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Phosphoinositide-Dependent Kinase 1 Provides Negative Feedback Inhibition to Toll-Like Receptor-Mediated NF-κB Activation in Macrophages[▽]

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Phosphoinositide-dependent kinase 1 (PDK-1) represents an important signaling component in the phosphatidylinositol 3-kinase (PI3K) pathway, which plays an essential role in controlling a coordinated innate immune response. Here, we show that mice with conditional disruption of PDK-1 specifically in myeloid lineage cells (PDK-1$^{\Delta myel}$ mice) show enhanced susceptibility to lipopolysaccharide (LPS)-induced septic shock accompanied by exaggerated liver failure. Furthermore, primary macrophages derived from PDK-1$^{\Delta myel}$ mice lack LPS- and Pam3CSK4-stimulated AKT activity but exhibit increased mRNA expression and release of tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6). Moreover, LPS- and Pam3CSK4-stimulated primary macrophages exhibit enhanced phosphorylation and degradation of IκBα. While immediate upstream Toll-like receptor 4 (TLR-4)-induced signaling, including IL-1 receptor (IL-1R)-associated protein kinase (IRAK) phosphorylation, is unaltered in the absence of PDK-1, macrophages from PDK-1$^{\Delta myel}$ mice exhibit prolonged ubiquitination of tumor necrosis factor receptor-associated factor 6 (TRAF-6) in response to LPS stimulation. These experiments reveal a novel PDK-1-dependent negative feedback inhibition of TLR-induced NF-κB activation in macrophages *in vivo*.

Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns (PAMPS), such as lipopolysaccharides (LPSs) (endotoxin) in the case of TLR-4, to trigger the inflammatory response necessary for defense against invading pathogens (8, 19, 28, 30). Binding of PAMPS to TLRs triggers the recruitment of the adaptor molecule myeloid differentiation primary response gene 88 (MyD88) to the cytoplasmic domain of the receptor, which initiates the association and phosphorylation of interleukin 1 receptor (IL-1R)-associated protein kinase 1 (IRAK-1). This induces the recruitment and association of tumor necrosis factor receptor-associated factor 6 (TRAF-6) to this complex. Formation of the IRAK-1/TRAF-6 complex leads to its dissociation from the receptor, ultimately promoting the activation of the inhibitor of κB kinase (IKK) complex (1).

The active IKK complex mediates phosphorylation of IκBα, thereby targeting it for proteasomal degradation (5, 6, 18). Degradation of IκBα enables the release and nuclear translocation of NF-κB, where it regulates the expression of genes involved in inflammation, apoptosis, and other key cellular processes (1).

Moreover, TLR signaling also activates mitogen-activated protein kinase (MAPK) kinases (MEK), leading to activation of c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK), which ultimately promote inflammatory-gene expression by activating transcription factor-activating protein 1 (AP-1) and early growth response factor 1 (EGR-1) (24–26, 37).

Another signaling branch activated by TLR-4 is the phosphatidylinositol 3-kinase (PI3K) signaling pathway, whose induction results in the activation of AKT, which stimulates NF-κB via GSK3β inhibition through the transcriptional coactivators cyclic AMP (cAMP) response element binding (CREB) and CREB binding protein (CBP) (39).

The role of TLR signaling in regulating innate immune response mechanisms and thereby stimulating an efficient adaptive immune response is well established (3, 40). However, tight regulation of TLR signaling is essential for an effective immune response, since excessive and prolonged activation of TLR-4 leads to endotoxic shock (56). Hence, to limit its hyperactivation, TLR-4 signaling must be dampened by a number of mechanisms. So far, IKKα, IRAK-M, suppressor of cytokine signaling 1 (SOCS-1), tripartite-motif-containing 30-α (TRIM30-α), cylindromatosis (CYLD), and the PI3K signaling cascade have been shown to inhibit TLR activation in an autoregulatory feedback loop (20, 33–36, 41, 51, 60).

Although it has been shown that TLR-4 signaling can activate PI3K to limit production of tumor necrosis factor alpha (TNF-α) (41) and that pharmacological blockade of PI3K results in enhanced activation of NF-κB (23), the exact molecular role of PI3K activation in TLR signaling remains controversial and is probably multifaceted. It has been shown that genetic

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disruption of p85α, the regulatory subunit of PI3K, in dendritic cells results in hyperactivation of p38 MAPK (20). More recently, pharmacological inhibition of GSK3β in monocytes has been demonstrated to increase the nuclear amounts of transcription factor NF-κB subunit p65 and also that of CREB interacting with the coactivator CBP (39). However, the role of PI3K signaling in TLR-4 signaling is complicated by reports indicating that PI3K can also stimulate NF-κB activation via IKK phosphorylation in a PDK-1-dependent fashion (53).

Induction of PI3K leads to activation of lipid signaling via the conversion of phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3). PIP3 levels are low in resting cells but are acutely increased in response to multiple stimuli that activate PI3K (20). On one hand, this leads to the recruitment and activation of PDK-1, which in turn regulates the phosphorylation of several members of the AGC kinase family, including p70 ribosomal protein S6 kinase (p70S6K), serum and glucocorticoid-inducible kinase (SGK), protein kinase C (PKC) isoforms, p90 ribosomal S6 kinase (RSK), protein kinase C-related kinases 1 and 2 (PRK1/2), and p21-activated kinase 1 (PAK1) at the residue Thr308 equivalent of AKT (49, 58). On the other hand, in other cell types, PIP3 can also propagate signals independently of PDK-1-mediated kinase activation, such as the regulation of ATP-sensitive potassium (K_{ATP}) channels and actin cytoskeleton degradation (47, 48).

Thus, in light of the controversial reports on the role of PI3K signaling in TLR-4 signaling, we aimed to specifically address the role of PDK-1-dependent signaling in macrophages *in vivo*. To this end, we generated and characterized mice with a targeted disruption of the PDK-1 gene specifically in myeloid lineage cells.

MATERIALS AND METHODS

Animals. All animal procedures were conducted in compliance with protocols approved by local government authorities and were in accordance with NIH guidelines. Mice were housed in groups of 3 to 5 at 22 to 24°C in a 12-h/12-h light/dark cycle with lights on at 6 a.m. The animals were fed a normal chow diet (Teklad Global Rodent no. T.2018.R12; Harlan, Germany) containing 53.5% carbohydrates, 18.5% protein, and 5.5% fat (12% of calories from fat). Genomic DNA was isolated from tail tips, and genotyping was performed by PCR. All experiments on mice were performed at 8 to 10 weeks of age.

Generation of PDK-1$^{\Delta myel}$ mice. *LysMCre* mice were mated with *PDK-1$^{lox/lox}$* mice, and a breeding colony was maintained by mating *PDK-1$^{lox/lox}$* with *LysMCre-PDK-1$^{lox/lox}$* mice. *PDK-1^{lox}* mice had been backcrossed for more than 10 generations on a C57BL/6 background, and *LysMCre* mice, initially established on a C57BL6/129sv background, had been backcrossed for 10 generations on a C57BL6 background before being intercrossed with *PDK-1^{lox}* mice. *LysM-Cre* mice had been previously demonstrated to promote Cre-mediated recombination of *loxP*-flanked genomic sequences with 95% efficiency in F4/80 monocytes, with 99% in neutrophils and with 16% in splenic dendritic cells, as well as with 31% in bone marrow dendritic cells (15). For simplicity, we have termed PDK-flox/flox LysMCre^{+} mice myeloid-lineage-specific PDK-1-deficient (PDK-1$^{\Delta myel}$) mice. *LysMCre* mice were genotyped by PCR as previously described (15), and *PDK-1$^{lox/lox}$* mice were genotyped by PCR with primers crossing the *loxP* site as previously described (10, 59).

Macrophage isolation and stimulation. (i) Peritoneal macrophages. Peritoneal macrophages were isolated by intraperitoneal injection of 2 ml of 4% thioglycolate in distilled water. Following peritoneal lavage after 4 days of injection, the harvested cells were plated in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin and streptomycin, and 2 mM glutamine. The cells were serum starved for 16 to 18 h and stimulated with 1 μg/ml of LPS for the indicated times in the experiments. Following peritoneal lavage, approximately 80% of the cells were stained positive for F4/80, whereas after being plated for a day, the purity of isolated macrophages used for the individual experiment was approximately 74% (data not shown).

(ii) Bone marrow-derived macrophages (BMDM). Mice were sacrificed by CO_2 anesthesia and rinsed in 70% (vol/vol) ethanol, and bone marrow was isolated from the femurs and tibias. After several washing steps, bone marrow cells were resuspended in RPMI 1640 (supplemented with 10% FCS, 1% glutamine, 1% penicillin-streptomycin, and 10 ng/ml recombinant macrophage colony-stimulating factor [M-CSF] [Peprotech]). The bone marrow cells were plated at a concentration of 1×10^6 to 2×10^6 cells/ml in RPMI 1640 (supplemented with 10% FCS, 1% glutamine, 1% penicillin-streptomycin, and 10 ng/ml recombinant M-CSF) on 10-cm bacterial petri dishes and differentiated for 7 to 10 days. Preceding all the experiments, macrophages were washed two times with sterile phosphate-buffered saline (PBS) and serum starved for 16 h.

