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A Role for APPL1 in TLR3/4-Dependent TBK1 and IKKε Activation in Macrophages

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Endosomes have important roles in intracellular signal transduction as a sorting platform. Signaling cascades from TLR engagement to IRF3-dependent gene transcription rely on endosomes, yet the proteins that specifically recruit IRF3-activating molecules to them are poorly defined. We show that adaptor protein containing a pleckstrin-homology domain, a phosphotyrosine-binding domain, and a leucine zipper motif (APPL)1, an early endosomal protein, is required for both TRIF- and retinoic acid–inducible gene 1–dependent signaling cascades to induce IRF3 activation. APPL1, but not early endosome Ag 1, deficiency impairs IRF3 target gene expression upon engagement of both TLR3 and TLR4 pathways, as well as in H1N1-infected macrophages. The IRF3-phosphorylating kinases TBK1 and IKKε are recruited to APPL1 endosomes in LPS-stimulated macrophages. Interestingly, APPL1 undergoes proteasome-mediated degradation through ERK1/2 to turn off signaling. APPL1 degradation is blocked when signaling through the endosome is inhibited by chloroquine or dynasore. Therefore, APPL1 endosomes are critical for IRF3-dependent gene expression in response to some viral and bacterial infections in macrophages. Those signaling pathways involve the signal-induced degradation of APPL1 to prevent aberrant IRF3-dependent gene expression linked to immune diseases. (The Journal of Immunology, 2015, 194: 3970–3983.)

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Abbreviations used in this article: APPL, adaptor protein containing a pleckstrin-homology domain, a phosphotyrosine-binding domain, and a leucine zipper motif; BMDM, bone marrow–derived macrophage; EEA1, early endosome Ag 1; IF, immunofluorescence; IP, immunoprecipitation; PH, pleckstrin homology; Poly(I:C), polyinosinic-polycytidylic acid; PTB, phosphotyrosine binding; RIG-I, retinoic acid–inducible gene 1; siRNA, small interfering RNA; WB, Western blot; WT, wild-type.

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pronounced in SHIP-1–deficient cells (23). However, signaling proteins that recruit TBK1 and IKKε to endosomes are unknown.

Adaptor protein containing a pleckstrin-homology (PH) domain, a phosphotyrosine-binding (PTB) domain, and a leucine zipper motif (APPL+) membrane structures, referred to as APPL endosomes, are defined as a subpopulation of early endosomes that play critical roles in cargo trafficking and signal transduction (24). They are defined as transient early endocytic intermediates distinct from classical EEA1+ endosomes and include APPL1, APPL2, and annexin A2 (24–26). APPL1 is a 709-aa endosomal protein initially characterized as an AKT-interacting candidate in a yeast two-hybrid screen (27). APPL1 binds the cytosolic region of a variety of membrane receptors, including the nerve growth factor receptor (TrkA), epidermal growth factor receptor, and adiponectin receptor, either directly or via the small PDZ-containing adaptor GIPC, and it acts as a signaling platform to assemble AKT- and MAPK-activating complexes (28–32). As a result, APPL1 deficiency triggers apoptosis during development and causes defective neuritogenesis (29, 32, 33). The PH domain of APPL1 helps to recruit proteins to cell membranes to target them to appropriate cellular compartments and/or to bring them to signaling molecules to trigger signal transduction. Yet, kinases recruited to endosomes through APPL1 have not been extensively characterized.

We show in this article that IKK-related kinases TBK1 and IKKε are recruited to APPL1 endosomes through the PH domain upon TLR3/4 activation in macrophages. As a result, APPL1, but not EEA1, endosomes are required for IRF3 phosphorylation and IRF3-dependent gene transcription. H1N1 infection in macrophages also relies on APPL1 to trigger gene expression through IRF3. Interestingly, APPL1 is degraded poststimulation through a proteasome-dependent pathway in TLR3/4-activated macrophages, as well as in H1N1-infected macrophages, to prevent the aberrant expression of IRF3 target genes. Therefore, our data define a molecular mechanism by which some viral and bacterial infections rely on APPL1 endosomes to properly activate IKK-related kinases, as well as highlight signal-induced degradation of APPL1 in activated macrophages as a mechanism to keep IRF3-dependent gene expression under control.

