IMMUNOLOGY

Inositol polyphosphate multikinase promotes Toll-like receptor–induced inflammation by stabilizing TRAF6

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Toll-like receptor (TLR) signaling is tightly controlled to protect hosts from microorganisms while simultaneously preventing uncontrolled immune responses. Tumor necrosis factor receptor–associated factor 6 (TRAF6) is a critical mediator of TLR signaling, but the precise mechanism of how TRAF6 protein stability is strictly controlled still remains obscure. We show that myeloid-specific deletion of inositol polyphosphate multikinase (IPMK), which has both inositol polyphosphate kinase activities and noncatalytic signaling functions, protects mice against polymicrobial sepsis and lipopolysaccharide-induced systemic inflammation. IPMK depletion in macrophages results in decreased levels of TRAF6 protein, thereby dampening TLR-induced signaling and proinflammatory cytokine production. Mechanistically, the regulatory role of IPMK is independent of its catalytic function, instead reflecting its direct binding to TRAF6. This interaction stabilizes TRAF6 by blocking its K48-linked ubiquitination and subsequent degradation by the proteasome. Thus, these findings identify IPMK as a key determinant of TRAF6 stability and elucidate the physiological function of IPMK in TLR-induced innate immunity.

INTRODUCTION

Toll-like receptors (TLRs) are microbe sensors that contribute to host defenses against invading pathogens. In immune cells, TLR activation induces inflammatory signaling pathways that ultimately lead to the production of proinflammatory cytokines (*1*). TLR4, for example, recognizes specific pathogen–associated molecules and initiates a series of inflammatory signal transduction pathways by recruiting MyD88 (myeloid differentiation primary response 88) adaptor proteins (*2*). The TLR4/MyD88 signaling complex subsequently interacts with interleukin-1 (IL-1) receptor–associated kinases (IRAKs), tumor necrosis factor (TNF) receptor–associated factor 6 (TRAF6), and transforming growth factor β–activated kinase 1 (TAK1) (*3*–*6*). These interactions lead to the activation of downstream effectors, such as c-Jun N-terminal kinase (JNK) and nuclear factor κB (NF-κB), and subsequent transcriptional induction of proinflammatory cytokines, including TNFα (*7*–*11*). Because TLRs are central to innate immunity, uncontrolled TLR activation can lead to the excessive immune responses observed in autoimmune and inflammatory diseases such as sepsis (*12*). Thus, TLR signaling is tightly regulated to ensure that immune responses are appropriate in magnitude (*13*).

Ubiquitination is the covalent attachment of ubiquitin to proteins by ubiquitin ligases and is a multifunctional protein modification that controls diverse biological phenomena including innate immunity. The covalent linkage of ubiquitin or a polyubiquitin chain to lysine 48 (K48) of a protein is a well-characterized signal that targets the ubiquitinated protein to degradation by the 26*S* proteasome (*14*, *15*). K63-linked ubiquitin can act as a scaffold that controls conformational changes and as molecular interactions (*16*). TRAF6 is a RING domain–containing ubiquitin ligase required for TLR signaling. After binding an activated TLR, TRAF6 undergoes K63-linked polyubiquitination (*17*, *18*). TRAF6 is essential for activation of the downstream effectors IκB kinase (IKK), mitogen-activated protein kinase (MAPK), and NF-κB (*8*, *10*, *19*–*21*), although the precise function of TRAF6 K63-linked autoubiquitination in the regulation of NF-κB activation is unclear (*22*–*25*). Deubiquitinating enzymes (for example, CYLD, A20, and MYSM1) that remove the K63-linked polyubiquitin from TRAF6 provide negative feedback regulation of TLR signaling (*26*–*30*). Recently, K48-linked ubiquitination of TRAF6 was found to facilitate TRAF6 degradation, effectively blocking TLR-dependent inflammatory signaling events (*12*, *31*–*35*). Still, the molecular mechanisms that regulate K48 ubiquitination and TRAF6 protein levels during TLR-dependent inflammatory responses remain unclear.

Inositol polyphosphate multikinase (IPMK) is an enzyme with broad substrate specificity that catalyzes the production of inositol polyphosphates (for example, inositol 1,3,4,5,6-pentakisphosphate) and phosphatidylinositol 3,4,5-triphosphates (*36*–*39*). In addition to its catalytic role in inositol phosphate metabolism, IPMK noncatalytically regulates major signaling factors including mechanistic target of rapamycin (mTOR), adenosine 5′-monophosphate–activated protein kinase (AMPK), p53, and serum response factor (SRF) (*40*–*44*). Thus, IPMK acts as a signaling hub in mammalian cells that coordinates the activity of various signaling networks (*45*, *46*). Accordingly, we asked whether IPMK may also play a critical role in TLR signaling and related innate immune responses. Here, we demonstrate that IPMK noncatalytically enhances TLR signaling by stabilizing TRAF6 in macrophages. Conditional deletion of IPMK in murine macrophages blunts TLR-mediated signaling and the induction of proinflammatory cytokines, rendering mice resistant to septic responses. We further show that dynamic interactions between IPMK and TRAF6 are critical for the control of K48-linked ubiquitination of TRAF6 in TLR signaling.

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RESULTS

Myeloid IPMK mediates experimental septic responses in vivo

To address the role of macrophage IPMK in controlling TLR-dependent inflammatory responses, we generated myeloid-specific *Ipmk*-deficient

mice by crossing *Ipmk* floxed mice with LysM-Cre mice (fig. S1A). We confirmed that the *Ipmk* gene was specifically deleted in bone marrow–derived macrophages (BMDMs) and peritoneal macrophages isolated from IPMK-deficient LysM-Cre^{+}*Ipmk*$^{fl/fl}$ (*Ipmk*$^{\Delta Mac}$) mice but was unaffected in control LysM-Cre^{-}*Ipmk*$^{fl/fl}$ mice (*Ipmk*^{WT}) (fig. S1, B to D). Myeloid cell population counts in the peritoneal cavity, mesenteric lymph nodes, and spleen of *Ipmk*^{WT} and *Ipmk*$^{\Delta Mac}$ mice were similar (fig. S2, A and B), suggesting that IPMK deletion does not affect the development or maturation of myeloid cells.

We first applied a cecal ligation and puncture (CLP)–induced sepsis model to trigger systemic inflammation. Only 11% of *Ipmk*^{WT} mice survived to day 8 post-CLP compared to 58% of *Ipmk*$^{\Delta Mac}$ mice (Fig. 1A). Consistent with this, *Ipmk*$^{\Delta Mac}$ mice showed lower levels of inflammatory cell infiltration in the liver and lung than did *Ipmk*^{WT} mice (Fig. 1, B and C). To confirm this apparent protective role of IPMK in TLR signaling, we measured the susceptibility of each mouse strain to endotoxic shock by injecting them with either a high, normally sublethal dose or a low, nonlethal dose of lipopolysaccharide (LPS). *Ipmk*$^{\Delta Mac}$ mice showed increased survival compared with *Ipmk*^{WT} mice after administration of a high dose of LPS (Fig. 1D) and exhibited less severe endotoxemia-associated symptoms after injection of a nonlethal (low) dose (Fig. 1, E to H). *Ipmk*$^{\Delta Mac}$ mice showed less LPS-induced hypothermia (Fig. 1E), a lesser degree of LPS-induced weight loss (Fig. 1F), and appetite suppression (Fig. 1G) than did *Ipmk*^{WT} mice, and they had smaller spleens than *Ipmk*^{WT} mice (Fig. 1H). This reduced inflammatory response of *Ipmk*$^{\Delta Mac}$ mice was also accompanied by a reduction in proinflammatory cytokines. LPS-treated *Ipmk*$^{\Delta Mac}$ mice showed lower serum concentrations of IL-6 and TNF than did *Ipmk*^{WT} mice (Fig. 1I). Consistent with this finding, levels of *Il-1*β, *Il-6*, and *Tnf*α gene expression in the lung and spleen were lower in *Ipmk*$^{\Delta Mac}$ mice (Fig. 1, J and K). Together, these results indicate that macrophage IPMK regulates sensitivity to TLR-induced inflammation.

Macrophage IPMK modulates TLR-induced inflammatory responses

To investigate whether IPMK affects inflammation in a cell-autonomous fashion, we isolated BMDMs from *Ipmk*$^{\Delta Mac}$ mice and their wild-type (*Ipmk*^{WT}) littermates and measured their TLR-induced inflammatory responses. We found that mRNA (Fig. 2A) and protein levels (Fig. 2B) of the proinflammatory cytokines IL-1β, IL-6, and TNFα were significantly reduced in LPS-stimulated BMDMs from *Ipmk*$^{\Delta Mac}$ mice compared with those from *Ipmk*^{WT} mice. We further found that phosphorylation of signaling molecules downstream of TLR4 was reduced in LPS-stimulated *Ipmk*$^{\Delta Mac}$ BMDMs compared with *Ipmk*^{WT} BMDMs (Fig. 2C and fig. S3). Similar defects in LPS-induced cytokine production downstream of TLR4 were also observed in IPMK-depleted RAW 264.7 macrophages (fig. S4, A to D). Expression, secretion, and signaling of proinflammatory cytokines in BMDMs stimulated with the TLR1 and TLR2 ligand Pam_3CSK_4 were also decreased in *Ipmk*$^{\Delta Mac}$ mice (Fig. 2, D to F). However, myeloid-specific depletion of IPMK had no effect on cytokine expression, secretion, or signaling in BMDMs stimulated with the TLR3 ligand polyinosinic:polycytidylic acid [poly (I:C)] (fig. S5, A to C). These results clearly show that IPMK is required for MyD88-dependent activation of TLRs and its subsequent induction of proinflammatory cytokine expression.

