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.**

Toll-like receptors are critical for innate and adaptive immune responses. They are type 1 transmembrane receptors involved in the detection of structurally conserved bacterial and viral components referred to as pathogen-associated molecular patterns (1). Although TLR4 senses LPS from Gram-negative bacteria, as well as endogenous molecules produced following tissue injury, TLR3 detects extracellular viral dsRNA (2–5). Activated TLR3 and TLR4 trigger the production of proinflammatory cytokines and type I IFN through NF-κB, MAPKs, and IRF3 (6). Yet, the identity and the intracellular trafficking of signaling proteins acting downstream of the receptors differ from one TLR to the other. Indeed, TLR4 signals through the Toll–IL-1R domain-containing adaptor molecules TIRAP (also referred to as Mal) and MyD88 at the plasma membrane to promote early NF-κB activation, and it is subsequently endocytosed to activate late NF-κB activation and IRF3 through TRAM and TRIF (also referred to as TICAM-1) from early endosomes (7). In contrast, TLR3 appears to signal exclusively from endosomes (8, 9).

IKK-related kinases TBK1 and IKKε are activated upon TLR3 and TLR4 engagement with their ligands and promote type I IFN production though IRF3 phosphorylation (10–12). Both IKK-related kinases are assembled by distinct scaffold proteins, namely TANK (also referred to as I-TRAF), NAP1, SINTBAD, and optineurin, which are all critical for TBK1 activation (13–18). These scaffold proteins compete for binding to the C-terminal part of TBK1 and IKKε and provide specificity in cellular responses to bacterial and viral infections by assembling discrete TBK1- and IKKε-containing complexes in distinct cellular compartments (10, 19–21). Indeed, although TANK promotes TBK1 activation and IFN-β production upon viral infection, SINTBAD and NAP1 activate autophagy against cytosolic bacteria as TBK1-associated proteins (21, 22). Yet, the cellular localization and intracellular trafficking of these TBK1- and IKKε-containing complexes upon TLR activation remain poorly defined. Although TRIF, TRAM, and TRAF3, as well as the TBK1-interacting protein TAPE, are recruited to endosomes upon signaling, it remains unclear whether TBK1 and IKKε themselves also localize in endosomes upon TLR3 and TLR4 activation. Such a hypothesis may be relevant because TBK1 partially localizes to early endosome Ag 1 $(EEA1)^+$ endosomes upon TLR3 engagement in bone marrow–derived macrophages (BMDMs), and this cellular localization is more

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Received for publication June 25, 2014. Accepted for publication February 3, 2015.

This work was supported by grants from the Belgian National Funds for Scientific Research, TELEVIE, the Belgian Federation against Cancer, the King Baudouin Foundation, the University of Liege (Concerted Research Action Program BIO-ACET and Fonds Spéciaux), the Inter-University Attraction Pole 7/12 (the Federal Ministry of Science), the Centre Anti-Cancéreux, the Leon Fredericq Foundation, and the Walloon Excellence in Life Sciences and Biotechnology. P.C. and E.D. are Research Associates, and A.C. is a Senior Research Associate at the Belgian National Funds for Scientific Research.

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The online version of this article contains supplemental material.

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-1, 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.

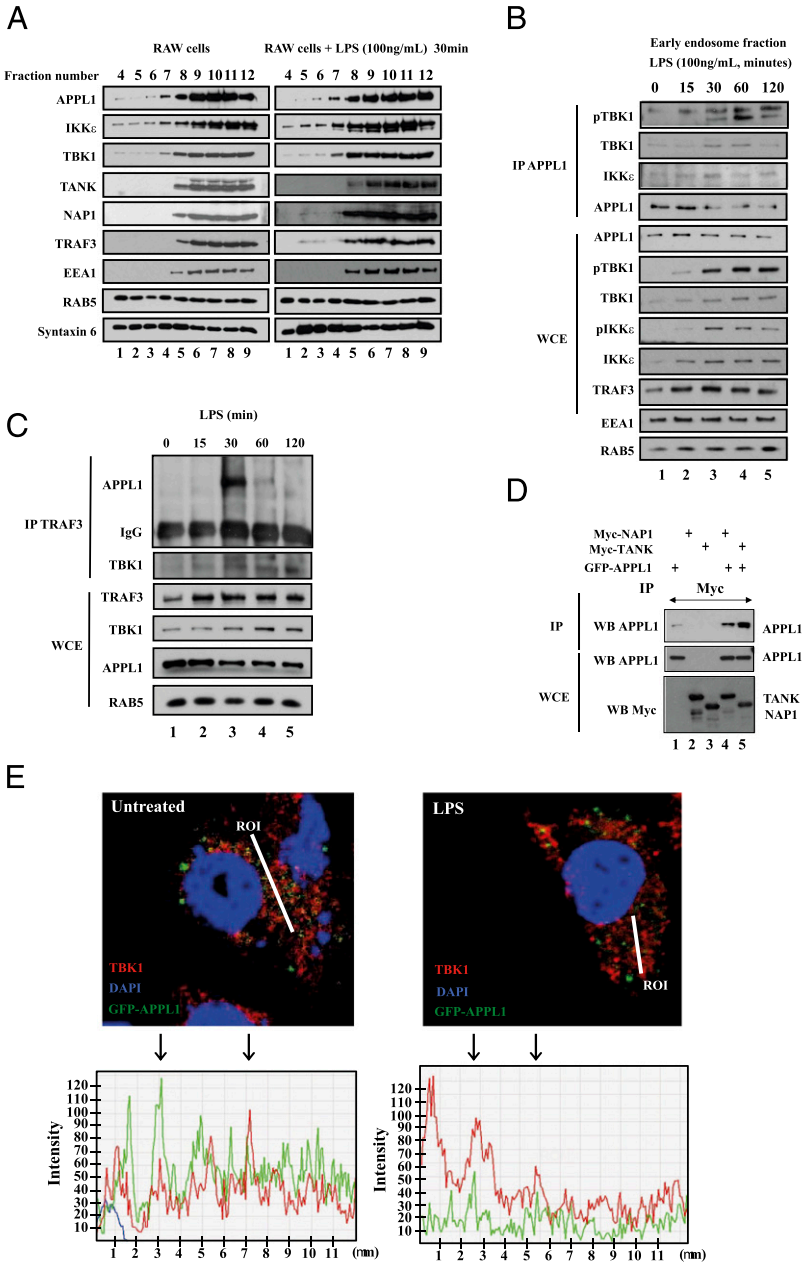

FIGURE 1. IKKε and TBK1 are recruited to APPL1 endosomes upon TLR3 and TLR4 engagement. (**A**) TBK1 and IKKε and their scaffold proteins TANK and NAP1 coelute with endosomal markers. Fractionated extracts from untreated or LPS-stimulated RAW 264.7 cells (*left* and *right panels*, respectively; see *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). MG132 was from A&E Scientific (Marcq, Belgium). BX795, U0126, and GSK1120212 inhibitors were from Axon Medchem (Reston, VA), Cell Signaling (Temecula, CA), and Selleckchem (Boston, MA), respectively.

Polyclonal anti-HA, anti-ERK1/2, anti-TANK, anti-Myc, anti-IRF3, and anti-IκBα 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-TANK Ab used in Western blot (WB) analyses was described previously (35). The anti-EEA1, 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 IKKε 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.

