin Immunoprecipitation—ChIP assays were performed using the ChIP-IT™ Express kit from Active Motif according to the supplier’s instructions. Briefly, BMDM (5 × 10^6 cells) from wild-type or Pellino1[F397A] mice were stimulated with 10 μg/ml poly(I:C), then cross-linked for 10 min with 1% formaldehyde at room temperature and the cells lysed. The chromatin was then enzymatically digested to an average size of ~300 bp, and the “sheared” chromatin incubated with shaking overnight at 4 °C with 2 μg of control IgG (Active Motif) or anti-IRF3 (Santa Cruz Biotechnology SC15991X) and 25 μl of protein G magnetic beads. The immune-precipitated DNA was purified with the MiniElute PCR purification kit from Qiagen, then incubated with primers (100 nM) in a total volume of 20 μl using the PerfeCT SYBR Green Fast mix from Quanta Biosciences. The precipitated DNA was amplified and measured by real-time quantitative PCR. qPCR values were determined using the ΔΔ Cycle Threshold (CT) method using IgG and the constitutively expressed gene hypoxanthine phosphoribosyl transferase as an internal control. The following primers were used: CHIP Ifnβ for 5′-GCC AGG AGC TTG AAT AAA ATG-3′; CHIP Ifnβ rev 5′-CTG TCA AAG GCT GCA GTG AG-3′.

Histopathology—This was carried out on tissue sections as described previously (18). Images were analyzed and processed using Photoshop software (CS3; Adobe).

Statistical Analysis—The statistical significance of results was calculated using the two-tailed Student’s t test using GraphPad Prism software.

Cell Culture, Stimulation, Lysis, and Immunoblotting—This is described under supplemental materials.

RESULTS

Generation of Pellino1[F397A] Knock-in Mice—Each Pellino isoform comprises a Forkhead-associated (FHA) domain connected to a region of anti-parallel β-sheet, termed the wing appendage (19), which is followed by a RING domain that carries the E3 ligase activity. The three-dimensional structure of the Pellino1 RING domain is unknown. Moreover, attempts to construct a homology model by threading its amino acid sequence onto known RING finger structures failed (data not shown), probably because of the low sequence similarity between the Pellino1 RING domain and other known RING domain structures (supplemental Fig. S1A). However, we noticed that the first, second, and fourth pairs of zinc-coordinating residues are equivalent to those in the RING domain of the E3 ubiquitin ligase c-Cbl, namely 533CPM536C, 367CG369H, and 396CPF398C of Pellino1, and propose that the third pair corresponds to 386HGT389H (supplemental Fig. S1A). In the structure of Cbl complexed to the E2-conjugating enzyme UbcH7, the Ile and Phe residues within the first and fourth Zn-coordinating Cys pair (CKIC and CPFC) mediate contacts with UbcH7 (also known as UBE2L3) (20). Thus, mutation of the equivalent residues in mouse Pellino1 (Met-335 and Phen-397) would be expected to disrupt the interaction between the Pellino1 RING domain and E2-conjugating enzymes. Consistent with this prediction, we found that the mutation of Phen-397 to Ala abolished the E3 ligase activity of bacterially expressed Pellino1 measured in the presence of the E1-activating enzyme (UBA1, also called UBE1) and the E2-conjugating enzyme Ubc13-Uev1a (also called UBE2N-UBE2V1) (supplemental Fig. S1B). We therefore made a knock-in mouse, in which wild type Pellino1 was replaced by the E3 ligase-inactive mutant Pellino1[F397A] (supplemental Fig. S1, C and D). The adults were of similar size to wild-type littermates with no obvious phenotypic differences when kept in the relatively sterile conditions of the animal unit (results not shown). The Pellino1[F397A] knock-in mice were born at a slightly lower frequency than that expected from normal Mendelian inheritance (supplemental Fig. S1E).

The Pellino1 protein was expressed in all mouse tissues examined (supplemental Fig. S1F), consistent with earlier measurements of mRNA levels in different tissues (21). However, the level of expression of the Pellino1[F397A] mutant was lower than that of the wild type protein in every tissue studied (supplemental Fig. S1F), suggesting that the E3 ligase activity of Pellino1 may be required for its stability.

The Effect of Poly(I:C) and LPS on the Expression of Pellino1 in BMDM from Pellino1[F397A] and Wild Type Mice—The stimulation of BMDM with poly(I:C) or LPS increases the expression of the Pellino1 protein, which begins after 2 h and reaches a maximum between 4 and 6 h (15). As found for wild type Pellino1, the level of the Pellino1[F397A] protein in BMDM from the “knock-in” mice also increased markedly after stimulation with the TLR3 ligand poly(I:C) or the TLR4 agonist LPS (Fig. 1A). Similar to other tissues (supplemental Fig. S1F), the level of the Pellino1[F397A] protein was lower than wild type Pellino1 in BMDM, either before or after stimulation with poly(I:C) or LPS (Fig. 1A). In contrast, the poly(I:C)- or LPS-stimulated production of the mRNA encoding the Pellino1[F397A] mutant or wild type Pellino1 was similar (Fig. 1B), indicating that the reduced amount of the Pellino1[F397A] protein either results from less efficient translation of the mRNA or from decreased stability of the protein. These experiments also established that the E3 ligase activity of Pellino1 was not required for the poly(I:C) or LPS-stimulated transcription of the pel1 gene.

