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CaMKIV-Dependent Preservation of mTOR Expression Is Required for Autophagy during Lipopolysaccharide-Induced Inflammation and Acute Kidney Injury

Xianghong Zhang,* Gina M. Howell,* Lanping Guo,* Richard D. Collage,* Patricia A. Loughran,*++ Brian S. Zuckerbraun,* and Matthew R. Rosengart*,‡

Autophagy, an evolutionarily conserved homeostasis process regulating biomass quantity and quality, plays a critical role in the host response to sepsis. Recent studies show its calcium dependence, but the calcium-sensitive regulatory cascades have not been defined. In this study, we describe a novel mechanism in which calcium/calmodulin-dependent protein kinase IV (CaMKIV), through inhibitory serine phosphorylation of GSK-3β and inhibition of FBXW7 recruitment, prevents ubiquitin proteosomal degradation of mammalian target of rapamycin (mTOR) and thereby augments autophagy in both the macrophage and the kidney. Under the conditions of sepsis studied, mTOR expression and activity were requisite for autophagy, a paradigm countering the current perspective that prototypically, mTOR inhibition induces autophagy. CaMKIV–mTOR-dependent autophagy was fundamentally important for IL-6 production in vitro and in vivo. Similar mechanisms were operant in the kidney during endotoxemia and served a cytoprotective role in mitigating acute kidney injury. Thus, CaMKIV–mTOR-dependent autophagy is conserved in both immune and nonimmune/parenchymal cells and is fundamental for the respective functional and adaptive responses to septic insult. The Journal of Immunology, 2014, 193: 2405–2415.

Autophagy is an ancient homeostatic process that regulates cellular biomass quantity, quality, and distribution through the recycling of cytoplasmic proteins and organelles (1, 2). But autophagy may also be acutely induced as an adaptive response to stress that enables cells to reuse cytoplasmic components to support vital functions. In the setting of nutrient deprivation, the mechanisms orchestrating this dynamic process involve the main growth rheostat mammalian target of rapamycin (mTOR) and AMP-activated protein kinase (AMPK), a sensor of cellular energy status (2–8). Prototypically, AMPK, sensing reduced cellular energy (i.e., elevated AMP), inhibits mTOR, a negative regulator of autophagy, thereby inducing autophagy (3, 8–10).

Although the quintessential function of autophagy is to digest and recycle portions of the cytoplasm during starvation, recent data support a fundamental role in innate immunity as an antimicrobial effector. In macrophages (Mφ), LPS induces autophagy through mechanisms dependent on TLR4, Toll/IL-1R domain-containing adapter inducing IFN-β, RIP1, and p38 MAPK (14, 16). Biologically, it has been shown to be important in the elimination of intracellular microbes, contribute to MHC class II presentation, and assist pattern recognition receptors by delivering cytosolic pathogen-associated molecular patterns to endosomal TLRs (15, 17, 18). Interestingly, nonimmune cells, such as hepatocytes and renal tubular cells, also exhibit TLR signaling and respond to the stress of sepsis or LPS by inducing autophagy (19–24). However, a complete understanding of the signaling mechanisms regulating autophagy during sepsis and the functional role of autophagy in cell survival and recovery of organ function has yet to be developed.

The calcium/calmodulin-dependent protein kinases (CaMK), a family of serine/threonine kinases responsive to intracellular Ca2+ concentration [Ca2+]i, are integral to the immune response, mediating Ca2+-dependent Mφ function and regulating septic inflammation (25–27). Recently, we and others have defined regulatory roles of this multifunctional family in autophagy. CaMKKβ, in response to elevations in cytosolic calcium, functions as an upstream kinase for AMPK-dependent autophagy (28). In the Mφ, LPS induces a CaMKI–AMPK complex that regulates autophagy (29). In this circumstance, however, CaMKI–AMPK-dependent autophagy occurs independent of mTOR inhibition. These data suggest that for some cell types, such as the Mφ, concomitant autophagy and mTOR activity are necessary for specific cellular function.

In this study, we further characterize the regulatory role of the CaMK during autophagy, focusing upon the downstream CaMKIV. Our data suggest that CaMKIV negatively regulates ubiquitin-mediated degradation of mTOR and titrates basal mTOR expression, which serves a permissive role for LPS-induced autophagy. This mechanism is not specific to the Mφ, as it is also observed in the kidney during endotoxemia and appears to serve a cell-specific functional and adaptive response to the stress of sepsis.