Gene expression analysis. Quantitative gene expression analysis was performed as previously described (17). For quantitative gene expression analysis, total cellular RNA was isolated using a Qiagen RNeasy Kit (Qiagen, Germany). The RNA was reverse transcribed with EuroScript Reverse Transcriptase (Eurogentec, Belgium) and amplified using TaqMan Universal PCR Master Mix and NO AmpErase UNG with TaqMan Assay-on-Demand kits (Applied Biosystems, CA). Relative expression of target mRNAs was determined using standard curves based on macrophages, and samples were adjusted for total RNA content by hypoxanthine guanine phosphoribosyl transferase 1 (Hprt1) RNA quantitative PCR. Calculations were performed by a comparative method ($2^{-\Delta\Delta CT}$). Quantitative PCR was performed on an ABI-Prism 7900 sequence detector (Applied Biosystems, Germany). Assays were linear over 4 orders of magnitude.

Analysis of cytokine levels and AST levels. TNF-α and IL-6 levels in the primary cell culture supernatant and blood serum were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's guidelines (R&D Systems, Inc., MN). Serum levels of aspartate transaminase (AST) were determined in the diagnostic laboratory using standard protocols.

LPS-induced septic shock. Mice at the age of 8 to 10 weeks were challenged by intraperitoneal injection of a sublethal dose of LPS (50 μg/g bodyweight; from *Escherichia coli* serotype O55:B5) in pyrogen-free PBS. At all times, animals were housed in sterile rooms, using autoclaved cages, food, and water.

Flow cytometry. Myeloid cells were isolated from the peritoneal cavity, bone marrow, spleen, and lymph node. Isolated myeloid cells were treated with red cell lysis buffer (1.3 M NH_4Cl, 169 mM Tris). The cells were then resuspended in PBA (PBS, 0.5% bovine serum albumin [BSA], 0.02% Na-acid). The cells were stained with phycoerythrin (PE)-conjugated Gr-1, fluorescein isothiocyanate (FITC)-conjugated F4/80, allophycocyanin (APC)-conjugated CD11c, and APC-conjugated CD45R/B220 antibodies (Becton Dickinson) and subsequently analyzed on a FACSCalibur using the Cell Quest program (Becton Dickinson). Dead cells were labeled with propidium iodide and excluded from the analysis. The percentage of cells was analyzed.

TUNEL assay. A terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay was performed using the manufacturer's guidelines for animal tissues (Dead END Fluorometric TUNEL System; Promega).

Western blot analysis. Protein isolation from the cells and Western blot analysis were performed as previously described (10). The antibodies used in this study were PDK-1 (611070; BD), insulin receptor β subunit (IRβ) (sc-711; Santa Cruz Biotechnology Inc.), phospho-AKT (Ser473) (9271; Cell Signaling) and Akt (9272; Cell Signaling), phospho-GSKβ (9331; Cell Signaling), GSKβ (9315; Cell Signaling), phospho-p44/42 (9106; Cell Signaling), p44/42 (9102; Cell Signaling), phospho-SAPK/JNK (9251; Cell Signaling), SAPK/JNK (9252; Cell Signaling), phospho-p38 (9216; Cell Signaling), p38 (9212; Cell Signaling), phospho-IκBα (Ser32/36) (9246; Cell Signaling), IκBα (9246; Cell Signaling), phospho-IRAK-1 (4361; Cell Signaling), IRAK-1 (4362; Cell Signaling), TRAF-6 (sc-7221; Santa Cruz Biotechnology Inc.), Ub_n (sc-8017; Santa Cruz Biotechnology Inc.), anticalnexin C terminal (575 to 593) (208880; Calbiochem), and β-actin (clone AC-15) (A5441; Sigma). Quantification of changes in optical density was performed with Quantity One (Bio-Rad Laboratories, Munich, Germany).

siRNA transfections. Small interfering RNAs (siRNAs) (650 pmol; Silencer Select siRNA negative control, 4390846; AKT1, S62215; AKT2, S62219; Applied Biosystems, CA) were transferred to a 4-mm cuvette (Bridge, Providence, RI) and incubated for 3 min with 4×10^6 BMDM in 100 μl Optimem (Invitrogen, Frederick, MD) before electroporation in a Gene Pulser X cell + CE module (Bio-Rad, Hercules, CA). The pulse conditions were as follows: square wave, 1,000 V, 2 pulses, and 0.5-ms pulse length. Seventy-two to 96 h after electroporation, RNA interference (RNAi) efficiency was tested using quantitative real-time PCR, and silenced BMDM were used for functional assays.

Statistical analysis. Data were analyzed for statistical significance using a two-tailed Student's *t* test.

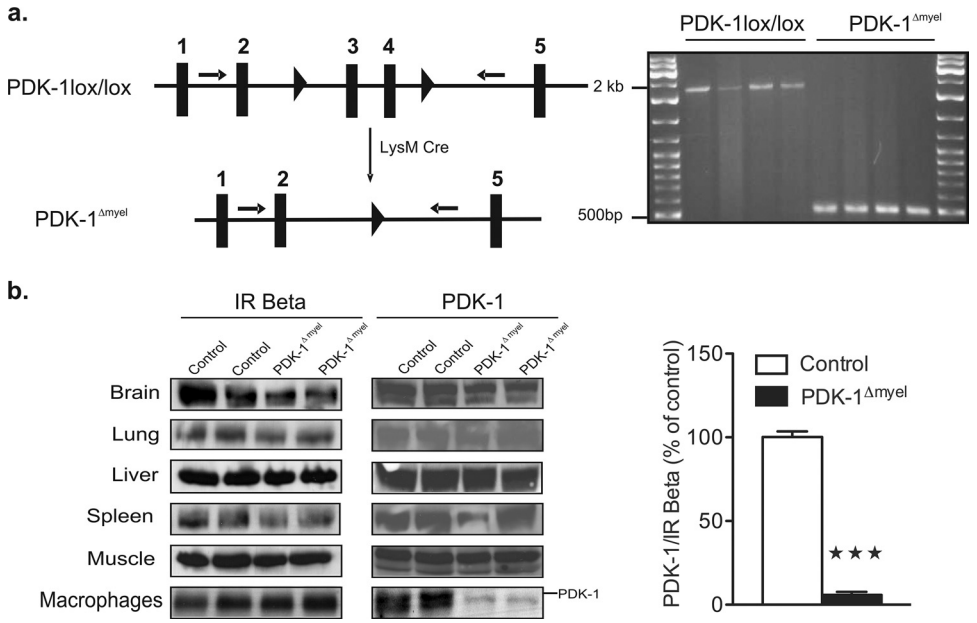

FIG. 1. Generation and characterization of PDK-1$^{\Delta myel}$ mice. (a) Schematic representation of Cre-mediated recombination of the PDK-1 allele (left) and confirmation of deleted exons in macrophages by deletion PCR (right). The diagram illustrates the positions of exons 1 to 5 and the *loxP* sites. The arrows indicate the positions of primers used for genotyping. (b) Western blot analysis of PDK-1 and IR-β (loading-control) expression in thioglycolate-elicited primary macrophages of control and PDK-1$^{\Delta myel}$ mice (left). Also shown is densitometric analysis of PDK-1 protein expression in macrophages from control and PDK-1$^{\Delta myel}$ mice (right) ($P \leq 0.001$).

RESULTS

Myeloid lineage-restricted PDK-1 deficiency does not affect development and maintenance of myeloid cells. In order to generate mice lacking PDK-1 in myeloid lineage cells, we crossed mice expressing the Cre recombinase under the control of the lysozyme M promoter (LysMCre) with mice homozygous for a *loxP*-flanked PDK-1 allele (*PDK-1$^{fl/fl}$*) (15, 59). Double-heterozygous *LysMCre$^{+/-}$ PDK-1$^{fl/+}$* mice were further intercrossed with *PDK-1$^{fl/fl}$* mice to yield *LysMCre$^{+/-}$ PDK-1$^{fl/fl}$* mice (*PDK-1$^{\Delta myel}$* mice). Littermates lacking expression of the Cre recombinase (*PDK-1$^{fl/fl}$*) were used as controls throughout the study. PDK-1$^{\Delta myel}$ mice were born at the expected Mendelian ratio (data not shown). It was previously demonstrated that the LysMCre transgene allows efficient and specific deletion of *loxP*-flanked alleles in macrophages and granulocytes and, to a lesser extent, in dendritic cells (15). PCR analysis of DNA extracted from peritoneal macrophages revealed deletion of the *loxP*-flanked exons 3 and 4 of the PDK-1 gene exclusively in PDK-1$^{\Delta myel}$ mice (Fig. 1a). Moreover, immunoblot analysis revealed a 95% reduction of PDK-1 protein in lysates of peritoneal macrophages from PDK-1$^{\Delta myel}$ mice compared to cells from control mice, whereas the expression of PDK-1 was unaltered in brain, lung, liver, spleen, and skeletal muscle of these mice (Fig. 1b). Taken together, these experiments demonstrate the efficient disruption of PDK-1 in macrophages of PDK-1$^{\Delta myel}$ mice.