IPMK regulates TRAF6 stability through K48-linked polyubiquitination

Next, we sought to determine whether the hypoinflammatory responses of IPMK-depleted macrophages were caused by the elevated expression of negative regulators of TLR signaling. We found that this was not the case; BMDMs isolated from *Ipmk*$^{\Delta Mac}$ mice and *Ipmk*^{WT} littermates showed comparable levels of the TLR signaling inhibitors RNF216 (RING finger protein 216), TANK (TRAF family member–associated NF-κB), and TNFAIP3 (TNFα-induced protein 3) (fig. S6A) (*12*, *27*, *47*). Moreover, we found that IPMK depletion had no significant effects on the expression of the signaling molecules TLR4, MyD88, or TRIF (Toll/IL-1 receptor domain–containing adapter-inducing interferon-β) at mRNA (fig. S6B) or protein levels (fig. S6C).

Having previously shown that the loss of IPMK reduces phosphorylation of IKK and other downstream effectors of TLRs without altering receptors or upstream signaling adaptors, we next focused on TRAF6, an adaptor protein engaged in MyD88-dependent downstream signaling in macrophages (*6*, *12*, *21*). We found a significant reduction of TRAF6 protein in BMDMs from *Ipmk*$^{\Delta Mac}$ compared with those from *Ipmk*^{WT} mice (Fig. 3A), and TRAF6 protein was similarly decreased in IPMK-depleted RAW 264.7 macrophages compared with scrambled RNA–transfected RAW 264.7 cells (Fig. 3B). TRAF6 mRNA levels were comparable in *Ipmk*^{WT} and *Ipmk*$^{\Delta Mac}$ BMDMs (Fig. 3C), suggesting that IPMK regulates the stability of TRAF6 protein rather than its expression. To measure TRAF6 protein stability, we monitored TRAF6 levels in the presence of the protein synthesis inhibitor cycloheximide. TRAF6 protein was degraded more rapidly in *Ipmk*$^{\Delta Mac}$ BMDMs than in *Ipmk*^{WT} BMDMs (Fig. 3D), implying that IPMK regulates TRAF6 protein turnover. Consistent with this, degradation of TRAF6 protein in *Ipmk*$^{\Delta Mac}$ BMDMs was prevented by the proteasome inhibitor MG-132 (Fig. 3E).

K48-linked ubiquitination of TRAF6 is important for TRAF6 degradation, especially in the context of TRAF6-mediated inhibition of TLR-dependent inflammatory responses (*31*–*35*). Therefore, we tested whether IPMK protects TRAF6 from K48-linked ubiquitination. IPMK overexpression markedly suppressed K48-linked ubiquitination of TRAF6 in human embryonic kidney (HEK) 293T cells (Fig. 3F). In addition, loss of IPMK enhanced K48-linked ubiquitination of endogenous TRAF6 in both BMDMs and RAW 264.7 macrophages (Fig. 3, G and H). Together, these results indicate that IPMK regulates the stability of TRAF6, a key signaling adaptor in the TLR signaling pathway, by regulating its K48-linked ubiquitination.

To determine the functional significance of the reduction in TRAF6 caused by IPMK depletion, we examined the effects of TRAF6 reconstitution in IPMK-depleted macrophages. Overexpression of TRAF6 in IPMK-depleted RAW 264.7 macrophages increased both LPS-stimulated TLR4 activation (Fig. 3I) and proinflammatory cytokine expression (Fig. 3J). Collectively, these results suggest that the inhibition of TLR-mediated inflammatory responses induced by IPMK depletion depends on IPMK-mediated regulation of TRAF6 protein levels.

IPMK-TRAF6 regulation mediates TLR signaling independent of IPMK catalytic activity

We next asked whether the catalytic activity of IPMK is required for its regulation of TLR signaling. In *Ipmk*$^{\Delta Mac}$ BMDMs, reconstitution of either wild-type IPMK (WT-IPMK) or the catalytically inactive IPMK mutant IPMK-K129A (KA-IPMK) (*38*) equally restored TRAF6 protein levels (Fig. 4, A and B) and enhanced LPS-induced proinflammatory cytokine expression and phosphorylation of TLR signaling components (Fig. 4, C and D). We further examined the influence of IPMK catalytic activity on K48-linked ubiquitination of TRAF6 and found that KA-IPMK notably suppressed K48-linked ubiquitination to the same extent, as did WT-IPMK (Fig. 4E). Reconstitution of