Materials and Methods
Reagents and Abs
Ultra-pure LPS (Escherichia coli 0111:B4) was obtained from List Biologicals (Campbell, CA). ST0609 (7-oxo-7H-benzimidazo[2,1-a]benzene) isouquinoline-3-carboxylic acid–acetic acid was obtained from Calbiochem.
(San Diego, CA) and used at concentrations of 10–20 μM. STO609 is highly specific for CaMKII; it has an in vitro IC50 of 0.13–0.38 μM for CaMKII and 32 μM for CaMK II with little or no inhibition of CaMKI, CaMK IV, protein kinase A, protein kinase C, ERK, or myosin L-chain kinase (30). MG132 was purchased from Calbiochem (San Diego, CA) and used at a concentration of 10 μM. Abs for mTOR, p-Ser 9–GSK3β, GSK3β, p-Thr389 p70 S6 kinase, and p-Ser235/236 S6 were obtained from Cell Signaling Technology (Danvers, MA). Abs for ATG7, ATG5-12, LC3B, ubiquitin, FBXW7, actin, and tubulin were obtained from Abcam (Cambridge, MA).

**Animal experimentation**

We performed all animal experiments in accordance with the National Institutes of Health guidelines under protocols approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. We purchased 6–8-week-old C57BL/6J and B6.129 × 1-Camk4tm1Tch1 (CaMKIVfl/fl) male mice from The Jackson Laboratory (Bar Harbor, ME) (26, 31, 32). The CaMKIVfl/fl strain is derived from a C57BL/6l background; these mice are viable, fertile, normal in size, and do not display any gross physical or behavioral abnormalities. We randomly assigned groups of mice from each strain to a specific experiment.

Mice were anesthetized using a mixture of isoflurane and oxygenbled in at 3.5 l/min. LPS (List Biologicals) dissolved in D/Nase/RNase-free distilled water (1 mg/ml) was instilled i.p. at a concentration of 1.5 or 5 mg/kg. Animals were euthanized 18 or 48 h after LPS. Blood was isolated by tracardiac puncture, and the kidneys were harvested. Whole blood was stored overnight at 4˚C and the serum isolated by centrifugation at 5000 g for 10 min.

For all studies, one investigator performed the surgical experimentation and collected the samples. The data were then analyzed by an investigator blinded to the specific treatment.

**Cell isolation and treatment**

The murine Mβ cell line RAW 264.7 (American Type Culture Collection, Rockville, MD) was grown in DMEM (BioWhittaker, Walkersville, MD) supplemented with 10% FCS (Sigma-Aldrich, San Diego, CA), 50 U/ml penicillin, and 50 μg/ml streptomycin (Cellgro Mediatech. Kansas City, MO). Peritoneal Mβ (pMβ) were isolated from C57BL/6 mice by lavaging the peritoneal cavity with five 5-ml (25 ml total) aliquots of 4˚C PBS (26, 33). The lavage was centrifuged at 300 × g for 5 min, and the cells were resuspended in RPMI 1640 supplemented with 10% FCS, 50 U/ml penicillin, and 50 μg/ml streptomycin and plated. Selected cell populations were treated with STO609 (10 μM) for 1–4 h or MG132 (10 μM) for 6 h, as described in the figure legends. DMSO, at concentrations similar to comparative experiments, was used as an appropriate control and never exceeded 0.2%. Selected cells were then treated with LPS (100 ng/ml) for the durations described in the figure legends.

**Small interfering RNA**

RAW 264.7 Mβ (2 × 105), murine peritoneal Mβ (1 × 105), or HCK-8 (2 × 103) were plated in 0.5 ml growth medium (without antibiotics) in 24-well plates, resulting in 30, 80, or 30% confluence, respectively. Nontarget and mTOR small interfering RNA (siRNA) were obtained from Dharmacon (Lafayette, CO). We used the Smartpool siRNA from Dharmacon that incorporates four separate siRNA sequences for each targeted protein was determined by immunoblot. All experiments and cell number determinations were performed in triplicate.

**Plasmid construction and transfection**

Plasmids encoding a constitutively active CaMKIV (CaMKIV-dCT) or a kinase-inactive CaMKIV-dCTK75E mutant were generous gifts from Dr. Douglas Black (Yale University, New Haven, CT) (26). CaMKIV-dCT contains a C-terminally truncated version of the CaMKIV encoding gene, truncated to 317 and an N-terminal FLAG epitope. CaMKIV-dCTK75E was constructed by changing lysine 75 to glutamate in CaMKIV, which negatively affects ATP binding at the catalytic site. For transient transfection, RAW 264.7 cells or pMβ were seeded in a 24-well plate at 3 × 104 or 1 × 105 cells/well. After 2 h of adhesion, Mβ were transfected with 1 μg plasmid CaMKIV-dCT or CaMKIV-dCTK75E using the Lipofectamine 2000 reagent according to the instructions specified by the manufacturer (Life Technologies, Carlsbad, CA). Following transfection, cells were handled as detailed in the figure legends.

**Cellular protein extraction**

Total cellular protein was extracted at 4˚C in 500 μl lysis buffer (29, 33). Protein concentration was determined using a bicinchoninic acid protein assay (Pierce, Rockford, IL).