Since PDK-1 has been shown to be essential for embryonic development of mice and is implicated in the regulation of cell proliferation, cell cycle progression, and control of apoptosis (45, 59), we next determined if PDK-1 deficiency alters myeloid cell populations. To this end, we performed fluorescence-activated cell sorting (FACS) analyses using markers for granulocytes (Gr-1), macrophages (F4/80), dendritic cells (CD11c), and B cells (B220) to determine the abundance of these cells in different organs. Apart from the slightly, but not significantly, increased expression of Gr-1 in bone marrow of the PDK-1$^{\Delta myel}$ mice, the percentages of different myeloid cell populations, reflected by expression of Gr-1, F4/80, and CD11c, as well as B220-positive B lymphocytes in different in immune tissues, such as bone marrow, spleen, lymph node, and the peritoneal cavity, showed no significant variations between control and PDK-1$^{\Delta myel}$ mice (Fig. 2), indicating that PDK-1-dependent signaling is dispensable for granulocyte and macrophage development and maintenance *in vivo*.

PDK-1$^{\Delta myel}$ mice exhibit increased susceptibility to septic shock. Since it has been demonstrated that blockade of the PI3K pathway leads to enhanced LPS response (23), we directly tested the performance of PDK-1$^{\Delta myel}$ mice in an endotoxin challenge. To this end, PDK-1$^{\Delta myel}$ mice and littermate controls were intraperitoneally injected with a sublethal dose of LPS. Whereas only 20% of the control mice died within the test period of 6 days, the same dose resulted in 90% lethality among PDK-1$^{\Delta myel}$ mice (Fig. 3a). Next, we determined TNF-α concentrations in sera of PDK-1$^{\Delta myel}$ and control mice before and 30 h after administration of LPS. Consistent with the increased mortality among PDK-1$^{\Delta myel}$ mice upon endotoxin challenge, these animals exhibited significantly elevated serum TNF-α concentrations 30 h after LPS administration compared to controls (Fig. 3b).

Although proinflammatory response mechanisms are critical for the clearance of pathogens, a prolonged increase of proinflammatory mediators can often have deleterious effects on the host and contributes to the development of septic shock (7, 16). If not resolved, excessive inflammation ultimately triggers

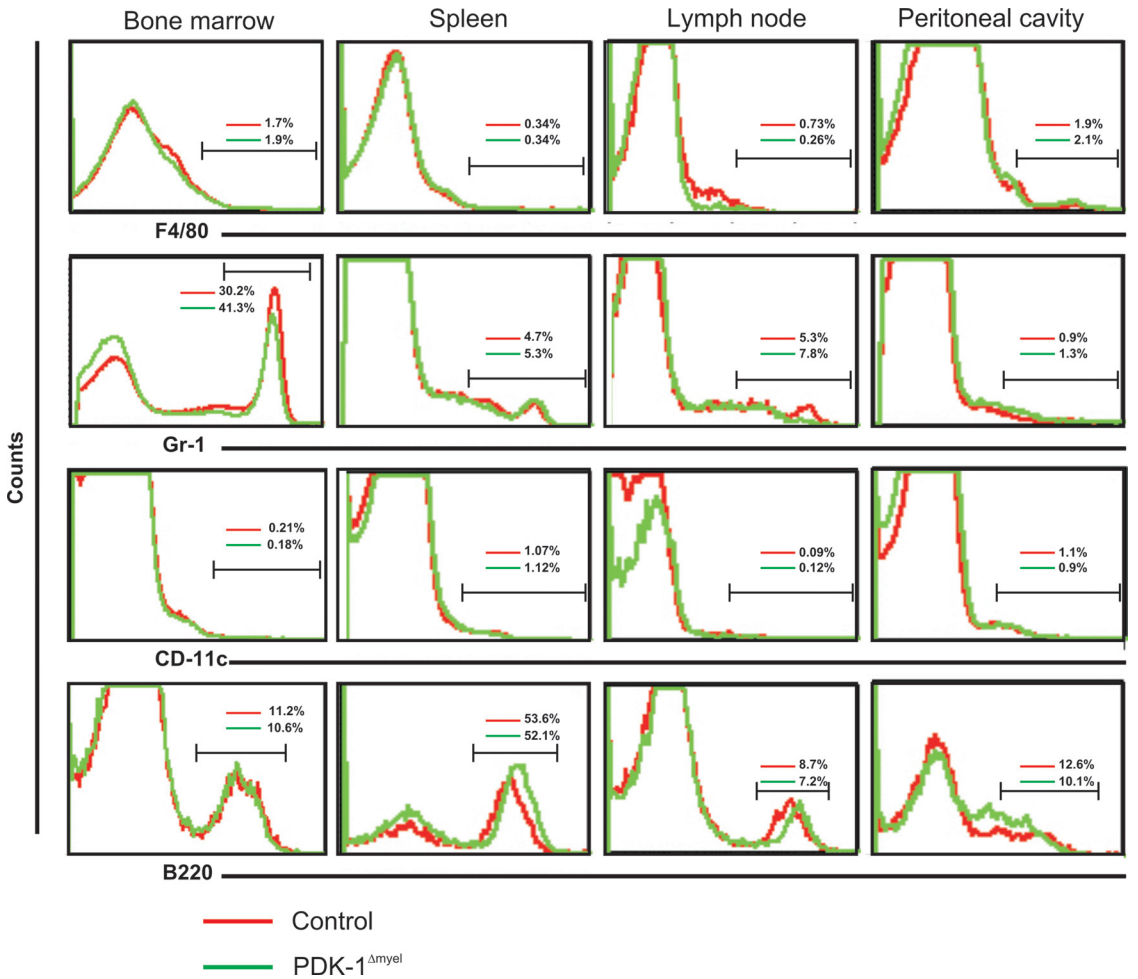

FIG. 2. PDK-1 is not essential for maintenance of myeloid cell populations. FACS analyses were performed on bone marrow, spleen, lymph nodes, and peritoneal cavity to determine the populations of myeloid cells in control and PDK-$1^{\Delta myel}$ animals. The histogram plots are representative from individual animals. Control, $n = 4$; PDK-$1^{\Delta myel}$, $n = 4$.

the onset of enhanced liver injury followed by multiple organ failure (55).

To analyze the effect of myeloid cell-restricted PDK-1 deficiency on endotoxin-induced liver damage, we next determined serum concentrations of the liver injury marker AST before and after LPS challenge in control and PDK-$1^{\Delta myel}$ mice and, to rule out any transgene-related side effects, in mice heterozygous for the LysMCre transgene (LysMCre$^{+/-}$) (Fig. 3c). Under basal conditions, there was no difference in the serum levels of AST in control, LysMCre$^{+/-}$, and PDK-$1^{\Delta myel}$ mice (Fig. 3c). However, 30 h after LPS administration, PDK-$1^{\Delta myel}$ mice displayed significantly increased serum AST compared to both control strains (Fig. 3c). Furthermore, histological analysis revealed the occurrence of increased numbers of apoptotic foci and polymorphonuclear inflammatory cells and enhanced vacuolar degeneration of hepatocytes in livers of PDK-$1^{\Delta myel}$ compared to control and LysMCre$^{+/-}$ mice (Fig. 3d). The observation of increased liver injury in PDK-$1^{\Delta myel}$ mice was further substantiated by TUNEL analysis of apoptotic cells. Here, LPS treatment significantly enhanced the number of TUNEL-positive, apoptotic cells in liver sections of PDK-$1^{\Delta myel}$ mice compared to control mice and LysMCre$^{+/-}$ mice (Fig. 3e). Overall, myeloid cell-restricted PDK-1 deficiency leads to a systemic susceptibility to endotoxin-induced sepsis, reflected by increased liver damage and, subsequently, increased apoptosis in hepatic tissue.