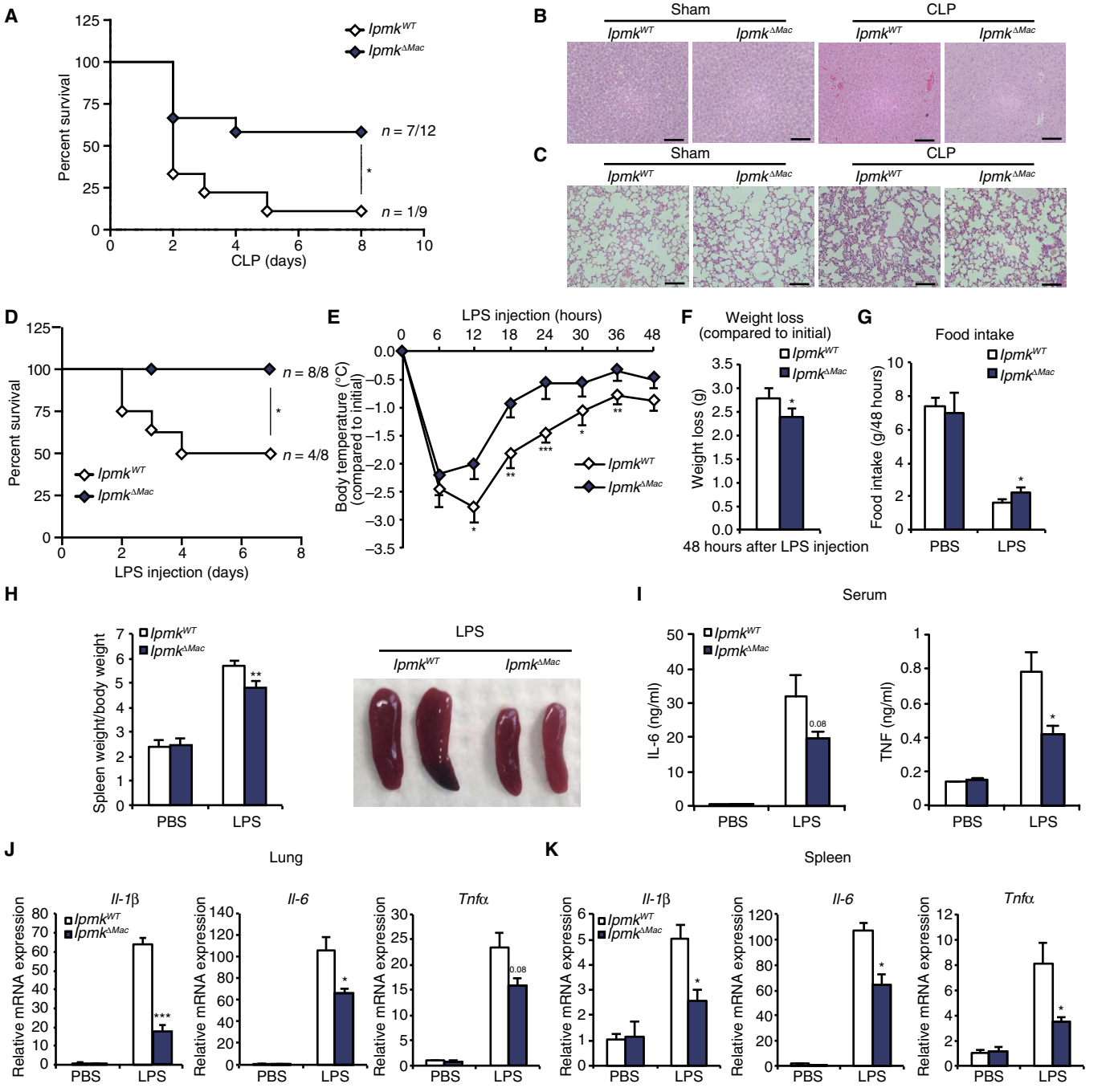

Fig. 1. Depletion of IPMK in myeloid cells protects against septic responses. (**A**) Survival rate of $lpmk^{WT}$ ($n = 12$) and $lpmk^{\Delta Mac}$ ($n = 9$) mice, compiled from two independent experiments after severe CLP-induced sepsis. (**B** and **C**) Livers (B) or lungs (C) of $lpmk^{WT}$ and $lpmk^{\Delta Mac}$ mice were harvested 20 hours after CLP or sham operation, sectioned, and stained with hematoxylin and eosin (H&E). Representative images from three mice per group are shown. Scale bars, 100 μm. (**D**) Survival rate of $lpmk^{WT}$ ($n = 8$) and $lpmk^{\Delta Mac}$ ($n = 8$) mice challenged with LPS [30 mg/kg, intraperitoneally (ip)], compiled from two independent experiments. (**E** to **G**) $lpmk^{WT}$ ($n = 21$) and $lpmk^{\Delta Mac}$ ($n = 25$) mice challenged with LPS (4.5 mg/kg, intraperitoneally), compiled from three independent experiments. LPS-induced changes in body temperature over time (E), reduction in body weight 48 hours after injection of LPS (F), and LPS-induced reduction in food intake over time (G). PBS, phosphate-buffered saline. (**H**) LPS-induced changes in the weight and size of spleens from $lpmk^{WT}$ ($n = 7$) and $lpmk^{\Delta Mac}$ ($n = 7$) mice 48 hours after exposure to LPS (4.5 mg/kg, intraperitoneally). (**I**) Serum concentrations of IL-6 and TNF were measured 6 hours after LPS (4.5 mg/kg, intraperitoneally) injection. (**J** and **K**) Expression of *Il-1β*, *Il-6*, and *Tnfα* mRNA was quantitated by reverse transcription quantitative polymerase chain reaction (RT-qPCR) in lung (J) and spleen (K) 6 hours after LPS (4.5 mg/kg, intraperitoneally) injection. Three mice per group were analyzed from two independent experiments (I to K). In all experiments, $lpmk^{WT}$ littermates served as controls for $lpmk^{\Delta Mac}$ mice. Data are means ± SE. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, log-rank test (A and D) or Student's *t* test.

WT- or KA-IPMK in *Ipmk*$^{\Delta Mac}$ BMDMs also resulted in a decrease in K48-linked ubiquitination (Fig. 4F). These results suggest that the effects of IPMK on TRAF6 protein levels and TLR-mediated inflammatory signaling do not require its catalytic activity.

IPMK prevents TRAF6 degradation through protein-protein interaction

We next investigated whether IPMK enhances TRAF6 stability through a direct protein-protein interaction. IPMK pull-down experiments in

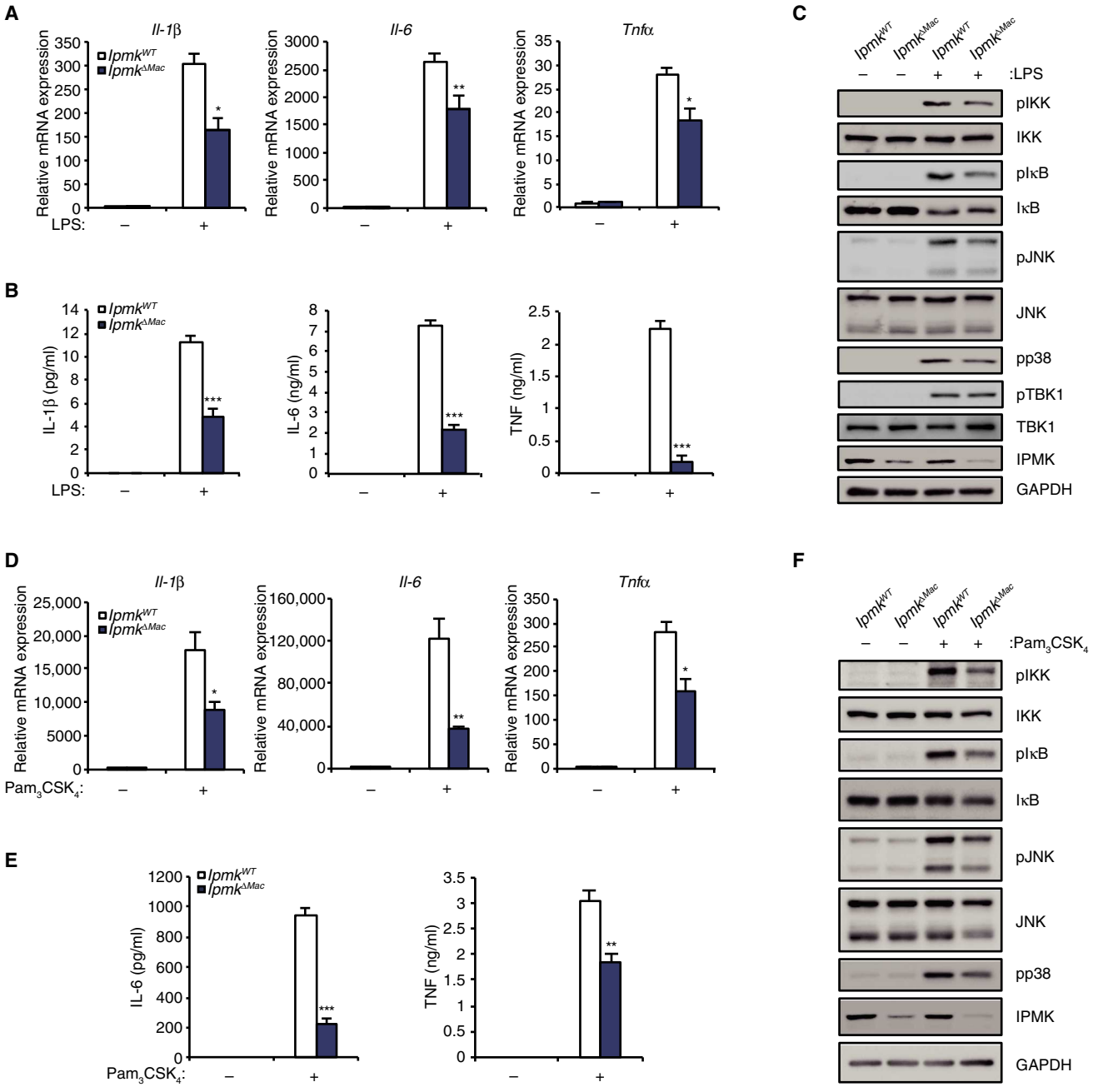

Fig. 2. IPMK depletion in macrophages blunts TLR-dependent inflammatory responses. (**A**) mRNA expression of the proinflammatory cytokines *Il-1β*, *Il-6*, and *Tnfα* was quantified by RT-qPCR in BMDMs 6 hours after stimulation with LPS (100 ng/ml). (**B**) Secreted levels of the cytokines IL-1β, IL-6, and TNF in BMDM culture medium were measured by enzyme-linked immunosorbent assay (ELISA) 6 hours after stimulation with LPS (100 ng/ml). (**C**) Phosphorylation of signaling molecules was analyzed by immunoblotting lysates of BMDMs stimulated for 2 hours with LPS (100 ng/ml). (**D**) mRNA levels of the proinflammatory cytokines *Il-1β*, *Il-6*, and *Tnfα* were quantified by RT-qPCR in BMDMs 6 hours after stimulation with Pam_3CSK_4 (100 ng/ml). (**E**) Secreted levels of the cytokines IL-6 and TNF in BMDM culture medium were measured by ELISA 6 hours after stimulation with Pam_3CSK_4 (100 ng/ml). (**F**) Phosphorylation of signaling molecules in BMDMs stimulated for 2 hours with Pam_3CSK_4 (100 ng/ml) was analyzed by immunoblotting. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. In all BMDM studies, *Ipmk*^{WT} littermates served as controls for *Ipmk*$^{\Delta Mac}$ mice. Data are representative of at least three independent experiments (A to F) and are presented as means ± SE ($n = 3$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, Student's *t* test.