**Immunoprecipitation**

Five microliters Ab was added to 500 μg isolated cellular protein within lysis buffer and incubated at 4˚C overnight (29). Thirty microliters 50% slurry prewashed Protein G PLUS-Agarose (Santa Cruz Biotechnology, Dallas, TX) were then added to each sample, followed by incubation for an additional 2 h at 4˚C. The samples were spun at 14,000 × g for 4 times in lysis buffer. Samples were then resuspended in 30 μl lysis buffer for future analysis.

**Immunoblot**

Total cellular lysate was electrophoresed in a 6, 10, or 15% SDS-PAGE gel (based on protein of interest) and transferred to a Hybond-ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The membrane was blocked for 1 h at room temperature with 5% milk and incubated with primary Ab for overnight at 4˚C. Blots were then incubated in a HRP-conjugated secondary Ab against the primary Ab at RT for 1 h. The blots were developed using the SuperSignal chemiluminescent substrate (Thermo Scientific, Rockford, IL) and exposed on KAR-5 film (Eastman Kodak, Rochester, NY). All gels were rebotted for total protein to confirm equal loading or to assess knockdown after RNA interference (RNAi). Densitometry was performed by the National Institutes of Health Image J64 program (National Institutes of Health, Bethesda, MD).

**RNA preparation and RT-PCR**

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA), according to the instructions specified by the manufacturer. The mRNA level was determined by quantitative RT-PCR. Briefly, 1 μg total RNA was used for the first-strand cDNA synthesis, which was carried out by using a RT system kit, according to the instructions of the manufacturer (Promega). cDNA (1 μl) was used as a template in PCR reactions, and the PCR reaction mixture at a 25 μl volume contained 12.5 μl 2× Brilliant II SYBR Green QPCR Master Mixes (Agilent Technologies, Santa Clara, CA), 1 μl cDNA, and 1 μl mTOR sense and antisense primer sets (SuperArray Bioscience). After denaturing at 95˚C for 15 min, the amplification protocol consisted of 40 cycles of 95˚C for 30 s, followed by 60˚C for 60 s. Expression levels of mRNA were normalized by the housekeeping gene GAPDH.

**In vitro kinase assay**

Recombinant (1 μg) GSK3α/β fusion protein (27 kD) was incubated in the presence or absence of activated p-Thr389–CaMKIV (25 ng) for 10 min at 30˚C with the following additions: 10 mM MgCl2, 0.2 mM ATP, 1 mM CaCl2, and 1 μM CaM. Reactions were terminated by boiling in SDS–2–ME dissociation solution, subjected to 10% SDS-PAGE, and probed with p-Ser9–GSK3β and anti-CaMKIV. Total GSK3β was evaluated by Coomassie blue R250 staining.

**Microscopy**

pMβ cells were plated on sterilized glass slides and allowed to adhere for 2 h at 37˚C. Cells underwent three washes with PBS and were then fixed in 4% paraformaldehyde at 4˚C. For in vivo experiments, kidneys were flushed with PBS and then perfused with 2% paraformaldehyde. After 2-h fixation, samples were transferred to 30% sucrose for 24 h with a total of three sucrose changes. The samples were cryopreserved in liquid nitrogen cooled 2-methylbutane and stored at −80˚C until sectioning to 5 μm thickness. Immunostaining for in vivo and in vitro was performed as follows. Samples were permeabilized in 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) at RT, and then the BSA was removed with three washes of PBS. The samples were then blocked with incubation with 2% BSA in PBS for 1 h at RT. For experiments with cells, samples were then incubated with rabbit anti-FBXW7 (Abcam) or rabbit p-Ser9–GSK3β (Cell Signaling Technology) for 60 min at RT. In vivo samples stained with anti-LC3-II/Ab (Novus; 5 μg/ml) in PBB plus 0.1% Triton X-100 at 4˚C. After washing three times with PBB, cells were incubated with a Cy3 secondary Ab (goat anti-rabbit; 1:1000; Jackson ImmunoResearch Laboratories) plus Alexa Fluor 488 phallolidin (1:250). In vivo, the slides were rinsed, and HOECHST stain (1 mg/100 ml bisbenzimide) was applied for 30 s. Coverslips or cover glass were adhered with gelvatol, a water-soluble mounting media,
and slides were placed at 4˚C overnight before imaging. Samples were viewed at ×20 original magnification with a 2-fold digital zoom with a numeric aperture of 0.8 on an Olympus Fluoview 1000 (Olympus America, Center Valley, PA). Imaging conditions were maintained at identical settings within each Ab-labeling experiment with original gating performed using the negative control. Quantification was performed using Metamorph (Molecular Devices, Sunnyvale, CA) (34). For transmission electron microscopy, kidneys were flushed with PBS and subsequently perfused and fixed with 2% glutaraldehyde in 0.1 mol/l phosphate buffer (pH 7.4), followed by 1% OsO4. The tissue was sectioned into 2-cm cubes and then postfixed with 1% OsO4 and 1% K2Fe(CN)6 for 1 h. Samples were washed three times with PBS and then dehydrated in a graded series of 30–100% ethanol before incubation in 3× 1-h incubations of Polybed 812 epoxy resin. Samples were cured at 37˚C for 24 h followed by 48 h at 65˚C. Ultrathin sections of 60 nm were then cut by a microtome (Leica Ultracut R; Leica Microsystems), collected on 200 mesh grids that were then poststained with 2% uranyl acetate in 50% methanol for 10 min, followed by 1% lead citrate for 7 min, and analyzed using a JEM 1011CX electron microscope (JEOL, Peabody, MA). Images were acquired digitally from a randomly selected pool of 10–15 fields for each experimental condition (34). Sample processing and imaging occurred at the Center for Biological Imaging at the University of Pittsburgh.

