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TLR4-Mediated AKT Activation Is MyD88/TRIF Dependent and Critical for Induction of Oxidative Phosphorylation and Mitochondrial Transcription Factor A in Murine Macrophages

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Mitochondria play a critical role in cell survival and death. Mitochondrial recovery during inflammatory processes such as sepsis is associated with cell survival. Recovery of cellular respiration, mitochondrial biogenesis, and function requires coordinated expression of transcription factors encoded by nuclear and mitochondrial genes, including mitochondrial transcription factor A (T-fam) and cytochrome c oxidase (COX, complex IV). LPS elicits strong host defenses in mammals with pronounced inflammatory responses, but also triggers activation of survival pathways such as AKT pathway. AKT/PKB is a serine/threonine protein kinase that plays an important role in cell survival, protein synthesis, and controlled inflammation in response to TLRs. Hence we investigated the role of LPS-mediated AKT activation in mitochondrial bioenergetics and function in cultured murine macrophages (B6-MCL) and bone marrow-derived macrophages. We show that LPS challenge led to increased expression of T-fam and COX subunits I and IV in a time-dependent manner through early phosphorylation of the PI3K/AKT pathway. PI3K/AKT pathway inhibitors abrogated LPS-mediated T-fam and COX induction. Lack of induction was associated with decreased ATP production, increased proinflammatory cytokines (TNF-α), NO production, and cell death. The TLR4-mediated AKT activation and mitochondrial biogenesis required activation of adaptor protein MyD88 and Toll/IL-1R domain-containing adaptor-inducing IFN-β. Importantly, using a genetic approach, we show that the AKT1 isoform is pivotal in regulating mitochondrial biogenesis in response to TLR4 agonist. *The Journal of Immunology, 2012, 188: 2847–2857.

**Abbreviations used in this article:** BMDM, bone marrow-derived macrophage; COX, cytochrome c oxidase; ETC, electron transfer chain; FCCP, carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone; MnSOD, manganese superoxide dismutase; mROS, mitochondrial reactive oxygen species; NRF-1, nuclear respiratory factor 1; OxPhos, oxidative phosphorylation; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1α; ROS, reactive oxygen species; T-fam, mitochondrial transcription factor A; TMRM, tetramethylrhodamine-methylster; TRIF, Toll/IL-1R domain-containing adapter-inducing IFN-β; WT, wild-type.

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LPS and Mitochondrial Bioenergetics

Dria, upregulation of ETC capacity, and generation of new mitochondria later in the disease process after acute insults such as hypoxemia or sepsis are important for survival (6–8). The exact mechanism underlying the recovery of bioenergetic failure after inflammatory insult is not well studied.

LPS stimulation under specific conditions leads to activation of the pro-survival cascade followed by growth and differentiation. For example, it has been shown that LPS may serve as a signal to promote preconditioning and to protect against reperfusion injury (9). This bifunctionality of LPS is neither well understood nor studied. Among numerous pathways activated through TLR-mediated signaling, the PI3K/AKT pathway plays a central role in the regulation of cell survival and proliferation (9, 10). Evidence suggests an important negative regulatory role for the PI3K/AKT pathway in innate immune responses to TLR agonists (11). Although growth factor-mediated PI3K activation and the subsequent AKT activation is well characterized, TLR-mediated AKT activation and its role in inflammation and mitochondrial recovery in response to TLR4 agonists is not well defined. It was shown that certain TLRs, such as TLR4, require the adaptor protein MyD88 and Toll/IL-1R domain-containing adapter-inducing IFN-β (TRIF) to interact and activate the PI3K/AKT pathway (12, 13). The PI3K/AKT pathway plays both proinflammatory and anti-inflammatory roles in TLR signaling (11, 14). The PI3K/AKT pathway may be a link between immune response through TLRs and restoring energy metabolism through mitochondrial biogenesis.

Recovery of energy capacity by biogenesis requires coordinated expression of genes encoded by two independent genomes (nuclear and mitochondrial), including transcription factor activation and their binding to promoter consensus sequences in nuclear genes encoding mitochondrial proteins (7, 15). Among the transcriptional regulators, the mitochondrial transcription factor A (T-fam), a nuclear-encoded protein, governs mitochondrial gene expression (16, 17). In addition, the peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) and nuclear respiratory factors 1 and 2 (NRF-1 and -2) are involved in nuclear respiratory gene expression and serve to integrate mitochondrial gene expression with a wide range of cellular functions (17, 18).

The exact molecular mechanism of LPS-mediated bioenergetic recovery is not known. In this study, using a murine macrophage cell line and macrophages lacking MyD88/TRIF, we investigated the effect of LPS on mitochondrial bioenergetics. We show that recovery of cellular energy perturbation after LPS treatment is closely related to PI3K/AKT activation, and inhibition of this pathway is directly related to bioenergetic failure and cell death. Using a genetic approach, we show that the AKT1 isoform plays a critical role in mitochondrial bioenergetics in response to TLR4. We present data that indicate that TLR4-mediated AKT activation requires adaptor protein MyD88 and TRIF. Such activation is critical for T-fam upregulation, and increased expression and activity of both COX subunit I (mitochondrially encoded) and COX subunit IV (nuclear encoded).