LPS-induced alteration in cell composition in the immune tissues and peritoneal cavity. The induction of endotoxic shock results in changes in cell populations in the immune tissues, such as bone marrow, spleen, and peritoneal cavity (13). Hence, we determined F4/80$^+$ macrophages, Gr-1$^+$ granulocytes, CD11c$^+$ dendritic cells, and B220$^+$ B cells in the immune tissues and peritoneal cavities of control and PDK-$1^{\Delta myel}$ mice 24 h following LPS injection. Although the percentage of Gr-1$^+$ granulocytes in bone marrow and peritoneal cavity tended to be increased in PDK-$1^{\Delta myel}$ mice compared to controls and that of B220$^+$ B cells was slightly reduced in spleens of PDK-$1^{\Delta myel}$ mice compared to controls, these differences did not reach statistical significance between the two genotypes. Moreover, the percentages of cells positive for all other markers analyzed were similar in the bone marrow, spleen, lymph nodes, and peritoneal cavities of control and PDK-$1^{\Delta myel}$ mice following LPS injection (Fig. 4). Taken together, these data suggest that the LPS-induced alterations in

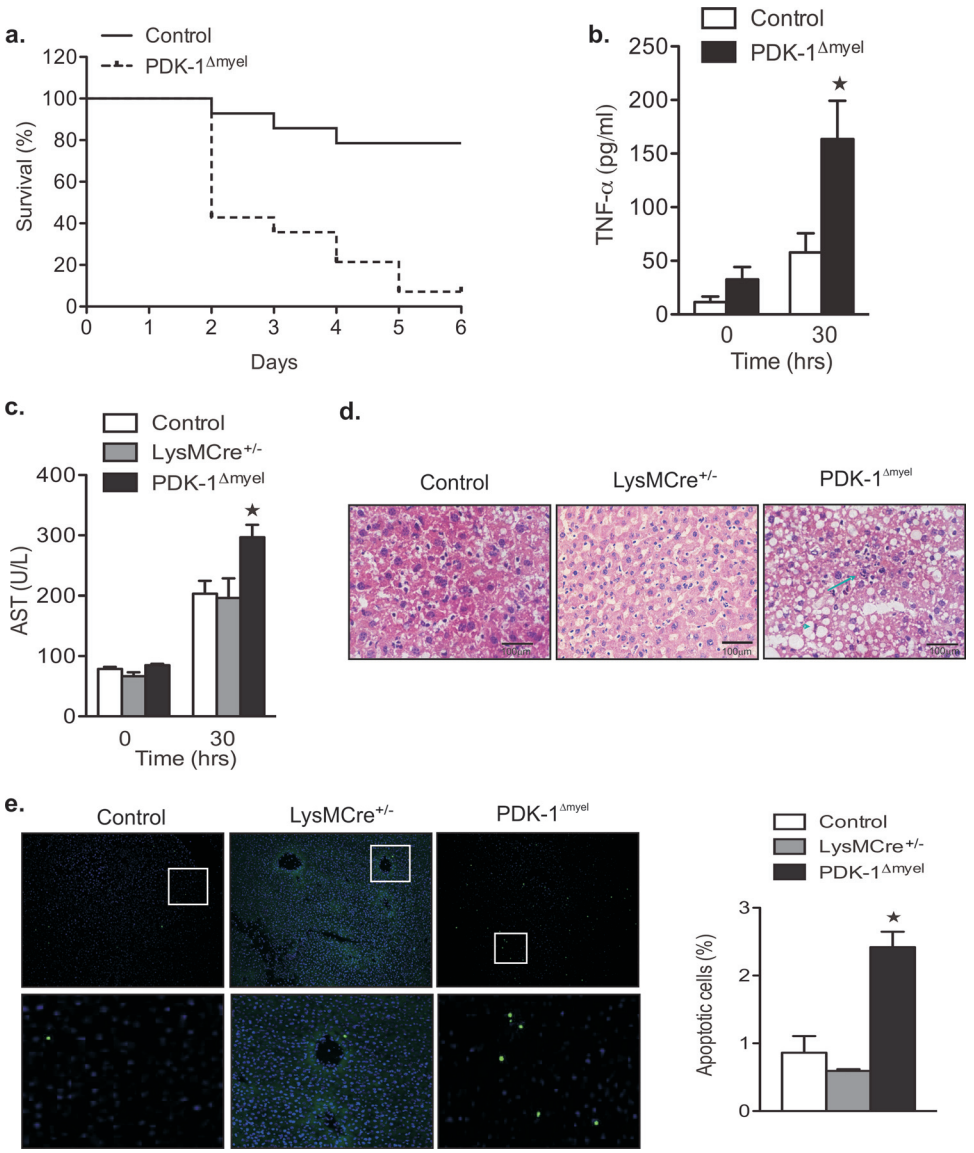

FIG. 3. PDK-1$^{\Delta myel}$ mice exhibit increased susceptibility to septic shock. (a) Systemic response to *in vivo* LPS injection; survival rates of control ($n = 14$) and PDK-1$^{\Delta myel}$ ($n = 14$) mice. (b) Serum TNF-α levels in control and PDK-1$^{\Delta myel}$ mice before and 30 h after intraperitoneal injection of LPS. Control, $n = 14$; PDK-1$^{\Delta myel}$, $n = 13$. (c) Serum AST levels before and 30 h following intraperitoneal injection of LPS. Control, $n = 15$; LysMCre$^{+/-}$, $n = 3$; PDK-1$^{\Delta myel}$, $n = 13$. (d) Hematoxylin and eosin staining of liver sections 30 h after intraperitoneal injection of LPS. The arrow indicates the foci of apoptotic cells and inflammatory cellular infiltrates, and the arrowhead indicates vacuolar degeneration of hepatocytes. (e) TUNEL staining of liver tissue sections from control, LysMCre$^{+/-}$ and PDK-1$^{\Delta myel}$ mice after 30 h of peritoneal injection of LPS (top) and quantification of apoptosis (bottom). Control, $n = 3$; LysMCre$^{+/-}$, $n = 3$; PDK-1$^{\Delta myel}$, $n = 3$. The results are means plus standard errors of the mean (SEM); *, $P \leq 0.05$.

myeloid cell compartments occurred largely to comparable extents in control and PDK-1$^{\Delta myel}$ mice.

PDK-1 deficiency causes enhanced LPS- and Pam3CSK4-stimulated expression and secretion of inflammatory cytokines by macrophages. It has previously been demonstrated that predominantly cytokines produced by macrophages contribute to the development of severe sepsis upon systemic bacterial infection (42). Therefore, we isolated primary peritoneal macrophages from control and PDK-1$^{\Delta myel}$ mice to investigate the role of PDK-1 in inflammatory gene expression ex vivo. These cells were then stimulated with LPS or Pam3CSK4, and inflammatory gene expression was determined by real-time PCR. Strikingly, this analysis revealed that LPS-evoked, TLR-4-dependent expression of the key proinflammatory cytokines, such as TNF-α and IL-6, and the chemokine CCL2 was enhanced in PDK-1-deficient macrophages compared to controls (Fig. 5a). An augmented proinflammatory response was also reflected by enhanced release of TNF-α and IL-6 protein from LPS-stimulated macrophages of PDK-1$^{\Delta myel}$ mice compared to controls (Fig. 5b). Similarly, PDK-1-deficient macrophages exhibited increased mRNA expression and release of IL-6 and TNF-a following TLR-2 activation by Pam3CSK4 (Fig. 5c and d).