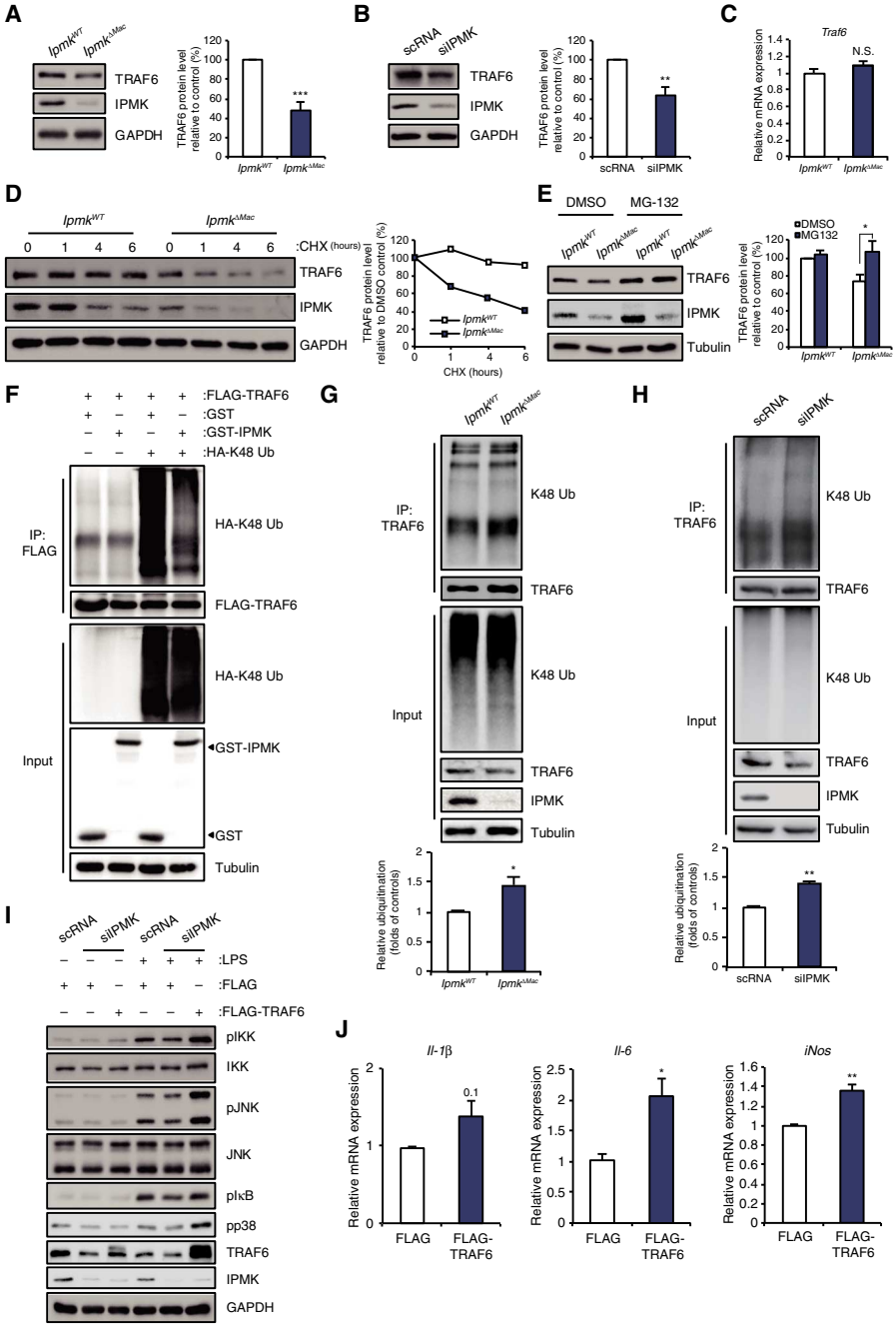

Fig. 3. IPMK deficiency reduces TRAF6 protein levels through K48 ubiquitination–dependent protein degradation, and overexpression of TRAF6 restores TLR signaling. (**A**) TRAF6 protein levels in BMDMs were measured by immunoblot analysis. (**B**) TRAF6 protein levels were measured in IPMK-depleted RAW 264.7 macrophages by immunoblot analysis. scRNA, scrambled RNA. (**C**) TRAF6 mRNA levels in BMDMs were measured by RT-qPCR. (**D**) Immunoblot analysis of TRAF6 protein in BMDMs treated with cycloheximide (CHX) (100 μg/ml) for the indicated times. (**E**) Immunoblot analysis of TRAF6 protein in BMDMs treated with MG-132 (10 μM) or dimethyl sulfoxide (DMSO) (vehicle control) for 8 hours. (**F**) HEK293T cells transiently cotransfected with hemagglutinin (HA)–K48 ubiquitin (Ub), FLAG-TRAF6, glutathione *S*-transferase (GST), or GST-IPMK expression plasmids. Forty-eight hours after transfection, cells were lysed and boiled at 95°C for 15 min and subjected to immunoprecipitation (IP) with an anti-FLAG antibody followed by an immunoblot analysis with anti-FLAG, anti-GST, or anti-HA antibodies. (**G**) Levels of endogenous TRAF6 K48 ubiquitination in $Ipmk^{WT}$ and $Ipmk^{\Delta Mac}$ BMDMs. The cells were lysed and boiled at 95°C for 15 min and subjected to immunoprecipitation with an anti-TRAF6 antibody followed by an immunoblot analysis with anti-K48 ubiquitin–specific antibodies. (**H**) Levels of endogenous TRAF6 K48 ubiquitination in IPMK-depleted RAW 264.7 macrophages. The cells were lysed and boiled at 95°C for 15 min and subjected to immunoprecipitation with an anti-TRAF6 antibody followed by an immunoblot analysis with anti-K48 ubiquitin–specific antibodies. (**I**) IPMK-depleted RAW 264.7 cells overexpressing FLAG-TRAF6 or vector control (FLAG only) were stimulated with LPS (100 ng/ml) for 2 hours, and phosphorylation levels of signaling molecules were assessed by immunoblotting. (**J**) IPMK-depleted RAW 264.7 cells overexpressing FLAG-TRAF6 or vector control (FLAG only) were stimulated with LPS (100 ng/ml) for 6 hours. mRNA levels of the proinflammatory cytokines *Il-1β*, *Il-6*, and *iNos* were measured by RT-qPCR. In all BMDM studies, $Ipmk^{WT}$ littermates served as controls for $Ipmk^{\Delta Mac}$ mice. All blots are representative of at least three independent experiments; densitometric quantitation results were normalized to controls. Data are means ± SE ($n = 3$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, Student's *t* test; N.S., not significant.