Materials and Methods

Chemicals

All chemicals were purchased from Sigma Chemical (St. Louis, MO) unless specified otherwise. Inhibitors (LY294002, wortmannin, okadaic acid) were purchased from Calbiochem (San Diego, CA). Ultrapure LPS was purchased from Invivogen (San Diego, CA). Anti-phospho-AKT and PI3K, total AKT, AKT1, AKT2, and β-actin Abs were purchased from Cell Signaling Technology (Beverly, CA). Abs against mitochondrial complexes, COX subunits I and IV (COX I and COX IV), were purchased from Mitosciences (Eugene, OR). Ab against manganese superoxide dismutase (MnSOD) was purchased from New England Biolab (Ipswich, MA). Anti-mouse IgG and anti-rabbit IgG HRP-linked Abs were purchased from Cell Signaling Technology (Beverly, CA), and anti-goat HRP was purchased from Bio-Rad (Hercules, CA).

Cell culture

The mouse macrophage cell line (B6-MCL) and MyD88/TRIF deficient cell line were generous gifts of Dr. Eicke Latz (University of Massachusetts Medical School, Worcester, MA). Both cell lines were generated from wild-type (WT) C57BL/6 mice as previously described (19), and MyD88/ TRIF2/2 macrophages were generated in the same background mice as previously described (20). B6-MCL and cells deficient in MyD88/TRIF were grown in IMDM supplemented with 10% heat-inactivated FCS, antibiotics, sodium pyruvate, nonessential amino acids, and 2-ME. RAW 264.7 cells were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in a 95% air, 5% CO2 humidified atmosphere at 37°C in RPMI medium supplemented with 1-glutamine, penicillin-streptomycin, and 10% FCS (Invitrogen, Carlsbad, CA).

Mice and macrophages

MyD88−/− and TRIF−/− mice were generated as previously described (21). AKT1−/− and AKT2−/− mice were generated as previously described (22, 23). All mice were backcrossed onto the C57BL/6 background at least 10 times. WT C57BL/6 mice were maintained at animal facilities at the University of Michigan (Ann Arbor, MI) and University of Pennsylvania (Philadelphia, PA). Animal studies were approved by the University of Michigan and University of Pennsylvania Committee on Use and Care of Animals.

Isolation of bone marrow-derived macrophages

Bone marrow-derived macrophages (BMDMs) from AKT1−/−, AKT2−/−, MyD88−/−, and TRIF−/− mice were prepared as described previously (24). In brief, femurs and tibias from 6- to 12-wk-old mice were dissected, and the bone marrow was flushed out. Bone marrow cells were cultured with IMDM supplemented with 30% L929 supernatant containing macrophage-stimulating factor, glutamine, sodium pyruvate, 10% heat-inactivated FBS (Life Technologies-BRL), and antibiotics for 5–7 d. Macrophages were replated at a density of 2 × 104 cells/well the day before the experiment.

Protein extraction and immunoblotting

Cells were harvested after the appropriate treatment and washed with PBS. Total cellular proteins were extracted after addition of a protease inhibitor mixture and antiphasatase I and II, by adding RIPA buffer (Sigma Aldrich). Protein concentrations of samples were measured with the BCA assay (Bio-Rad). Equal amounts of protein (5–30 μg) were mixed with sample volume of sample buffer (20% glycerol, 4% SDS, 10% 2-ME, 0.05% bromophenol blue, and 1.25 M Tris·HCl, pH 6.8), loaded on to a 10% SDS-polyacrylamide gel, and run at 40 mA for 3 h. Proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) for 30 min at 20 V using a SemiDry Transfer Cell (Bio-Rad). The polyvinylidene difluoride membrane was blocked with 5% dry milk in TBST (TBS with 0.1% Tween 20) for 1 h, washed, and incubated with primary Ab (diluted between 1/500 and 1/1000 in 5% dry milk in TBST) overnight at 4°C. The blots were washed four times with TBST and then incubated for 1 h with HRP-conjugated secondary anti-IgG Ab using a dilution between 1/5000 and 1/10000 in 5% dry milk in TBST. Membranes were washed four times in TBST. Immunoactive bands were visualized using a chemiluminescent substrate (ECL-Plus; GE Healthcare, Pittsburgh, PA). Quantitation of images was performed with Hybotl Cl film (Denville Scientific, Metuchen, NJ). OD analysis of signals was performed using ImageQuant software from Molecular Dynamics (version 5). Equal loading of the blots was shown by either total AKT, PI3K, or β-actin.

Cell viability

Cell viability was measured using the MTT assay as described previously (25). Cells equivalent to 1 × 103 ml−1 were seeded in 96-well cell culture plates and incubated for 24 h before treatment, as indicated in the Results. Absorbance was measured at 550 nm. Relative cell viability was calculated according to the formula: cell viability (%) = absorbance experimental/absorbance control × 100.

ATP assay

B6-MCL were cultured to 80% confluency and treated as indicated in each experiment. After completion of experiments, cells were collected by scraping and immediately stored in aliquots at –80°C until measurement.

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ATP was released using the boiling method, by addition of 300 μl boiling buffer (100 mM Tris–Cl [pH 7.75], 4 mM EDTA) and immediate transfer to a boiling water bath for 2 min (5). Samples were put on ice and sonicated for 10 s. Samples were diluted 500-fold, and 50 μl was used to determine the ATP concentration using the ATP bioluminescence assay kit HS II (Roche, Indianapolis, IN), according to the manufacturer’s protocol. Experiments were performed in triplicate, and data were standardized to the protein concentration using the DC protein assay kit (Bio-Rad).