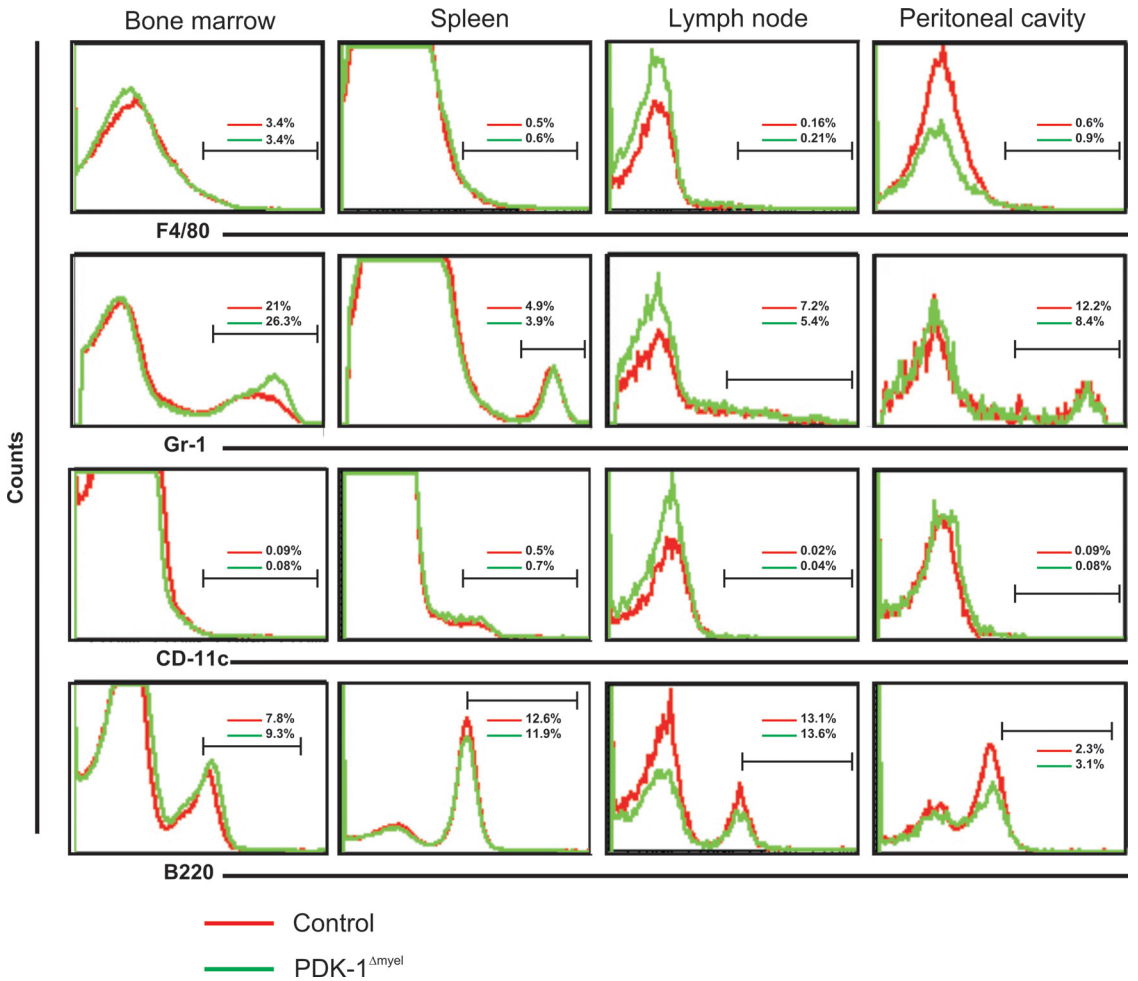

FIG. 4. Endotoxin-induced myeloid cell population changes in immune tissues. FACS analyses were performed on bone marrow, spleen, lymph nodes, and peritoneal cavity to determine the population of myeloid cells in control and PDK-$1^{\Delta myel}$ animals after 24 h of LPS challenge. The histogram plots shown are representatives from individual animals. Control, $n = 4$; PDK-$1^{\Delta myel}$, $n = 4$.

Taken together, these data clearly indicate that PDK-1 deficiency results in a clear amplification of not only TLR-4- but also TLR-2-mediated proinflammatory signaling in macrophages, which directly translates *in vivo* into enhanced susceptibility to septic shock.

Thus, these analyses demonstrated that PDK-1 acts as a negative regulator of TLR-2- and TLR-4-activated transcription of key proinflammatory cytokines and chemokines, all of which are well-characterized NF-κB and MAPK target genes.

PDK-1 deficiency does not alter LPS-stimulated MAPK activation in macrophages. The stimulation of TLR-4 signaling by LPS leads to the activation of both PI3K and MAPK signaling cascades (27, 41). PDK-1 plays an important role in the PI3K/AKT axis, since PDK-1-mediated phosphorylation of AKT at Thr308 is necessary for autophosphorylation events at Ser473 (4). Only after phosphorylation at this residue is AKT able to phosphorylate downstream targets, such as GSK3β (52). To test whether PDK-1 deficiency affects the LPS-stimulated activation of AKT, we isolated primary peritoneal macrophages of control and PDK-$1^{\Delta myel}$ mice. While in control cells LPS rapidly stimulated AKT phosphorylation at Ser473, this effect was completely abolished in macrophages lacking PDK-1 (Fig. 6a).

It has been demonstrated that disruption of the regulatory subunit of PI3K, p85α, in dendritic cells leads to enhanced LPS-mediated activation of p38 MAPK (20). Since p38 and other MAPKs are central regulators of the inflammatory response (19), we determined the activation states of three members of the MAPK family in primary macrophages of control and PDK-$1^{\Delta myel}$ mice upon exposure to LPS. However, immunoblot analysis revealed no difference in kinetics or magnitudes of ERK, JNK, and p38 phosphorylation between control and PDK-1-deficient macrophages (Fig. 6b). These data indicate that the LPS-mediated activation of AKT is abolished in PDK-1-deficient macrophages, whereas MAPK signaling in response to LPS is unaltered.

PDK-1-deficient macrophages exhibit increased activation of the IKK/NF-κB axis upon TLR-4 and TLR-2 stimulation. In a resting cell, NF-κB is sequestered in the cytoplasm by its inhibitor, IκBα, whereas stimulation with either LPS or proinflammatory cytokines causes phosphorylation of IκBα, which is thereby targeted to proteasomal degradation (5, 6, 18). To

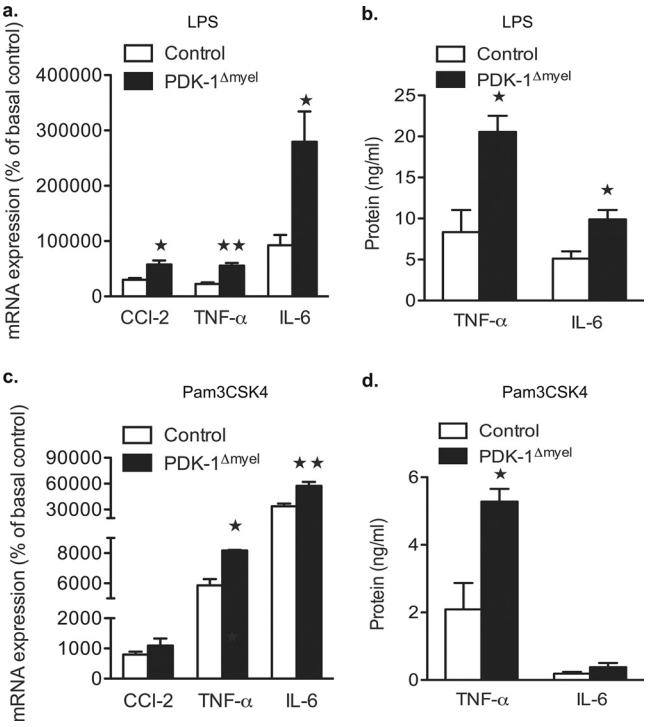

FIG. 5. PDK-1$^{\Delta myel}$ macrophages exhibit enhanced inflammatory response. (a) Relative mRNA expression analysis of CCL-2, TNF-α, and IL-6 in primary peritoneal macrophages derived from control and PDK-1$^{\Delta myel}$ mice after stimulation with LPS (1 μg/ml) for 24 h. Control, $n = 5$; PDK-1$^{\Delta myel}$, $n = 5$. (b) Cell culture supernatants of primary peritoneal macrophages derived from control and PDK-1$^{\Delta myel}$ mice were analyzed by ELISA for IL-6 and TNF-α protein content after 24 h of LPS (1 μg/ml) stimulation. Control, $n = 5$; PDK-1$^{\Delta myel}$, $n = 7$. (c) Relative mRNA expression analysis of CCL-2, TNF-α, and IL-6 in primary peritoneal macrophages derived from control and PDK-1$^{\Delta myel}$ mice after stimulation with Pam3CSK4 (50 ng/ml) for 24 h. Control, $n = 5$; PDK-1$^{\Delta myel}$, $n = 5$. (d) Cell culture supernatants of primary peritoneal macrophages derived from control and PDK-1$^{\Delta myel}$ mice were analyzed by ELISA for IL-6 and TNF-α protein content after stimulation with Pam3CSK4 (50 ng/ml) for 24 h. Control, $n = 5$; PDK-1$^{\Delta myel}$, $n = 5$. Results are all means ± SEM. *, $P \leq 0.05$; **, $P \leq 0.01$.

analyze the activation state of this pathway, LPS-regulated expression and phosphorylation of IκBα were determined in control and PDK-1-deficient macrophages (Fig. 7a). Both the amount of immunoreactive IκBα and its phosphorylation were unaltered in control and PDK-1-deficient macrophages in the basal state (Fig. 7a). Similarly, initial phosphorylation of IκBα and degradation occurred to similar extents in both cell types following LPS stimulation (Fig. 7a). However, while IκBα phosphorylation declined 90 min after LPS stimulation in control cells, followed by stabilization of IκBα expression, PDK-1-deficient cells exhibited significantly enhanced phosphorylation and degradation of IκBα until 120 min after LPS stimulation (Fig. 7a). These data indicate that PDK-1-deficient macrophages exhibit increased and prolonged activation of NF-κB-dependent signaling upon LPS stimulation.

Next, we decided to test the activation of TLR-2-dependent signaling in control and PDK-1-deficient macrophages. Consistent with the altered activation of AKT in response to TLR-4-stimulation of PDK-1-deficient macrophages, Pam3CSK4 also rapidly stimulated AKT phosphorylation at Ser473 in control cells, and this effect was abolished in macrophages lacking PDK-1 (Fig. 7b). Similar to LPS stimulation, there was no difference in basal IκBα levels upon Pam3CSK4 stimulation. However, also following Pam3CSK4 stimulation, phosphorylation of IκBα was significantly enhanced in PDK-1-deficient macrophages compared to control cells after 60 and 90 min and exhibited a trend toward enhanced phosphorylation of IκBα 120 min following TLR-2 activation (Fig. 7c). These data reveal that PDK-1 limits the phosphorylation of IκBα, not only after stimulation of TLR-4, but also following TLR-2 activation.