**Renal function parameters**

Renal function was determined by assaying serum for cystatin C, using an enzyme immunoassay kit (R&D Systems, Minneapolis, MN). Cystatin C has emerged as a more precise marker of glomerular filtration rate and has been validated in both human and murine studies (35, 36).

**Cytokine production**

IL-10, TNF-α, and IL-6 concentrations were quantified by an enzyme immunoassay kit (R&D Systems) that is based on a coated-well, sandwich enzyme immunoassay.

**Statistical analysis**

Statistical analyses were performed using Stata 12SE software (Stata, College Station, TX). Values are expressed as means ± SEM. Groups are compared by ANOVA. A p value <0.05 was considered statistically significant.

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**FIGURE 1.** Mφ LPS-induced autophagy is CaMKIV dependent. (A) Primary pMφ from C57BL/6 or CaMKIV−/− mice were exposed to LPS (100 ng/ml) for 30 min. Total cellular lysate (10 μg/lane) was isolated and the induction of ATG5-12, ATG7, LC3B, and actin assessed by immunoblot (representative blot of n = 4 independent experiments; n = 4 mice total per experimental condition for all four experiments combined). (B) pMφs were transfected with empty vector, a constitutively active CaMKIV (CaMKIV-dCT), or a kinase-deficient mutant (CaMKIV-dCTK75E) for 8 h. Total cellular lysate (10 μg/lane) was then isolated and analyzed for the expression of ATG5-12, ATG7, LC3B, total CaMKIV, and actin by immunoblot (representative blot of n = 3 independent experiments). (C) WT and CaMKIV−/− pMφs were exposed to LPS (100 ng/ml) for 30 min, and autophagy was assessed by electron microscopy. Top and bottom panels, Scale bars, 500 nm. Black arrowheads indicate double membrane autophagosomes/autophagolysosomes.
Results

Mφ LPS-induced autophagy is CaMKIV dependent

We have previously shown that CaMKIV is activated in Mφ exposed to LPS (26). In this study, we show that in Mφ responding to LPS, autophagy is dependent upon CaMKIV signaling. pMφ isolated from CaMKIV<sup>−/−</sup> mice (CaMKIV<sup>−/−</sup> pMφ) exhibited attenuated ATG5/12, ATG7, and LC3B in response to LPS stimulation, by comparison with wild-type (WT) pMφ (Fig. 1A). To investigate whether CaMKIV is sufficient to induce autophagy, we used a constitutively active CaMKIV plasmid (CaMKIV-dCT) and a kinase defective mutant (CaMKIV-dCTK75E) (26). As shown in Fig. 1B, CaMKIV-dCT strongly induced ATG5/12, ATG7, and LC3B, which was lesser in magnitude with CaMKIV-dCTK75E transfection. Electron microscopy shows reduced density of double-membrane autophagosomes in LPS-stimulated CaMKIV<sup>−/−</sup> pMφ, by comparison with similarly treated WT pMφ (Fig. 1C). These data support that active CaMKIV regulates LPS-induced autophagy in the Mφ.

CaMKIV regulates Mφ mTOR expression

The preponderance of data suggest that mTOR complex 1 (mTORC1) activity negatively regulates autophagy; and thus mechanisms, like AMPK activity, that inhibit mTORC1, induce autophagy (4, 9, 10, 37). However, we observed that CaMKIV<sup>−/−</sup> pMφ have reduced mTORC1 activity as shown by the reduced phosphorylation of p70 S6 kinase and p70 S6 kinase, and p70 S6 kinase, p-Ser235/236, p-S6, and actin expression was assessed by immunoblot (representative blot of n = 3 independent experiments; n = 4 mice total for all four experiments combined). (D) pMφ were transfected with empty vector, a constitutively active CaMKIV (CaMKIV-dCT), or a kinase-deficient mutant (CaMKIV-dCTK75E) for 8 h. Total cellular lysate (10 μg/lane) was then isolated and analyzed for the expression of mTOR and actin by immunoblot (representative blot of n = 3 independent experiments).