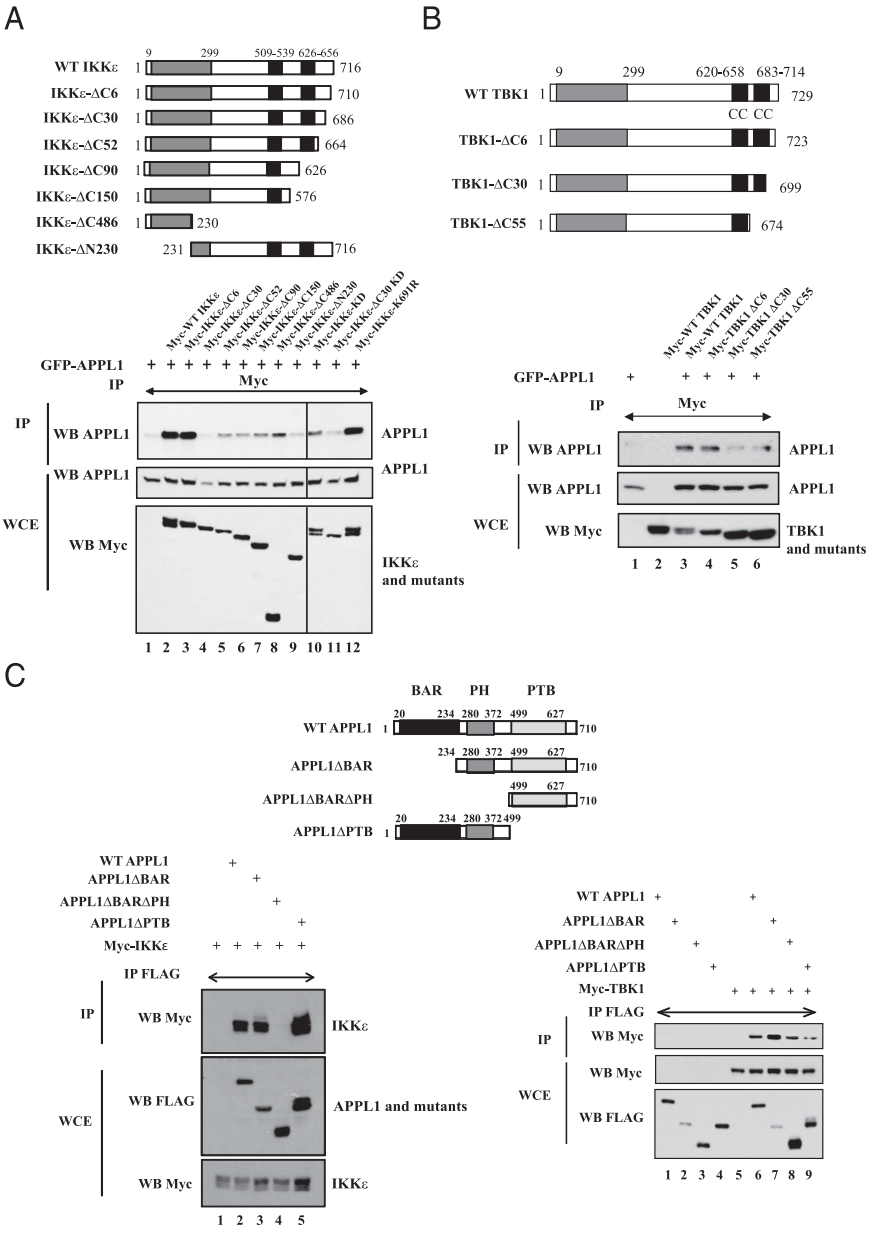

FIGURE 2. Identification of TBK1, IKKε, and APPL1 domains required for their interaction. (**A**) IKKε binds APPL1 through two distinct domains. HEK293 cells were transfected with the indicated expression plasmids, and the resulting cell extracts were subjected to anti-Myc IP, followed by an anti-APPL1 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 IKKε 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*).

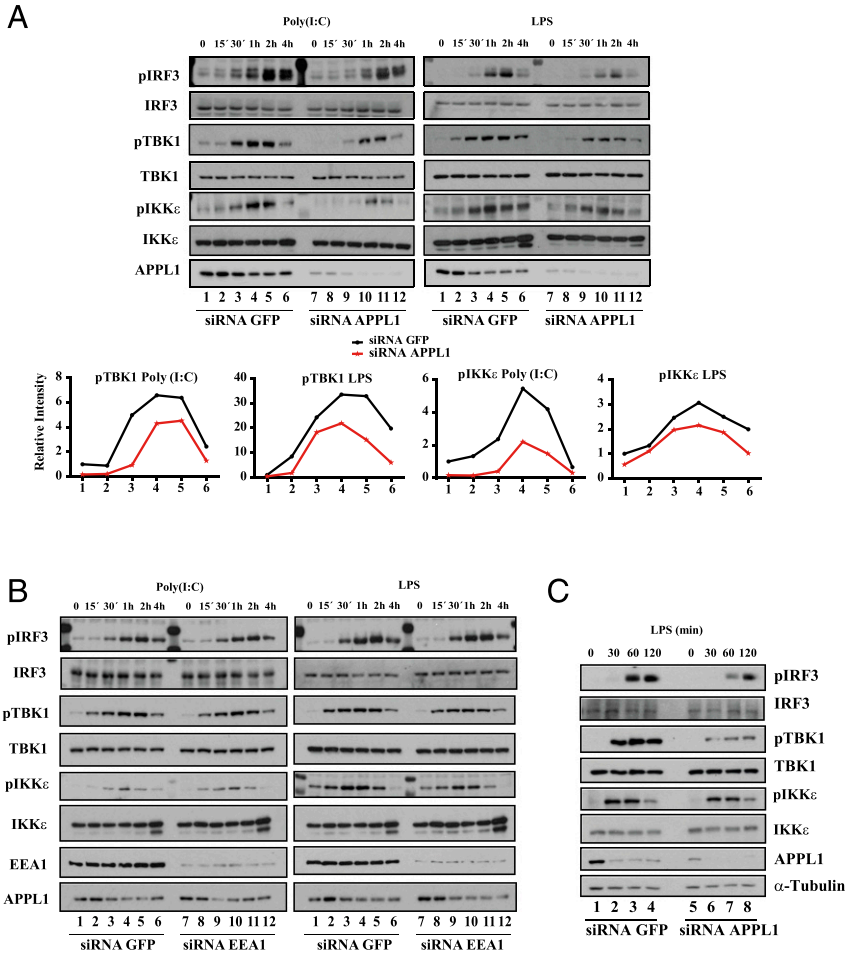

FIGURE 3. Impaired TLR3/4-mediated IRF3 phosphorylation in APPL1-deficient, but not in EEA1-deficient, macrophages. (**A**) Defective TBK1, IKKε, and IRF3 phosphorylation upon APPL1 deficiency in Poly(I:C)- or LPS-treated macrophages. Control or APPL1-deficient RAW 264.7 cells were left untreated or stimulated with Poly(I:C) (100 μg/ml) or LPS (100 ng/ml) for the indicated periods of time; the resulting cell extracts were subjected to WB analyses using the indicated Abs. Three independent experiments were carried out and systematically demonstrated a defective activation of both IKK-related kinases upon TLR3/4 engagement in APPL1-depleted cells. Quantification of both pTBK1 and pIKKε levels in all experimental conditions is illustrated for one representative experiment. (**B**) EEA1 is dispensable for LPS and Poly(I:C)-dependent TBK1, IKKε, and IRF3 phosphorylation. Control or APPL1-deficient RAW 264.7 cells were left untreated or stimulated with Poly(I:C) (100 μg/ml) or LPS (100 ng/ml) for the indicated periods of time. WB analyses were conducted on the resulting cell extracts (1% SDS) using the indicated Abs. (**C**) APPL1 deficiency impairs LPS-dependent TBK1 and IRF3 phosphorylation in BMDMs. Control or APPL1-depleted BMDMs were left untreated or stimulated with LPS for the indicated periods of time, and the resulting cell extracts were subjected to WB analyses using the indicated Abs.