The Poly(I:C)-stimulated Production of IFNβ mRNA and IFNβ Secretion Is Suppressed in BMDM from Pellino1[F397A] Knock-in Mice—The TRIF-dependent signaling network that initiates transcription of the gene encoding IFNβ requires the...
Pellino1 Regulates the Production of IFNβ

TBK1-catalyzed phosphorylation and activation of IRF3. Because TBK1 also activates the Pellino1 E3 ligase and increases its expression, we investigated whether the pathway leading to IFNβ gene transcription was affected in BMDM from the Pellino1[F397A] knock-in mice. These studies showed that the poly(I:C)-stimulated activation of TBK1 and IRF3 phosphorylation were similar in BMDM from wild type and Pellino1[F397A] mice (Fig. 2A), but the production of IFNβ mRNA in cells from the Pellino1[F397A] mice was reduced considerably after stimulation for 1–2 h with poly(I:C) (Fig. 2B). An even greater reduction in IFNβ mRNA production (Fig. 2C) and loss of IFNβ secretion (Fig. 2D) was observed between 6 and 24 h after stimulation with poly(I:C).

The secretion of IFNβ could only be detected reliably by ELISA 6 h after stimulation with poly(I:C) and the rate of secretion accelerated greatly thereafter (Fig. 2D). This second phase of IFNβ secretion is explained by the positive feedback autoimmune loop shown schematically in Fig. 3A. In the present study, the importance of the positive feedback loop was demonstrated by blocking it with the potent and specific Janus kinase (JAK) inhibitors, Ruxolitinib or Tofacitinib (supplemental Fig. S2), which prevent the phosphorylation of STAT1 at Tyr-701 catalyzed by JAKs (Fig. 3B). These compounds drastically decreased poly(I:C)-stimulated secretion of IFNβ after 6 h (Fig. 3C). A similar decrease in IFNβ secretion was observed in BMDM from mice that do not express the IFNAR (Fig. 3D). Nevertheless, a 100-fold increase in the level of IFNβ mRNA still occurred during the first 1–2 h of exposure to poly(I:C) in the presence or absence of Ruxolitinib or Tofacitinib (Fig. 3E), demonstrating that this initial phase of IFNβ production takes place independently of the positive feedback loop. A further 100-fold to 1000-fold increase in the level of IFNβ mRNA was observed after stimulation with poly(I:C) for 6 h or longer (Fig. 3E, note the Log scale of the ordinate), which was followed by the secretion of large amounts of IFNβ into the cell culture medium (Fig. 2D). This increase in IFNβ mRNA and secretion was dependent on the positive feedback loop, since it was prevented by either Ruxolitinib or Tofacitinib (Fig. 3, C and E).

The decreased production of IFNβ mRNA in BMDM from the Pellino1[F397A] mice 0.5–2 h after stimulation with poly(I:C) (Fig. 2B), demonstrated that Pellino1 plays an important role in the TRIF-TBK1 signaling pathway that is independent of the feedback loop. However, the more striking decrease in IFNβ mRNA production from 6 h onwards (Fig. 2C), suggested that Pellino1 might also drive the positive feedback loop. To investigate this possibility, we initially studied the poly(I:C)-stimulated formation of the mRNA encoding IRF7 and IFNa, which participate in the positive feedback loop by stimulating...
transcription of the IFNγ gene (IRF7) or by activating the IFNAR (IFNγ/H9251) (Fig. 3). These experiments showed that the poly(I:C)-stimulated production of IRF7 (Fig. 4A), IFNγ/H9251 (Fig. 4B), and IFNγ/H9251 mRNA (Fig. 4C), as well as IFNα secretion (Fig. 4D), were lower in BMDM from the Pellino1[F397A] knock-in mice than from wild type mice, demonstrating reduced production of components required for the operation of the positive feedback loop.

We also studied the gene encoding MX1, which is required for cells to set up a specific anti-viral state against influenza virus (24) and whose transcription is reported to be dependent on ISGF3 (see legend to Fig. 3) (25). The level of MX1 mRNA should therefore reflect the activity of the positive feedback loop (Fig. 2B). The poly(I:C)-stimulated transcription of MX1 was 60–70% lower in BMDM from the Pellino1[F397A] mice than wild type mice (Fig. 4E), supporting a role for Pellino1 in stimulating ISGF3-dependent gene transcription. A similar reduction was observed in the mRNA encoding other interferon-inducible genes, like interferon-stimulated gene 15 (ISG15) (Fig. 4F).

From the results presented thus far, it could be argued that the role of Pellino1 in driving the positive feedback loop was indirect and merely a consequence of the requirement of this protein during the initial phase of IFNγ/H9251 production. We therefore investigated the role of Pellino1 in the positive feedback loop directly by studying whether it was required for the IFNβ-
stimulated transcription of IFN-regulated genes. These experiments showed that the IFNβ-stimulated production of the mRNA encoding IRF7, MX1 and ISG15, as well as the chemokine CXCL10, was lower in BMDM from the Pellino1[F397A] mice compared with wild type mice (Fig. 5A), demonstrating that the reduced transcription of IFNβ-regulated genes is not explained by impaired function of the IFNAR, or by reduced activation of the JAKs or by their inability to phosphorylate STAT1. Thus Pellino1 exerts its effect on IFNβ-stimulated gene transcription at a step(s) that is distal to the activation of STAT1.

The increased phosphorylation of STAT1 in BMDM from the Pellino1[F397A] mice appears to be explained by the enhanced level of expression of STAT1 in these cells (Fig. 5B, second panel), and suggests that wild type Pellino1 may negatively regulate the expression of STAT1, perhaps to prevent the hyper-activation of the JAK-STAT1/2 pathway.