FIGURE 2. CaMKIV regulates mTOR expression. (A) Primary pMφ from C57BL/6 or CaMKIV<sup>−/−</sup> mice were exposed to LPS (100 ng/ml) for 30 min. Total cellular lysate (10 μg/lane) was isolated, and mTOR and actin expression were assessed by immunoblot (representative blot of n = 4 independent experiments in which two mice per group are shown; n = 8 total mice/experimental condition for all four experiments combined); lanes 1 and 2 (WT 1), lanes 3 and 4 (WT 2); lanes 5 and 6 (CaMKIV<sup>−/−</sup> 1); and lanes 7 and 8 (CaMKIV<sup>−/−</sup> 2). (B) Primary pMφ from C57BL/6 or CaMKIV<sup>−/−</sup> mice were exposed to LPS (100 ng/ml) for 30 min. Total cellular lysate (10 μg/lane) was isolated, and p-Thr<sup>389</sup>p70 S6 kinase, p70 S6 kinase, p-Ser<sup>240/244</sup>S6, p-Ser<sup>235/236</sup>S6, and actin expression was assessed by immunoblot (representative blot of n = 4 independent experiments; n = 4 mice total/group for all four experiments combined). (C) Primary pMφ were exposed to STO609 (10 μM) and LPS (100 ng/ml) for 4 h. Total cellular lysate (10 μg/lane) was isolated, and mTOR and tubulin expression was assessed by immunoblot (representative blot of n = 4 independent experiments; n = 4 mice total for all four experiments combined). (D) pMφ were transfected with empty vector, a constitutively active CaMKIV (CaMKIV-dCT), or a kinase-deficient mutant (CaMKIV-dCTK75E) for 8 h. Total cellular lysate (10 μg/lane) was then isolated and analyzed for the expression of mTOR and actin by immunoblot (representative blot of n = 3 independent experiments).

FIGURE 3. mTOR is necessary for Mφ autophagy. Primary pMφ were subjected to RNAi using nontarget (NT) or mTOR siRNA. After 72 h, cells were exposed to LPS (100 ng/ml) for 30 min. Total cellular lysate (10 μg/ml) was then isolated and analyzed for the expression of mTOR, ATG7, ATG5/12, LC3B, and tubulin by immunoblot (representative blot of n = 3 independent experiments; n = 3 mice total for all three experiments combined).
mTOR expression, both at baseline and after LPS stimulation, by comparison with WT pMφ (Fig. 2A). The reduced mTOR expression in CaMKIV−/− pMφ correlated with attenuated mTORC1 signaling, as evidenced by reduced p-Thr389 p70 S6 kinase and p-Ser235/236 S6 (Fig. 2B). Similarly, exposure to the CaMKK1/2 biochemical inhibitor STO609 acutely inhibited mTOR protein expression in pMφ (Fig. 2C). Alternatively, elevating CaMKIV activity through the transfection of active CaMKIV-dCT increased mTOR protein concentration (Fig. 2D). These data support that CaMKIV regulates mTOR expression.

mTOR is necessary for Mφ LPS-induced autophagy

These data led us to hypothesize that CaMKIV-dependent regulation of mTOR may play an important role in the induction of autophagy in Mφ responding to LPS. As shown in Fig. 3, RNAi of mTOR significantly inhibited mTOR expression in pMφ, by comparison with scrambled, nontargeted, control siRNA. The attenuated expression of mTOR correlated with reduced LPS-induced ATG7, ATG5/12, and LC3B. Thus, in Mφ, mTOR appears to be necessary for autophagy.

CaMKIV negatively regulates FBXW7- and GSK3-β–dependent ubiquitin-mediated degradation of mTOR in Mφ

We next explored potential mechanisms by which CaMKIV regulates mTOR expression. As shown in Fig. 4A, both CaMKIV−/− and WT pMφ expressed similar levels of mTOR mRNA, suggesting that CaMKIV regulation of mTOR occurs at a posttranscriptional level (Fig. 4A). Prior work has shown that FBXW7 targets mTOR for ubiquitination and proteosomal degradation (38, 39). As shown in Fig. 4B, treatment with the proteasome inhibitor MG132 increased the expression of mTOR in both WT and CaMKIV−/− pMφ (Fig. 4B). Similarly, MG132 restored the expression of mTOR that had been inhibited with STO609 biochemical CaMKK inhibition (Fig. 4C). The increased mTOR with MG132 was predominantly the ubiquitinated form of mTOR as shown in Fig. 4D. These data support that CaMKIV inhibits ubiquitin-dependent mTOR degradation.

Flügel et al. (38) described increased ubiquitin-mediated protein degradation of hypoxia inducible factor-1 after phosphorylation by GSK-3β and recruitment of the ubiquitin ligase and tumor suppressor F-box and WD protein FBXW7. GSK-3β is negatively regulated by inhibitory phosphorylation of serine 9 (40). Thus, we explored whether CaMKIV regulated FBXW7 and p-Ser9–GSK3β.