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 using a multiplicity of infection of 10 of the mouse-adapted swine H1N1 influenza strain in 1 ml serum-free DMEM containing 0.5 μg/ml 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 collected and washed once in SIM buffer (250 mM sucrose, 3 mM imidazole [pH 7.4], 1 mM EDTA). Cell pellets 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 fraction, were collected and adjusted to 40.6% (w/w) sucrose by mixing with 1.2 vol of 62% (w/w) sucrose solution in 3 mM imidazole (pH 7.4). A total of 3 ml this mixture was loaded in the bottom of a 13.2-ml ultracentrifuge tube. It was overlaid with 4.5 ml 35% (w/w) sucrose in 3 mM imidazole (pH 7.4) and 3 ml 25% sucrose (w/w) in 3 mM imidazole (pH 7.4), and the tube was filled with SIM buffer (8.5% [w/w] sucrose). The loaded gradient was subjected to centrifugation using an SW 40 Ti rotor (Beckman Coulter) at 35000 rpm overnight at 4°C. The early endosome fraction was collected from the 25–35% interphase and subjected to further analyses.

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 ≥15 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. Agarose beads were subsequently added for ≥2 h before collecting the immunoprecipitates. Collected beads were washed five times in the same nondenaturant buffer and boiled in Laemmli blue sample buffer. Immunofluorescence (IF) analyses also were conducted as described (39). Briefly, to reveal the colocalization of endogenous TBK1 with GFP-APPL1 in endosome structures, RAW 264.7 cells were seeded on coverslips in six-well plates, transfected with GFP-APPL1, fixed 24 h later with 4% paraformaldehyde, and permeabilized with 0.3% Triton X-100 for 10 min 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 Abs (Dako, Glostrup, Germany). To reveal the colocalization of APPL1 with RAB5, RAW 264.7 cells were seeded on coverslips in six-well plates, transfected with GFP-APPL1 or GFP A318D and with RFP-RAB5, and fixed 24 h later with 4% paraformaldehyde. DAPI staining was carried out to visualize nuclei. 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 EZNA 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 IKKε are recruited to APPL1 endosomes in LPS-stimulated macrophages

To gain insights into the subcellular localization of TBK1 and IKKε 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 IKKε (Fig. 1A). Interestingly, TBK1- and IKKε-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 IKKε. We next isolated enriched endosomal fractions from control or LPS-stimulated RAW 264.7 cells and showed that TBK1, IKKε, 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 IKKε 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 IKKε are recruited in APPL1 endosomes upon LPS signaling in macrophages.

Having defined APPL1 as a TBK1- and IKKε-associated protein, we next conducted additional IP experiments in HEK293 cells using a variety of IKKε and APPL1 mutants to precisely map domains required for their interaction. The binding of IKKε to APPL1 relies on its C-terminal domain (aa 687–710) because the

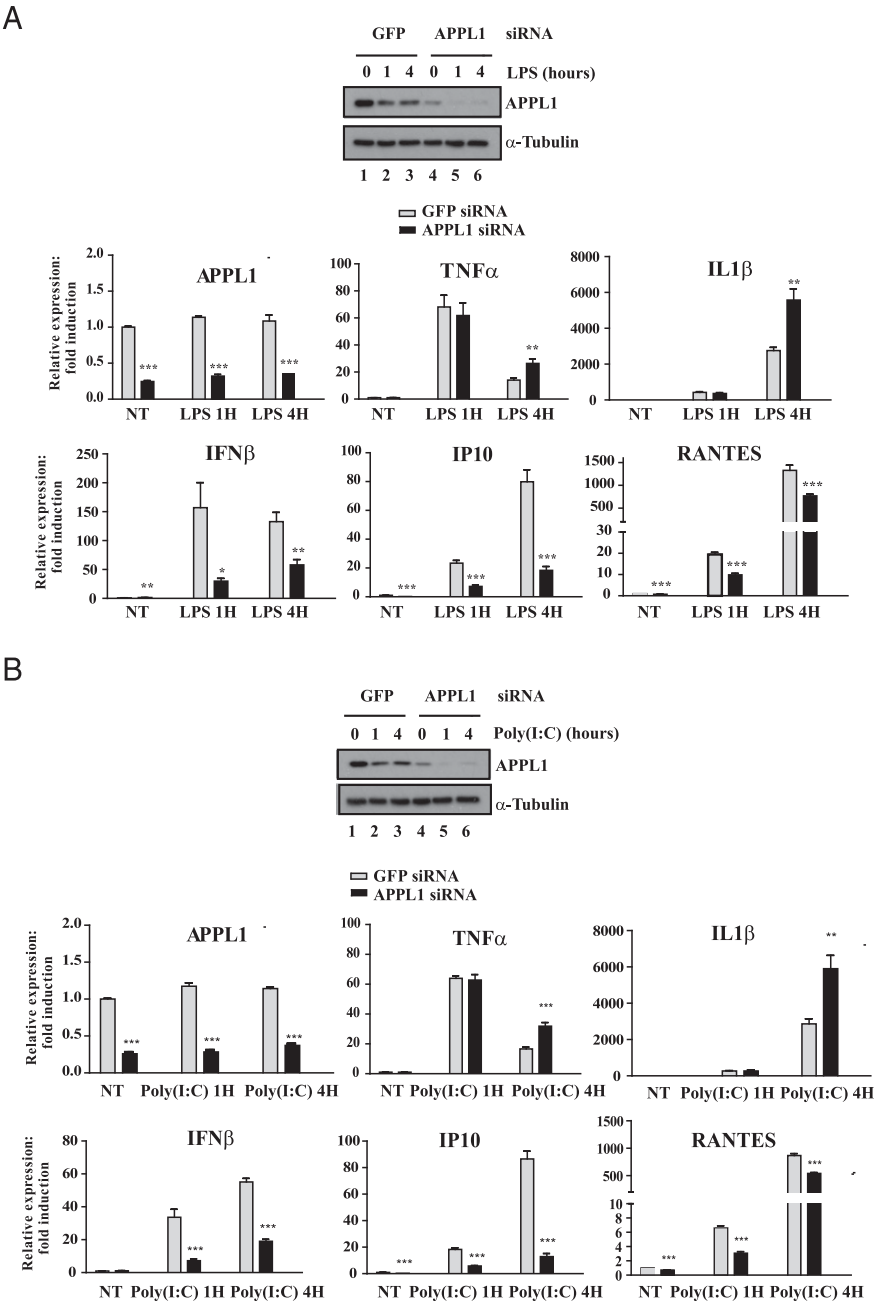

FIGURE 4. Induction of IRF3 target gene expression upon TLR3 and TLR4 activation relies on APPL1 in macrophages. Defective IRF3-dependent gene expression upon LPS (**A**) or Poly(I:C) (**B**) stimulation in APPL1-depleted macrophages. Control (GFP) or APPL1-deficient RAW 264.7 cells were left untreated or stimulated with LPS (100 ng/ml) or Poly(I:C) (100 μg/ml) for the indicated periods of time. Total RNA from the resulting cells was subjected to real-time PCR analyses to quantify mRNA levels of APPL1, IRF3-dependent target genes (IFNβ, IP10, and RANTES), and proinflammatory cytokines (TNFα and IL1β). 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 (mean ± SD), are shown. WB analyses to confirm siRNA-mediated APPL1 depletions are also shown. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, Student t test.

