Mammalian Target of Rapamycin Complex 2 (mTORC2) Negatively Regulates Toll-like Receptor 4-mediated Inflammatory Response via FoxO1*

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Activation of the PI3K pathway plays a pivotal role in regulating the inflammatory response. The loss of mTORC2 has been shown to abrogate the activation of Akt, a critical downstream component of PI3K signaling. However, the biological importance of mTORC2 in innate immunity is currently unknown. Here we demonstrate that rictor, a key component of mTORC2, plays a critical role in controlling the innate inflammatory response via its ability to regulate FoxO1. Upon LPS stimulation, both rictor-deficient mouse embryonic fibroblasts (MEFs) and rictor knockdown dendritic cells exhibited a hyperinflammatory phenotype. The hyperinflammatory phenotype was due to a defective Akt signaling axis, because both rictor-deficient MEFs and rictor knockdown dendritic cells exhibited attenuated Akt phosphorylation and kinase activity. Analysis of downstream Akt targets revealed that phosphorylation of FoxO1 was impaired in rictor-deficient cells, resulting in elevated nuclear FoxO1 levels and diminished nuclear export of FoxO1 upon LPS stimulation. Knockdown of FoxO1 attenuated the hyperinflammatory phenotype exhibited by rictor-deficient MEFs. Moreover, FoxO1 deletion in dendritic cells attenuated the capacity of LPS to induce inflammatory cytokine expression. These findings identify a novel signaling pathway by which mTORC2 regulates the TLR-mediated inflammatory response through its ability to regulate FoxO1.

PI3K is a heterodimeric enzyme that consists of a regulatory (p85) and a catalytic (p110) subunit (1). PI3K has the ability to phosphorylate both lipids and proteins. The activation of PI3K drives the generation of phosphatidylinositol 3,4,5-trisphosphate. A major downstream target of PI3K is the AGC family member and kinase Akt (Protein Kinase B). Upon the generation of phosphatidylinositol 3,4,5-trisphosphate, Akt is recruited to the plasma membrane through its pleckstrin homology domain. Sequential phosphorylation of Akt on both serine 473 and threonine 308 are required for full activation. Phosphorylation of Akt on threonine 308 is mediated by PDK1 (2, 3). Several kinases including DNA-activated Protein Kinase, integrin-linked kinase, and PKCβ have been proposed to be involved in the phosphorylation of Akt on serine 473 (4–6). Studies utilizing cells deficient in rictor, a component of the mammalian target of rapamycin complex 2 (mTORC2), have shown that mTORC2 is the primary kinase involved in phosphorylation of serine 473 on Akt. Upon insulin or serum stimulation, rictor-deficient MEFs displayed defective Akt Ser-473 phosphorylation compared with wild type cells (7–9).

mTOR can be present in two distinct complexes, mTORC1 and mTORC2 (8, 10). The mTORC1 complex consists of mTOR, raptor, mLST8, and Deptor, which is a negative regulator mTOR (11–13). mTORC1 is the target of the immune-suppressant macrolide drug rapamycin and functions in a wide variety of cellular processes including cell growth via its phosphorylation of two downstream targets, S6 kinase 1, and elf4E-binding protein (14). mTORC1 has been shown to play an important role in regulating the TLR-mediated innate inflammatory response and the production of type I interferons (15, 16). In contrast to mTORC1, the mTORC2 complex is typically insensitive to rapamycin and consists of mTOR, sin1, mLST8, and rictor (17, 18). Early studies assessing the biological importance of mTORC2 demonstrated that mTORC2 controlled the actin cytoskeleton and the regulation of PKCα (17). Subsequent studies have further demonstrated that rictor-deficient cells exhibited selective deficiencies in the activation/phosphorylation of downstream targets of Akt (18). Thus, although rictor-deficient cells have been reported to exhibit defective Akt phosphorylation and activity, the loss of rictor appears to only affect a subset of Akt substrates.