Mitochondrial membrane potential measurements

The mitochondrial membrane potential (ΔΨm) of intact cells was measured as described previously (26) with modifications. Cultured cells were washed with PBS and trypsinized. The protein concentration of cells was adjusted to 0.2 mg/ml in DMEM without phenol red (Life Technologies-Invitrogen, Carlsbad, CA) and not supplemented with FBS and antibiotics. Tetramethylrhodamine-methylene (20 mM; TMRM; T-668; Molecular Probes-Invitrogen) was added to the cell suspension. As a control, the ΔΨm was dissipated using 1 μM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazonine (FCCP) and 500 μM oligomycin. Cells were incubated at 37°C for 30 min in the dark under slow rotation. Yellow fluorescence (excitation, 532-nm laser; emission, 585 nm; band pass, 42 nm) was measured using a BD FACSc Array (BD Biosciences, San Jose, CA), and data were analyzed with WinMDI version 2.9 software.

Measurement of nitrite

The nitrite concentration in the culture media was used as a measure of NO production (27). After stimulation/incubation, the generation of NO in the cell culture supernatants was determined by measuring nitrite accumulation in the medium using Griess reagent (1% sulfanilamide and 0.1% N-[1-naphthyl]-ethylenedia mine dihydrochloride in 5% H3PO4; Sigma). One hundred microliters of culture supernatant and 100 μl Griess reagent were mixed and incubated for 5 min. The absorption was measured in an automated plate reader at 540 nm. Sodium nitrite (NaNO2, Sigma) was used to generate a standard curve for quantification. Background nitrite was subtracted from the experimental values. Results for each condition were obtained from three separate measurements of identically treated wells, and the data are derived from four independent experiments.

ELISA

Murine TNF-α and IL-6 cytokine levels were measured in cell culture supernatants according to the manufacturer’s instructions (ELISA DuoKits; R&D Systems) as previously described (25).

Measurement of COX activity

After appropriate treatments of cell homogenates in incubation buffer (250 mM sucrose, 20 mM K-HEPES [pH 7.4], 10 mM MgSO4, 2 mM KH2PO4, 1 mM PMSF, 10 mM KF, 2 mM EGTA, 2 mM oligomycin), COX activity was analyzed in a closed 200-μl chamber equipped with a micro Clark-type oxygen electrode (Oxygraph system; Hansatech) as previously described (5). Measurements were performed in the absence of nucleotides. Measurements were carried out with 2 μM COX at 25°C in the presence of 20 mM ascorbate and increasing amounts of cow heart cytochrome c from 0 to 40 μM. Oxygen consumption was recorded and analyzed with the Oxygraph plus software. Turnover number is defined as consumed O2 (μM)/min/protein (mg) (5). Protein concentration was determined with the DC protein assay kit (Bio-Rad).

mROS measurements

Cells were stimulated with LPS (100 ng/ml) in the presence or absence of wortmannin for different time points. As positive control, cells were treated with anticytokine A (5 μM) for 30 min. To measure mROS, we incubated cells with MitoSOX (Invitrogen) at a final concentration of 1 μM in the dark for 20 min at 37°C. Cells were collected by centrifugation, washed three times in PBS, and immediately resuspended in cold PBS and subjected to FACS analysis. MitoSOX Red was excited by laser at 488 nm, and the data were collected at forward scatter, side scatter, 585/42 nm (FL2), and 670LP (FL3) channel. Experiments were performed at least in triplicate. Data are presented as fold change in mean intensity of MitoSOX fluorescence when compared with PBS alone.

RNA extraction and quantitative reverse transcriptase/real-time PCR

Total RNA was extracted using Stat 60 (Iso-Tex Diagnostics, Friendswood, TX) and reverse transcribed using the Reverse Transcription System (Promega, Madison, WI). The primers for amplification of T-fam, AKT, PGC-1, NRF-1, COX IV, and a reference gene (β-actin) were used to amplify the corresponding cDNAs. Using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), we performed quantitative analysis of mRNA expression with a MX3000p instrument (Stratagene, Santa Clara, CA). PCR amplification was performed in a total volume of 20 μl containing 2 μl total cDNA and 20 pg primers (Invitrogen). The PCR amplification protocol consisted of an initial incubation step for 10 min at 95°C, followed by 45 cycles of denaturation at 95°C for 10 s, annealing for 20 s at 60°C, and extension at 72°C for 20 s. Relative mRNA levels were calculated after normalizing to β-actin. Data were analyzed using the unpaired, two-tailed Student t-test, and the results were expressed as relative fold change.

Statistical analysis

Statistical analyses were performed using SPSS software, version 18.0 (Chicago, IL). One-way ANOVA test and post hoc repeated-measures comparisons (least significant difference) were performed on all obtained data. ELISA, MITT, ROS, and quantitative real-time RT-PCR results were expressed as mean ± SEM. For all analyses, two-tailed p values <0.05 were considered significant.