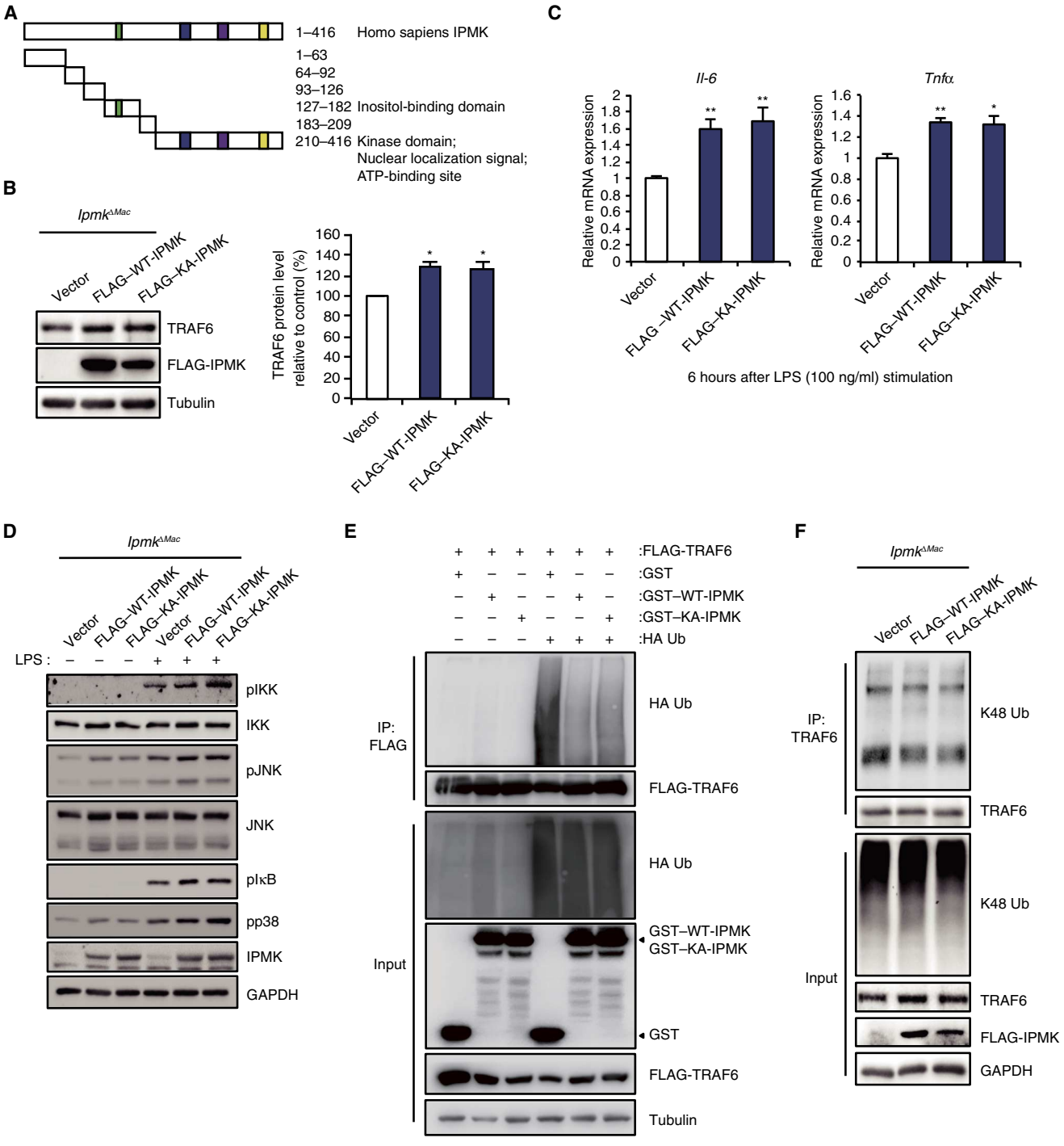

Fig. 4. IPMK regulates TRAF6 stability and TLR signaling independent of its catalytic activity. (**A**) Schematic depiction of human IPMK domains. IPMK fragments used for binding studies are indicated below, with numbers corresponding to the amino acids in the full-length protein. Key domains for inositol binding (green), kinase activity (SSLL in blue and IDF in yellow), and the nuclear localization signal sequence (purple). ATP, adenosine 5′-triphosphate. (**B**) *Ipmk*$^{\Delta Mac}$ BMDMs were either mock-transduced or transduced with FLAG–WT-IPMK or FLAG–KA-IPMK. TRAF6 protein levels were analyzed by immunoblotting and quantified densitometrically using tubulin as a normalization control. (**C**) *Ipmk*$^{\Delta Mac}$ BMDMs were either mock-transduced or transduced with FLAG–WT-IPMK or FLAG–KA-IPMK. Cells were stimulated with LPS (100 ng/ml) for 6 hours. mRNA levels of the proinflammatory cytokines *Il-6* and *Tnfα* were measured by RT-qPCR. (**D**) *Ipmk*$^{\Delta Mac}$ BMDMs were either mock-transduced or transduced with FLAG–WT-IPMK or FLAG–KA-IPMK. Cells were stimulated with LPS (100 ng/ml) for 2 hours, and phosphorylation of signaling molecules was detected by immunoblotting. (**E**) HEK293T cells were transfected with FLAG-TRAF6 and GST, GST–WT-IPMK, or GST–KA-IPMK, with or without HA–K48 ubiquitin. Forty-eight hours after transfection, cells were lysed and boiled at 95°C for 15 min and subjected to immunoprecipitation with an anti-FLAG antibody followed by an immunoblot analysis with anti-FLAG, anti-GST, or anti-HA antibodies. (**F**) Levels of endogenous TRAF6 K48 ubiquitination were measured in either mock-transduced or FLAG–WT-IPMK– or FLAG–KA-IPMK–transduced *Ipmk*$^{\Delta Mac}$ BMDMs. The cells were lysed and boiled at 95°C for 15 min and subjected to immunoprecipitation with an anti-TRAF6 antibody followed by an immunoblot analysis with anti-K48 ubiquitin–specific antibodies. All blots are representative of at least three independent experiments. Results are means ± SE ($n = 3$). *$P < 0.05$; **$P < 0.01$, one-way analysis of variance (ANOVA) followed by Tukey's post-test.

HEK293T cells overexpressing IPMK and TRAF6 revealed protein-protein interactions between IPMK and TRAF6 (Fig. 5A). The association of endogenous IPMK and TRAF6 was further confirmed by coimmunoprecipitation experiments in RAW 264.7 macrophages (Fig. 5B). The absence of IPMK signals in TRAF6 immunoprecipitates from $Ipmk^{\Delta Mac}$ BMDMs further points to the specificity of this IPMK-TRAF6 interaction (Fig. 5C). We were also able to show that this interaction occurs in vitro using recombinant IPMK and TRAF6 proteins (Fig. 5D), confirming direct protein-protein interactions.

We next created a series of truncated IPMK mutants to determine which IPMK domain is responsible for binding TRAF6 (Fig. 4A). This analysis showed that TRAF6 interacts with amino acids 93–182 and 210–416 of IPMK (Fig. 5E). We also found that IPMK fragment 93–182 functions as a dominant-negative mutant such that its overexpression prevents full-length IPMK from binding TRAF6 in HEK293T cells (Fig. 5F). Overexpression of this dominant-negative IPMK peptide (DN-IPMK) in wild-type BMDMs reduced TRAF6 protein levels (Fig. 5G) and increased K48-linked ubiquitination of TRAF6 (Fig. 5H). Overexpression of DN-IPMK also inhibited LPS-induced proinflammatory cytokine production and TLR signaling events (Fig. 5, I and J). Together, these data suggest that the direct association of IPMK with TRAF6 stabilizes TRAF6 protein by inhibiting its K48-linked ubiquitination, thereby enhancing TLR-dependent signaling.

TLR stimulation dissociates TRAF6 from IPMK

Thus far, we have identified a mechanism downstream of TLR signaling in which IPMK protects TRAF6 from K48-linked ubiquitination through direct protein-protein interactions. Because K48-linked ubiquitination of TRAF6 is important for the termination of TLR signaling (*33*), we further investigated the mode of interaction between IPMK and TRAF6 in response to TLR activation. We found that LPS treatment reduced the binding of GST-IPMK to FLAG-TRAF6 (Fig. 6A). We then confirmed that treatment of BMDMs and RAW 264.7 macrophages with LPS also caused the dissociation of endogenous IPMK from TRAF6 (Fig. 6, B and C), suggesting that TLR stimulation induces disassembly of the IPMK-TRAF6 complex.

To clarify the mechanism of this LPS-induced reduction in the IPMK-TRAF6 interaction, we examined which TRAF6 domain is responsible for IPMK binding. We applied an immunoprecipitation approach using truncated forms of TRAF6 and found that the major IPMK binding site is the N-terminal RING domain (TRAF6[1–132]) (Fig. 6, D and E), which is essential for the K63-linked ubiquitination of active TRAF6 (*18*). We tested whether IPMK may also affect K63-linked TRAF6 ubiquitination and observed that IPMK overexpression can suppress both K63- and K48-linked ubiquitination (fig. S7). Because the association of IRAK1 with the TRAF6 C-terminal domain is a key trigger for the K63-linked activation and the K48-linked degradation of TRAF6 in early TLR signaling (*32*), we assessed whether the LPS-induced dissociation of IPMK and TRAF6 is regulated by IRAK1. We found that IRAK1 overexpression interfered with the interaction between TRAF6 and IPMK, with TRAF6 becoming sequestered into a complex with IRAK1 itself (Fig. 6F). In addition, cells overexpressing TRAF6[1–289], which lacks the IRAK1-binding domain, exhibited a sustained interaction between IPMK and TRAF6[1–289] even after LPS treatment (Fig. 6G). These findings suggest that the interaction between TRAF6 and IRAK1 causes TRAF6 to dissociate from IPMK. Thus, proper TLR signaling requires a tight regulation of the dynamic association and dissociation of IPMK and TRAF6.

DISCUSSION

As a pleiotropic protein, IPMK is known to regulate various biological processes (for example, growth), acting either enzymatically to mediate the biosynthesis of inositol polyphosphates and phosphatidylinositol 3,4,5-trisphosphates (*36–39*, *48*) or noncatalytically to control key signaling factors (for example, mTOR and AMPK) and transcriptional activation (*40–44*, *49*). However, no previous study has explored the functional significance of IPMK in regulating the innate immune response. Here, we establish a physiologically important role for IPMK in regulating TRAF6 protein stability, showing that IPMK directly interacts with TRAF6 and protects it against K48-linked ubiquitination and subsequent degradation. We report that IPMK depletion in cultured macrophages reduces TRAF6 protein levels by increasing TRAF6 K48 ubiquitination. This, in turn, inhibits signaling events downstream of TLR, including MyD88-dependent TLR signaling and proinflammatory cytokine production. Consistent with this role, reconstitution of TRAF6 in IPMK-deficient macrophages restores TLR signaling and proinflammatory cytokine expression. We further demonstrate the functional significance of the IPMK-TRAF6 interaction for TLR signaling by showing that overexpression of a TRAF6-binding DN-IPMK fragment interferes with the IPMK-TRAF6 interaction. As is the case with IPMK deletion, this increases the levels of K48-linked ubiquitinated TRAF6 and destabilizes the TRAF6 protein, thereby reducing TLR signaling and proinflammatory cytokine production. Notably, protection of TRAF6 against K48-linked ubiquitination is independent of the inositol phosphate kinase and phosphatidylinositol kinase activities of IPMK. In a mouse model of polymicrobial sepsis and LPS-induced endotoxemia, myeloid-specific IPMK deletion significantly decreases mortality in mice and diminishes proinflammatory cytokine responses.

In response to TLR stimulation, TRAF6 is recruited by upstream regulators, such as MyD88-IRAK1 (*50*, *51*), and the subsequent engagement of TRAF6 activates downstream signaling effectors (*5*, *10*). According to our model (Fig. 6H), IPMK binds TRAF6 and protects it against K48 ubiquitination and subsequent degradation in unstimulated macrophages. Upon LPS stimulation, TRAF6 binds to upstream signaling activators such as IRAK1, thereby disrupting the interaction with IPMK. The reduced binding of IPMK to TRAF6, in turn, allows TRAF6 to participate in the transmission of downstream signaling events and to undergo TRAF6 K48 ubiquitination, which leads to the degradation of activated TRAF6. The loss of IPMK in macrophages thus increases TRAF6 K48 ubiquitination and degradation, limiting the activation of TLR signaling and blunting the production of inflammatory cytokines and their associated immune responses. Notably, our findings define a signaling role of macrophage IPMK by showing that IPMK as a novel TRAF6-binding factor regulates TLR signaling events by governing the stability of the TRAF6 protein.