TLR-mediated activation of the PI3K pathway has been shown to play an important role in regulating both pro- and anti-inflammatory cytokine production (19–22). Although PI3K activation can engage a diverse number of cell signaling pathways that directly or indirectly affect the inflammatory response, its ability to activate Akt is believed to play a predominant role. Studies by Androulidaki et al. (23) demonstrated that Akt1-deficient macrophages are unable to be tolerized by LPS and produce increased levels of proinflammatory cytokines when stimulated with LPS. Several downstream targets of Akt,
mTORC2 Regulates FoxO1

including mTORC1 and GSK3, have been identified to play pivotal roles in controlling the inflammatory response downstream of Akt. Inhibition of PI3K/Akt or the downstream kinases mTORC1 and GSK3 in TLR-stimulated cells has been shown to differentially control the levels of pro- and anti-inflammatory cytokines (15, 16, 19, 21, 24). Recent studies by Turnquist et al. (24) assessing the inflammatory properties of mTORC1 suggested that the ability of mTORC1 to control the inflammatory response is likely due to mTORC1 negatively regulating the activity of GSK3 in LPS-stimulated cells. Taken together, these findings highlight the importance of Akt signaling in controlling the inflammatory response.

In the present study, we investigated the role of rictor in the regulation of the TLR4-mediated inflammatory response. We demonstrate that the loss of rictor by genetic deletion or siRNA-mediated knockdown abrogated the TLR4-mediated phosphorylation of Akt on serine 473, resulting in reduced kinase activity. Rictor deficiency augmented the production of proinflammatory cytokines upon LPS stimulation. Analysis of multiple downstream substrates of Akt revealed that phosphorylation of mTORC1/S6K1 and GSK3 was not affected in TLR4-stimulated rictor-deficient MEFS. However, the phosphorylation of FoxO1 was impaired in both rictor-deficient MEFS and rictor knockdown DC. Previous studies have shown that FoxO1, a transcription factor negatively regulated by Akt, enhanced TLR4-mediated inflammation (25). Herein, we demonstrate that in the absence of rictor, the nuclear levels of FoxO1 were highly elevated, and cells were unable to export FoxO1 into the cytoplasm upon TLR4 stimulation. Complementation of rictor-deficient MEFS with a constitutively active Akt attenuated their hyperinflammatory phenotype and restored FoxO1 phosphorylation and nuclear/cytosolic shuttling. Knockdown of FoxO1 attenuated the hyperinflammatory phenotype exhibited by rictor-deficient MEFS stimulated with LPS. Moreover, FoxO1 KO DC exhibited attenuated levels of proinflammatory cytokines and increased production of IL-10. These findings identify a novel signaling pathway by which mTORC2 regulates the TLR4-mediated inflammatory response through FoxO1 regulation.

EXPERIMENTAL PROCEDURES

Medium, Reagents, and Mice—DC and MEFS were cultured in RPMI 1640 medium or DMEM (Invitrogen), respectively, and supplemented with 10% FBS (R10), 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 2 mM L-glutamine, 20 mM HEPES, 50 units/ml penicillin, and 50 μg/ml streptomycin. Ultra pure LPS from Escherichia coli 0111:B4 was purchased from Invivogen. The SGK-1 inhibitor (GSK 650394) was purchased from Trocres, and the PKC-α inhibitor (Gö 6976) was purchased from LC Laboratories. Lipofectamine RNAiMAX and Lipofectamine LTX were obtained from Invitrogen and used for the siRNA transfection studies. TransAM™ NF-κB, FoxO1 transcription factor ELISA kits, and the nuclear extraction kits were purchased from Active Motif. Akt (Ser-473) and Akt (Thr-308) ELISA kits were obtained from Cell Signaling Technology. Cytokine ELISA kits were purchased from eBioscience or R & D System. Myr-Akt plasmids were obtained from Addgene (catalog number 16244). FoxO1 and rictor-specific siRNA were purchased from Santa Cruz Biotechnology. All of the antibodies were obtained from Cell Signaling Technology with the exception of PKC-α (Ser-657; Millipore), total SGK1 (Santa Cruz), and SGK-1 (Ser-422; Santa Cruz). FOXO1/L/L mice were generously provided by Dr. DePinho (Harvard University) and bred with CD11c.Cre recombinase mice purchased from Jackson Laboratories. The experimental mice were CD11c.Cre⁺.FOXO1/L/L mice, and the control mice were CD11c.Cre⁻.FOXO1/L/L littermates.