Results

LPS treatment induces COX upregulation at the protein and level

Because macrophages play a central role in recognition of TLR ligands and initiating inflammatory responses, as well as ROS production, we have chosen murine macrophages to determine the effects of LPS on the ETC. To show reproducibility in independent systems, we conducted most experiments in two different cell lines. Murine macrophage cells (B6-MCL) were generated as previously described (19). We also confirmed the results using RAW 264.7 cells. In all experiments, cells were grown to reach 80% confluency. B6-MCL cells were treated with 100 ng/ml ultrapure LPS (to assure only TLR4-mediated signaling) for different periods as indicated. Because the exact effects of LPS on the ETC and mitochondrial transcription factors in immune cells are unknown, we first investigated the kinetic responses of important members of the ETC, including COX subunits I and IV, as well as mitochondrial transcription factors NRF-1 and T-fam, in response to LPS stimulation. Total cell lysates were subjected to immunoblotting using specific Abs against COX subunits I and IV (mitochondrially encoded) and IV (nuclear encoded), NRF-1, and T-fam. As shown in Fig. 1A, LPS induction expression of both subunits of COX in a time-dependent manner. We observed increased protein expression for COX IV starting at 3 h after LPS challenge, whereas increased expression of T-fam was observed as early as 90 min after treatment. LPS stimulation had only a minimal effect on NRF-1 protein expression at a later time point (after 24 h; Fig. 1A). Fig. 1B summarizes the densitometric analysis of four different experiments. These data indicate that LPS increased protein expression of COX, and that there was a time-dependent protein expression of mitochondrial transcription factors in response to LPS. To assess the dose-dependent upregulation of COX I, we treated cells with different log concentrations of LPS for 6 h. Supplemental Fig. 1 shows a dose-dependent but nonlinear response to LPS in terms of COX I upregulation. Fig. 1C shows means of relative gene expression for T-fam, COX IV, and NRF-1 after 2 h LPS treatment. Although relative gene expression increased in response to LPS for all these genes, there was more variation in terms of protein expression as determined by immunoblotting.
LPS alters mitochondrial membrane potential, COX activity, and ATP production

The mitochondrial membrane potential ($\Delta \Psi_m$) is generated through pumping of protons across the mitochondrial inner membrane by ETC complexes I, III, and IV (COX) producing the proton motive force used by ATP synthase to generate ATP. Previous studies have shown that $\Delta \Psi_m$ of monocytes is decreased in severe sepsis, and that this is associated with cell death (29). We analyzed the kinetic response of $\Delta \Psi_m$ in response to LPS treatment using a membrane potential-sensitive fluorescent probe (TMRM) and flow cytometry. As controls, the $\Delta \Psi_m$ was dissipated using 1 mM FCCP, resulting in a left shift of TMRM fluorescence, or increased $\Delta \Psi_m$ using 0.5 mM oligomycin with a right shift of fluorescence (Fig. 2A). LPS treatment led to an early (at 30 min) reduction in TMRM fluorescence indicating a decreased $\Delta \Psi_m$ (Fig. 2B) followed by a recovery of $\Delta \Psi_m$ starting at 3 h after LPS treatment (Fig. 2C). At 6 h after LPS treatment, $\Delta \Psi_m$ recovered and was even slightly higher compared with untreated cells (Fig. 2D), consistent with increased protein expression for COX subunits at this time point (Fig. 1A, 1B).

Next, we asked whether LPS-mediated increases in protein expression for COX I/IV are associated with a change in both the activity of COX and changes of ATP content of cells. Murine macrophages (B6-MCL) were cultured in the presence of LPS (100 ng/ml) for 6 h as COX I and T-fam levels peaked at that time point (Fig. 1B). After solubilization, COX activity was measured by adding increasing amounts of its substrate cytochrome c. As shown in Fig. 2E, LPS treatment led to a 56% increase of COX activity at maximal turnover.

To further assess the link between the change of protein expression in response to LPS treatment and changes in $\Delta \Psi_m$, we measured the ATP content of B6-MCL cells. Fig. 2F shows the time-dependent changes of ATP levels after LPS treatment of B6-MCL cells with a significant increase of ATP after 6 h and a further increase after 12 h. At 3 h, ATP levels were slightly decreased, then increased >3-fold and 5-fold after 6 and 12 h, respectively. These data confirm the relationship between cellular ATP and mitochondrial OxPhos capacity represented in the form of COX activity and expression.

**LPS treatment activates both PI3K and AKT**

The PI3K and AKT pathways are involved in many important cellular processes including cell growth, cell survival, and apoptosis (30). Because of the central role of mitochondria as mediators of cell survival and apoptosis, we investigated the effect of LPS-mediated AKT activation on the upregulation of T-fam, as well as COX I. First, we examined the time-dependent phosphorylation of PI3K and AKT. B6-MCL cells were challenged with LPS (100 ng/ml) for different time points as indicated (Fig. 3A). Total cell lysates were subjected to Western blot analysis using a specific Ab against the phosphorylated form of the regulatory domain of PI3K (phospho-p85) and an Ab detecting the Ser473-phosphorylated form of AKT. Fig. 3A shows rapid phosphorylation of p85 5 min after LPS challenge followed by AKT phosphorylation on Ser473. Next, we explored specific inhibitors of the PI3K/AKT pathway and their effect on LPS-mediated p85 and AKT phosphorylation. B6-MCL cells were treated with either LY294002 or wortmannin 30 min before LPS challenge or kept in media without treatment. Fig. 3B shows that both inhibitors LY294002 and wortmannin prevented phosphorylation of p85 and AKT, indicating that LPS-induced AKT phosphorylation is PI3K dependent.