The LPS-stimulated Production of IFNβ Is Reduced in BMDM from Pellino1[F397A] Mice—The LPS-stimulated phosphorylation of TBK1 and IRF3 was similar in BMDM from Pellino1[F397A] and wild type mice (supplemental Fig. S3A). Stimulation with LPS for 1–2 h produced much higher levels of IFNβ mRNA than stimulation for the same length of time with poly(I:C), presumably explaining why IFNβ secretion could be measured by ELISA at these relatively early time points. The IFNβ mRNA produced after stimulation with LPS for 1–2 h was 60% lower in Pellino1[F397A] mice than in wild type mice, similar to the percentage reduction in IFNβ observed after stimulation with poly(I:C) for the same time (Fig. 3B). However, in contrast to poly(I:C), the LPS-stimulated production of IFNβ mRNA and secretion peaked after 1–2 h and then declined rapidly (supplemental Fig. S3, B and C). Moreover, in contrast to poly(I:C), the LPS-stimulated secretion of IFNβ was similar in BMDM from wild type mice and IFNAR-deficient mice (supplemental Fig. S3D), showing that LPS does not engage the positive feedback loop that enhances IFNβ production. This is consistent with the finding that LPS does not stimulate the secretion of IFNα (26). The transient LPS-stimulated increase in IFNβ mRNA and failure to engage the positive feedback loop may result from the desensitization of the LPS receptor and/or from the presence of an inhibitor(s) of IFNβ production produced via the MyD88-dependent signaling pathway.

Reduced Secretion of IFNβ in Bone Marrow-derived Dendritic Cells (BMDC) from Pellino1[F397A] Mice—To investigate whether the requirement for Pellino1 was specific to BMDM or
was also observed in other myeloid cells, further studies were carried out using bone-marrow-derived dendritic cells (BMDC). Similar to BMDC, the poly(I:C)-stimulated (supplemental Fig. S4A) and LPS-stimulated (supplemental Fig. S4B) secretion of IFNβ was reduced in BMDC from the Pellino1[F397A] mice relative to wild type mice. Compared with BMDC, the poly(I:C)-stimulated secretion of IFNβ was delayed and lower in BMDC from wild type mice (supplemental Fig. S4A). As found in BMDC, prolonged stimulation of BMDC with poly(I:C) (supplemental Fig. S4C) or LPS (supplemental Fig. S4D) induced a large increase in the expression of the Pellino1 protein.

**Reduced Production of Type 1 Interferons in Sendai Virus-infected MEFs**—MEFs express low levels of TLR3 and other TLR receptors. Therefore to investigate whether Pellino1 plays a role in the production of IFNβ via the cytoplasmic dsRNA receptors RIG1 and MDA5 (27), we infected MEFs with Sendai virus containing defective-interfering genomes, which strongly stimulate IFNβ production in these cells (28). The binding of dsRNA to RIG1/MDA5 activates a signaling pathway that converges with the poly(I:C)-TRIF-dependent pathway at the level of TBK1 (29). The production of IFNβ mRNA (Fig. 6A) and IFNβ secretion (Fig. 6B) after infection with Sendai virus was reduced by 90% in MEFs from Pellino1[F397A] mice compared with wild type controls. The production of CXCL10 (an established marker of viral infection) (Fig. 6C), as well as IFNα4 mRNA (Fig. 6D), were also reduced in MEFs from the knock-in mice, but induction of the mRNA encoding the pro-inflammatory cytokine IL-12 (Fig. 6E) or Pellino1 itself (Fig. 6F) was similar to wild type mice.

The expression of the Sendai virus nucleocapsid protein was not decreased in MEFs from the knock-in mice up to 6 h and increased from 12–24 h (Fig. 6G), indicating that the decreased production of IFNβ in MEFs from the Pellino1[F397A] mice was not caused by the failure of the virus to infect MEFs from the Pellino1[F397A] mice.

**Defective Recruitment of IRF3 to the IFNβ Promoter in BMDC or MEFs from Pellino1[F397A] Mice**—To investigate whether Pellino1 affected the interaction of IRF3 with the IFNβ promoter, we immunoprecipitated IRF3 from cell extracts and quantitated the amount of the IFNβ promoter associated with the immunoprecipitates by RT-PCR. These studies showed that stimulation with poly(I:C) enriched the association of IRF3 with IFNβ promoters in BMDC from wild type mice but not from Pellino1[F397A] mice (Fig. 7A). This was observed after 1 h and sustained for at least 4 h. Similar results were obtained when Sendai virus-infected MEFs were used instead of poly(I:C)-stimulated BMDC (Fig. 7B). These ChIP experiments show that Pellino1 stimulates IFNβ gene transcription by promoting the binding of IRF3 to the IFNβ promoter.

**Comparison of the Phenotypes of the Pellino1[F397A] Mice and Pellino1−/− Mice**—In contrast to the present study, poly(I:C)- or LPS-stimulated IFNβ mRNA production was reported to be similar in BMDC from Pellino1−/− and wild type mice (40), and instead, BMDC from Pellino1−/− mice showed greatly reduced production of pro-inflammatory cytokines after stimulation with poly(I:C) or LPS. We therefore also investigated the TRIF-dependent signaling network that leads to the production of pro-inflammatory cytokines in BMDC from Pellino1[F397A] mice.