IKKε-ΔC6 mutant, but not the IKKε-ΔC30 mutant, bound APPL1 in HEK293 cells (Fig. 2A). Accordingly, additional IKKε mutants lacking C-terminal amino acids (IKKε-ΔC52, -90 and -150) failed to strongly bind APPL1 (Fig. 2A). Moreover, the IKKε-ΔN230 mutant and the kinase-dead version of IKKε did not efficiently associate with APPL1 (Fig. 2A). Therefore, the kinase domain of IKKε 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 IKKε, whereas both the Bin/Amphiphysin/Rvs and PTB sequences were dispensable (Fig. 2C). Interestingly, the APPL1ΔBin/Amphiphysin/RvsΔPH mutant that failed to bind IKKε still bound TBK1, whereas the APPL1 ΔPTB mutant associated only weakly with APPL1 (Fig. 2C). Thus, APPL1 recruits TBK1 and IKKε to endosomes through distinct domains.

APPL1 endosomes are required for TLR3/4-dependent IRF3 phosphorylation

APPL1 is a TBK1- and IKKε-associated protein, yet it was unclear whether those interactions play any role in TBK1- and IKKε-dependent pathways. Therefore, we explored whether APPL1 deficiency had any impact on TLR3/4-dependent TBK1 and IKKε activation in macrophages. APPL1-depleted, but not EEA1-depleted, RAW 264.7 cells did not properly activate TBK1 and IKKε upon stimulation by LPS or Poly(I:C), a synthetic analog of viral dsRNA (Fig. 3A, 3B, respectively). Of note, the defective phosphorylation of IKKε 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 IKKε 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 postinduction did not rely on APPL1 (Fig. 5). Taken together, our data suggest that APPL1-containing endosomes are required to activate TBK1 and IKKε 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

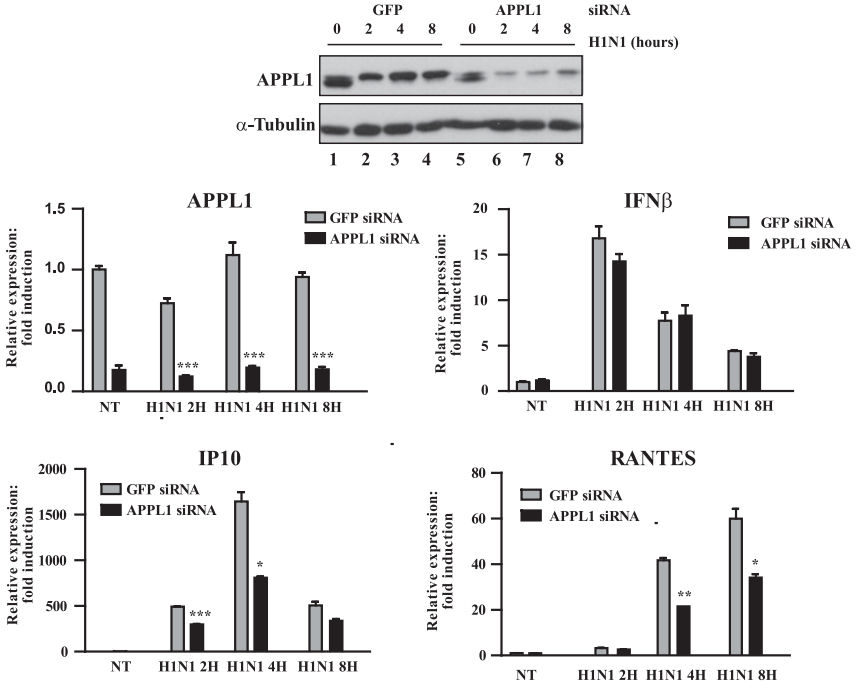

FIGURE 5. APPL1 controls the expression of some IRF3 target genes upon H1N1 infection in macrophages. Control or APPL1-depleted BMDMs infected or not with the H1N1 virus for the indicated periods of time were subjected to WB and real-time PCR analyses to assess APPL1 protein levels (*top panel*) or mRNA levels of APPL1, IFNβ, IP10, or RANTES (*middle* and *bottom panels*). The abundance of all transcripts in noninfected cells was set to 1, and their levels in other experimental conditions were relative to that after normalization with GAPDH. Data from two independent experiments, performed in triplicates (mean ± SD), are shown. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, Student *t* test.