PDK-1-mediated inhibition of the IKK/NF-κB axis is dependent on AKT. Upon activation, PDK-1 phosphorylates and activates, among other serine/threonine kinases, the downstream kinase AKT, which stimulates NF-κB via GSK3β inhibition (39). To test whether the observed activation of proinflammatory gene expression and IκBα phosphorylation and degradation in PDK-1-deficient macrophages is mediated downstream of AKT, we analyzed the effect of RNAi-mediated knockdown of AKT-1 or AKT-2 in BMDM from C57BL6 mice. Whereas both siRNAs (AKT-1 [Fig. 8a] and AKT-2 [data not shown]) mediated efficient knockdown of their respective target mRNAs, only silencing of AKT-1 led to significantly enhanced TNF-α and IL-6 mRNA expression and secretion upon stimulation with LPS (Fig. 8b and c). In line with enhanced proinflammatory gene expression, compared to control BMDM, the AKT-1-deficient BMDM showed enhanced relative phosphorylation and reduced stabilization of IκBα following LPS stimulation (Fig. 8d).

PDK-1 does not control TNF-α-mediated activation of the IKK/NF-κB axis. Aside from TLR ligands, another potent inducer of IKK-dependent signaling is TNF-α. It mediates its effects via activation of tumor necrosis factor receptor (TNFR) and recruitment of adaptor protein tumor necrosis factor receptor type 1-associated DEATH domain protein (TRADD) and TRAF-2 (38). TNF-α-mediated activation of the IKK complex is therefore independent of MyD88 and TRAF-6. To address the question of whether PDK-1-mediated limitation of IKK-dependent signaling is specific for TLR-dependent upstream signaling, we stimulated primary peritoneal macrophages with TNF-α. However, in contrast to the observed overactivation upon LPS or Pam3CSK4 stimulation, TNF-α stimulation did not reveal any differences in mRNA expression of CCL-2, IL-6, and TNF-α between PDK-1-deficient and control macrophages (Fig. 9a). Consistent with the unaltered mRNA expression, the secretion of IL-6 was also unchanged in control and PDK-1$^{\Delta myel}$ macrophages following TNF-α stimulation (Fig. 9b). To test whether PDK-1 deficiency affects the TNF-α-stimulated activation of AKT, we isolated primary peritoneal macrophages of control and PDK-1$^{\Delta myel}$ mice. Here, TNF-α rapidly induced AKT phosphorylation in control cells, and this effect was slightly, but not significantly, reduced in macrophages lacking PDK-1 (Fig. 9c). Furthermore, in contrast to enhanced and prolonged IκBα degradation upon LPS and Pam3CSK4 stimulation in the absence of PDK-1, PDK-1 deficiency did not affect the kinetics of IκBα phosphorylation and degradation in response to TNF-α (Fig. 9d).

Enhanced TRAF-6 ubiquitination in the absence of PDK-1. To further delineate the molecular basis of specifically en-

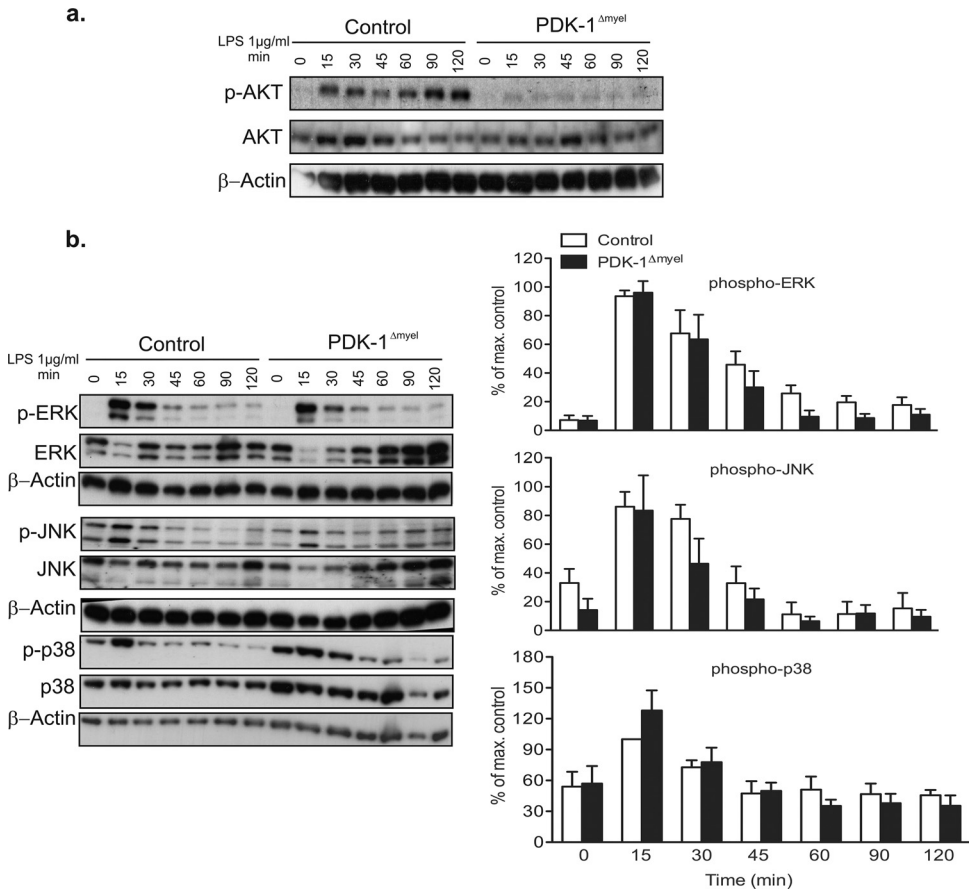

FIG. 6. PDK-1 deficiency does not alter LPS-stimulated MAPK activation in macrophages. (a) Peritoneal macrophages of PDK-1$^{\Delta myel}$ and control mice were stimulated with LPS (1 μg/ml) for the indicated times, and phospho-AKT (p-AKT) levels were measured by Western blotting. The Western blots are representative of three independent experiments. (b) Peritoneal macrophages of PDK-1$^{\Delta myel}$ and control mice were stimulated with LPS (1 μg/ml) for the indicated times, and MAPK pathway activation was determined by Western blotting (left). Densitometrical analysis of the Western blots is shown on the right. The data represent the means and SEM from four independent experiments. Representative Western blots are shown.

hanced TLR-mediated proinflammatory gene expression in PDK-1-deficient macrophages, we next analyzed the activation states of signaling components further upstream of NF-κB, since, notably, PDK-1-deficient macrophages exhibit enhanced IκBα activation in response to LPS and Pam3CSK4, but not TNF-α (Fig. 7a and c and 9d). Upstream signaling cascades of these stimuli converge at the level of the IKK complex. While TLR-4 and -2 both signal via TRAF-6, the TNFR employs the adaptor TRAF-2 to activate IKKs (11, 43). Assuming that the difference in the activation of these adaptor molecules might be culminating in the activation of the IKK complex, we next investigated the activation status of IRAK and TRAF-6 upon LPS stimulation in both PDK-1-deficient and control macrophages. Expression and LPS-induced phosphorylation of IRAK-1 were unaltered in macrophages of PDK-1$^{\Delta myel}$ and control mice upon stimulation with LPS (Fig. 10a). However, the LPS-induced ubiquitination of endogenous TRAF-6 was specifically enhanced in PDK-1-deficient macrophages, in both magnitude and kinetics (Fig. 10b). Collectively, these data suggest that PDK-1 transduces the signal downstream of TLR-4 independently of IRAK but by limiting the ubiquitination of TRAF-6.

DISCUSSION

The negative regulation of TLR4 signaling by PI3K in innate immune cells has been well documented (19). In previous studies using chemical inhibitors, such as wortmannin and LY294002, it was shown that blockage of the PI3K pathway enhances the LPS-stimulated inflammatory response in both monocytes and dendritic cells (20, 23). In follow-up studies, Fukao et al. could show that mice deficient for the regulatory subunit p85α of PI3K exhibit impaired clearance of enterobacteria *in vivo*, further supporting a critical role for PI3K in the control of innate immune responses (21). Specifically, these data suggest an important role for PI3K signaling in limiting an exaggerated inflammatory response to maintain homeostasis of the immune system (20). Apart from p85α, the roles of individual downstream components of PI3K signaling in control of inflammatory cytokine expression and action remain elusive.