Western Blot and Cytokine Analysis—Wild type and rictor-deficient MEFS were plated at a concentration of 5 × 10⁵ in Costar 6-well plates and rested overnight. After LPS (1 μg/ml) stimulation, the cells were lysed with radioimmune precipitation assay buffer (Sigma) for immunoblot. After generation, DC were plated at a concentration of 1 × 10⁶ in 12-well Costar plate and rested overnight. Protein concentrations were determined using a bicinchoninic acid protein assay kit (Thermo Co.), and samples were run on NuPage Novex 4–12 Bis-Tris gel (Invitrogen). Images were acquired using the Kodak Image Station 4000MM system. For cytokine analysis, DC or MEFS were plated at a concentration of 5 × 10⁵ in Costar 96-well plates and stimulated with LPS (1 μg/ml). Cell-free supernatants were harvested 20 h after LPS stimulation and assayed for cytokine levels according to the manufacturer’s protocol (R & D Systems).

AKT Kinase Assay Kit—The Akt assay kit was purchased from Cell Signaling (9840). The assay was carried out following the manufacturer’s protocol. Briefly, wild type and rictor-deficient MEFS were plated in Costar 6-well plates at a concentration of 1 × 10⁶ in DMEM (Invitrogen), supplemented with 10% FBS (R10), 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 2 mM L-glutamine, 20 mM HEPES, 50 units/ml penicillin, and 50 μg/ml streptomycin. After resting overnight, the cells were stimulated with LPS (1 μg/ml) for 0 min, 30 min, and 1 h. The medium was aspirated off, and the cells were washed with cold PBS twice before adding the cell lysis buffer (Cell Signaling). The cells were then sonicated and centrifuged to remove cellular debris. Lysates were then incubated overnight with a Sepharose bead-conjugated Akt antibody (Cell Signaling) at 4 °C along with gentle agitation. The lysates were incubated with the GSK3 fusion protein for 30 min. Western blots were probed for phospho-GSK3 α/β (Ser-9) (Cell Signaling) and total AKT (Cell Signaling) to establish equal loading. For densitometry analysis, phospho-GSK bands were first normalized to total AKT and then to the WT null condition.

Generation of Bone Marrow-derived Dendritic Cells—Bone marrow was flushed from the femur and tibiae of 6-week-old female mice using sterile Hanks’ balanced salt solution (Invitrogen) and homogenized by passage through an 18.5-gauge syringe repeatedly. The cells were washed in PBS (Invitrogen), centrifuged at 1500 rpm for 5 min, and resuspended in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS (R10), 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 2 mM L-glutamine, 20 mM HEPES, 50 units/ml penicillin, 50 μg/ml streptomycin, and 5 ng/ml recombinant murine-GM-CSF (referred to as R10F). The cells were then plated at a concentration of 2 × 10⁶ cells/10 ml medium in 100-mm Petri dishes. Three days after harvest, 10 ml of R10F with 5 ng/ml rm-GM-CSF was
added to each plate. On day 6, half of the medium was removed from each plate, and the cells in the extracted volume were centrifuged and resuspended in 10 ml of fresh R10F with 5 ng/ml rm-GM-CSF. On day 8, the steps of day 6 were repeated. On day 10, the nonadherent cell were harvested by collecting the supernatant, and purity was checked by typing DC markers (>90%).

Transfection—DC were transfected with nontargeting control (Ctrl) siRNA (sc-37007), siRNA-rictor (sc-61479), or siRNA-FoxO1 (sc-35383) from Santa Cruz using Lipofectamine LTX (Invitrogen) following the manufacturer’s protocol. The transfections of the myr-Akt plasmid were performed using Lipofectamine LTX (Invitrogen) following the manufacturer’s protocol. For transfection studies, the levels of total rictor and FoxO1 were assessed by Western blot 3 days after transfection. For cytokine expression studies, transfected cells were stimulated with LPS 3 days post-transfection, and cell-free supernatants were assayed by ELISA 20 h after the addition of LPS (R & D Systems).