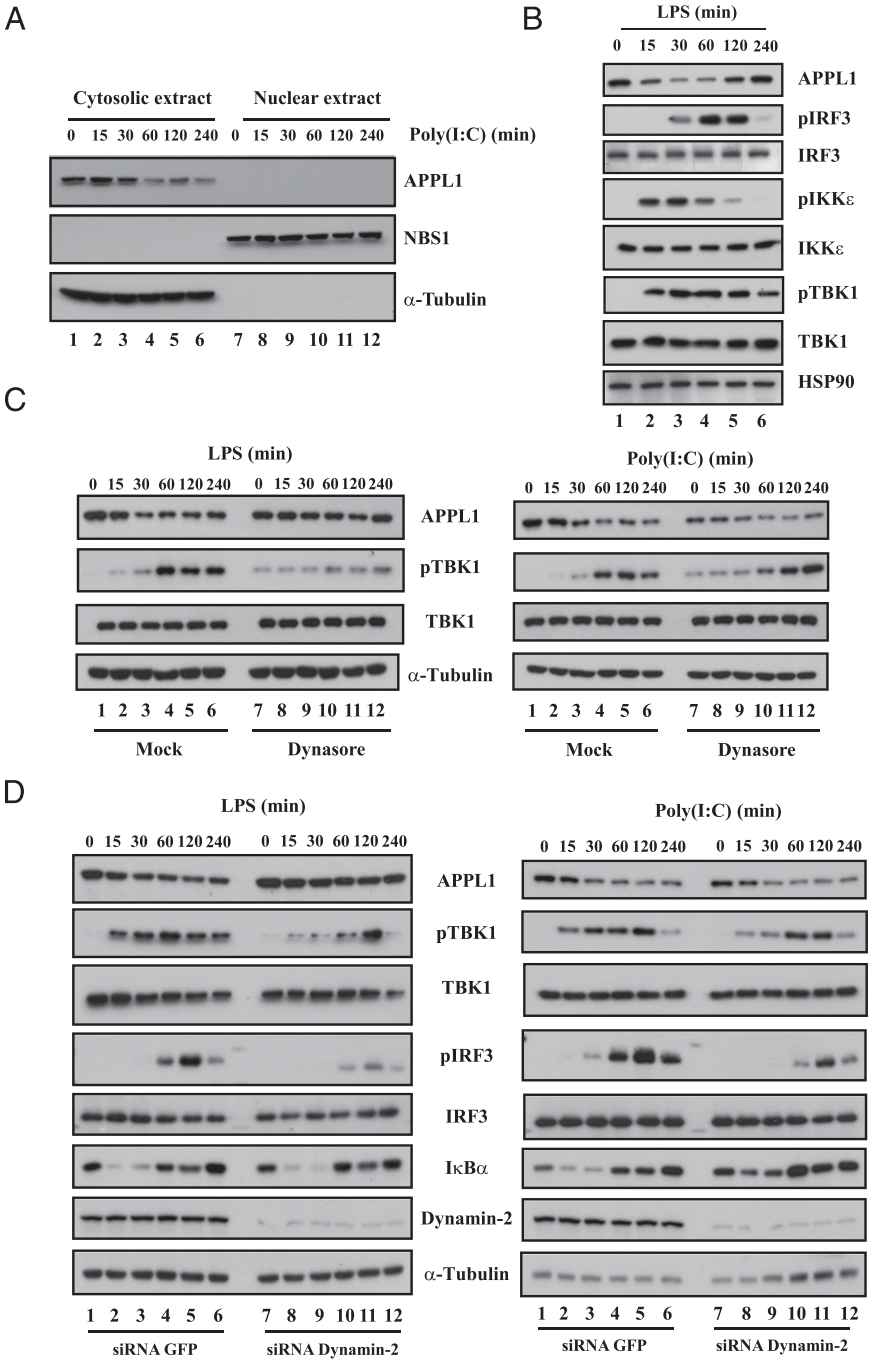

FIGURE 6. APPL1 degradation upon TLR3 and TLR4 engagement. (**A**) APPL1 does not translocate into the nucleus upon TLR3 activation. RAW 264.7 cells were left untreated or stimulated with Poly(I:C) (100 μg/ml) for the indicated periods of time. Cytoplasmic and nuclear extracts from the resulting cells were subjected to WB analyses using the indicated Abs. NBS1 and α-tubulin were used as nuclear and cytoplasmic markers, respectively. (**B**) LPS signaling triggers APPL1 degradation in untransformed macrophages. BMDMs were left untreated or stimulated with LPS (100 ng/ml) for the indicated periods of time, and the resulting cell extracts were subjected to WB analyses. (**C**) Dynasore prevents LPS-dependent, but not Poly(I:C)-dependent, APPL1 degradation in macrophages. RAW 264.7 cells were pretreated with dynasore (100 μM) for 2 h in a serum-free medium and subsequently were left untreated or stimulated for the indicated periods of time with LPS (100 ng/ml) or Poly(I:C) (100 μg/ml). Resulting cell extracts were subjected to WB analyses. (**D**) Dynamin-2 deficiency impairs Poly(I:C)- and 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.

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 signaling but not Poly(I:C)-dependent signaling or APPL1 degradation (Fig. 6D). Of note, IRF3 phosphorylation upon Poly(I:C) stimulation was impaired upon dynamin-2 deficiency (Fig. 6D), presumably because Poly(I:C) internalization is required to promote IRF3 phosphorylation.

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.

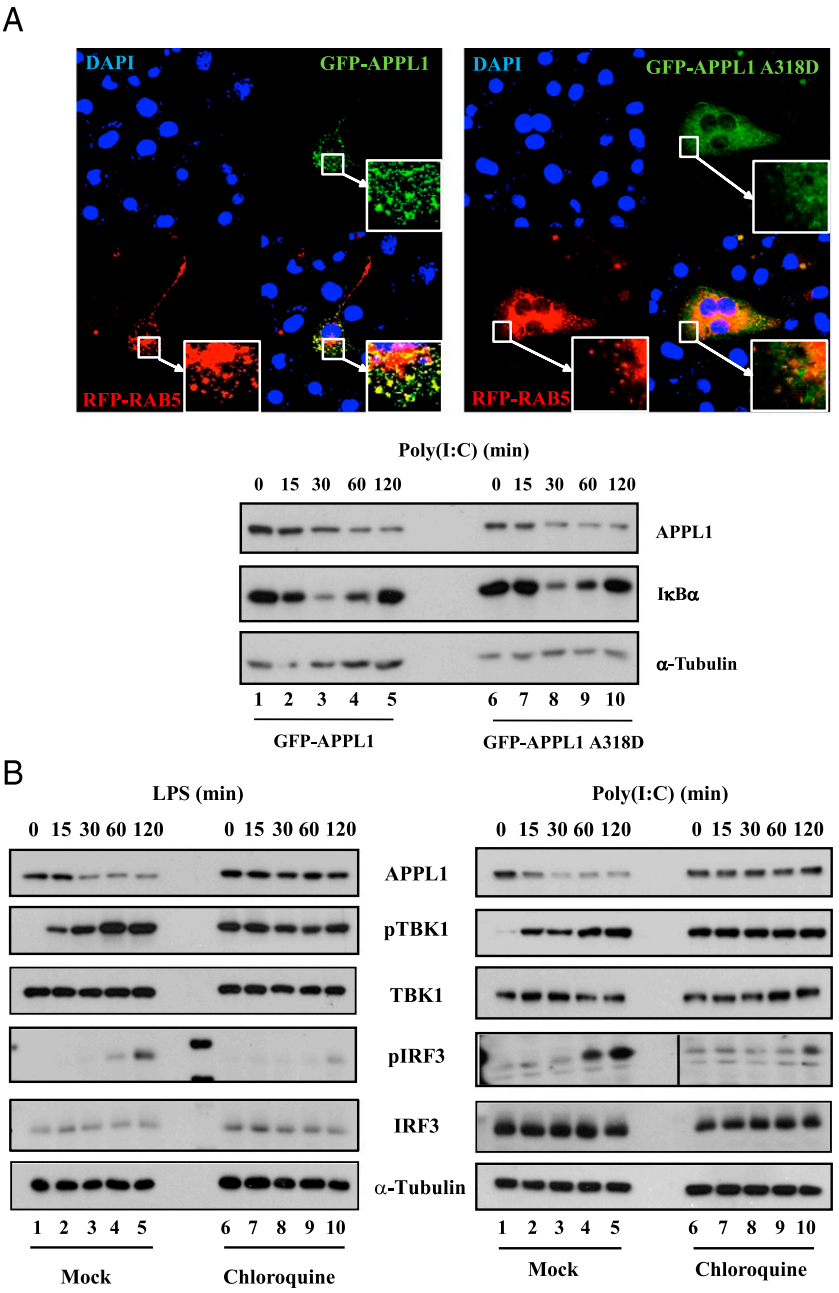

FIGURE 7. Blockage of LPS- and Poly(I:C)-dependent APPL1 degradation by chloroquine. (**A**) APPL1 binding to RAB5 is dispensable for LPS- or Poly(I:C)-dependent APPL1 degradation. RAW 264.7 cells were transfected with RFP-RAB5 and GFP-APPL1 or with the A318D APPL1 mutant. The resulting cells were subjected to IF analyses. APPL1 colocalization with RAB5 in endosome structures is shown as yellow dots (*upper panels*). 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. (**B**) Chloroquine blocks LPS- and Poly(I:C)-dependent TBK1 activation and 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 the anti-pIRF3 Ab cross-reacts with the m.w. marker. The black line was added where images from a single experiment were joined for the anti-pIRF3 blot to hide signals due to cross-reactivity of this Ab with the m.w. marker.