In this study, we demonstrated that mice with myeloid cell-specific disruption of PDK-1, a crucial downstream component of the PI3K signaling cascade (59), are prone to LPS-induced septic shock. Furthermore, macrophages isolated from these animals show enhanced expression and secretion of proinflam-

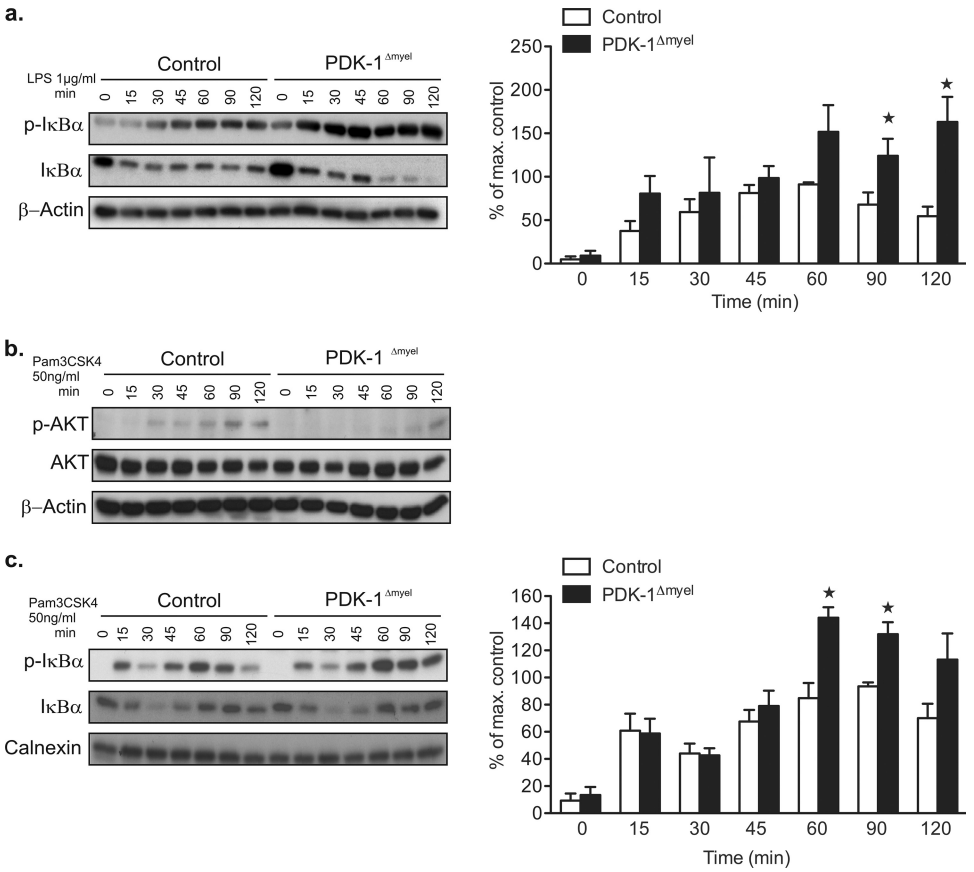

FIG. 7. PDK-1-deficient macrophages exhibit increased IKK activation upon LPS stimulation. (a) Peritoneal macrophages of PDK-1$^{\Delta myel}$ and control mice were stimulated with LPS (1 μg/ml) for the indicated times, and phospho-IκBα and IκBα levels were measured by Western blotting (left). Densitometric analysis of the Western blots from four independent experiments are shown on the right. Representative Western blots are shown. The results are means plus SEM; ⋆, $P \leq 0.05$. (b) Peritoneal macrophages of PDK-1$^{\Delta myel}$ and control mice were stimulated with Pam3CSK4 (50 ng/ml) for the indicated times, and phospho-AKT levels were measured by Western blotting. The Western blots are representative of three independent experiments. (c) Peritoneal macrophages of PDK-1$^{\Delta myel}$ and control mice were stimulated with Pam3CSK4 (50 ng/ml) for the indicated times, and IκBα levels were measured by Western blotting (left). Densitometric analysis of the Western blots of four independent experiments is shown on the right. Representative Western blots are shown. The results are means plus SEM; ⋆, $P \leq 0.05$.

matory cytokines in response to TLR-2 and -4 stimulation. On a molecular level, PDK-1-deficient macrophages exhibit enhanced ubiquitination of TRAF-6, which subsequently leads to overactivation of the IKK/NF-κB axis, reflected by enhanced phosphorylation and degradation of IκBα. Notably, although our data focus on macrophages as the main effector cell type in LPS-induced septic shock, the potential roles of other myeloid cells, such as granulocytes (where the LysMCre transgene also deletes efficiently) should be considered. However, these experiments unequivocally reveal a critical role for PDK-1 in limiting TLR-induced signaling in macrophages.

Previous studies in cultured cells suggested that AKT, one of the downstream substrates of PDK-1, limits proinflammatory gene expression through inhibition of MAPK activation (23). AKT can inhibit activation of p38 MAPK by at least two mechanisms. First, it phosphorylates apoptosis signal-regulating kinase 1 (ASK1), which inhibits activation of MAPK kinases, MKK-3 or -6, the upstream regulators of p38 in L929 cells (31). Second, AKT has been shown to directly block the activity MEKK3, at least in endothelial cells (22). It has also been reported that the PI3K-AKT pathway negatively regulates JNK and ERK activity in dendritic cells (24, 46). However, we showed that LPS stimulation of macrophages promotes similar activation of MAPK signaling cascades, including p38, JNK, and ERK, in the presence or absence of PDK-1. Thus, the negative regulation of inflammatory cytokines by PDK-1 in peritoneal macrophages appears to be independent of its effect on MAPK activity, pointing to a differential, cell-type-specific regulation of p38 MAPK via the PI3K pathway in dendritic cells and macrophages. Moreover, these data indicate that in macrophages, AKT is not a physiologically relevant regulator of MAPK activation *in vivo*.

Aside from the control of MAPK activation, TLR-mediated signal transduction leads to the activation of the NF-κB signaling pathway to regulate inflammatory signaling (19). Here, the canonical pathway controls inflammatory responses in macrophages and requires activation of IKKβ (2, 29). Activation of IKKβ by TLR signaling promotes the degradation of IκBα and enables the nuclear translocation of NF-κB (29). We showed that upon LPS and Pam3CSK4 stimulation, both of

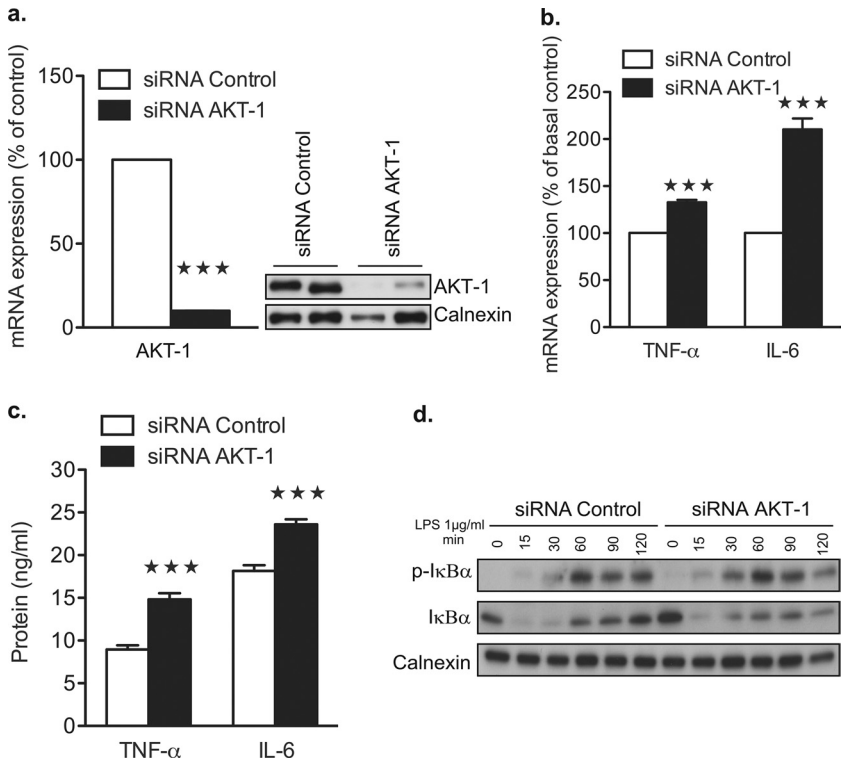

FIG. 8. AKT-1-deficient BMDM exhibit enhanced inflammation upon LPS stimulation. (a) Relative mRNA expression analysis of AKT-1 in silenced BMDM. The results are means plus SEM of three independent transfections; ***, $P \leq 0.001$ (left). Western blot analysis of AKT and calnexin (loading control) in silenced BMDM. (b) Relative mRNA expression analysis of TNF-α and IL-6 in silenced BMDM stimulated with LPS (1 μg/ml) for 24 h. The results are means plus SEM of three independent experiments in triplicate; ***, $P \leq 0.001$. (c) Cell culture supernatants of silenced BMDM were analyzed by ELISA for IL-6 and TNF-α protein content after stimulation with LPS (1 μg/ml) for 24 h. The results are means plus SEM of three independent experiments in triplicate; ***, $P \leq 0.001$. (d) Silenced BMDM were stimulated with LPS (1 μg/ml) for the indicated times, and phospho-IκBα and IκBα levels were measured by Western blotting. The Western blots are representative of three independent experiments.

which recruit the adaptor protein MyD88 to their receptors (TLR-4 and TLR-2) for signal transduction, peritoneal macrophages isolated from PDK-$1^{\Delta myel}$ mice exhibit enhanced phosphorylation of IκBα and thereby enhanced activation of IKK-dependent signaling. Thus, we define TLR-2 and TLR-4 activated PDK-1 signaling as a negative regulator of NF-κB activation in macrophages *in vivo*. The notion that PDK-1-dependent signaling limits NF-κB activation upstream of IKKβ-mediated phosphorylation and degradation of IκBα provides several novel insights into the mechanisms of autoregulatory MyD88-mediated feedback inhibition.