Luciferase Activity Assay—As previously described, the 3×IRS-pGL2-luciferase reporter (Addgene) is driven by three copies of insulin response sequence, which is recognized by FoxO1(26). The 3×IRS-pGL2-luc reporter was obtained along with the pCMV β-galactosidase reporter from Addgene. MEFs were plated at a concentration of 1 × 10⁶ cells/ml in complete DMEM (described above) in 12-well plates, rested overnight, and transfected utilizing Lipofectamine LTX (Invitrogen). The cells were transfected with 0.1 μg of 3×IRS luciferase reporter and 10 ng of pCMV β-galactosidase reporter. The Promega luciferase reporter assay system was utilized and was performed according to the manufacturer’s protocol. Six hours post-transfection, the cells were treated with LPS (1 μg/ml). After an additional 18 h, the medium was aspirated off, and the cells were washed twice with ice-cold PBS and then lysed for the assay. Luciferase activity was represented as a ratio of luciferase to β-galactosidase activity.

Statistical Analysis—Statistical significance between groups was evaluated by the analysis of variance and the Turkey multiple comparison test using the InStat program (GraphPad, San Diego, CA). Differences between groups were considered significant at the level of p < 0.05.

RESULTS

Loss of Rictor Results in Abrogated Phosphorylation of Akt and Elevated Production of Inflammatory Cytokines by LPS-stimulated Cells—Akt has been shown to play a critical role in the regulation of the TLR response (19). Because mTORC2 is required for the full activation of Akt, we sought to define a role for mTORC2 in TLR4-mediated inflammatory activity. The genetic deletion of rictor, a key component of mTORC2, results in embryonic lethality (9, 27). Thus, to determine the role of rictor in regulating the inflammatory response, we initially employed the use of rictor-deficient MEFs (9). To ensure that the absence of rictor did not result in an altered mTORC1 expression profile, we monitored the levels of mTOR, raptor, mLST8, and rictor in wild type and rictor-deficient MEFs (Fig. 1, A and B). As compared with wild type cells, the absence of rictor did not affect the levels of mTOR, raptor, or mLST8 in rictor-deficient cells. Moreover, the levels of mTOR, rictor, raptor, or mLST8 were not noticeably affected upon stimulation with LPS (Fig. 1, A and B).

To date, no studies have described a role for rictor in the phosphorylation of Akt on serine 473 in TLR-stimulated innate immune cells (8, 9). Thus, we monitored the phosphorylation levels of Akt on serine 473 in LPS-stimulated MEFs and DC. Stimulation of wild type MEFs with LPS induced the phosphorylation of Akt on serine 473, whereas no detectable levels of Akt phosphorylation on serine 473 were observed in rictor-deficient MEFs stimulated with LPS (Fig. 1, C and D). We also assessed the phosphorylation of Akt in LPS-stimulated DC treated with rictor siRNA. As demonstrated in Fig. 1E, DC treated with siRNA targeting rictor resulted in a greater than 90% reduction in the cellular levels of rictor, as compared with DC treated with control siRNA. An assessment of Akt phosphorylation showed that DC treated with rictor siRNA exhibited a severe defect in their ability to phosphorylate Akt on serine 473, as compared with LPS-stimulated DC treated with control siRNA (Fig. 1F). To directly assess Akt activity, we utilized an Akt activity assay in which Akt was immunoprecipitated and then co-cultured with a peptide fragment of GSK3α/β, a well characterized substrate of Akt. Interestingly, although both nonstimulated, rictor-deficient and wild type MEFs exhibited similar levels of Akt activity, exposure to LPS resulted in a large discrepancy in Akt activity between the wild type and rictor-deficient MEFs. The loss of rictor dramatically reduced the kinase activity of Akt in LPS-stimulated cells (Fig. 1G). Treatment of wild type MEFs with the PI3K inhibitor LY294002 diminished Akt activity upon LPS stimulation, illustrating that the induction of Akt activity by LPS is PI3K-dependent in MEFs (Fig. 1G).