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**FIGURE 1.** IKKε and TBK1 are recruited to APPL1 endosomes upon TLR3 and TLR4 engagement. (A) TBK1 and IKKε and their scaffold proteins TANK and NAP1 coelute with endosomal markers. Fractionated extracts from untreated or LPS-stimulated RAW 264.7 cells (left and right panels, respectively; see Materials and Methods for details) were subjected to WB analyses using the indicated Abs. (B) LPS triggers the recruitment of both endogenous TBK1 and IKKε to APPL1 endosomes. Cell extracts from early endosome fractions of untreated or LPS-stimulated RAW 264.7 cells were subjected to anti-APPL1 IP, followed by anti-pTBK1, anti-TBK1, anti-IKKε, and anti-APPL1 WB analyses (top four blots). Cell extracts (WCE) also were subjected to WB analyses using the indicated Abs. (C) TRAF3 and APPL1 bind at the endogenous level in enriched endosome fractions from LPS-stimulated macrophages. Cell extracts from early endosome fractions of untreated or LPS-stimulated RAW 264.7 cells were subjected to anti-TRAF3 IP, followed by anti-TBK1 and anti-APPL1 WB analyses (top two blots). WCE extracts also were subjected to WB analyses using the indicated Abs. (D) TANK and NAP1 are APPL1-interacting proteins. HEK293 cells were transfected with the indicated expression plasmids, and the resulting cell extracts were subjected to anti-Myc IP, followed by an anti-APPL1 WB (top blot). Cell extracts also were subjected to WB analyses using the anti-APPL1 or anti-Myc Ab (middle and bottom blots). (E) TBK1 and APPL1 partially colocalize in macrophages. RAW 264.7 cells were transfected with GFP-APPL1 and were left untreated or stimulated with LPS for 30 min (upper left and upper right panels, respectively). Both endogenous TBK1 and ectopically expressed GFP-APPL1 were visualized by IF. Profiles of the relative intensities of the two fluorophores along the respective white lines (lower panels). Arrows depict zones of colocalization of TBK1 with GFP-APPL1. ROI, region of interest.
Materials and Methods

Cell culture, biological reagents, and treatments

HEK293 and RAW 264.7 cells were cultured as previously described (15, 34). LPS and R848 were purchased from InvivoGen (Toulouse, France), and polyinosinic-polycytidylic acid [Poly(I:C)], dynasore, and chloroquine were from Sigma (St. Louis, MO). Cell Signaling (Temecula, CA), andSELLex (Boston, MA), respectively.

Polyclonal anti-HA, anti-ERK1/2, anti-TANK, anti-Myc, anti-IRF3, and anti-IkB Abs, as well as monoclonal anti-Myc Abs, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-FLAG Abs and beads were purchased from Sigma. The polyclonal anti-TBK1, anti-IKKε, anti-pERK1/2, and anti-pIRF3 rabbit Abs, as well as anti-pTBK1 (serine 172), anti-pIKKε (serine 172), and anti-APPL1 monoclonal rabbit Abs, were from Cell Signaling/Millipore. The polyclonal anti-TBK1, anti-IKKε, anti-pERK1/2, and anti-pIRF3 rabbit Abs, as well as anti-pTBK1 (serine 172), anti-pIKKε (serine 172), and anti-APPL1 monoclonal rabbit Abs, were from Cell Signaling/Millipore. The polyclonal anti-TBK1 Abs used in Western blot (WB) analyses was described previously (35). The anti-EA1, anti-Rab5, anti-Rab7, and anti-syntaxin 6 Abs were part of the Vesicle Trafficking Antibody Sampler Kit (Cell Signaling). The siGENOME SMARTpool-Mouse Appl1 small interfering RNA (siRNAs) and the siGENOME Set of 4 Mouse Appl1 siRNAs were purchased from Dharmacon (Lafayette, CO). Dynamin-2, NEMO, TRAF3, Cul1, and Rbx1 siRNAs also were from Dharmacon.

Myc-tagged wild-type (WT) IKKε and the IKKε-ΔC6, IKKε-ΔC30, and IKKε-ΔC52 mutants were described previously, as was the kinase-dead construct (15). The other mutants (IKKε-ΔC90, IKKε-ΔC150, IKKε-ΔC486, IKKε-ΔN230, IKKε-ΔN300, and IKKε-ΔC30) and kinase-dead mutants were generated by PCR, whereas the IKKe K691R construct was generated by site-directed mutagenesis. The GFP-APPL1 construct was a generous gift from Dr. Pietro DeCamilli (Howard Hughes Medical Institute, Yale University, New Haven, CT). The GFP-APPL1 A318D mutant was generated by site-directed mutagenesis. The RFP-RAB5 construct was from Addgene (Cambridge, MA). Myc-TANK and Myc-NAP1 constructs were generated by subcloning both coding sequences into the pCMV-Myc expression plasmid (Clontech, Palo Alto, CA).

Mouse strains and generation of BMDMs

The Ikkε KO and control littermate mouse strains were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed at the Animal Facility of the University of Liege. BMDMs were generated as described previously (36). Briefly, bone marrow cells, isolated from femur and tibia, were stimulated with M-CSF–containing medium (RPMI 1640 supplemented with 20% L929 cell supernatant and 10% FCS) for differentiation of bone marrow progenitors to naive macrophages on nontissue culture–coated bacterial petri dishes (∼7 × 10^6 cells/dish) for 7 d. Differentiation media were replenished, and adherence was checked under the microscope at day 4.