**Inhibition of the PI3K/AKT pathway abrogates LPS-mediated upregulation of COX and T-fam**

We next assessed the effect of inhibition of the PI3K/AKT pathway on protein expression for COX I and T-fam. B6-MCL cells were treated with vehicle (DMSO), LY294002, or wortmannin 30 min before LPS challenge. Immunoblotting on total cell lysates was performed using specific Abs against NRF-1, COX I, and T-fam. Fig. 4 shows that pretreatment with wortmannin inhibited LPS-mediated upregulation of both COX I and T-fam with a minimal effect on NRF-1 expression. Similar results were observed in the presence of LY294002 (Supplemental Fig. 2). We observed an increase in expression for both proteins when cells were exposed to wortmannin or LY294002 alone. In the presence of AKT inhibitors, however, LPS-mediated protein expression for T-fam and COX I was strongly diminished. These data suggest that the activation of the PI3K/AKT pathway is required for LPS-mediated upregulation of COX subunit I and T-fam. It is known that protein phosphatase 2A plays a pivotal role in dephosphorylation of AKT, and thereby diminishes its activity (31). To confirm that phosphorylation of AKT plays a critical role in LPS-mediated upreg-
ulation of COX I and T-fam, we evaluated the effect of a protein phosphatase 2A inhibitor (okadaic acid) to prevent the dephosphorylation of AKT. Cells were incubated with okadaic acid (10 nM) 30 min before LPS treatment for different time points. As expected, okadaic acid treatment led to AKT phosphorylation alone and to a prolonged activation of AKT in the presence of LPS (data not shown). Next, we evaluated the effect of okadaic acid in the presence and absence of LPS on COX I. Okadaic acid pretreatment led to an enhanced LPS effect in terms of COX I upregulation (Fig. 4C), supporting the role of active AKT in this process.

To further confirm that the AKT pathway is a distinct intracellular signal and specific to LPS-mediated upregulation of COX I and T-fam, we assessed the effect of LPS challenge in the presence or absence of LY294002 or wortmannin. B6-MCL cells were cultured and pretreated with either inhibitor for 30 min before LPS treatment. A 6-h LPS treatment duration was chosen because it produced a robust induction of MnSOD and COX I expression. MnSOD expression was analyzed by immunoblotting of total cell lysates using a specific Ab. As shown in Fig. 4D (lower panel), neither of the two inhibitors prevented the LPS-mediated protein expression for MnSOD. Interestingly, the presence of wortmannin alone induced MnSOD, and a similar effect was found for LY294002. These data suggest that LPS-mediated upregulation of MnSOD versus COX I and T-fam occurs through distinct intracellular signal transduction pathways independent of the PI3K/AKT pathway.

**AKT inhibition and failure of COX I and T-fam upregulation in response to LPS is associated with increased TNF-α, NO, and cell death, and decreased ROS and ATP levels**

It has been suggested that the AKT pathway can play a positive and a negative role in TLR-mediated cytokine production (11, 13). We investigated the effect of AKT inhibition on the response of LPS stimulation in our cell system. LPS stimulation (100 ng/ml for 24 h) enhanced TNF-α secretion in supernatants, but secretion of TNF-α was significantly higher (p < 0.05) in pretreated cells with wortmannin (Fig. 5A). Some studies suggested that NO may induce mitochondrial biogenesis (NRF-1, T-fam, and COX) and MnSOD expression (35, 36). Hence we investigated whether the inhibition of the AKT pathway in LPS-stimulated macrophages
changes the NO levels in these cells. Fig. 5B shows the effect of LPS in the presence and absence of wortmannin on cumulative nitrite production 24 h after LPS treatment as an indirect measure of NO production. As expected, LPS treatment alone resulted in a 50% increase of nitrite levels. Interestingly, wortmannin alone had no effect on nitrite production, whereas wortmannin in combination with LPS led to a 2-fold increase in nitrate levels (Fig. 5B). LPS stimulation of macrophages induces production of ROS such as peroxides and superoxide. ROS play an important role in cellular functions via the activation of signaling cascades, and it has been suggested that ROS affect mitochondrial biogenesis, morphology, and function (37). Hence we investigated the effect of LPS stimulation in the presence and absence of wortmannin. Murine macrophages (B6-MCL) were treated with LY294002 (50 ng/ml) or wortmannin (100 ng/ml) for 30 min before adding LPS (100 ng/ml) to the media for 6 h. After incubation, whole-cell extracts were prepared and 20 μg total protein was subjected to SDS-PAGE and Western blot analysis for NRF-1, COX I, and β-actin as loading control. The presence of wortmannin reduced mROS. However, this mROS reduction was associated with increased cell death (Fig. 5E). These data suggest that mitochondrial biogenesis is required for mROS production. By inhibiting the AKT pathway, mitochondrial re-

generation is diminished, leading to decreased mROS production and an increase in cell death. Next, we assessed whether the inhibition of the AKT pathway translates into changes of ATP content of the cells. B6-MCL cells were cultured and pretreated for 30 min with either LY294002 or wortmannin before stimulation with LPS (100 ng/ml) for 6 h. As shown in Fig. 5D, ATP levels were significantly decreased in the presence of both inhibitors (LY294002 or wortmannin), whereas LPS treatment led to an increase in cellular ATP content. To further explore the biological effect of inhibition of AKT and subsequent failure of upregulation of protein expression for COX I and T-fam, we assessed cell viability using the tetrazolium dye (MTT) assay (25). Fig. 5E shows that LPS treatment alone at the concentration applied throughout this study did not change viability. Using AKT pathway inhibitors LY294002 or wortmannin led to a minimal decrease in cell viability, whereas LPS challenge in the presence of wortmannin reduced mROS.
of AKT pathway inhibitor led to a significant loss of cell viability (p < 0.001). Collectively, these data suggest that LPS-mediated upregulation of COX requires PI3K/AKT activation, and that this activation is important in the prevention of cell death and excessive proinflammatory mediators (NO and TNF-α).