Poly(I:C) activates the canonical IKKs (IKKα and IKKβ) and mitogen-activated protein (MAP) kinases and the production of pro-inflammatory cytokines (30), via a TRIF-dependent signaling pathway that is reported to require receptor-interacting protein 1 (RIP1) and its polyubiquitylation (31, 32). To study RIP1, we captured the polyubiquitylated protein formed in poly(I:C)-stimulated BMDC on immobilized NEMO, a protein that binds to Lys-63-linked and linear polyubiquitin chains (18). We found that the poly(I:C)-stimulated polyubiquitylation of RIP1 was similar in BMDC from Pellino1[F397A] and wild type mice (Fig. 8A). Similarly MRT67307, a potent inhibitor of the IKK-related protein.
Pellino1 Regulates the Production of IFNβ

FIGURE 7. IFNβ promoters associated with IRF3 are reduced in cells from Pellino1[F397A] knock-in mice. A, BMDM from Pellino1[F397A] or wild type mice were stimulated with poly(I:C) (10 μg/ml) for the times indicated, lysed and ChIP assays performed after immunoprecipitating IRF3 from the extracts with a specific anti-IRF3 antibody (or with anti-IgG as a control). The enrichments of IFNβ promoters in the immunoprecipitates were then quantitated as described under “Experimental Procedures.” B, same as A except that MEFs were infected with Sendai virus (SeV) (100 HA/ml). The results are shown ± S.D. for triplicate determinations using MEFs from three wild type and three Pellino1[F397A] mice at each time point. Similar results were obtained in three independent experiments.

We also found that the poly(I:C)-stimulated phosphorylation of the IKKβ substrate p105 (35, 36) and the phosphorylation of p38 MAP kinase was not impaired in BMDM from the Pellino1[F397A] mice (Fig. 8A, lower panel) or in the presence of MRT67307 (Fig. 8B, lower panel). More detailed analysis revealed that the transient activation of IKKβ (studied by either the phosphorylation of p105 or the degradation of IkBα), p38 MAPK, and JNK, actually occurred more rapidly in poly(I:C)-stimulated BMDM from the Pellino1[F397A] mice than wild type mice (Fig. 8C). The poly(I:C)-stimulated production of the mRNAs encoding the pro-inflammatory cytokines IL-12 and TNFα mRNA did not differ significantly in BMDM from Pellino1[F397A] and wild type mice from 6–24 h after stimulation, although there was a significant reduction in the secretion of these cytokines and in the production of IL-10 (supplemental Fig. S5).

The LPS-stimulated phosphorylation of p105, the degradation of IkBα and the activation of MAP kinases was similar in BMDM from Pellino1[F397A] and wild type mice (supplemental Fig. S6A), but the production of IL-6 and IL-12 mRNA and the secretion of IL-6, IL-12, and TNFα were reduced significantly (supplemental Fig. S6B).

The decreases in poly(I:C) and LPS-stimulated pro-inflammatory cytokine production that we observe in BMDM from Pellino1[F397A] mice could be explained, at least in part, by the decreased production of IFNβ, because IL-6 mRNA production was reduced in the spleens of IRF3−/− mice compared with wild type mice after LPS injection in vivo (37), while production of IL-12 mRNA was decreased in macrophages from IFNβ−/− mice compared with wild type mice (38).

Other Phenotypes of the Pellino1[F397A] Mice—6-Month-old Pellino1[F397A] mice showed enlarged mandibular lymph nodes, moderate to marked periductular infiltrates of lymphoid cells in salivary glands, and mild to moderately increased amounts of lymphoid tissue around the airways of the lung, the pelvis (not shown) and blood vessels in the kidney. Multifocal moderate periductular lymphoid infiltrates were also observed in the pancreas of several Pellino1[F397A] mice and aggregates of lymphoid cells around the blood vessels in the liver of two Pellino1[F397A] mice were also observed (supplemental Fig. S7). Enlarged lymph nodes were observed in Pellino1−/− mice that were more than 6-months-old, but we did not observe any enlargement in the spleens of 6-month-old Pellino1[F397A] mice, which have been reported for Pellino1−/− mice of the same age.

DISCUSSION

It is well established that the activation of TLR3, TLR4, RIG1, and MDA5 induces the activation of TBK1, which then phosphorylates IRF3 leading to its dimerization and translocation to the nucleus where it stimulates transcription of the gene encoding IFNβ (39). These findings did not, however, exclude the possibility that other proteins that are produced and/or activated by this signaling pathway might also be important for IFNβ gene transcription and in this paper we identify Pellino1 as a new and important component of this pathway. We have previously shown that TBK1 and IKKe activate Pellino1 and increase its expression in response to poly(I:C) and LPS (15), and we now show that IFNβ mRNA production and IFNβ secretion is reduced in BMDM or BMDC from mice expressing the E3-ligase inactive Pellino1[F397A] mutant instead of the wild type protein. Indeed the reduction in IFNβ secretion is comparable to that observed in BMDC from IRF3−/− mice (37).

The production of IFNβ mRNA and IFNβ secretion induced by infection of MEFs with Sendai virus was also greatly suppressed in the Pellino1[F397A] “knock-in” mice. Thus Pellino1 plays an important role in the production of IFNβ by viruses that signal via TLR3 and/or cytoplasmic RNA receptors.

Pellino1 did not enhance the poly(I:C)-stimulated transcription of the IFNβ gene in BMDM by facilitating the phosphorylation of TBK1 at Ser-172 or IRF3 at Ser-396 (Fig. 2), and we now show that IFNβ expression in response to poly(I:C) and LPS (15), and the phosphorylation of TBK1 at Ser-172 or IRF3 at Ser-396 (Fig. 2), and the possibility that other proteins that are produced and/or activated by this signaling pathway might also be important for IFNβ gene transcription and in this paper we identify Pellino1 as a new and important component of this pathway. We have previously shown that TBK1 and IKKe activate Pellino1 and increase its expression in response to poly(I:C) and LPS (15), and we now show that IFNβ mRNA production and IFNβ secretion is reduced in BMDM or BMDC from mice expressing the E3-ligase inactive Pellino1[F397A] mutant instead of the wild type protein. Indeed the reduction in IFNβ secretion is comparable to that observed in BMDC from IRF3−/− mice (37).