FIGURE 2. Identification of TBK1, IKKe, and APPL1 domains required for their interaction. (A) IKKe binds APPL1 through two distinct domains. HEK293 cells were transfected with the indicated expression plasmids, and the resulting cell extracts were subjected to anti-Myc IP, followed by an anti-APPL1 WB (upper panel). Crude cell extracts were subjected to anti-APPL1 and anti-Myc WBs as well (lower panel). The black line was added where images from a single experiment were joined. (B) The C-terminal part of TBK1 is required for binding to APPL1. HEK293 cells were transfected with the indicated expression plasmids, and the resulting cell extracts were subjected to anti-Myc IP, followed by an anti-APPL1 WB (upper panel). Crude cell extracts also were subjected to anti-APPL1 and anti-Myc WB (lower panel). (C) APPL1 binds TBK1 and IKKe through distinct domains. HEK293 cells were transfected with the indicated expression plasmids, and the resulting cell extracts were subjected to anti-FLAG IP, followed by an anti-Myc WB (top blot). Crude cell extracts also were subjected to anti-FLAG and anti-Myc WB (middle and bottom blots).
Differentiated macrophages were plated on tissue culture–coated dishes (4.10^6 cells/10-cm dish) in RPMI 1640 a day before transfection at day 6 and/or stimulation at day 7.

**Viral infection of macrophages**

The mouse-adapted variant of A/Swine/Iowa/4/1976 (H1N1) was described previously (37). Infection of primary cultures was performed in triplicate and in a multiplicity of infection of 10 of the mouse-adapted swine H1N1 influenza strain in 1 ml serum-free DMEM containing 0.5 μm TPCK trypsin (Sigma-Aldrich, Munich, Germany), 1% penicillin-streptomycin, and 0.6% Fungizone (amphotericin B). Infected wells were collected at the indicated times, lysed, and stored at −80°C.

**Isolation of endosome fractions**

Endosome isolation was performed on a step sucrose gradient, as previously described (25, 38). In brief, after being washed in cold PBS, cells were resuspended in SIM buffer supplemented with phosphatase inhibitors and protease inhibitors and incubated on ice for 20 min for cells to be swollen before the homogenization step. Next, cells were broken by a douncer with 30 strokes. Nuclei were removed by centrifugation for 10 min at 2000 g at 4°C. The resulting cell homogenates, referred to as the postnuclei supernatant (PNS), were subsequently added for 2 h before collecting the immunoprecipitates. Collected beads were washed five times in the same non-detergent buffer and boiled in Laemmli blue sample buffer.

**Immunoprecipitation and immunofluorescence**

Immunoprecipitation (IP) involving ectopically expressed and/or endogenous proteins was performed as previously described (15). For the detection of endogenous polyubiquitinated APPL1 adducts in denaturing conditions, cells were lysed in 1% SDS, and lysates were boiled for 5 min. The resulting lysates were diluted 10 times in an nondenaturant buffer composed of 50 mM Tris (pH 8), 150 mM NaCl, and 1% Nonidet P-40. For IP, lysates were incubated with Abs overnight at 4°C, followed by 45 min of incubation at room temperature. Cells were incubated with the primary anti-TBK1 Ab overnight at 4°C, followed by 45 min of incubation at room temperature with secondary goat anti-rabbit Alexa Fluor 568–conjugated IgG. Images were acquired on a Leica SP5 inverted confocal microscope microscope (Leica Microsystems, Wetzlar, Germany).

**Total RNA extraction and real-time PCR**

Total RNA was extracted using the EZNATM Total RNA kit (Omega Bio-Tek, Norcross, GA), and cDNA was synthesized using the RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas, Glen Burnie, MD). Subsequent PCRs were carried out using the Power SYBR Green PCR Master Kit (Applied Biosystems, Foster City, CA) on the LightCycler 480 (Roche).
Primers, whose sequences are available upon request, were designed using the Primer Express software.

Results

**TBK1 and IKKe are recruited to APPL1 endosomes in LPS-stimulated macrophages**

To gain insights into the subcellular localization of TBK1 and IKKe in macrophages, we biochemically fractionated extracts from RAW 264.7 cells and performed WB analyses with Abs directed against a variety of organelle-specific markers. As expected, TANK and NAP1 perfectly coeluted with TBK1 and IKKe (Fig. 1A). Interestingly, TBK1- and IKKe-containing fractions also were enriched with endosomal proteins, namely APPL1 and EEA1 (Fig. 1A), suggesting that endosomes may act as signaling platforms for both TBK1 and IKKe. We next isolated enriched endosomal fractions from control or LPS-stimulated RAW 264.7 cells and showed that TBK1, IKKe, and TRAF3 were all recruited to endosomes in LPS-treated macrophages (Fig. 1B). We also immunoprecipitated APPL1 from those endosomal fractions and noticed that TBK1, its activated form (pTBK1), and IKKe bound APPL1 in an LPS-dependent manner (Fig. 1B). Endogenous TRAF3 and APPL1 also interacted in an LPS-dependent manner in enriched endosome fractions (Fig. 1C). Of note, NAP1 and TANK bound APPL1 in transfected HEK293 cells (Fig. 1D). Moreover, endogenous TBK1 partially colocalized with GFP-APPL1 in both untreated and LPS-stimulated RAW 264.7 cells, as judged by IF analyses (Fig. 1E). Therefore, both TBK1 and IKKe are recruited in APPL1 endosomes upon LPS signaling in macrophages.