**LPS-mediated AKT activation and subsequent COX I and T-fam upregulation are MyD88/TRIF dependent**

Although several studies have shown that LPS stimulation leads to PI3K/AKT activation, the mechanism by which LPS activates AKT is not well understood and remains controversial. In addition, PI3K/AKT pathway activation can occur in response to diverse growth factors and ligand stimulation through diverse receptor activation (30). Macrophages from MyD88/TRIF−/- mice are defective in many TLR4-mediated responses, such as LPS-induced secretion of cytokines (e.g., IL-6, TNF-α, and IL-1β) (38). A previous study has shown that LPS-mediated AKT activation is TLR4/MyD88 dependent and requires PI3K activation (13). Therefore, we assessed whether the LPS-mediated increased expression for COX I and T-fam requires MyD88/TRIF adaptor proteins. Phenotypes of WT and MyD88/TRIF−/- cells were verified by a range of functional parameters, including responsiveness to TLR4 and cytokine production (Supplemental Fig. 3). We stimulated side-by-side WT and MyD88/TRIF−/- cells with LPS for different time points to assess AKT activation. Fig. 6A shows phosphorylation of p85 and AKT in response to LPS stimulation in WT and MyD88/TRIF−/- cells (90 min, 3 h, and 6 h). As shown, WT cells responded to LPS stimulation with increased expression of both T-fam and COX I, whereas MyD88/TRIF−/- cells failed to upregulate either one of them. These data suggest that LPS-induced activation of the PI3K/AKT pathway is dependent on the presence of MyD88/TRIF adaptor protein. To determine whether the lack of AKT activation in MyD88/TRIF−/- would parallel the effect on LPS-mediated T-fam and COX I expression, we stimulated WT and MyD88/TRIF−/- cells side by side with LPS for different time points (90 min, 3 h, and 6 h). As shown in Fig. 6B, WT cells responded to LPS treatment with increased expression of both T-fam and COX I, whereas MyD88/TRIF−/- cells failed to upregulate either one of them. These data suggest that LPS-induced phosphorylation of AKT on Ser473 requires the presence of MyD88 and TRIF adaptor proteins, and that the LPS-induced expressions of T-fam and COX I require activation of the PI3K/AKT pathway. Next, we asked whether absence of either MyD88 or TRIF would be sufficient to prevent LPS-mediated PI3K/AKT pathway activation and subsequent T-fam and COX expression. BMDMs from WT and macrophages deficient for either MyD88 and MyD88/TRIF would be sufficient to prevent LPS-mediated PI3K/AKT pathway activation and subsequent T-fam and COX expression.
or TRIF were stimulated with LPS for the indicated times. As shown, LPS-mediated PI3K/AKT pathway activation required both MyD88 and TRIF. In macrophages lacking TRIF, we observed an attenuated and delayed response in AKT phosphorylation, whereas cells lacking MyD88 did not respond to LPS with AKT phosphorylation (Fig. 6C). Similarly, WT macrophages responded to LPS with an increased expression for T-fam (Fig. 6D) and COX IV (Fig. 6E), whereas macrophages deficient in MyD88 or TRIF did not increase expression for T-fam (Fig. 6D) and COX IV (Fig. 6E).

Expression of AKT1 is pivotal in LPS-mediated COX upregulation in BMDMs

Three AKT isoforms have been described with distinct biological functions. To further investigate mechanistically which AKT isoform plays a major role in the LPS response and mitochondrial biogenesis in macrophages, we used cells derived from knockout mice lacking AKT1 or AKT2. Whereas AKT1 and AKT2 are ubiquitously expressed, including immune cells, endothelial cells, and in hematopoietic cells, AKT3 expression is largely confined to the testes and brain (39–41). Thus, we focused on AKT1 and AKT2 isoforms. BMDMs from mice deficient in AKT1, AKT2, or WT mice were cultured side by side under equal conditions. Cells were treated with LPS (100 ng/ml) for different time points, and whole-cell extracts were subjected to immunoblotting. As seen in Fig. 7A, LPS stimulation of BMDMs isolated from WT AKT1−/− and AKT2−/− led to rapid phosphorylation of p85 and AKT phosphorylation on phospho-Ser473. This Ab detects phosphorylated AKT on Ser473 regardless of the isoforms expressed (either AKT1, AKT2, or AKT3). Next, we used isoform-specific AKT1 and AKT2 Abs to detect the level of AKT1 and AKT2 expression in WT, AKT1−/−, and AKT2−/− BMDMs. Fig. 7B confirmed the lack of expression of AKT1 protein in AKT1−/− and of AKT2 in AKT2−/− macrophages, respectively. As described earlier, in our cell model, we detected the highest expression for COX 6 h after LPS treatment. Thus, immunoblotting was done using the 6 h time point after LPS treatment. Fig. 7C shows that the cells lacking AKT1 had severe impairment in COX I and COX IV expression in WT after 6 h, TRIF−/− and MyD88−/− cells did not show any expression of COX IV.