As shown in Fig. 5A, CaMKIV−/− pMφ exhibited increased FBXW7 expression and reduced inhibitory p-Ser9–GSK3β (i.e., increased kinase activity due to a loss of inhibitory serine 9 phosphorylation) by comparison with WT pMφ, which appropriately correlated with reduced mTOR expression (Fig. 4B). Similarly, biochemical CaMKK inhibition with STO609 increased FBXW7 and decreased p-Ser9–GSK3β (Fig. 5B), which again mirrored the
CaMKIV regulates mTOR and autophagy in the kidney

We next explored whether these mechanisms were operant in other organs responding to the stress of sepsis and the functional ramifications. Similar to pMφ, kidneys isolated from CaMKIV−/− mice exhibited reduced expression of mTOR, which correlated with reduced p-Ser9-GSK3β (Fig. 7A). However, both WT and CaMKIV−/− kidneys exhibited similar and low expression of FBX7. The loss of renal mTOR was associated with reduced renal autophagy during endotoxemia (Figs. 7B). Representative immunoblot shows significantly less LC3B expression in the renal cortexes of CaMKIV−/− animals than of WT animals 18 h after LPS (p < 0.001) (Fig. 7B). CaMKIV−/− kidneys, by contrast to WT kidneys, exhibited less immunofluorescence and a less pronounced punctate staining pattern for LC3 (Fig. 7B). Electron microscopy illustrates numerous multilaminar autophagosomes and autophagolysosomes in the renal cortex of WT animals after endotoxemia. These structures are notably reduced in the kidneys of CaMKIV−/− animals, which exhibit swollen, damaged mitochondria lacking prototypical architectural features (Fig. 7B). We confirmed that mTOR expression is necessary for LPS-induced autophagy using mTOR siRNA and human proximal renal tubular (HKC-8) cells. As shown in Fig. 7C, RNAi of mTOR attenuated LPS-induced autophagy in HKC-8 cells by comparison with NT siRNA. Thus, in the kidney as well, CaMKIV preserves mTOR expression, which is necessary for LPS-induced autophagy.

Recent literature suggests that autophagic mechanisms directed at removal of damaged mitochondria, or mitophagy, provide a critical role in the kidney, where autophagy is a mediator of renal ischemia-reperfusion injury and a target for therapeutic interventions in sepsis.
FIGURE 6. CaMKIV and mTOR regulate macrophage IL-6 production. (A) RAW 264.7 cells were exposed to mTOR siRNA. After 72 h, they were exposed to LPS (100 ng/ml) for 18 h. Supernatant was harvested and assayed for IL-6 by ELISA (n = 3 independent experiments). Data are mean ± SEM. Total cellular lysate was subjected to immunoblot of mTOR or actin (n = 3 independent experiments). (B) RAW 264.7 cells were exposed to LPS (100 ng/ml) for 18 h either in the presence or absence of DMSO, KN93 (10 μM), STO690 (10 μM), or rapamycin (50 ng/ml). Supernatant was harvested and assayed for TNF-α, IL-6, and IL-10 by ELISA (n = 3 independent experiments). Data are mean ± SEM. (C) pMø were harvested from WT and CaMKIV−/− mice and exposed to LPS (100 ng/ml) for 18 h. Supernatant was harvested and assayed for IL-6 by ELISA (n = 3 independent experiments; n = 8 mice total/group for all three experiments combined). Data are mean ± SEM. (D) IL-6 concentrations after endotoxemia comparing WT and CaMKIV−/− mice (n = 3 independent experiments, n = 12 mice total/group for all three experiments combined). Data are mean ± SEM.
FIGURE 7. CaMKIV regulates mTOR and autophagy in the kidney. (A) Immunoblot analysis of renal cortex lysate detecting mTOR, p-Ser\(^{9}\)-GSK3\(\beta\), FBXW7, GSK3\(\beta\), and tubulin from WT and CaMKIV\(^{-/-}\) mice (representative blot of \(n = 4\) independent experiments in which two mice per group are shown; \(n = 8\) mice total/group for all four experiments combined). Corresponding densitometry blots compare mTOR and p-Ser\(^{9}\)-GSK3\(\beta\) from WT and CaMKIV\(^{-/-}\) mice. (B) The effect of endotoxemia (LPS 5 mg/kg) on autophagy in the renal cortices of WT and CaMKIV\(^{-/-}\) mice was assessed by immunoblot for LC3B (16 kD). Representative blot is shown at 18-h time point from \(n = 4\) independent experiments in which three mice per group are shown (\(n = 12\) mice total/group for all four experiments combined). Immunofluorescent microscopy (original magnification \(\times 20\)) of renal cortex in WT and CaMKIV\(^{-/-}\) mice harvested at 18 h after LPS (5 mg/kg). LC3 (Cy3, red), actin (Alexa 488, green), and nucleus (Hoechst, blue). Top panel scale bars, 20 \(\mu\)m. Representative images of \(n = 3\) independent experiments (\(n = 9\) mice total/group for all three experiments combined). Transmission electron microscopy (5 \(\times\) 10\(^4\) and 5 \(\times\) 10\(^5\)) of renal cortex of WT and CaMKIV\(^{-/-}\) mice 18 h after endotoxemia. Middle panel, Scale (Figure legend continues)
cytoprotective role in protecting against acute kidney injury (AKI), which primarily affects the mitochondria-rich proximal tubule cells (42). We observed that CaMKIV−/− mice experienced greater AKI than WT mice. As shown in Fig. 8, endotoxemic CaMKIV−/− mice, by comparison with their WT counterparts, exhibited significant elevations in cystatin C concentration (992 versus 655 ng/ml; p = 0.002). These data create a paradigm in which CaMKIV maintains mTOR expression which is fundamental for the induction of autophagy and protection against AKI during endotoxemia (Fig. 9).