Having defined APPL1 as a TBK1- and IKKe-associated protein, we next conducted additional IP experiments in HEK293 cells using a variety of IKKe and APPL1 mutants to precisely map domains required for their interaction. The binding of IKKe to APPL1 relies on its C-terminal domain (aa 687–710) because the...
IKKe-ΔC6 mutant, but not the IKKe-ΔC30 mutant, bound APPL1 in HEK293 cells (Fig. 2A). Accordingly, additional IKKe mutants lacking C-terminal amino acids (IKKe-ΔC52, -90 and -150) failed to strongly bind APPL1 (Fig. 2A). Moreover, the IKKe-ΔN230 mutant and the kinase-dead version of IKKe did not efficiently associate with APPL1 (Fig. 2A). Therefore, the kinase domain of IKKe is also required to properly bind APPL1. Interestingly, the C-terminal part of TBK1 also was essential for binding to APPL1 because the TBK1-ΔC6 mutant, but not the TBK1-ΔC30 mutant, interacted with APPL1, as judged by co-IP (Fig. 2B). Similar experiments conducted with APPL1 mutants indicated that the PH domain of APPL1 was required for binding to IKKe, whereas both the Bin/Amphiphysin/Rvs and PTB sequences were dispensable (Fig. 2C). Interestingly, the APPL1ΔBin/Amphiphysin/RvsΔPH mutant that failed to bind IKKe still bound TBK1, whereas the APPL1 ΔPTB mutant associated only weakly with APPL1 (Fig. 2C). Thus, APPL1 recruits TBK1 and IKKe to endosomes through distinct domains.

APPL1 endosomes are required for TLR3/4-dependent IRF3 phosphorylation
APPL1 is a TBK1- and IKKe-associated protein, yet it was unclear whether those interactions play any role in TBK1- and IKKe-dependent pathways. Therefore, we explored whether APPL1 deficiency had any impact on TLR3/4-dependent TBK1 and IKKe activation in macrophages. APPL1-depleted, but not EEA1-depleted, RAW 264.7 cells did not properly activate TBK1 and IKKe upon stimulation by LPS or Poly(I:C), a synthetic analog of viral dsRNA (Fig. 3A, 3B, respectively). Of note, the defective phosphorylation of IKKe seen in APPL1-depleted cells was more pronounced upon Poly(I:C) stimulation compared with LPS stimulation. As a result, LPS- and Poly(I:C)-dependent IRF3 phosphorylation was impaired upon APPL1 deficiency (Fig. 3A). Importantly, APPL1 deficiency in BMDMs markedly impaired TBK1 and IRF3 phosphorylation upon LPS stimulation, thus demonstrating that our findings are relevant in untransformed cells (Fig. 3C). In agreement with our data in RAW 264.7 cells, APPL1 was less critical for IKKe phosphorylation upon TLR4 engagement (Fig. 3C).

Because IRF3 phosphorylation through both TLR3/4-dependent pathways in macrophages relied on APPL1, the expression of TBK1 and IRF3 target genes, namely IP-10, IFN-β, and RANTES (40), was defective in APPL1-depleted RAW 264.7 cells subjected to LPS or Poly(I:C) treatment (Fig. 4). In contrast, the induction of TNF-α and IL-1β mRNA expression through both TLR3 and TLR4 pathways was higher upon APPL1 deficiency in RAW 264.7 cells (Fig. 4). In agreement with a dispensable role for EEA1 in TLR3/4 and IRF3-dependent gene transcription, EEA1-depleted RAW 264.7 cells properly expressed IRF3 target genes upon treatment with LPS or Poly(I:C) (Supplemental Fig. 1).

To explore whether APPL1 also was involved in IRF3 activation through TLR-independent pathways in macrophages, we infected control and APPL1-depleted BMDMs with the influenza H1N1 virus, which triggers IFN production through the retinoic acid-inducible gene 1 (RIG-1)-dependent pathway (41). Again, APPL1 deficiency interfered with the induction of both IP-10 and RANTES mRNA levels in H1N1-infected cells (Fig. 5). However, IFN-β production postinfection did not rely on APPL1 (Fig. 5). Taken together, our data suggest that APPL1-containing endosomes are required to activate TBK1 and IKKe through TLR3/4 to promote IRF3-dependent gene transcription.

TLR3/4 ligands trigger APPL1 degradation in macrophages
While addressing the role of APPL1 endosomes in TLR-dependent signaling pathways, we systematically noticed that cytoplasmic APPL1 protein levels decreased upon stimulation with LPS or Poly(I:C) (Figs. 3A–C, 4). H1N1 infection also triggered the disappearance of a slower-migrating form of APPL1 (Fig. 5). It did not result from a nuclear translocation of APPL1 upon signaling, because it was not detected in nuclear extracts, even after prolonged Poly(I:C) stimulation (Fig. 6A). APPL1 degradation poststimulation also was observed in BMDMs subjected to LPS treatment (Figs. 3C, 6B). To elucidate the mechanisms by which APPL1 is degraded upon signaling, we first explored whether TLR4 internalization from the cytoplasmic membrane to early endosomes, a process that critically relies on GTPase dynamin, was required (7). Dynasore, a highly specific dynamin inhibitor (42), impaired LPS-dependent TBK1 phosphorylation and blocked the signal-induced APPL1 degradation (Fig. 7A, 7B). However, degradation of APPL1 upon stimulation with Poly(I:C) or H1N1 virus infection remained dependent on GTPase dynamin (Fig. 7C, 7D). This suggests that the cytoplasmic to early endosomal trafficking of APPL1 is a common feature of both TLR3/4-dependent and TLR3/4-independent pathways that requires GTPase dynamin for efficient APPL1 degradation.
degradation (Fig. 6C). Therefore, TLR4 intracellular trafficking is required for LPS-dependent TBK1 activation and APPL1 degradation. Interestingly, TBK1 phosphorylation and APPL1 degradation upon TLR3 engagement were not impaired by dynasore (Fig. 6C), which is in agreement with the fact that TLR3 is localized in endosomes and does not rely on dynamin to signal. Consistently, siRNA-mediated dynamin-2 depletion in macrophages also blocked LPS-dependent TBK1 and IRF3 phosphorylation and interferes with LPS-induced, but not Poly(I:C)-induced, APPL1 degradation. Control or dynamin-2-deficient RAW 264.7 cells were left untreated or stimulated with LPS (100 ng/ml) or Poly(I:C) (100 μg/ml) for the indicated periods of time, and the resulting cell extracts were subjected to WB analyses.