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 upon Poly(I:C) or LPS stimulation (Fig. 8B). Interestingly, bafilomycin A1, an inhibitor of vacuolar H^+ ATPases that prevents the fusion between autophagosomes and lysosomes, had the opposite effects; it enhanced Poly(I:C)- and LPS-dependent TBK1 phosphorylation and APPL1 degradation (Fig. 8C). Thus, APPL1 degradation is linked to TBK1 activation and is proteasome dependent, but not lysosome dependent, upon TLR activation.

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 BMDMs isolated from IKKε-deficient mice, suggesting that IKKε is dispensable for APPL1 degradation (Supplemental Fig. 2A). Similarly, IKKε 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 IKKε 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 IKKε are dispensable for TLR4-mediated APPL1 degradation.

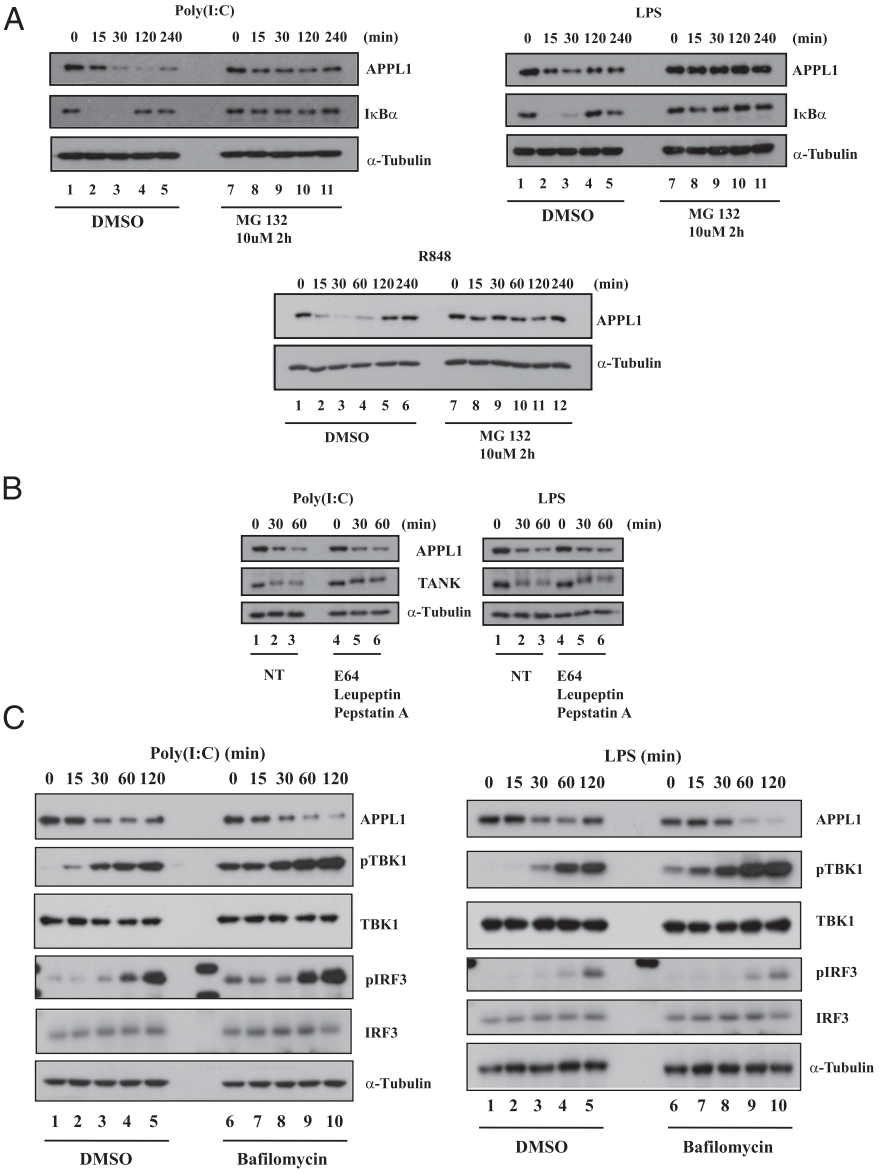

FIGURE 8. Proteasome-mediated APPL1 degradation upon TLR engagement. (**A**) APPL1 is degraded through the proteasome upon TLR3, TLR4, and TLR7/8 engagement in macrophages. RAW 264.7 cells were preincubated with DMSO (control vehicle) or MG132 (10 μM) for 2 h. Subsequently, cells were left untreated or stimulated with Poly(I:C) (100 μg/ml), LPS (100 ng/ml), or R848 (10 μg/ml), and WB analyses were carried out, using the indicated Abs, on the resulting cell extracts (1% SDS). (**B**) APPL1 degradation upon TLR3 and TLR4 activation does not rely on lysosomes. RAW 264.7 cells were left untreated (NT) or incubated with E64 (20 μM), pepstatin A (10 μM), and leupeptin (50 μM) for 2 h, and the resulting cells were left unstimulated or treated with Poly(I:C) (100 μg/ml) or LPS (100 ng/ml) for the indicated periods of time. Total cell extracts (1% SDS) were subjected to WB analyses using the indicated Abs. (**C**) Bafilomycin enhances LPS-dependent TBK1 activation and APPL1 degradation. RAW 264.7 cells were preincubated with DMSO (control vehicle) or bafilomycin (100 nM) for 2 h; subsequently, the cells were left untreated or stimulated with Poly(I:C) (100 μg/ml) or LPS (100 ng/ml), and WB analyses were carried out on resulting cell extracts using the indicated Abs (1% SDS).

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 degradation upon TLR3/4 engagement in macrophages.

To explore whether APPL1 is modified by polyubiquitination to be degraded upon signaling, we looked for polyubiquitin chains on APPL1 in cells stimulated with LPS by co-IP. Cell extracts were made under denaturing conditions to rule out the detection of polyubiquitin chains on any APPL1-associated proteins. Again, MG132 prevented an LPS-dependent decrease in APPL1 protein levels (Fig. 9C). Of note, MG132 also interfered with LPS-dependent ERK1/2, but not TBK1, phosphorylation. Interestingly, polyubiquitinated forms of APPL1 were detected in MG132-pretreated cells (Fig. 9C). Those polyubiquitinated forms of APPL1 also were detected in LPS-treated cells but only when the proteasome was inhibited. Importantly, the pharmacological inhibition of MEK1/2-ERK1/2 interfered 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