Fine regulation of TLR signaling pathways serves to balance the magnitude and duration of the inflammatory response, thus preventing aggressive immune responses that lead to unwanted host damage (*52*). TRAF6 is a key signal transducer that is essential for TLR-mediated innate immunity (*53*, *54*), highlighting the significance of mechanisms that regulate TRAF6. Deubiquitinases such as CYLD, A20, and MYSM1 have been identified as key components for the termination of TRAF6 activity by removing K63-linked polyubiquitin chains from TRAF6 (*26–30*). The physiological relevance of such negative regulation of TRAF6 activity is buttressed by the persistent cytokine production and hyperinflammatory innate immune responses observed in knock out mouse models (*30*, *47*, *55–58*). Unlike the precise control of

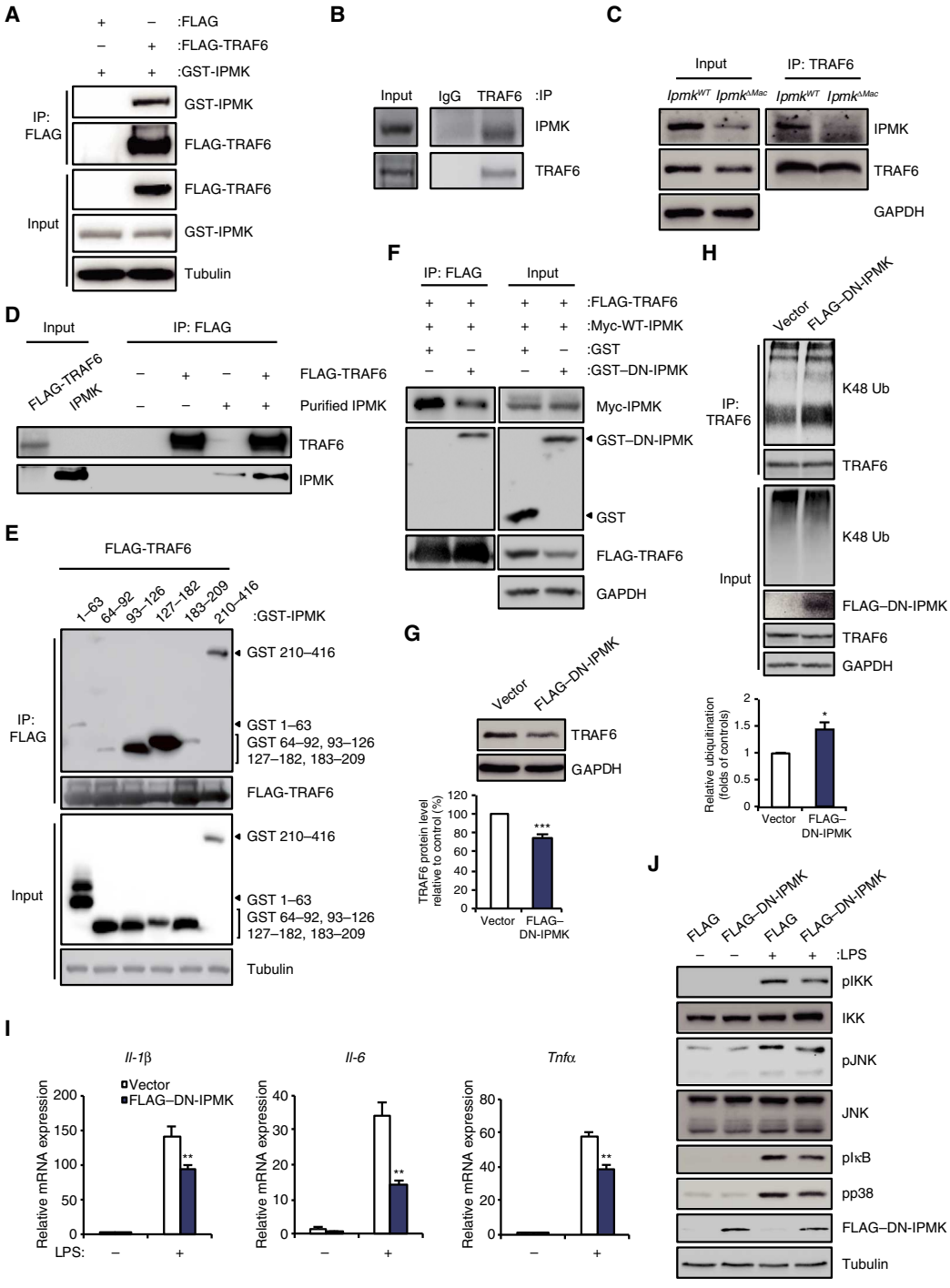

Fig. 5. IPMK, a TRAF6-binding protein, regulates TRAF6 protein stability. (**A**) HEK293T cells were cotransfected with GST-IPMK and FLAG-TRAF6 or vector control (FLAG only), followed by immunoprecipitation and immunoblot analysis. IgG, immunoglobulin G. (**B**) TRAF6 was immunoprecipitated from RAW 264.7 macrophages, and coimmunoprecipitated IPMK was detected by immunoblot analysis. (**C**) TRAF6 was immunoprecipitated from *Ipmk*$^{\Delta Mac}$ and littermate *Ipmk*^{WT} BMDMs, and coimmunoprecipitated IPMK was detected by immunoblot analysis. (**D**) Recombinant IPMK and in vitro translated FLAG-TRAF6 were coincubated and then immunoprecipitated and analyzed by immunoblotting. (**E**) Mapping the IPMK domain responsible for binding TRAF6. GST, GST-IPMK, or GST-IPMK fragments were pulled down from HEK293T cells cotransfected with FLAG-TRAF6. TRAF6 proteins in IPMK pull-down experiments were detected by immunoblotting. (**F**) HEK293T cells cotransfected with plasmids encoding FLAG-TRAF6, Myc-IPMK, and either GST or GST–DN-IPMK were subjected to immunoprecipitation and immunoblot analysis. (**G** to **J**) Wild-type BMDMs were mock-transduced or transduced with FLAG–DN-IPMK. TRAF6 protein levels were determined by immunoblot analysis and quantified densitometrically using GAPDH as a normalization control (G). Levels of K48-linked ubiquitinated TRAF6 were measured by immunoprecipitation and immunoblot analysis and quantified densitometrically using GAPDH as a normalization control (H). Cells were stimulated with LPS (100 ng/ml) for 6 hours, after which *Il-1β*, *Il-6*, and *Tnfα* mRNA levels were measured by RT-qPCR (I). Cells were stimulated with LPS (100 ng/ml) for 2 hours, after which phosphorylation of signaling molecules was analyzed by immunoblotting (J). All blots are representative of at least three independent experiments. Results are means ± SE ($n = 3$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, Student's t test.