To explore whether APPL1 binding to Rab5 was required for APPL1 degradation in Poly(I:C)-stimulated macrophages, we expressed WT APPL1 or the A318D APPL1 mutant, which fails to bind Rab5 (43), in RAW 264.7 cells. We first noticed that WT APPL1, but not the A318D APPL1 mutant, strongly colocalized with Rab5 in endosome structures, as judged by IF analyses (Fig. 7A). The A318D APPL1 mutant was similarly degraded upon Poly(I:C) stimulation (Fig. 7A), which suggests that APPL1 binding to Rab5 is dispensable for Poly(I:C)-dependent APPL1 downregulation. Remarkably, chloroquine, which accumulates in endosomes and lysosomes and prevents endosomal acidification (44), also blocked LPS- and Poly(I:C)-mediated APPL1 degradation, as well as IRF3 phosphorylation (Fig. 7B). Of note, the signal-induced TBK1 phosphorylation also was impaired, at least because chloroquine enhanced basal phosphorylated levels of TBK1 (Fig. 7B). Taken together, our data suggest that TLR4 engagement triggers APPL1 degradation in macrophages, a process that relies on signal-induced TLR4 internalization. Our results also suggest that LPS-dependent APPL1 degradation occurs only when TBK1 phosphorylation is enhanced upon signaling.
APPL1 degradation upon TLR3/4 or TLR7/8 engagement is proteasome dependent

To learn more about the mechanisms underlying APPL1 degradation upon TLR3/4 engagement, we pretreated RAW 264.7 cells with MG132, a proteasome inhibitor, and noticed that APPL1 degradation upon stimulation with Poly(I:C), LPS, or R848 (a TLR7/8 agonist) was blocked (Fig. 8A). In contrast, pretreatment of RAW 264.7 cells with a combination of lysosomal inhibitors (E64, pepstatin A, and leupeptin) had no effect on APPL1 degradation in macrophages. RAW 264.7 cells were pretreated with chloroquine (200 µg/ml) for 2 h and then left untreated or stimulated with LPS (100 ng/ml) or Poly(I:C) (100 µg/ml) for the indicated periods of time. Total cell extracts (1% SDS) were subjected to WB analyses. Note that endogenous APPL1 was barely detectable with this exposure time. RAW 264.7 cells expressing GFP-APPL1 or the A318D APPL1 mutant were left unstimulated or treated with Poly(I:C) (100 µg/ml); the resulting total cell extracts (1% SDS) were subjected to WB analyses, as indicated (lower panel). Note that endogenous APPL1 was barely detectable with this exposure time. RAW 264.7 cells expressing GFP-APPL1 or the A318D APPL1 mutant were left unstimulated or treated with Poly(I:C) (100 µg/ml); the resulting total cell extracts (1% SDS) were subjected to WB analyses, as indicated (lower panel).