The production of IFNβ mRNA and IFNβ secretion induced by infection of MEFs with Sendai virus was also greatly suppressed in the Pellino1[F397A] “knock-in” mice. Thus Pellino1 plays an important role in the production of IFNβ by viruses that signal via TLR3 and/or cytoplasmic RNA receptors.

Pellino1 did not enhance the poly(I:C)-stimulated transcription of the IFNβ gene in BMDM by facilitating the phosphorylation of TBK1 at Ser-172 or IRF3 at Ser-396 (Fig. 2A and supplemental Fig. S3A), but was needed for IRF3 to interact with the IFNβ promoter. This effect could already be observed after 1 h (Fig. 7A), which is earlier than any increase in the expression of the Pellino1 protein can be detected (15), implying that the effects are likely to be mediated by the covalent modification of Pellino1, presumably its phosphorylation.

The expression of Pellino1[F397A] in cells from the knock-in mice was much lower than wild type Pellino1. It is therefore unclear whether the failure of the mutant protein to stimulate IFNβ gene transcription results from the mutation of Phe-397 to Ala, the low level at which the mutant is expressed or both. However, reduced expression of the mutant Pellino1 alone seems less likely to account for the results because poly(I:C)- or LPS-stimulated IFNβ mRNA production was reported to be unimpaired in BMDM from Pellino1 knock-out mice (40). This suggests that the F397A mutation underlies, at least in part, the failure to stimulate IFNβ gene transcription. We also cannot exclude the possibility that the mutation of Phe-397 to Ala destroys another function of the RING-like domain of Pellino1 that is independent of its E3 ligase activity. It is also possible that this mutation induces a conformational change that affects an unknown function associated with the FHA domain or “Wing...
Appendage." Nevertheless our results identify an important new function associated with the Pellino1 protein, namely its ability to stimulate IFNβ/gene transcription. Why IFNβ mRNA production is not defective in BMDM from Pellino1/F397A mice is unknown, but compensation by Pellino2, Pellino3, or even another E3 ligase when Pellino1 is not expressed would be the simplest explanation. Such compensation clearly does not occur in cells from the Pellino1[F397A] knock-in mice despite its low level of expression compared with the wild type protein.

Pellino1 not only stimulates the initial phase of IFNβ formation, but also the positive feedback loop by which the small amounts of IFNβ formed initially then amplify IFNβ production (Fig. 3A), and which is critical for the anti-viral immune response (41). In this pathway, IFNβ stimulates the JAK-STAT pathway leading to the phosphorylation of STAT1 and STAT2 (42, 43), which is followed by the formation of a complex between the STAT1/2 heterodimer and IRF9 to form the transcription factor ISGF3. ISGF3 can then stimulate the transcription of many proteins including those that participate in the positive feedback loop, such as IRF7. IRF7 can activate the IFNβ gene promoter, either directly or as a heterodimeric complex with IRF3 (39), or by stimulating the transcription of other proteins that participate in the positive feedback loop, such as IFNα (Fig. 3A). We found that the IFNβ-stimulated activation of STAT1 was not impaired in BMDM from Pellino1[F397A] mice (Fig. 5B), indicating that Pellino1 stimulates the positive feedback loop at a later step(s) in this

FIGURE 8. Poly(I:C)-stimulated polyubiquitylation of RIP1 and the activation of the canonical IKK complex and MAP kinases is unimpaired in BMDM from Pellino1[F397A] mice. BMDM from wild type (WT) Pellino1 or Pellino1[F397A] mice were stimulated with poly(I:C) (10 μg/ml) for the times indicated and extracted in lysis buffer containing 100 mM iodoacetamide to prevent deubiquitylation. A, cell lysates (12 mg protein) were incubated for 2 h at 4 °C with 30 μg of immobilized wild type (WT) Halo-NEMO or the polyubiquitin binding-defective mutant Halo-NEMO[D311N] (D/N) in a total volume of 6 ml. The resin was washed four times with 1 ml of lysis buffer containing 500 mM NaCl, once with 1 ml of 10 mM Tris/HCl (pH 8.0) and proteins captured by the immobilized NEMO were released by denaturation in SDS, subjected to SDS-PAGE and in the upper panel immunoblotted with anti-RIP1. The supernatant (SN) from the Halo-NEMO "pull-down" (20 μg of protein) was subjected to SDS-PAGE, transferred to a PVDF membrane and immunoblotted with anti-tubulin as a loading control. In the lower panel 40 μg of cell extract protein was denatured in SDS, subjected to SDS-PAGE, transferred to a PVDF membrane and immunoblotted with the antibodies indicated. B, same as A except that BMDM from wild-type Pellino1 mice were incubated for 1 h with (+) or without (−) 2 μM MRT67307 prior to stimulation with poly(I:C). The * in A and B denotes the position of the unmodified form of RIP1. C, BMDM from wild type or Pellino1[F397A] mice were stimulated with poly(I:C) (10 μg/ml) for the times indicated. The cells were lysed, and 30 μg of cell lysate protein was subjected to SDS-PAGE and immunoblotted with the antibodies in A, as well as with an antibody that recognizes the active phosphorylated form of JNK and all forms of IkBα and p38 MAPK.
Pellino1 Regulates the Production of IFNβ

pathway. For example, it may act in concert with IRF7 or the IRF7/IRF3 heterodimer to stimulate their interaction with the IFNβ gene (Fig. 3A).