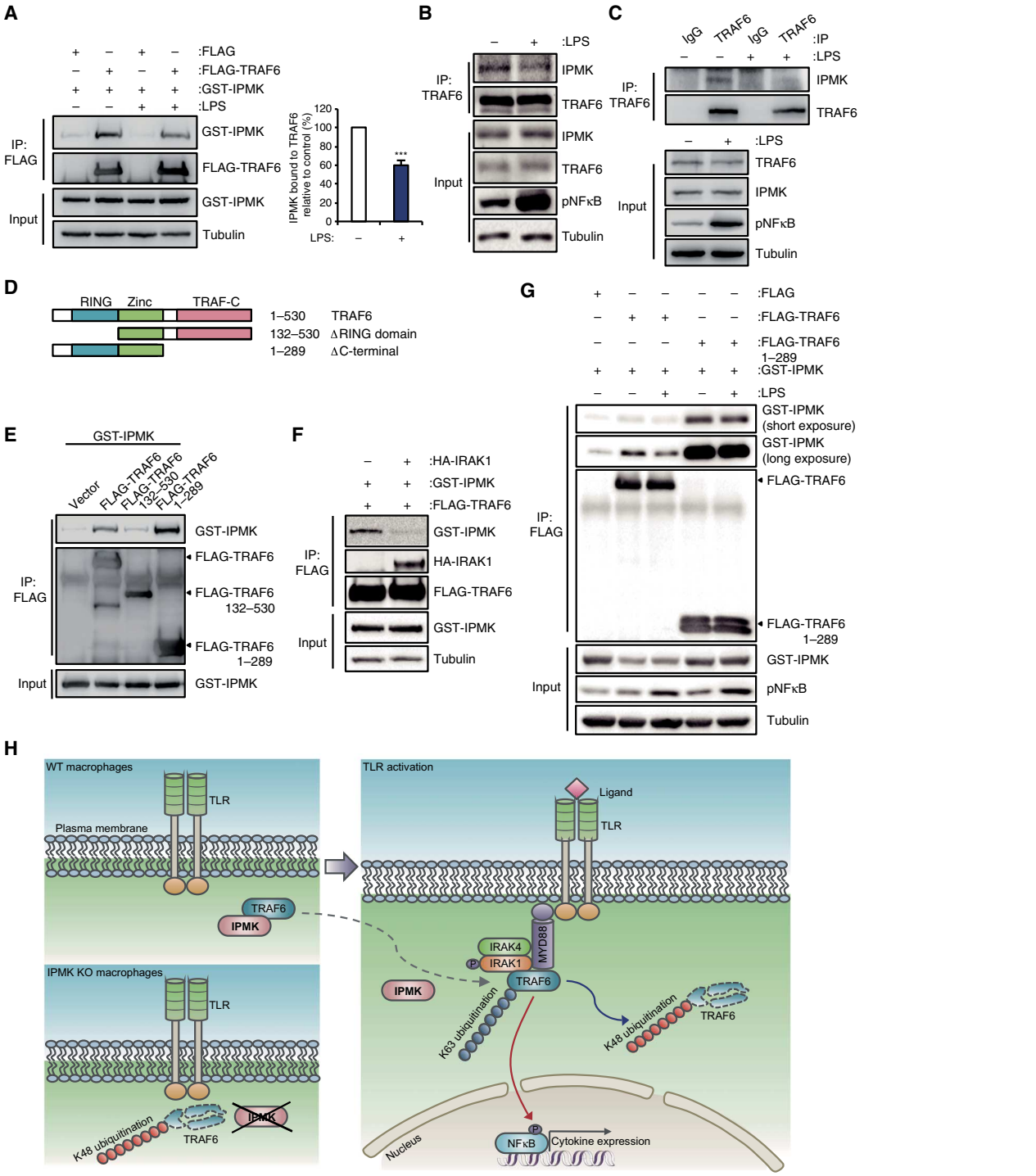

Fig. 6. IPMK-TRAF6 binding is negatively regulated by IRAK1. (**A**) HEK293T-TLR4 cells were transfected with GST-IPMK and FLAG-TRAF6 or vector control (FLAG only). Cells were stimulated with LPS (100 ng/ml) for 15 min, followed by immunoprecipitation and immunoblot analysis. Densitometric quantitation of GST-IPMK bound to FLAG-TRAF6 was normalized to immunoprecipitated FLAG-TRAF6. (**B** and **C**) Wild-type BMDMs (B) and RAW 264.7 macrophages (C) were incubated with or without LPS (100 ng/ml) for 15 min, followed by immunoprecipitation and immunoblot analysis. (**D**) TRAF6 contains a RING domain, zinc finger repeats, and a C-terminal TRAF (TRAF-C) domain. (**E**) HEK293T cells were transfected with GST-IPMK or FLAG-TRAF6 fragments (RING domain–deleted 132–530 and N-terminal 1–289), followed by immunoprecipitation and immunoblot analysis. (**F**) HEK293T cells were transfected with GST-IPMK, FLAG-TRAF6, HA-IRAK1, or vector control (HA only). HA-IRAK1 and GST-IPMK in each FLAG-TRAF6 immunoprecipitate were detected by immunoblotting. (**G**) HEK293T-TLR4 cells were transfected with GST-IPMK and FLAG-TRAF6 or FLAG-TRAF6[1–289] and incubated with or without LPS (100 ng/ml) for 15 min. GST-IPMK in each FLAG immunoprecipitate was detected by immunoblotting. Data are representative of at least three independent experiments. (**H**) Model depicting the regulation of TLR signaling by IPMK. In unstimulated macrophages, IPMK binds TRAF6 and protects it from K48 ubiquitination and subsequent degradation. Upon TLR activation, TRAF6 dissociates from IPMK and engages IRAK1, thereby transducing TLR signaling cascades. IPMK-deficient conditions in macrophages lead to increased K48 ubiquitination and degradation of TRAF6. Data in (A) are means ± SE ($n = 3$). ***$P < 0.001$, Student's t test. KO, knockout.

K63-dependent TRAF6 functions, the mechanisms underlying TRAF6 protein stability are unclear. A few recent studies have clearly suggested that dynamic regulation of TRAF6 protein levels is a key mode of immunoregulation. TRIM38, a member of the tripartite motif–containing (TRIM) protein superfamily, has been shown to promote K48-linked polyubiquitination of TRAF6, thus resulting in its proteasomal degradation and amplified activation of TLR signaling (*33*). WWP1 is another TRAF6-binding E3 ligase that promotes K48-linked polyubiquitination and subsequent proteasomal degradation of TRAF6 in response to LPS treatment (*34*). Numblike (NUMBL) has also been identified as a TRAF6-binding partner that down-regulates TRAF6 protein by shortening its half-life (*31*). Our demonstration that IPMK serves as a physiological factor for controlling TRAF6 protein stability thus emphasizes the need for further efforts to precisely resolve the complex molecular interactions among IPMK, TRAF6, and other known factors.

In conclusion, the functional interaction between IPMK and TRAF6 described here represents a previously unrecognized mechanism, demonstrating how IPMK promotes TLR-induced inflammation by stabilizing TRAF6. We demonstrate that this function is independent of the catalytic activity of IPMK and is instead mediated by dynamic protein-protein interactions between IPMK and TRAF6. Thus, beyond its catalytic role in inositol phosphate metabolism and its scaffolding role in various signaling pathways (*37*, *40*–*42*, *48*), IPMK also acts as an important point of control for TLR-dependent immunoregulation. We further expect that therapeutics that modulate the levels of IPMK or its binding to TRAF6 will be useful in the management of uncontrolled inflammatory diseases.

MATERIALS AND METHODS

Animal experiments

Animal protocols were performed in accordance with the guidelines approved by the Korea Advanced Institute of Science and Technology Animal Care and Use Committee. Myeloid cell–specific IPMK knockout mice were generated by crossing *Ipmk* floxed mice (*40*) with LysM-Cre mice (#004781; The Jackson Laboratory). Male mice were used for experiments at 8 to 9 weeks of age. In all experiments, including BMDM studies, $Ipmk^{WT}$ littermates served as controls for $Ipmk^{\Delta Mac}$ mice. All mice were bred and housed under specific pathogen–free conditions in a 12-hour light-dark cycle. They received food and water ad libitum.

CLP model of sepsis and LPS-induced endotoxemia

The CLP model of sepsis was performed as described previously (*59*). Briefly, mice were anesthetized, and the cecum was exteriorized, ligated, and punctured once through and through midway between the ligation using a 21-gauge needle. Sham-operated mice received cecal ligation only. The abdomen was closed in two layers, and the mice were resuscitated by subcutaneous injection of 1.0 ml of prewarmed normal saline. For histological analyses, mice were sacrificed 20 hours after surgery, and lung and liver were removed. Lung and liver tissues were fixed in 10% formalin (Sigma-Aldrich) overnight at room temperature, embedded in paraffin, cut into 5-μm sections, and stained with H&E (Sigma-Aldrich).

For LPS-induced endotoxemia, mice were challenged with an intraperitoneal injection of LPS [*Escherichia coli* serotype O127:B8 (L3137) or O26:B6 (L3755), Sigma-Aldrich] in 0.9% saline at two doses: a high dose (30 mg/kg) to monitor mortality and a lower, nonlethal dose (4.5 mg/kg). Food intake, weight loss, and rectal temperature were subsequently monitored for 48 hours (*56*, *60*). Mice were sacrificed 6 hours after injection for cytokine quantification in plasma and mRNA expression in tissues. Blood samples from mice were clotted and centrifuged, and serum was stored at −80°C until analyzed. IL-1β, IL-6, and TNF in serum and cell culture supernatants were analyzed by sandwich ELISA using a BD OptEIA ELISA kit (BD Biosciences).

Plasmid construction

pCMV-GST full-length IPMK and fragments were constructed as described previously (*42*). pRK5-HA-Ubiquitin-K48 (#17605) and pRK5-HA-Ubiquitin-K63 (#17606) constructs were purchased from Addgene. pcDNA3.0 FLAG-TRAF6 and fragments were gifts from E.-K. Jo and S. Y. Lee. pCEP-HA-IRAK1 was a gift from Y.-J. Song.

Cell culture and transfection

Reagents were obtained from the indicated sources as follows: high-glucose Dulbecco's modified Eagle's medium (DMEM) (Biowest); RPMI 1640 medium (Thermo Fisher Scientific); fetal bovine serum (FBS; Atlas Biologicals); Dulbecco's PBS (DPBS; Welgene); sodium pyruvate, Hepes, penicillin/streptomycin, 0.25% trypsin-EDTA, and Lipofectamine LTX (Invitrogen); Pam_3CSK_4, poly (I:C), blasticidin, and HygroGold (InvivoGen); recombinant mouse macrophage colony-stimulating factor (M-CSF) (R&D Systems); and cycloheximide, MG-132, and LPS (Sigma-Aldrich).

BMDMs were isolated from mouse femurs and tibias and differentiated for 6 days on non–culture-treated petri dishes in BMDM medium [RPMI 1640, 10% FBS, recombinant M-CSF (30 ng/ml), 1 mM sodium pyruvate, 2 mM L-glutamine, penicillin/streptomycin (100 μg/ml)]. Adherent BMDMs were detached on day 6, plated in 10-cm or multiwell plates, and analyzed the next day for protein and gene expression by immunoblotting and RT-qPCR, respectively. RAW 264.7 and HEK293 T cells were grown in high-glucose DMEM supplemented with 10% FBS, 2 mM L-glutamine, and penicillin/streptomycin (100 μg/ml). HEK293 T cells stably expressing TLR4 (HEK293T-TLR4) were selected by growing transfected cells in DMEM/10% FBS supplemented with blasticidin (10 μg/ml) and ultrapure hygromycin (50 μg/ml; HygroGold). For transient transfection of HEK293T and HEK293T-TLR4 cells, jetPRIME reagent (Polyplus) was used according to the manufacturer's protocol. Lipofectamine LTX was used for the introduction of small interfering RNA (siRNA) into RAW 264.7 cells, as described by the manufacturer (Invitrogen). IPMK and scrambled siRNAs were purchased from Bioneer Co. IPMK siRNA sequences are as follows: sense: 5′-CAGAGAGGUCCUAGUUAAUUUCA-3′; antisense: 5′-AGUGAAAUUAACUAGGACCUCUCUGUU-3′.