Discussion

Autophagy is an evolutionarily conserved lysosomal mechanism that enables cells to conserve and maintain cellular biomass quality and quantity by targeting damaged or unused proteins and even organelles for degradation (1, 2, 8, 43, 44). Though essential for tissue homeostasis, evidence is accumulating that it may be acutely activated by cells in response to multiple forms of stress. The mechanisms regulating this complex process and the ramifications of any defects are just being elucidated. In this study, we describe a novel mechanism in which CaMKIV, by inhibiting GSK3β activity and FBXW7 recruitment, prevents ubiquitin proteosomal degradation of mTOR and thereby directly augments autophagy in both the Mø and the kidney. Under the conditions of sepsis studied, mTOR expression and activity are requisite for autophagy, a paradigm counteracting the current perspective that prototypically, mTOR inhibition induces autophagy. Observed in both Mø (i.e., immune cells) and the kidney (i.e., parenchymal cells), this CaMKIV–mTOR-dependent autophagic mechanism is conserved across several cell types and fundamental for the respective functional (e.g., Mø cytokine production) and adaptive responses (e.g., renal tubular cell cytoprotection) to septic insult.

Our data complement prior investigations that describe mechanisms by which the family of multifunctional CaMK regulate autophagy. Using carcinoma cell lines and NIH3T3 fibroblasts, Hoyer-Hansen et al. (28) observed that calcium-mobilizing agents (e.g., vitamin D, thapsigargin) inhibited the activity of mTOR, a negative regulator of autophagy, and induced the accumulation of autophagosomes in a Beclin-1 and ATG-7 manner. This process was mediated by CaMKKβ and AMPK (28). Other studies have identified a CaMKKα/β–AMPK pathway in which CaMKKα/β functions as an indirect AMPK kinase (4, 45). In Mø, LPS induces endoplasmic reticulum stress and calcium mobilization that we have shown leads to the activation of all members of the CaMK cascade (26, 29, 33, 46). We have previously shown that LPS-induced CaMKI and -IV activity in Mø are CaMKKα/β dependent (26, 29, 33). Recently, we described a CaMKIα–AMPK pathway rapidly activated as an early autophagic response in Mø exposed to LPS (29). We now include CaMKIV that like its sibling CaMKI also regulates autophagy. Thus, we propose a paradigm in which dual CaMKKα/β–CaMKI and CaMKKα/β–CaMKIV transduction pathways, responding to calcium mobilization, mediate distinct aspects of autophagy. Collectively, our data support a functional role for all members of the CaMK family during autophagy.

Our prior and these new data highlight that CaMKI and -IV augment autophagy independent of mTOR inhibition. The mTOR typically serves as a negative regulator of autophagy, and as a consequence, initiation of autophagy is largely dependent on release of mTOR inhibition (3, 9, 10). Indeed, AMPK-mediated inhibition of mTOR has been fairly well established as a mechanism that induces autophagy in a variety of cells and experimental models (3, 4, 9, 10, 37). Studies have explored the use of pharmacologic mTOR inhibitors, such as rapamycin, which potently inhibit downstream mTOR signaling and thereby induce autophagy (47–49). However, in this study, CaMKIV invoked an mTOR-dependent mechanism to directly augment autophagy. Loss of CaMKIV or CaMK activity (i.e., STO609) reduced mTOR expression and TORC1 activity, which attenuated autophagy. Restoration of active CaMKIV induced TORC1 signaling and mTOR-dependent LC3B expression. Finally, mTOR siRNA inhibited LPS-induced autophagy in both Mø and renal tubular cells. This dependence of autophagy on mTOR was also observed in renal tubular cells during endotoxemia. In our prior study, CaMKIα regulated AMPK-dependent autophagy independent of mTOR inhibition (29). Thus, in both immune and nonimmune cells, CaMK-dependent autophagy appears to require mTOR, at least in the context of sepsis.