MEK1/2-ERK1/2 signaling is required for TLR3/4-dependent APPL1 degradation

To further explore the molecular mechanisms underlying APPL1 degradation, we next assessed whether any known TLR3/4-activated kinase could promote APPL1 downregulation. LPS triggered APPL1 degradation in BMDCs isolated from IKKe-deficient mice, suggesting that IKKe is dispensable for APPL1 degradation (Supplemental Fig. 2A). Similarly, IKKe deficiency in RAW 264.7 cells did not have any impact on LPS-mediated APPL1 degradation (Supplemental Fig. 2B). Moreover, APPL1 degradation upon TLR4 activation was also TBK1 independent in RAW 264.7 cells (Supplemental Fig. 2C). Finally, the pharmacological inhibition of both TBK1 and IKKe totally abolished LPS-dependent IRF3 phosphorylation but did not impact ERK1/2 activation or APPL1 degradation (Supplemental Fig. 2D). Therefore, both IKK-related kinases TBK1 and IKKe are dispensable for TLR4-mediated APPL1 degradation.
We next assessed the role of MEK1/2 and ERK1/2 in APPL1 degradation by pretreating RAW 264.7 cells with U0126, a pharmacological MEK1/2-ERK1/2 inhibitor. As expected, LPS-dependent ERK1/2 activation was totally abolished in U0126-treated cells (Fig. 9A). Interestingly, APPL1 levels did not decrease upon TLR3 or TLR4 engagement in cells pretreated with the MEK1/2-ERK1/2 inhibitor (Fig. 9A). The pretreatment of RAW 264.7 cells with GSK1120212, another structurally unrelated MEK1/2 inhibitor, also prevented LPS- or Poly(I:C)-dependent APPL1 degradation (Fig. 9B). In contrast, MK-2206, a specific AKT inhibitor, did not affect APPL1 degradation in LPS- or Poly(I:C)-stimulated RAW 264.7 cells (Supplemental Fig. 3A). Moreover, the NF-κB–activating IKK complex also was dispensable, because NEMO deficiency did not interfere with APPL1 degradation in LPS- or Poly(I:C)-stimulated macrophages (Supplemental Fig. 3B). Taken together, our data indicate that the pharmacological inhibition of MEK1/2-ERK1/2 interferes with APPL1 disappearance and APPL1 polyubiquitination in unstimulated or LPS-treated RAW 264.7 cells (Fig. 9C). Therefore, MEK1/2-ERK1/2 are required for LPS-dependent APPL1 degradation by promoting its polyubiquitination. APPL1 degradation prevents aberrant IRF3-dependent gene transcription upon TLR4 engagement

To examine the biological relevance of APPL1 degradation post-stimulation, we assessed the expression levels of several IRF3 target genes upon TLR3 engagement when APPL1 degradation was blocked. As expected, the TBK1/IKKε pharmacological inhibitor BX795 interfered with Poly(I:C)-dependent IRF3 phosphorylation and, consequently, with IFN-β transcription; however, it did not
impact on APPL1 degradation (Fig. 10A, 10B). In contrast, pre-treatment with the MEK1/2-ERK1/2 inhibitor GSK1120212, which blocked APPL1 degradation upon TLR3 engagement, enhanced mRNA levels of IFN-β, RANTES, and IP-10 (Fig. 10A, 10B). Of note, pretreatment with both BX795 and GSK1120212 inhibitors also interfered with the induction of IFN-β, RANTES, and IP-10 (Fig. 10A, 10B). We carried out similar analyses in control and APPL1-depleted BMDMs and noticed that MEK1/2-ERK1/2 inhibition potentiated LPS-dependent IRF3 phosphorylation and, consequently, enhanced the mRNA levels of IRF3 target genes (Fig. 10C, 10D). Therefore, APPL1 degradation poststimulation prevents aberrant IRF3-dependent gene transcription.

**LPS tolerance involves a blockage of APPL1 degradation upon TLR4 engagement**

To explore whether APPL1 plays any role in LPS tolerance in macrophages, we pretreated RAW 264.7 cells with LPS and subjected them to a second round of LPS stimulation. As expected, the transcriptional induction of IFN-β, RANTES, and IP-10 upon TLR4 engagement was totally abolished in cells pretreated with LPS, which reflects LPS tolerance (Fig. 11A). Interestingly, pretreated cells showed decreased protein levels of APPL1 but higher activated levels of TBK1, IKKe, and IRF3 compared with unstimulated cells (Fig. 11B). Moreover, the LPS-induced APPL1 degradation was severely impaired when cells were subjected to the second round of LPS stimulation (Fig. 11B). Therefore, LPS tolerance involves defective APPL1 degradation upon TLR4 engagement in macrophages.

**Discussion**

Cellular responses to viral and bacterial infections involve the activation of IKK-related kinases TBK1 and IKKe to promote IRF3 phosphorylation and to produce type I IFN and chemokines. We show that APPL1 endosomes are required to trigger an IRF3-dependent gene transcription upon TLR3/4 engagement by recruiting TBK1 and IKKe to endosomes in macrophages. The transcriptional induction of some IRF3 target genes upon infection with the H1N1 virus in macrophages also relies on APPL1. Moreover, we
also demonstrate that TLR3/4 engagement involves proteasome-mediated APPL1 degradation through ERK1/2 to prevent an aberrant IRF3-dependent gene expression.

Endosomes are essential for LPS-dependent IRF3 phosphorylation, yet details on the subtype of endosomes required for IFN production upon viral and bacterial infections in macrophages were lacking (7). In this article, we define APPL1, but not EEA1, endosomes as intracellular structures required for TBK1 activation and subsequent IRF3-dependent gene induction in macrophages activated by TLR3/4 ligands or infected by the H1N1 virus. Interestingly, we also demonstrated that IRF3, but not NF-κB, activation upon TLR3/4 engagement critically relies on APPL1 endosomes in macrophages. Indeed, although the expression of IRF3-dependent target genes was impaired in APPL1-deficient macrophages subjected to LPS or Poly(I:C) stimulation, the signal-induced IκB degradation and expression of proinflammatory cytokines remained intact. Therefore, our data strongly suggest that APPL1 is dispensable for TLR3/4-dependent NF-κB activation in macrophages. A previous report demonstrated that APPL1 was required, as a TRAF2-binding protein, for basal, but not TNF-α-induced, NF-κB activation in HEK293 cells, at least by promoting NIK stability and p65 nuclear import (45). It remains to be examined whether APPL1 also promotes NIK stability and p65 nuclear translocation to support basal NF-κB activity in macrophages. In any case, it is unlikely that APPL1 promotes TLR-dependent IFN production through NIK stabilization. Indeed, NIK does not enhance, but rather...
limits, type I IFN production in BMDMs without altering TBK1 and IKKe activation upon stimulation with a variety of TLR ligands (46).