Viruses have evolved many strategies to evade the host immune system, including the production of proteins that inactivate components of the interferon defense system. It is therefore of interest that a viral form of Pellino has recently been identified as an open reading frame in the entomopoxivirus Melanoplus sanguinipes (44). Like the mammalian Pellinos, the viral Pellino contains an N-terminal Forkhead-Associated (FHA) domain, but lacks the “wing appendage” or the RING domain. This viral Pellino may therefore exert its effect by sequestering activators and/or substrates of the insect Pellino, which are thought to bind to the FHA domain. Indeed, the entomopoxviral Pellino has been reported to interact with mammalian IRAK1 and to compete with mammalian Pellino3 for binding to IRAK1 (44). It would be interesting to know whether viruses that infect mammalian cells encode E3 ligase-deficient forms of Pellino and whether they can also suppress the production of type 1 IFNs.

The signaling pathway activated by poly(I:C) bifurcates “downstream” of TRIF. One branch of this pathway leads to the phosphorylation of IRF3 and the production of IFNβ, and the other to the activation of the canonical IKKs (IKKα and IKKβ) and mitogen-activated protein (MAP) kinases and the production of pro-inflammatory cytokines (30). The latter pathway is reported to require the expression of RIP1 and its polyubiquitylation (31, 32). Here, we found that the poly(I:C)-stimulated polyubiquitylation of RIP1, the activation of the canonical IKKs, the phosphorylation of their substrates and the activation of p38α MAP kinase and JNKs were not impaired in BMDM from the Pellino1[F397A] knock-in mice. Consistent with these findings MRT67307, a potent inhibitor of the IKK-related kinases, did not impair the poly(I:C)-stimulated polyubiquitylation of RIP1 or the activation of the canonical IKKs and MAP kinases in BMDM at concentrations that prevent the phosphorylation of IRF3 (Fig. 8B), the activation of Pellino1’s E3 ligase activity and the increase in its expression (15). On the contrary, MRT67307 actually enhanced poly(I:C)-stimulated RIP1 polyubiquitylation (Fig. 8B), suggesting that the IKK-related protein kinases may negatively regulate another E3 ligase(s) that polyubiquitylates RIP1 and/or activate a RIP1 deubiquitylase. Taken together, these experiments show that the E3 ligase activity of Pellino1 is not required for the poly(I:C)-stimulated polyubiquitylation of RIP1 or the activation of the canonical IKKs and MAP kinases in BMDM.

The poly(I:C)/TLR3-stimulated polyubiquitylation of RIP1 and activation of the canonical IKK complex were reported to be severely impaired in MEFs from the Pellino1−/− mice and pro-inflammatory cytokine production was also greatly reduced (40). In contrast, we not only failed to observe suppression of poly(I:C)-stimulated RIP1 polyubiquitylation, and the activation of the canonical IKK complex and MAP kinases in BMDM from the Pellino1[F397A] mice but also in poly(I:C) stimulated MEFs from Pellino1[F397A] (supplemental Fig. S8). Therefore, the discrepancy from the results obtained previously with Pellino1−/− MEFs is not explained by a cell-specific difference. It is possible that the loss of the entire Pellino1 protein removes an unknown function of this protein, distinct from its E3 ligase activity, which is required for the poly(I:C)/TLR3-stimulated activation of NFκB and MAP kinases.

Acknowledgments—We thank Dr. Anne O’Garra for providing bone marrow from the IFNAR−/− mice, Dr. Rick Randall for valuable discussion, and the MRC-Protein Phosphorylation Unit’s (PPL) DNA Sequencing Service (coordinated by Nicholas Helps), DNA cloning team (coordinated by Mark Peggie and Rachel Toth), and tissue culture team (coordinated by Kirsten McLeod), for outstanding technical support.