In addition to being operant in vitro, our data support the in vivo importance of these mechanisms in modifying early cell-specific responses to sepsis. In a murine model of endotoxemia CaMKIV–mTOR autophagy was necessary for IL-6 production, which mirrored our in vitro Mø results. We believe that TORC1 activity, and not merely the structural presence of mTOR, underlies our observations as functional inhibition of mTOR with rapamycin similarly attenuated IL-6 production. This perspective is supported by other independent studies (50). Recently, it has been suggested that autophagy and inflammation are intimately linked (41). In Mø, a spatial coupling of mTOR and autophagy into a TOR–autophagy spatial coupling compartment (TASCC) augments the secretory phenotype and facilitates the mass synthesis of secretory proteins, 

Bars, 2 µm. Bottom panel. Scale bars, 500 nm. Representative images of n = 3 independent experiments (n = 9 mice total/group for all three experiments combined). (C) Human proximal renal tubular epithelial (HKC-8) cells were subjected to RNAi using nontarget (NT) or mTOR siRNA. After 72 h, cells were exposed to LPS (10 ng/ml) for 30 min. Total cellular lysate (10 µg/lane) was then isolated and analyzed for the expression of mTOR, LC3B, and tubulin by immunoblot (representative blot of three independent experiments).

![Figure 8](http://www.jimmunol.org/)
such as cytokines (41). Disrupting the TASCC suppresses the synthesis of IL-6 and IL-8 (41). We view this paradigm as explanatory for the mTOR dependency of CaMKIV regulated autophagy. Though awaiting further investigation, we hypothesize that CaMKIV, by regulating mTOR and thus autophagy, orchestrates construction of the TASCC, which is essential for optimal immune cell function.

These mechanisms we initially described in Mφs were shown to be equally relevant to renal tubular cells. AKI is arguably the most prevalent form of organ dysfunction during severe sepsis. Recent literature suggests a critical cytoprotective role for autophagy in both toxin-mediated and ischemia-reperfusion-induced AKI (42). Autophagic mechanisms directed at removal of damaged mitochondria, or mitophagy, are considered of particular importance in protecting against AKI, which primarily affects the mitochondria-rich proximal tubule cells (42). Renal tubular cells express TLRs, are responsive to LPS stimulation, and mobilize intracellular calcium with exposure to a variety of stresses (51–54). Our data suggest that CaMKIV receives these calcium transients induced by sepsis to mediate mTOR-dependent autophagy. These mechanisms serve an adaptive response as loss of CaMKIV correlated by sepsis to mediate mTOR-dependent autophagy. These mechanisms we initially described in Mφs were shown to be equally relevant to renal tubular cells. AKI is arguably the most prevalent form of organ dysfunction during severe sepsis. Recent literature suggests a critical cytoprotective role for autophagy in both toxin-mediated and ischemia-reperfusion-induced AKI (42).

In summary, CaMKIV, by inhibiting GSK3β and FBXW7-mediated ubiquitination and proteosomal degradation of mTOR, maintains mTOR expression (Fig. 9). The preservation of mTORmediated ubiquitination and proteosomal degradation of mTOR, and delayed functional recovery during endotoxemia.

FIGURE 9. CaMKIV is essential for autophagy during endotoxemia. CaMKIV inhibition of GSK-3β activity and FBXW7 recruitment prevents ubiquitin-mediated proteosomal degradation of mTOR and thereby directly augments autophagy in both the Mφ and the kidney. These mechanisms are cytoprotective in the kidney and important for immune cell function.

such as cytokines (41). Disrupting the TASCC suppresses the synthesis of IL-6 and IL-8 (41). We view this paradigm as explanatory for the mTOR dependency of CaMKIV regulated autophagy. Though awaiting further investigation, we hypothesize that CaMKIV, by regulating mTOR and thus autophagy, orchestrates construction of the TASCC, which is essential for optimal immune cell function.

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In summary, CaMKIV, by inhibiting GSK3β and FBXW7-mediated ubiquitination and proteosomal degradation of mTOR, maintains mTOR expression (Fig. 9). The preservation of mTOR expression and TORC1 activity is essential for LPS-induced autophagy in Mφs and renal tubular cells. These CaMKIV-dependent processes appear to be operant in vivo, lending support to the hypothesis that CaMKIV signaling mediates the autophagic response to sepsis. The observation that CaMKIV regulates autophagy in both immune and nonimmune cells, underscores the need to further study these mechanisms in the context of organ-specific injury and dysfunction in response to septic insult. Furthermore, additional studies are needed to translate these cellular events to organ physiology and the host response, in attempts to identify the clinical utility of CaMK and autophagic modulation during inflammatory states.

Disclosures
The authors have no financial conflicts of interest.

References