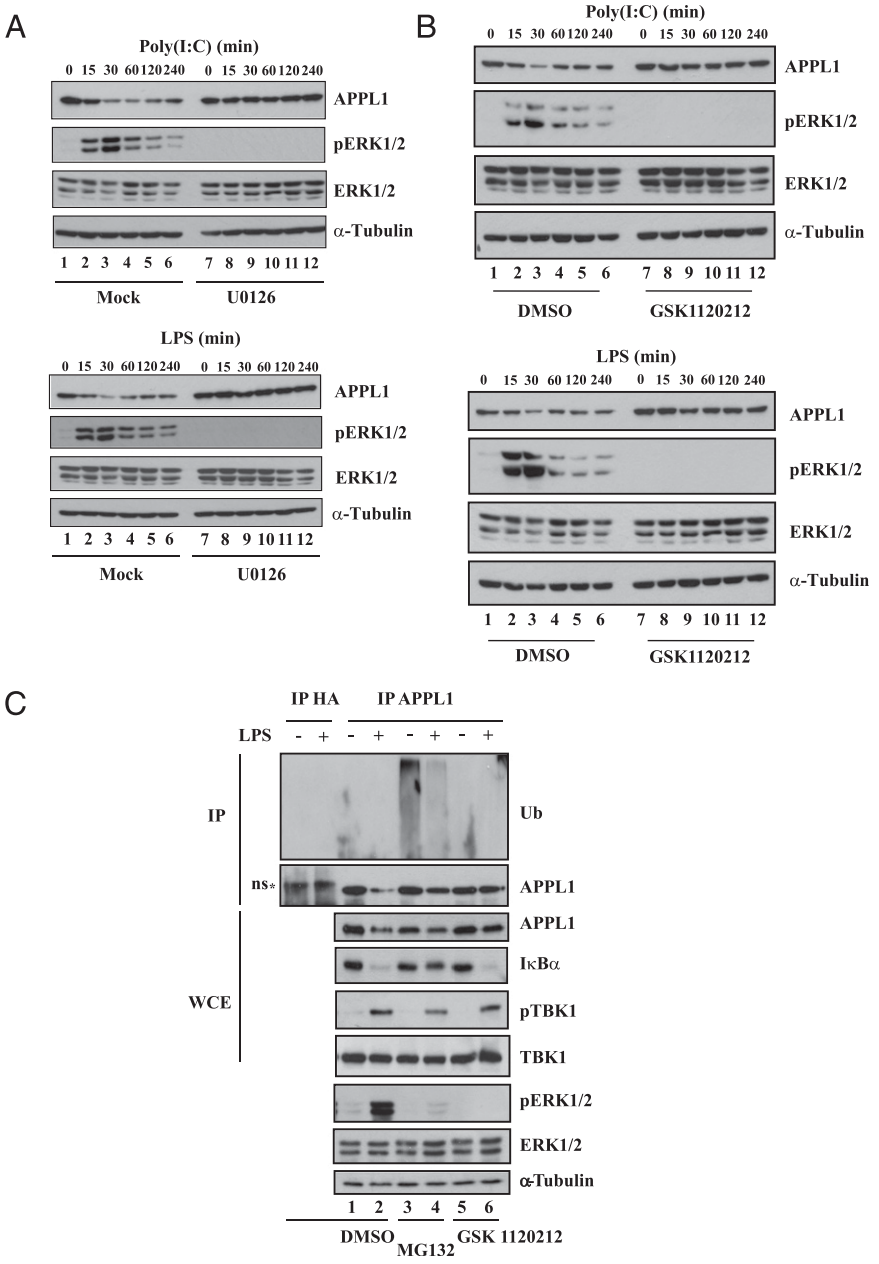

FIGURE 9. APPL1 degradation upon TLR3 and TLR4 engagement requires MEK1/2-ERK1/2. (**A** and **B**) The pharmacological inhibition of MEK1/2-ERK1/2 interferes with TLR3/4-mediated APPL1 degradation in macrophages. Control RAW 264.7 cells (Mock) or cells pretreated for 2 h with U0126 (20 μM) (A) or GSK1120212 (10 μM) (B) were left unstimulated or subjected to LPS (100 ng/ml) or Poly(I:C) (100 μg/ml) treatment for the indicated periods of time. The resulting cell extracts (1% SDS) were subjected to WB analyses, as indicated. (**C**) LPS signaling promotes APPL1 polyubiquitination through a MEK1/2-ERK1/2-dependent pathway. RAW 264.7 cells were pretreated with DMSO (control vehicle), MG132 (20 μM), or GSK1120212 (10 μM) for 2 h, as indicated, and were stimulated or not with LPS (100 ng/ml) for 30 min. Cell extracts (1% SDS) were diluted 10 times, and anti-HA (negative control) or anti-APPL1 IP was carried out. The resulting immunoprecipitates were subjected to an anti-ubiquitin WB to reveal polyubiquitinated forms of APPL1 (*top row*). Immunoprecipitates also were subjected to an anti-APPL1 WB (*second row*). Anti-IκBα, anti-pERK1/2, anti-ERK1/2, anti-pTBK1, anti-TBK1, and α-tubulin WBs on crude cell extracts are also shown. ns, nonspecific band.

impact on APPL1 degradation (Fig. 10A, 10B). In contrast, pretreatment 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, IKKε, 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 IKKε 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 IKKε 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