Our experiments extend previously published findings from overexpression studies, which indicate that PDK-1 activates NF-κB signaling via IKKβ activation (53). Our findings revealed exaggerated proinflammatory gene expression and increased susceptibility to septic shock in mice lacking PDK-1 in macrophages. Concordantly, administration of a GSK3β inhibitor potently suppressed the proinflammatory response in mice receiving lipopolysaccharide and protected them from endotoxin shock (39). In this study, GSK3β regulated inflammatory responses by differentially affecting the nuclear amounts of transcription factors NF-κB subunit p65 and CREB interacting with the coactivator CBP (39). Since PDK-1–AKT-dependent phosphorylation of GSK3β reduces GSK3β kinase activity, these experiments are consistent with PDK-1-mediated inhibition of NF-κB activation. However, inhibition of PDK-1 in our model exaggerates IκBα phosphorylation and degradation, acting at a level upstream of GSK3β-controlled cofactor recruitment and AKT-1. Thus, our experiments clearly revealed the existence of multiple, PDK-1-dependent mechanisms to dampen MyD88-dependent NF-κB activation.

Phosphorylation of AKT at Ser 473 is essential for overall activation of AKT, and this is enabled by PDK-1-mediated phosphorylation at the Thr308 residue of AKT (4). Stimulation of macrophages with TNF-α in the absence of PDK-1 leads to reduced but not a complete loss of AKT Ser473 phosphorylation (Fig. 9c). This could be caused by MAPK-dependent mTOR activation, bypassing PDK-1 dependency (12, 50).

Interestingly, in our study, PDK-1 exerted its negative regulatory effect on IKK complex activation by limiting the activation of TRAF-6, and not that of IRAK-1. Although Cao et al. reported the requirement of TRAF-6 for TAK1-mediated phosphorylation and activation of the JNK-p38 pathway upon IL-1β-dependent activation (11), Chen et al. proposed that LPS-dependent activation of TRAF-6 does not affect the phosphorylation and activation of MAPK JNK, p38, and ERK (14). These experiments indicate differential, cell-type-specific requirements of TRAF-6 to activate MAPK signaling. More-

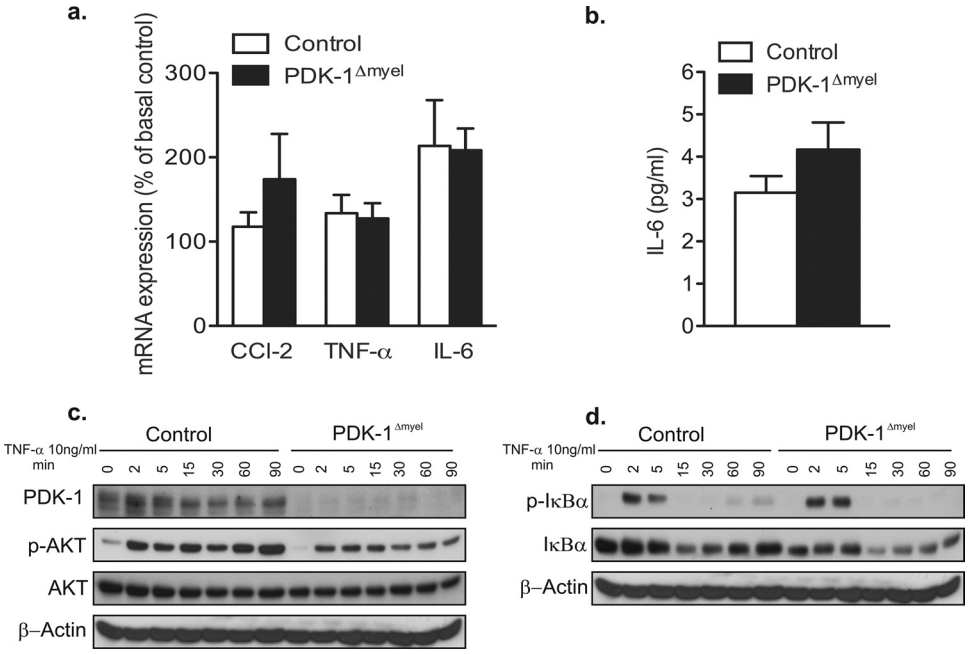

FIG. 9. PDK-1-deficient macrophages exhibit unaltered IKK activation upon TNF-α stimulation. (a) Relative mRNA expression analysis of CCL-2, TNF-α, and IL-6 in primary peritoneal macrophages derived from control and PDK-1$^{\Delta myel}$ mice after stimulation with TNF-α (10 ng/ml) for 24 h. Control, $n = 5$; PDK-1$^{\Delta myel}$, $n = 5$. The results are means plus SEM. (b) Cell culture supernatants of primary peritoneal macrophages derived from control and PDK-1$^{\Delta myel}$ mice were analyzed by ELISA for IL-6 protein content after stimulation with TNF-α (10 ng/ml) for 24 h. Control, $n = 5$; PDK-1$^{\Delta myel}$, $n = 5$. (c) Peritoneal macrophages of PDK-1$^{\Delta myel}$ and control mice were stimulated with TNF-α (10 ng/ml) for the indicated times, and PDK-1 and phospho-AKT levels were measured by Western blotting. The Western blots are representative of three independent experiments. (d) Peritoneal macrophages of PDK-1$^{\Delta myel}$ and control mice were stimulated with TNF-α (10 ng/ml) for the indicated times, and phospho-IκBα and IκBα levels were measured by Western blotting. The Western blots are representative of three independent experiments.

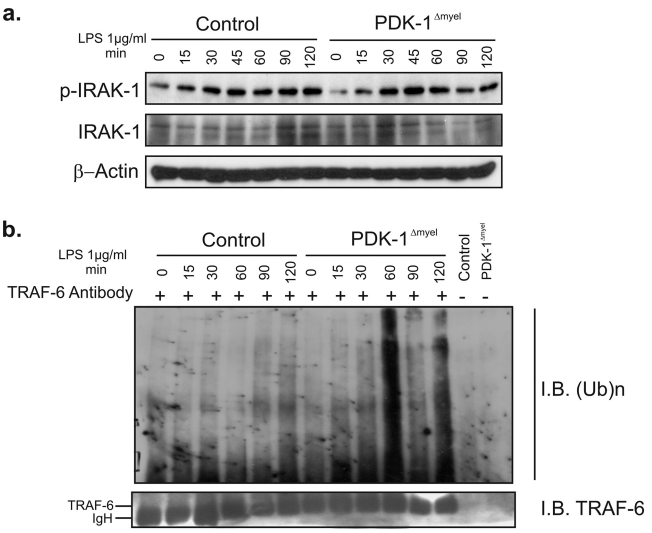

FIG. 10. PDK-1-deficient macrophages exhibit enhanced ubiquitination of TRAF6 upon LPS stimulation. (a) Peritoneal macrophages of PDK-1$^{\Delta myel}$ and control mice were stimulated with LPS (1 μg/ml) for various times, and phospho-IRAK-1 and IRAK-1 levels were measured by Western blotting. The Western blots are representative of three independent experiments. (b) Following stimulation with LPS, TRAF-6 was immunoprecipitated from whole-cell lysates of primary peritoneal macrophages of PDK-1$^{\Delta myel}$ and control mice using TRAF-6 antibody. Ubiquitination of immunoprecipitated TRAF-6 was then determined by ubiquitin antibody (Ub)n. The Western blots (I.B.) are representative of three independent experiments.

over, our results suggest that PDK-1 is essential for specifically limiting the activation of IKK-dependent signaling independent of MAPK activation in our cellular model.

Our experiments can be explained by multiple models for PDK1-dependent control of TRAF-6 ubiquitination and function. PDK-1-mediated inhibition of TRAF-6 could be directly controlled by TRAF-6 phosphorylation either by PDK-1 itself or by PDK-1-dependent kinases, such as AKT, PKC, SGK, GSK3β, and p70S6K. Indeed enhanced IκBα phosphorylation in the absence of AKT-1 supports the idea that at least part of the effect of PDK-1 deficiency to exaggerate proinflammatory gene expression is caused by reduced AKT-1 activation in the absence of PDK-1 (Fig. 8d). Since TRAF-6 ubiquitination and activity are controlled by the deubiquitinating enzymes CYLD and A20 (54, 57), PDK-1 or downstream kinases, such as AKT-1, could modulate the activities of these two deubiquitinating enzymes. It is also worth mentioning that although A20 has been implicated in removing K63-linked ubiquitin chains from TRAF-6, deletion of A20 in macrophages exaggerates both TNF-α- and LPS-stimulated NF-κB activation (9, 57). In contrast to A20 deletion, inactivation of CYLD results selectively in enhanced LPS-stimulated, but not TNF-α-stimulated, NF-κB activation, similar to what was observed in macrophages lacking PDK-1 (60). Thus, future investigations will analyze the role of PDK-1-dependent signaling in the control of CYLD activity. Whatever the molecular nature of PDK1-dependent control of TRAF-6 may ultimately prove to be, the

present study has identified a key negative regulatory function of PDK-1 for TLR signaling in myeloid cells.

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