Distinct subcellular localization of TBK1 and IKKe may be a way to achieve substrate specificity (19). It is believed that multiple TBK1 and IKKe adaptors recruit both kinases to distinct complexes to trigger cellular responses to a variety of viral and bacterial pathogens. In this context, phosphorylated TBK1 is localized in mitochondria in response to cytoplasmic viral DNA in HeLa cells but not in macrophages (47). We show in this study that phosphorylated TBK1 is recruited to APPL1 endosomes upon TLR3/4 engagement in macrophages. In contrast to some other kinases, such as AKT, TBK1 lacks any PH domain; therefore, it relies on a PH domain–containing protein to be recruited to cell membranes. Our data defined APPL1 as one protein that tethers TBK1 and IKKe to endosomes to trigger cell signaling through TLR3/4 in macrophages. Interfering with APPL1 levels impairs TBK1 and IRF3 phosphorylation, presumably by deregulating TBK1 subcellular localization. Therefore, APPL1 positively regulates type 1 IFN production in macrophages.

APPL1 is involved in multiple signaling pathways, yet none of them trigger APPL1 degradation. We show in this study that TLR3/4 activation triggers TBK1 phosphorylation, as well as APPL1 degradation, in macrophages. APPL1 downregulation also occurs upon TLR7 engagement, a pathway known to trigger TBK1 and IKKe activation (48). Therefore, APPL1 degradation is intimately connected to TBK1 activation through multiple TLRs. We also show that APPL1 degradation occurs in H1N1-infected macrophages. The H1N1 virus promotes TBK1 and IRF3 activation once bound to RIG-1, a cytoplasmic helicase that signals through MAVS, a mitochondrial adaptor protein (49). The functional link between the endosomal APPL1 protein and the RIG-1–dependent pathway that relies on the mitochondrial MAVS protein to promote IRF3 activation is not known. Although we clearly show that APPL1 is modified before undergoing degradation in H1N1-infected cells, the detailed mechanism by which the RIG-1–dependent pathway ultimately targets APPL1 deserves further investigation.

Dynamin-2 deficiency, which abolishes LPS-mediated TBK1 phosphorylation, prevents APPL1 degradation. In contrast, bafilomycin, which enhances LPS-dependent TBK1 phosphorylation, also potentiates APPL1 degradation. Yet, TBK1 deficiency does not impact on TLR3/4-mediated APPL1 degradation.
our data suggest that APPL1 degradation is a mechanism to turn off TBK1 and IRF3 phosphorylation to prevent aberrant type 1 IFN production in TLR signaling (50). This hypothesis is supported by our data showing enhanced IRF3-dependent gene transcription when APPL1 degradation posttranslational modification is specifically blocked. APPL1 degradation critically relies on ERK1/2, but not on TBK1 and IKKe, activation. Indeed, APPL1 degradation upon TLR engagement still occurs when TBK1 and IKKe are pharmacologically blocked. In contrast, APPL1 degradation does not occur upon MEK1/2-ERK1/2 inhibition. Therefore, these data indicate that TBK1/IKKε and MEK1/2-ERK1/2 activation occur through parallel signaling pathways. They also indicate that MEK1/2-ERK1/2, rather than TBK1/IKKε, are the sensors for APPL1 degradation. Although ERK1/2 promotes APPL1 polyubiquitination and subsequent degradation, it is unclear which substrate(s) is targeted by ERK1/2 to negatively regulate APPL1 protein levels. Mutations of S401, which were identified as phosphorylated residues on APPL1 (51), do not prevent LPS and Poly(I:C)-dependent APPL1 degradation (data not shown). It remains to be seen whether APPL1 phosphorylation is modified upon TLR signaling and if so, whether this posttranslational modification regulates its proteasome-mediated degradation. Because a slower-migrating form of APPL1 is detected in H1N1-infected macrophages, it is indeed possible that APPL1 itself is targeted before undergoing proteasome-dependent degradation. Yet, we cannot rule out the possibility that ERK1/2 targets another protein, possibly the E3 ligase itself, to trigger APPL1 polyubiquitination.

Identifying the E3 ligase required for APPL1 degradation will shed more light on the mechanisms by which macrophages tightly regulate type 1 IFN synthesis upon viral or bacterial infection. Our data indicate that protein synthesis is required, because APPL1 does not undergo degradation upon LPS or Poly(I:C) stimulation in RAW 264.7 cells pretreated with cycloheximide (data not shown). Yet, the canonical NF-kB-activating pathway is dispensable because NEMO deficiency did not interfere with APPL1 degradation upon TLR signaling in RAW 264.7 cells (Supplemental Fig. 3). Moreover, A20, an NF-kB-induced protein, as well as TRAF3, both described as TBK1- and IKKe-interacting proteins (52, 53), are also dispensable for APPL1 degradation upon TLR signaling (data not shown, Supplemental Fig. 4A). Importantly, Cul1 and Rbx1, two key subunits of the F-box family of E3 ligases (54), are not involved in APPL1 degradation (Supplemental Fig. 4B, 4C). Therefore, an unbiased screening approach is required to identify the E3 ligase that promotes APPL1 degradation upon TLR signaling.