FIGURE 6. LPS-induced COX I and T-fam expression is MyD88/TRIF dependent. (A) WT murine macrophages and MyD88−/− TRIF−/− cells were cultured side by side in the presence and absence of LPS (100 ng/ml) for 30 or 60 min. Total cell lysates were prepared, and 20 μg total protein was subjected to SDS-PAGE and Western blot analysis using phospho-epitope–specific Abs for p85 and AKT (Ser473). Equal loading of protein was confirmed using total p85 and AKT Abs. (B) WT murine macrophages and MyD88/TRIF−/− cells were cultured side by side in the presence and absence of LPS for 90 min, 3 h, and 6 h. Total cell lysates were prepared and 20 μg total protein was subjected to SDS-PAGE and Western blot analysis using Ab against T-fam and COX I. Equal loading of protein was confirmed using β-actin Ab. WT cells responded to LPS stimulation with a significant increase in T-fam starting at 90 min throughout 6 h after treatment with LPS. Upregulation of COX I in WT was seen starting at 3 h after LPS stimulation. In contrast, MyD88/TRIF−/− cells did not respond to LPS with increased expression for T-fam and COX I. (C) BMDMs isolated from WT, TRIF−/−, or MyD88-deficient mice were cultured in parallel with LPS for 5, 30, and 60 min. Total cell lysates were prepared, and 20 μg total protein was subjected to SDS-PAGE and Western blot analysis using phospho-epitope–specific Abs for p85 and AKT (Ser473). Equal loading was confirmed using total AKT and total p85 Abs. LPS treatment evoked a weak and delayed AKT phosphorylation in TRIF−/− cells, but MyD88-deficient cells did not show any response to LPS treatment. (D) BMDMs isolated from WT, TRIF−/−, or MyD88-deficient mice were either kept in media or treated with LPS for 3 h. Twenty micrograms of total protein was subjected to SDS-PAGE and Western blot analysis using T-fam Ab. Equal loading was confirmed using β-actin Ab. Although LPS treatment led to an increase in T-fam expression in WT, neither TRIF−/− nor MyD88−/− cells responded with an increased expression for T-Fam. (E) BMDMs isolated from WT, TRIF−/−, or MyD88-deficient mice were either kept in media or treated with LPS for 3 and 6 h. Twenty micrograms of total protein was subjected to SDS-PAGE and Western blot analysis using a COX IV Ab. Equal loading was confirmed using total AKT Ab. Although LPS treatment led to an increase in COX IV expression in WT after 6 h, TRIF−/− and MyD88−/− cells did not show any expression of COX IV.
AKT1 isoform plays a central role in LPS-mediated COX I and IV expression, whereas AKT2 contributes only to COX IV upregulation by LPS in BMDMs.

Discussion

In this study, we analyzed the molecular mechanisms underlying the LPS-mediated upregulation of ETC components focusing on COX and mitochondrial transcription factors (T-fam and NRF-1) as markers of mitochondrial biogenesis. We show that LPS-mediated upregulation of COX and T-fam is dependent on AKT phosphorylation (Figs. 1, 3), and that inhibition of the PI3K/AKT pathway leads to abrogation of this effect (Fig. 3). Increased expression of COX subunits I and IV in response to LPS challenge was associated with restoration of COX activity and increased cellular ATP levels (Fig. 2). Inhibition of the PI3K/AKT pathway abrogated the effect of LPS on both COX and T-fam, and led to decreased ATP production and increased cell death. We also show that the effect of LPS on both COX and T-fam is dependent on MyD88 and TRIF adaptor proteins. LPS challenge of MyD88/ TRIF knockout cells failed to phosphorylate AKT and upregulate T-fam and COX (Fig. 6A, 6B). Cells lacking MyD88 did not respond to LPS with AKT activation, whereas macrophages deficient in TRIF showed a delayed AKT activation (Fig. 6C). Neither MyD88 nor TRIF deficient cells showed an increase in T-fam or COX expression (Fig. 6D, 6E). These data suggest that MyD88/ TRIF is required both for AKT phosphorylation and for LPS-mediated increased expression of COX and T-fam. Importantly, macrophages from AKT1−/− mice showed a lack of response to LPS in terms of upregulation of COX I and IV (Fig. 7C). These data collectively indicate that AKT1 regulates and maintains mitochondrial function in response to TLR4 agonists.

Mitochondria are highly dynamic organelles and essential in providing most cellular ATP. They have emerged as important integrators of several vital signaling cascades (42). Mitochondrial membrane potential (ΔΨm) builds the proton motive force for generation of ATP through OxPhos and is critical for the integrity of mitochondrial function. Bacteria and several bacterial products such as LPS or bacterial toxins have been shown to affect ΔΨm and alter the OxPhos, as well as mROS production (3, 43, 44). We show in this study the dynamic changes of ΔΨm of murine macrophages in response to LPS, an initial decrease of ΔΨm followed by a delayed restoration of ΔΨm that mirrored the upregulation of COX (Figs. 1, 2). This process may be specifically important in the response of macrophages to bacterial products and may facilitate the required energy for cytokine production, as well as phagocytic activity of these cells. One recent study found that mROS production in response to specific TLRs is required for phagocytic activity of macrophages (3). Another study found that AKT1 and AKT2 isoforms regulate the intracellular ROS content in hematopoietic stem cells necessary for their function and proliferation (40).