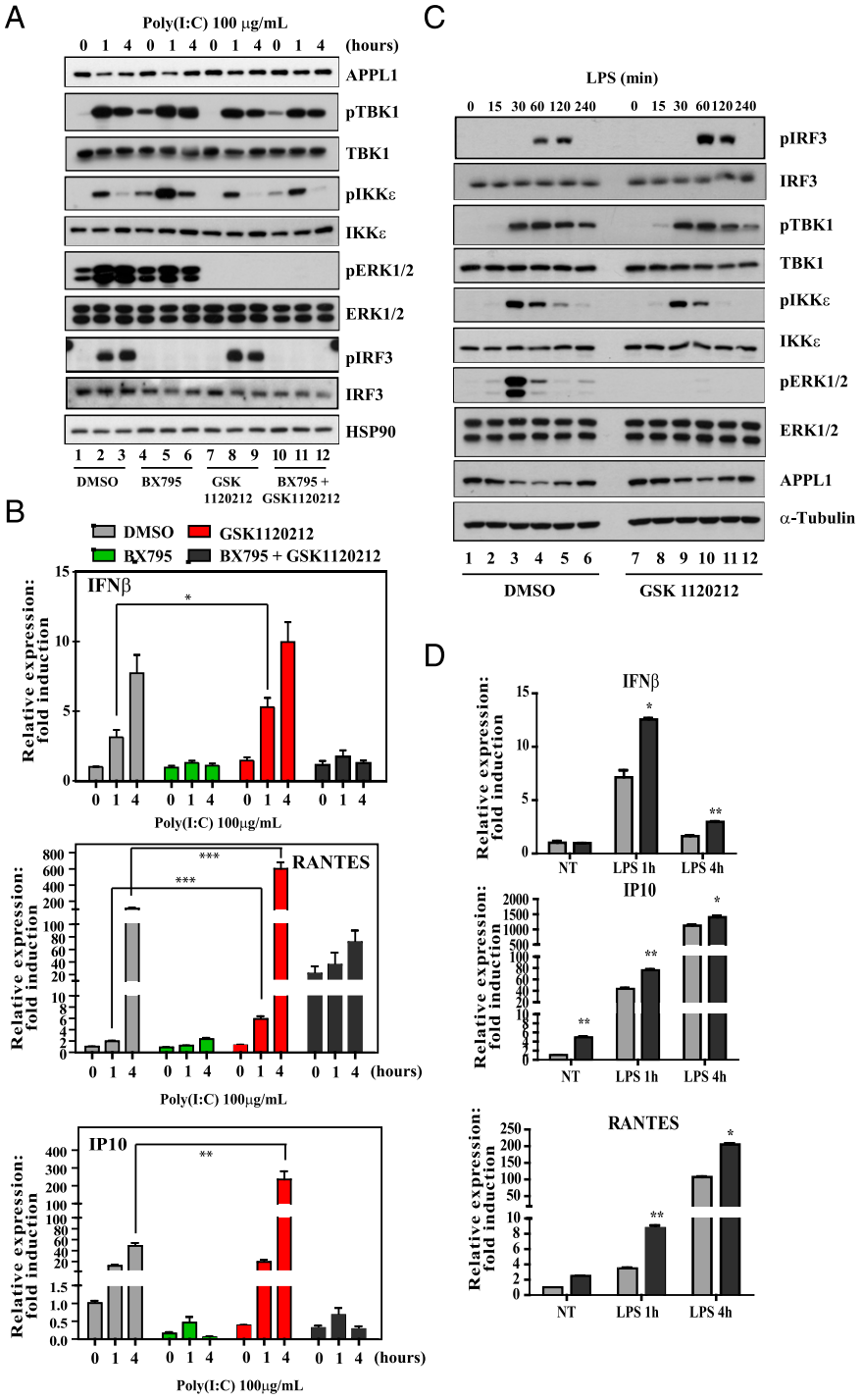

FIGURE 10. APPL1 degradation upon TLR signaling prevents excessive IRF3-dependent gene transcription. (**A** and **B**) MEK1/2 inhibition enhances IRF3-dependent gene transcription upon TLR3 engagement. RAW 264.7 cells were pretreated with DMSO (control vehicle) or with the indicated inhibitors; subsequently, the cells were left untreated or stimulated with Poly(I:C) (100 μg/ml) for the indicated periods of time. (A) WBs were carried out on cell extracts from the resulting cells. (B) mRNA levels of the indicated IRF3 target genes were assessed by real-time PCR in untreated and Poly(I:C)-stimulated cells in which TBK1/IKKε and/or MEK1/2 activities were inhibited or not. 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 (mean ± SD), are shown. (**C**) Enhanced LPS-dependent IRF3 activation in BMDMs pretreated with a MEK1/2-ERK1/2 inhibitor. Control or BMDMs pretreated with GSK1120212 (10 μM) for 2 h were left unstimulated or treated with LPS (100 ng/ml) for the indicated periods of time. WB analyses were carried out with cell extracts from the resulting cells using the indicated Abs. (**D**) Enhanced LPS-dependent expression of IRF3 target genes in untransformed macrophages in which MEK1/2-ERK1/2 activities are blocked. Control or BMDMs preincubated with GSK1120212 (10 μM) for 2 h were left unstimulated or incubated with LPS (100 ng/ml) for the indicated periods of time. Real-time PCR was carried out with total RNA extracted from the resulting cells. Data from two independent experiments, performed in triplicates (mean ± SD), are plotted as in (B). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, Student t test.

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 IκBα 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

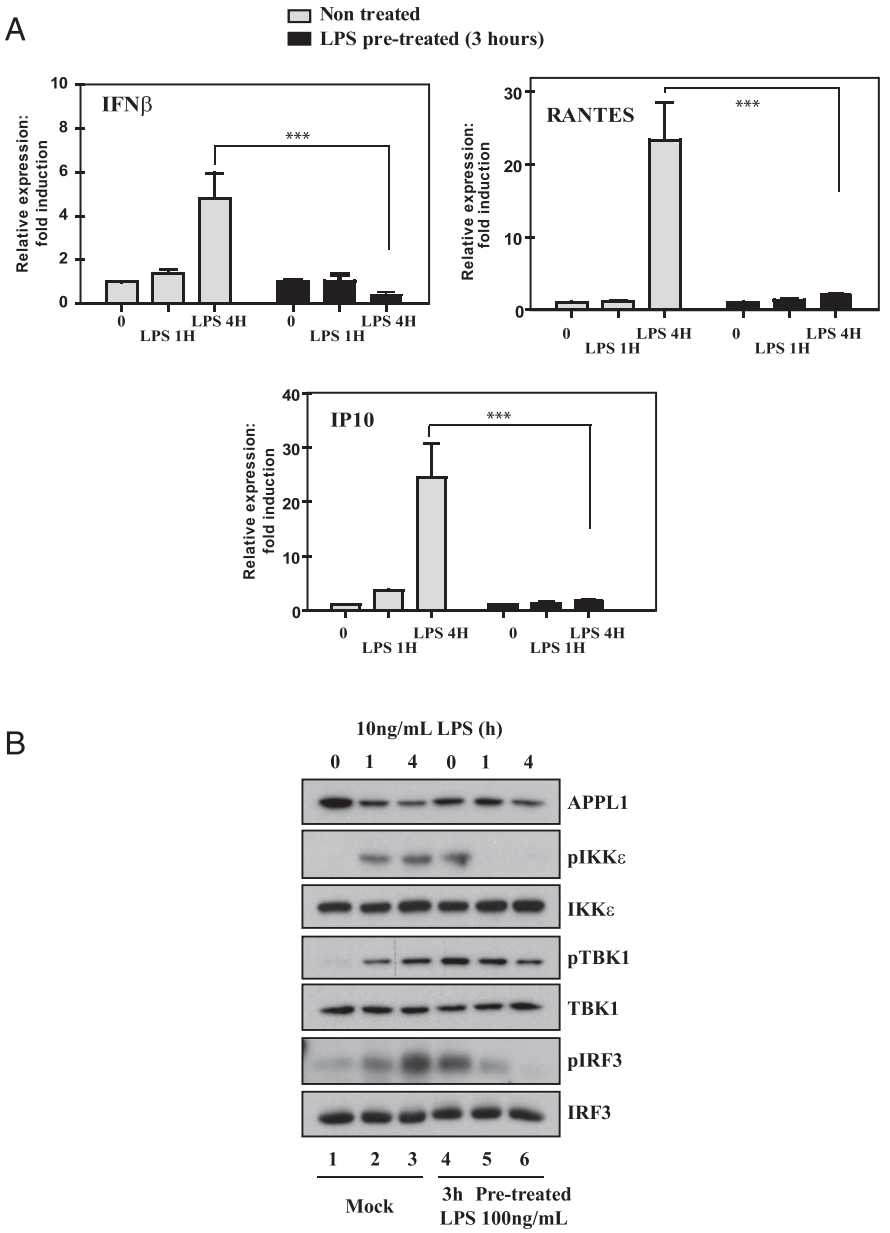

FIGURE 11. LPS-dependent APPL1 degradation is impaired upon LPS tolerance. (**A**) Blockage of IRF3-dependent gene transcription in macrophages subjected to two rounds of LPS stimulation. mRNA levels of IFNβ, RANTES, and IP-10 were assessed by real-time PCR in control versus LPS-pretreated RAW 264.7 cells that were subjected or not to a second round of LPS stimulation. 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 (mean ± SD), are shown. (**B**) LPS tolerance interferes with APPL1 degradation upon TLR4 engagement. Control or LPS-pretreated RAW 264.7 cells were left unstimulated or subjected to a second round of LPS stimulation; the resulting cell extracts were subjected to WB analyses using the indicated Abs. ***$p < 0.001$, Student t test.

limits, type I IFN production in BMDMs without altering TBK1 and IKKε activation upon stimulation with a variety of TLR ligands (46).

Distinct subcellular localization of TBK1 and IKKε may be a way to achieve substrate specificity (19). It is believed that multiple TBK1 and IKKε 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 IKKε 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 IKKε 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. Taken together,

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 poststimulation is specifically blocked.

APPL1 degradation critically relies on ERK1/2, but not on TBK1 and IKKε, activation. Indeed, APPL1 degradation upon TLR engagement still occurs when TBK1 and IKKε 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 defined 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-κB–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-κB–induced protein, as well as TRAF3, both described as TBK1- and IKKε-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 Fbox 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).

Acknowledgments

We thank the Interdisciplinary Cluster of Applied Genoproteomics Imaging and Flow Cytometry Platform for performing IF analyses.

Disclosures

The authors have no financial conflicts of interest.

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