Taken together, our data establish a link between TLR signaling and APPL1 endosomes for type I IFN production upon some viral or bacterial infection in macrophages and demonstrate that an appropriate immune response involves the signal-induced degradation of APPL1. This pathway is critical to prevent aberrant IRF3-dependent gene expression linked to immune diseases, such as systemic lupus erythematosus and rheumatoid arthritis (55, 56).

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Disclosures

The authors have no financial conflicts of interest.

References

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References


Figure S1: EEA1 is dispensable for the expression of IRF3 target genes upon TLR3/4 engagement in macrophages. Control or EEA1-depleted RAW 264.7 cells (siRNA GFP and EEA1, respectively) were untreated (“NT”) or stimulated with LPS (100 ng/mL) or with Poly(I:C) (100 µg/mL) for 1 or 4 hours (“1H” and “4H”, respectively) and total RNAs from the resulting cells were subjected to Real-time PCR analyses to follow-up mRNA levels of EEA1 and IRF3-dependent target genes (IFNβ, IP10 and Rantes). The abundance of all transcripts in control and unstimulated cells was set to 1 and their levels in other experimental conditions were relative to that after normalization with GAPDH. Data from three independent experiments performed in triplicates (means ± S.D.) are shown. (*= p < 0.05; **=p <0.01; ***= p<0.001, t-student test).
**Figure S2: APPL1 degradation upon TLR4 engagement does not rely on IKKε and TBK1.**

**A.** The pharmacological inhibition of both TBK1 and IKKε impairs LPS-dependent IRF3 phosphorylation but not ERK1/2 activation and APPL1 degradation. RAW 264.7 cells were pre-incubated with DMSO (control vehicle) or with BX795 (1 µM) for one hour and subsequently untreated or stimulated with LPS for the indicated periods of time. Cell extracts were subjected to western blot analyses using the indicated antibodies.

**B.** APPL1 protein levels decrease upon LPS stimulation in BMDMs isolated from control or IKKε-deficient mice. Cell extracts from untreated or LPS-stimulated BMDMs of the indicated genotype were subjected to WB analyses using the indicated antibodies.

**C. and D.** IKKε (B) or TBK1 (C) deficiency does not impact on LPS-dependent APPL1 degradation in macrophages. RAW 264.7 cells were transfected with the indicated siRNAs and subsequently unstimulated or treated with LPS (100 ng/mL) for the indicated periods of time. Total cell extracts from resulting cells were subjected to WB analyses using the indicated antibodies.
Figure S3: AKT and NEMO are dispensable for LPS- and Poly(I:C)-dependent APPL1 degradation. A. APPL1 downregulation occurs upon AKT inhibition in LPS- or Poly(I:C)-treated macrophages. RAW 264.7 cells were treated with the AKT inhibitor MK-2206 (1 µM) or with the control vehicle (“Mock”) for 2 hours and subsequently left unstimulated or treated with LPS (100 ng/mL) or with Poly(I:C) (100 µg/mL) for the indicated periods of time. Anti-APPL1, -pAKT and -AKT WBs were carried out on the resulting cell extracts (SDS 1%). B. NEMO deficiency does not impact on APPL1 degradation upon TLR3/4 engagement. Control (“GFP”) or NEMO-deficient RAW 264.7 cells were untreated or stimulated with LPS (100 ng/mL) or with Poly(I:C) (100 µg/mL) for the indicated periods of time. Anti-APPL1, -NEMO and -α-tubulin WBs were carried out on the resulting cell extracts (SDS 1%).
Figure S4: TRAF3, Cul1 and Rbx1 are dispensable for LPS- and Poly(I:C)-dependent APPL1 degradation. A. TRAF3 deficiency does not impact on APPL1 downregulation upon TLR3 engagement. Control ("GFP") or TRAF3-deficient RAW 264.7 cells were untreated or stimulated with Poly(I:C) (100 µg/mL) for the indicated periods of time. Anti-APPL1, -TRAF3 and -α-tubulin WBs were carried out on the resulting cell extracts (SDS 1%). B. and C. APPL1 degradation upon TLR3 engagement occurs in Cul1- or Rbx1-depleted macrophages. Control ("GFP"), Cul1- or Rbx1-deficient RAW 264.7 cells were untreated or stimulated with Poly(I:C) for the indicated periods of time. On the top, total RNAs were subjected to Real-time PCR analyses to follow-up mRNA levels of Cul1 (B) or Rbx1 (C). The abundance of all transcripts in control and unstimulated cells was set to 1 and their levels in other experimental conditions were relative to that after normalization with GAPDH. At the bottom, protein extracts (SDS 1%) of the resulting cells were subjected to WB analyses, as indicated.