COX biogenesis is a critical part of mitochondrial biogenesis. Among the 13 subunits of COX, the largest subunits, COX I–III, are encoded by the mitochondrial DNA and regulated at the transcriptional level by T-fam (45). COX is the terminal enzyme of the ETC and plays a pivotal role in electron transfer to molecular oxygen, and generation and maintenance of ΔΨm and ATP. COX activity, as well as expression, is tightly regulated by metabolic cellular demand (3, 42). Our laboratory has shown that TNF-α challenge leads to rapid inhibition of COX, and this inhibition is due to tyrosine phosphorylation of the catalytic subunit I of COX (5). This finding may explain, at least in part, the observed metabolic switch from aerobic to glycolytic energy metabolism during sepsis. However, not all septic patients experience organ failure with a poor outcome. Thus, additional mechanisms may be essential for the recovery from sepsis. Our current data indicate that LPS stimulation of murine macrophages leads to 20-fold increase in expression of COX I (Fig. 1A), and that such an increase was associated with a 56% increase in COX activity.

Evidence suggest that nonsurvivors of sepsis exhibit more extensive mitochondrial damage and failure of cellular energetics compared with survivors as determined from muscle biopsies of critically ill patients (46). Interestingly, Staphylococcus aureus-induced sepsis in mice with a lower bacterial load led to a recovery from bioenergetic failure with orderly (sequential) induction of NRF-1, NRF-2, PGC-1, and T-fam m-RNAs in the liver of septic animals (47), whereas animals with sepsis induced with a higher bacterial count failed to recover and exhibited lack of mitochondrial biogenesis. We have observed that treatment of macrophages with a higher dose of LPS (>1 μg/ml) failed to increase expression for COX I (Supplemental Fig. 1) and T-fam. In contrast with Haden et al.’s observation (47), we show expression at both the gene and protein levels that murine macrophages exhibit higher baseline NRF-1 mRNA expression and minimal change in response to LPS treatment as compared with PGC-1α (data not shown), T-fam, and COX I and IV. It should be
We propose that the enhanced CM-H2DCFDA probe in the adaptor protein MyD88/TRIF (Fig. 6A, 6B). Previously, it has not sufficient to upregulate COX in response to LPS. Our results ligands (13). However, PI3K activation in absence of AKT1 was apoptosis (30). It has been shown that the PI3K/AKT pathway is important cellular pathways including cell growth, migration, and response in AKT1 observations because our data mechanistically suggest that sup-
more susceptible to apoptosis and exhibited defective clearance AKT1 with severe atherosclerosis in a mice model of coronary particular, COX. Previously, AKT1 has been implicated as a crit-
promote survival (51, 52). It has been shown that mammalian cells energetics necessary to carry out the killing of pathogens and to death (Fig. 5E).

LPS challenge results in activation of the PI3K/AKT pathway, which is required for activation of survival pathways (48). Both PI3K and AKT pathway activation have been reported to be associated with TLR2, TLR3, TLR4, and TLR5 in different cells (13, 49). Although it has been suggested that the activation of the PI3K/AKT pathway has a negative regulatory function on TLR ligation, the level of interaction between the TLRs and the PI3K/AKT pathway is not well understood (13, 49, 50).

Some studies suggested that the inhibition of this pathway leads to decreased phosphatase activity, resulting in an increased production of inflammatory mediators such as IL-1β and IL-6 with an adverse effect on cell survival (11). Our data indicate that inhibition of AKT pathway in presence of LPS increases proinflammatory cytokines (IL-6 and TNF-α), ROS, NO, and decreased cell survival in murine macrophages, and these findings paralleled the lack of mitochondrial biogenesis. Although we have observed increased ROS (both cellular and mROS) in response to LPS, the presence of wortmannin led to an increased fluorescence in CM-H2DCFDA (data not shown) but a decrease in the MitoSox signal.

We propose that the enhanced CM-H2DCFDA probe in the presence of wortmannin is due to increased peroxynitrite in the cytoplasm because of cell death processes. As shown in Fig. 5C, wortmannin led to a decreased mROS, but also increased cell death (Fig. 5E).

Moreover, in macrophages, TLR ligation initiates autophagy, a process that provides metabolic intermediates to maintain bioenergetics necessary to carry out the killing of pathogens and to promote survival (51, 52). It has been shown that mammalian cells with hyperactive AKT accumulate ATP, whereas cells with reduced AKT activity exhibit markedly reduced ATP content (53). Such effects have been explained by the role of activated AKT on mitochondria-associated heoxinokase and glycolysis (54). Our data indicate that presence of AKT1 and its activation in response to LPS is critical for the upregulation of mitochondrial complexes, in particular, COX. Previously, AKT1 has been implicated as a critical regulator in homeostasis of hematopoietic stem cell function and ROS (40). Interestingly, another study associated the loss of AKT1 with severe atherosclerosis in a mice model of coronary artery disease (55).

Macrophages of animals lacking AKT1 were more susceptible to apoptosis and exhibited defective clearance of dead foam cells (55). Our study sheds some light on these observations because our data mechanistically suggest that suppressed mitochondrial energetics cause an impaired stress response in AKT1−/− macrophages. PI3K is involved in many important cellular pathways including cell growth, migration, and apoptosis (30). It has been shown that the PI3K/AKT pathway is activated in response to growth factors but also in response to TLR ligands (13). However, PI3K activation in absence of AKT1 was not sufficient to upregulate COX in response to LPS. Our results indicate that the PI3K/AKT pathway is required for upregulation of mitochondrial OxPhos and T-fam, and both processes require the adaptor protein MyD88/TRIF (Fig. 6A, 6B).

been shown that MyD88-deficient animals exhibit poorer outcome when infected with various microorganisms that were associated with an enhanced bacterial burden (11, 56–58). This paradigm, at least in part, may be explained by their inability to enhance mitochondrial biogenesis in response to microbial stimuli.

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Disclosures

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