REFERENCES
1. Flannery, S., and Bowie, A. G. (2010) The interleukin-1 receptor-associated kinases: critical regulators of innate immune signaling. Biochemical Pharmacology 80, 1981–1991
2. Xia, Z. P., Sun, L., Chen, X., Pineda, G., Jiang, X., Adhikari, A., Zeng, W., and Chen, Z. J. (2009) Direct activation of protein kinases by unanchored polyubiquitin chains. Nature 461, 114–119
3. Oshiumi, H., Matsumoto, M., Funami, K., Akazawa, T., and Seya, T. (2003) TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. Nat. Immunol. 4, 161–167
4. Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyma, M., Okabe, M., Takeda, K., and Akira, S. (2003) Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science 301, 640–643
5. Yamamoto, M., Sato, S., Mori, K., Hoshino, K., Takeuchi, O., Takeda, K., and Akira, S. (2002) Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. J. Immunol. 169, 6668–6672
6. Fitzgerald, K. A., McWhirter, S. M., Faia, K. L., Rowe, D. C., Latz, E., Golenbock, D. T., Coyle, A. J., Liao, S. M., and Maniatis, T. (2003) IKKε silon and TBK1 are essential components of the IRF3 signaling pathway. Nat. Immunol. 4, 491–496
7. Hemmi, H., Takeuchi, O., Sato, S., Yamamoto, M., Kaisho, T., Sanjo, H., Kawai, T., Hoshino, K., Tsukeda, K., and Akira, S. (2004) The roles of two Iakappab kinase-related kinases in lipopolysaccharide and double stranded RNA signaling and viral infection. J. Exp. Med. 199, 1641–1650
8. McWhirter, S. M., Fitzgerald, K. A., Rosains, I., Rowe, D. C., Golenbock, D. T., and Maniatis, T. (2004) IFN-regulatory factor 3-dependent gene expression is defective in Tbk1-deficient mouse embryonic fibroblasts. Proc. Natl. Acad. Sci. U.S.A. 101, 233–238
9. Ng, S. L., Friedman, B. A., Schmid, S., Gertz, J., Myers, R. M., Tenoever, B. R., and Maniatis, T. (2004) IFN-regulatory factor 3-dependent gene expression is defective in Tbk1-deficient mouse embryonic fibroblasts. Proc. Natl. Acad. Sci. U.S.A. 101, 233–238
10. Grousshans, J., Schrorner, F., and Nüsslein-Volhard, C. (1999) Oligomerization of Tube and Pelle leads to nuclear localization of dorsal. Mech. Dev. 81, 127–138
11. Haghayeghi, A., Sarac, A., Czerniecki, S., Grosshans, J., and Schock, F. (2010) Pellino enhances innate immunity in Drosophila. Mech. Dev. 127, 301–307
12. Ordonez, A., Smith, M., Windheim, M., Peggie, M., Carrick, E., Morrice, N., and Cohen, P. (2008) The IRAK-catalysed activation of the E3 ligase function of Pellino isoforms induces the Lys-63-linked polyubiquitination of IRAK1. Biochem. J. 409, 43–52
13. Schaauvliege, R., Janssens, S., and Beyaert, R. (2006) Pellino proteins are a family of IRAK1. Biochem. J. 409, 43–52
14. Smith, H., Peggie, M., Campbell, D. G., Vandelooere, M., Carrick, E., and Cohen, P. (2009) Identification of the phosphorylation sites on the E3 ubiquitin ligase Pellino that are critical for activation by IRAK1 and IRAK4. Proc. Natl. Acad. Sci. U.S.A. 106, 4584–4590
15. Smith, H., Liu, X. Y., Dai, L., Goh, E. T., Chen, A. T., Xi, J., Seh, C. C., Qureshi, I. A., Lescar, J., Ruedl, C., Gourlay, R., Morton, S., Hough, J., Qureshi, I., Lescar, J., Ruedl, C., Gourlay, R., Morton, S., Hough, J., Qureshi, I., Lescar, J., Ruedl, C., Gourlay, R., Morton, S., Hough, J., Qureshi, I., Lescar, J., Ruedl, C., Gourlay, R., Morton, S., Hough, J., Qureshi, I., Lescar, J., Ruedl, C., Gourlay, R., Morton, S., Hough, J., Qureshi, I., Lescar, J., Ruedl, C., Gourlay, R., Morton, S., Hough, J., Qureshi, I., Lescar, J., Ruedl, C., Gourlay, R., Morton, S., Hough, J., Qureshi, I., Lescar, J., Ruedl, C., Gourlay, R., Morton, S., Hough, J., Qureshi, I., Lescar, J., Ruedl, C., Gourlay, R., Morton, S., Hough, J., Qureshi, I., Lescar, J., Ruedl, C., Gourlay, R., Morton, S., Hough, J., Qureshi, I., Lescar, J., Ruedl, C., Gourlay, R., Morton, S., Hough, J., Qureshi, I., Lescar, J., Ruedl, C., Gourlay, R., Morton, S., Hough, J., Qureshi, I., Lescar, J., Ruedl, C., Gourlay, R., Morton, S., Hough, J., Qureshi, I., Lescar, J., Ruedl, C., Gourlay, R., Morton, S., Hough, J., Qureshi, I., Lescar, J., Ruedl, C., Gourlay, R., Morton, S., Hough, J., Qureshi, I., Lescar, J., Ruedl, C., Gourlay, R., Morton, S., Hough, J., Qureshi, I., Lescar, J.
McIver, E. G., Cohen, P., and Cheung, P. C. (2011) The role of TBK1 and IKKe in the expression and activation of Pellino 1. Biochem. J. 434, 537–548
16. Goh, E. T., Arthur, J. S., Cheung, P. C., Akira, S., Toth, R., and Cohen, P. (2012) Identification of the protein kinases that activate the E3 ubiquitin ligase Pellino 1 in the innate immune system. Biochem. J. 441, 339–346
17. Weighardt, H., Jusek, G., Mages, J., Lang, R., Hoebe, K., Beutler, B., and Holzmann, B. (2004) Identification of a TLR4- and TRIF-dependent activation program of dendritic cells. Eur. J. Immunol. 34, 558–564
18. Nanda, S. K., Venigalla, R. K., Ordureau, A., Patterson-Kane, J. C., Powell, D. W., Toth, R., Arthur, J. S., and Cohen, P. (2011) Polyubiquitin binding to ABIN1 is required to prevent autoimmunity. J. Exp. Med. 208, 1215–1228
19. Lin, C. C., Huoh, Y. S., Schmitz, K. R., Jensen, L. E., and Ferguson, K. M. (2007) The Mx1 gene program for robust type-I interferon induction. J. Biol. Chem. 282, 10952–10956
20. Zheng, N., Wang, P., Jeffrey, P. D., and Pavletich, N. P. (2000) Structure of the Toll-like receptor gene expression and endotoxin shock. Biochem. Biophys. Res. Commun. 206, 860–866