Lentivirus production and transduction of cells

The plasmid pCDH-MCS-T2A-copGFP-MSCV (CD525A-1; System Biosciences) was used as a backbone for the expression of genes of interest. Lentiviruses were generated in HEK293T cells by transfecting the lentiviral plasmids together with packaging vectors (pRSV-Rev and pMDLg/pRRE) and envelope-expressing plasmid (pMD2.G), as described previously (*61*). Lentiviruses were added to BMDMs on day 3 and again on day 4, and BMDMs were incubated until day 5.

Immunoblotting, immunoprecipitation, GST pull-down, and in vivo ubiquitination assays

For immunoblot analyses, cells were washed twice with PBS and lysed in lysis buffer consisting of 1% NP-40, 120 mM NaCl, 40 mM tris-HCl (pH 7.4), 1.5 mM sodium orthovanadate, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, and protease inhibitor cocktail (Roche). Protein concentrations were determined by the Bradford protein assay

(Bio-Rad) or bicinchoninic acid assay (Thermo Fisher Scientific). Immunoprecipitation and GST pull-down assays were performed as described previously (*42*).

In vivo ubiquitination assays were performed as described previously (*28*, *62*). Briefly, cells were lysed in an SDS lysis buffer consisting of 2% SDS, 150 mM NaCl, 10 mM tris-HCl (pH 8.0), 20 mM *N*-ethylmaleimide, 2 mM sodium orthovanadate, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, and protease inhibitor cocktail. Lysed cell samples were boiled at 95°C for 15 min and sheared by sonication; thereafter, 10 volumes of GST pull-down buffer were added, and samples were rotated for 30 min at 4°C. Lysates were then cleared by centrifugation at 13,000 rpm for 30 min, and 1 mg of supernatants was immunoprecipitated with a specific antibody.

Antibodies against the following proteins were obtained from the indicated sources: phospho-IKK (2697), IKK (2370), phospho–NF-κB (3033), NF-κB (8242), phospho-IκB (2859), IκB (4814), phospho-p38 (4511), p38 (9212), phospho-JNK (4668), phospho-TBK1 (5483), TBK1 (3013), K48 ubiquitin (8081), GST (2622), and β-actin (4970) (Cell Signaling); anti-JNK1/2 (554285; BD Pharmingen); TRAF6 (sc-7221), TLR4 (sc-293072), MyD88 (sc-8196), GAPDH (sc-32233), and normal rabbit IgG (sc-2027) (Santa Cruz Biotechnology); HA (MMS101R; Covance); FLAG (F1804) and tubulin (T5109) (Sigma-Aldrich); K48 ubiquitin (05-1307; Millipore); TRAF6 (ab33915; Abcam); and TRIF (NB120-13810; Novus Biologicals). The anti-rabbit IPMK antibody used was raised against a mouse IPMK peptide corresponding to amino acids 295–311 (SKAYSTHTKLYAKKHQS; Covance) containing an added N-terminal cysteine (*40*). Horseradish peroxidase–conjugated secondary antibodies (NCL1460KR) were purchased from Thermo Fisher Scientific.

RNA isolation and RT-qPCR

Total RNA was isolated from cells or tissues using the TRI Reagent (Molecular Research Center) according to the manufacturer's protocol. First-strand complementary DNA was synthesized from 1 to 3 μg of total RNA using reverse transcriptase (Invitrogen and Enzynomics). RT-qPCR analyses were performed using SYBR Green Master Mix (Toyobo) and the StepOnePlus Real-Time PCR System (Applied Biosystems). Expression levels of genes of interest were normalized to those of a housekeeping gene and are presented as fold changes over baseline using the $\Delta\Delta C_t$ method. Primer sequences for qPCR are as follows: *18s* (forward: 5′-CGCTTCCTTACCTGGTTGAT-3′; reverse: 5′-GAGCGACCAAAGGAACCATA-3′), *Ipmk* (forward: 5′-CCAAAATATTATGGCATCTG-3′; reverse: 5′-TATCTTTACATCCATTATAC-3′), *Il-1*β (forward: 5′-GCCTCGTGCTGTCGGACC-3′; reverse: 5′-TGTCGTTGCTTGGTTCTCCTTG-3′), *Il-6* (forward: 5′-ATGAACAACGATGATGCACTT-3′; reverse: 5′-TATCCAGTTTGGTAGCATCCAT-3′), *Tnf*α (forward: 5′-CACAAGATGCTGGGACAGTGA-3′; reverse: 5′-GAGGCTCCAGTGAATTCGGA-3′), *iNos* (forward: 5′-AATCTTGGAGCGAGTTGTGG-3′; reverse: 5′-CAGGAAGTAGGTGAGGGCTTG-3′), *Ifn*β (forward: 5′- CTGGCTTCCATCATGAACAA-3′; reverse: 5′-CATTTCCGAATGTTCGTCCT-3′), *Traf6* (forward: 5′-GCAGTGAAAGATGACAGCGTGA-3′; reverse: 5′-TCCCGTAAAGCCATCAAGCA-3′), *Tnfaip3* (forward: 5′-AAACCAATGGTGATGGAAACTG-3′; reverse: 5′-GTTGTCCCATTCGTCATTCC-3′), *Tank* (forward: 5′-GAGCTACAGCAAAAGACTGA-3′; reverse: 5′-TTGAGACCCTTGGCGGATTC-3′), *Rnf216* (forward: 5′- AGTTTCCATTTGAGGAGCTGACA-3′; reverse: 5′-AACACTGCCTCCTGGGCATAT-3′), *Tlr4* (forward: 5′- CAGTGGTCAGTGTGATTGTGG-3′; reverse: 5′-TTCCTGGATGATGTTGGCAGC-3′), *Myd88* (forward: 5′- CACCTGTGTCTGGTCCATT-3′; reverse: 5′-AGGCTGAGTGCAA- ACTTG-3′), and *Trif* (forward: 5′- CAGGACCTCAGCCTCTCATT-3′; reverse: 5′-TCACTCTGGAGTCTCAAG-3′).

Flow cytometry

Flow cytometry assay was performed as described previously (*30*). Briefly, cells from mesenteric lymph nodes, spleen, and peritoneal cavity were isolated and stained with anti-mouse F4/80 allophycocyanin (APC) (17-4801), anti-mouse CD11b fluorescein isothiocyanate (11-0112), and anti-mouse CD11c phycoerythrin (12-0114) antibodies (eBioscience) and anti-mouse Gr-1 APC antibody (553129) (BD Pharmingen) diluted 1:200 in fluorescence-activated cell sorting (FACS) buffer (DPBS containing 1% FBS and 2 mM EDTA) for 30 min at 4°C. Stained cells were washed twice with 2 ml of FACS buffer. Flow cytometry data were acquired using a FACSDiva flow cytometer (BD Biosciences) and analyzed using the FlowJo software (Tree Star).

Statistical analysis

Differences between averages were analyzed using a two-tailed Student's *t* test or one-way ANOVA followed by Tukey's post-test. Data are expressed as means ± SE.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/3/4/e1602296/DC1

fig. S1. Generation of myeloid lineage-specific conditional IPMK-null mice.
fig. S2. Validation of myeloid lineage-specific conditional IPMK-null mice.
fig. S3. IPMK depletion in macrophages blunts TLR-dependent inflammatory responses.
fig. S4. IPMK depletion in RAW 264.7 macrophages down-regulates TLR-dependent inflammatory responses.
fig. S5. IPMK depletion in macrophages does not alter TLR3-dependent inflammatory responses.
fig. S6. Upstream TLR signaling regulators are not altered in IPMK-depleted macrophages.
fig. S7. Overexpression of IPMK reduces TRAF6 ubiquitination.

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Acknowledgments: We thank S. H. Snyder, S. F. Kim, A. C. Resnick, H.-K. Lee, Y.-J. Song, I. H. Choi, S. Y. Lee, J. Choi, and C. H. Chung for providing reagents and offering helpful comments. **Funding:** This work was supported by the National Research Foundation of Korea (NRF-2013M3C7A1056102 to S.K.). **Author contributions:** E.K., R.H.S., and S.K. designed the experiments and analyzed the results. E.K. carried out the experiments. E.K., J.B., S.L., S.J.P., H.A., M.G.K., and J.E.P. performed the animal experiments. W.K., J.-M.Y., S.-J.K., S.-H.L., and E.-K.J. provided reagents and comments. E.K. and S.K. wrote the manuscript. R.H.S. and S.K. supervised the research. **Competing interests:** S.K., E.K., and J.B. are listed on the patent entitled "Pharmaceutical composition for preventing or treating inflammatory diseases comprising inositol polyphosphate multikinase inhibitor as an active ingredient" (submission date: 4 January 2017; application number: 10-2017-0001164; country: Republic of Korea; issuing institution: Korea Advanced Institute of Science and Technology). The other authors declare that they have no competing interests. **Data and materials availability:** All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

Submitted 20 September 2016
Accepted 24 February 2017
Published 21 April 2017
10.1126/sciadv.1602296

Citation: E. Kim, J. Beon, S. Lee, S. J. Park, H. Ahn, M. G. Kim, J. E. Park, W. Kim, J.-M. Yuk, S.-J. Kang, S.-H. Lee, E.-K. Jo, R. H. Seong, S. Kim, Inositol polyphosphate multikinase promotes Toll-like receptor–induced inflammation by stabilizing TRAF6. *Sci. Adv.* **3**, e1602296 (2017).