21. Thomas, K. E., Galligan, C. L., Newman, R. D., Fish, E. N., and Vogel, S. N. (2006) Contribution of interferon-β to the murine macrophage response to the toll-like receptor 4 agonist, lipopolysaccharide. J. Biol. Chem. 281, 31119–31130
22. Honda, K., Ohba, Y., Yanai, H., Negishi, H., Mizutani, T., Takaoka, A., Tay, C., and Taniguchi, T. (2005) Spatiotemporal regulation of MyD88-IRF-7 signaling for robust type-I interferon induction. Nature 434, 1035–1040
23. Honda, K., Yanai, H., Negishi, H., Asagiri, M., Sato, M., Mizutani, T., Shimada, N., Ohba, Y., Takaoka, A., Yoshida, N., and Taniguchi, T. (2005) IRF-7 is the master regulator of type I interferon-dependent immune responses. Nature 434, 772–777
24. Tumpey, T. M., Sztur, K. J., Van Hoeven, N., Katz, J. M., Kochs, G., Haller, O., García-Sastre, A., and Stauhehi, P. (2007) The Mx1 gene protects mice against the pandemic 1918 and highly lethal human H5N1 influenza viruses. J. Virol. 81, 10818–10821
25. Tenoever, B. R., Ng, S. L., Chua, M. A., McWhirter, S. M., García-Sastre, A., and Maniatis, T. (2007) Multiple functions of the IKK-related kinase IKKe in interferon-mediated antiviral immunity. Science 315, 1274–1278
26. Gautier, G., Humbert, M., Deauvieau, F., Scuiller, M., Hiscott, J., Bates, E. E., Trinchieri, G., Caux, C., and Garrone, P. (2005) A type I interferon autocrine-paracrine loop is involved in Toll-like receptor-induced interleukin-12p70 secretion by dendritic cells. J. Exp. Med. 201, 1435–1446
27. Yang, K., Shi, H., Qi, R., Sun, S., Tang, Y., Zhang, B., and Wang, C. (2006) Hsp90 regulates activation of interferon regulatory factor 3 and TBK-1 stabilization in Sendai virus-infected cells. Mol. Biol. Cell. 17, 1461–1471
28. Strahle, L., Garicin, D., and Kolakofsky, D. (2006) Sendai virus defective-interfering genomes and the activation of interferon-β. Virology 351, 101–111
29. Perry, A. K., Chow, E. K., Goodnough, J. B., Yeh, W. C., and Cheng, G. (2004) Differential requirement for TANK-binding kinase-1 in type I interferon responses to toll-like receptor activation and viral infection. J. Exp. Med. 199, 1651–1658
30. Kawai, T., and Akira, S. (2010) The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nature Immunology 11, 373–384
31. Cussen-Hermance, N., Khurana, S., Lee, T. H., Fitzgerald, K. A., and Kel-lier, M. A. (2005) RIP1 mediates the TRIF-dependent toll-like receptor 3- and 4-induced NF-κB activation but does not contribute to interferon regulatory factor 3 activation. J. Biol. Chem. 280, 36560–36566
32. Meylan, E., Burns, K., Hofmann, K., Blanchetave, V., Martinon, F., Kellier, M., and Tschopp, J. (2004) RIP1 is an essential mediator of Toll-like receptor 3-induced NF-κB activation. Nat. Immunol. 5, 503–507
33. Miettinen, M., Sareneva, T., Jukunen, I., and Matikainen, S. (2001) IFNs activate toll-like receptor gene expression in viral infections. Genes Immun 2, 349–355
34. Clark, K., Peggie, M., Plater, L., Sorcek, R. J., Young, E. R., Madwed, J. B., Hough, J., McIver, E. G., and Cohen, P. (2011) Novel cross-talk within the IKK family controls innate immunity. Biochem. J. 434, 93–104
35. Lang, V., Symons, A., Wattson, S. J., Janzen, J., Soneji, Y., Beinke, S., Howell, S., and Ley, S. C. (2004) ABIN-2 forms a ternary complex with TPL-2 and NF-κB p105 and is essential for TPL-2 protein stability. Mol. Cell Biol. 24, 5235–5248
36. Waterfield, M., Jin, W., Reiley, W., Zhang, M., and Sun, S. C. (2004) Β1 kinase is an essential component of the Tpl2 signaling pathway. Mol. Cell Biol. 24, 6040–6048
37. Sakaguchi, S., Negishi, H., Asagiri, M., Nakajima, C., Mizutani, T., Takaoka, A., Honda, K., and Taniguchi, T. (2003) Essential role of IRF-3 in lipopolysaccharide-induced interferon-β gene expression and endotoxin shock. Biochem. Biophys. Res. Commun. 306, 860–866
38. Thomas, K. E., Galligan, C. L., Newman, R. D., Fish, E. N., and Vogel, S. N. (2006) Contribution of interferon-β to the murine macrophage response to the toll-like receptor 4 agonist, lipopolysaccharide. J. Biol. Chem. 281, 31119–31130
39. Honda, K., Takaoka, A., and Taniguchi, T. (2006) Type I interferon [cor-rected] gene induction by the interferon regulatory factor family of transcription factors. Immunity 25, 349–360
40. Chang, M., Jin, W., and Sun, S. C. (2009) Pel1 facilitates TRIF-dependent Toll-like receptor signaling and proinflammatory cytokine production. Nature Immunology 10, 1089–1095
41. Sato, M., Suzumori, H., Hata, N., Asagiri, M., Ogasawara, K., Nakao, K., Nakaya, T., Katsuki, M., Nagushi, S., Tanaka, N., and Taniguchi, T. (2000) Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-α/β gene induction. Immunity 13, 539–548
42. Marie, I., Durbin, J. E., and Levy, D. E. (1998) Differential viral induction of distinct interferon-α genes by positive feedback through interferon regulatory factor-7. EMBO J. 17, 6660–6669
43. Sato, M., Hata, N., Asagiri, M., Nakaya, T., Taniguchi, T., and Tanaka, N. (1998) Positive feedback regulation of type I IFN genes by the IFN-inducible transcription factor IRF-7. FEBS Lett. 441, 106–110
44. Griffin, B. D., Mellett, M., Campos-Torres, A., Kinsella, G. K., Wang, B., and Moynagh, P. N. (2011) A poxviral homolog of the Pellino protein inhibits Toll and Toll-like receptor signaling. Eur. J. Immunol. 41, 798–812
45. Doyle, S. E., O’Connell, R., Vaidya, S. A., Chow, E. K., Yee, K., and Cheng, G. (2003) Toll-like receptor 3 mediates a more potent antiviral response than Toll-like receptor 4. J. Immunol. 170, 3565–3571