We next assessed whether the loss of rictor affected the LPS-mediated inflammatory response. Stimulation of rictor-deficient MEFs with LPS resulted in significantly enhanced production of IL-6 and IL-8, as compared with wild type control MEFs stimulated with LPS (p < 0.01) (Fig. 2, A and B). To confirm and extend these results, we also assessed the LPS-induced inflammatory response in dendritic cells exhibiting a knockdown in the cellular levels of rictor. As shown in Fig. 1E, DC treated with siRNA targeting rictor exhibited a reduction in total cellular levels of rictor, as compared with DC treated with control siRNA. Moreover, LPS-stimulated DC treated with rictor siRNA produced significantly elevated levels of IL-1β, IL-6, TNF-α, and IL-12, as compared with LPS-stimulated DC treated with control siRNA (p < 0.01) (Fig. 2, C–F). These data demonstrate that rictor plays a fundamental role in controlling the inflammatory response of LPS-stimulated cells.

We then asked whether complementation of rictor-deficient cells with a constitutively active Akt would attenuate the hyper-inflammatory response exhibited by rictor-deficient MEFs stimulated with LPS. Transfection of rictor-deficient cells with a constitutively active Akt (Myr-Akt) resulted in enhanced levels of Akt, as compared with rictor-deficient cells transfected with an empty vector control plasmid (Fig. 2G). In addition, treatment of Myr-Akt transfected MEFs with LPS over 6 h did not result in altered expression of Akt levels, demonstrating stable transfection (Fig. 2G). LPS stimulation of rictor-deficient
cells expressing an empty vector control plasmid did not exhibit any significant difference in their ability to produce IL-6, as compared with nontransfected rictor-deficient cells stimulated with LPS (Fig. 2H). However, rictor-deficient cells expressing a constitutively active Akt produced significantly less IL-6 when stimulated with LPS, as compared with LPS-stimulated rictor-deficient cells expressing an empty control vector ($p < 0.001$) (Fig. 2H). Thus, the complementation of rictor-deficient cells with a constitutively active Akt attenuated their hyperinflammatory phenotype upon LPS stimulation.

Several Key Mediators of Inflammation Downstream of Akt Are Not Affected in Rictor-deficient Cells Stimulated with LPS—

Several downstream targets of Akt have been demonstrated to be critical in regulating the innate inflammatory response. Thus, we screened the phosphorylation and activity of several downstream targets of Akt to determine the cause of the hyper-inflammatory phenotype of rictor-deficient cells stimulated with LPS. The constitutively active serine/threonine kinase GSK3-β can be inactivated via the ability of Akt to phosphorylate GSK3-β on serine 9, and this phospho-mediated inactivation of GSK3-β results in suppression of the ability of LPS to induce inflammatory cytokines (21, 28). Thus, we determined whether rictor-deficient MEFs exhibited a defect in their ability to phosphorylate GSK3-β when stimulated with LPS. An evaluation of the levels of phosphorylated GSK3-β in LPS-stimulated, rictor-deficient cells revealed similar levels and kinetics of GSK3-β phosphorylation, as compared with the levels observed in wild type cells stimulated with LPS (Fig. 3A).

The Akt signaling pathway is also responsible for the phosphorylation of mTOR, which is a component of both mTORC1 and mTORC2. Therefore, any defects in the levels of mTOR phosphorylation would likely result in defective activity downstream of mTORC1. Moreover, because mTORC1 inhibition has been demonstrated to augment the production of inflammatory cytokines by LPS-stimulated cells, a defect in mTOR phosphorylation could potentially explain the hyperinflammatory phenotype exhibited by rictor-deficient cells stimulated with LPS (15, 16, 24). However, as shown in Fig. 3B, the levels of mTOR phosphorylation were similar in both wild type and rictor-deficient MEFs stimulated with LPS (15, 16, 24). However, as shown in Fig. 3B, the levels of mTOR phosphorylation were similar in both wild type and rictor-deficient MEFs stimulated with LPS.

Recent studies by Lee et al. (29) have shown that mTORC2 is involved in the regulation of NF-κB in TCR/CD28-stimulated CD4$^+$ T cells. Because of the importance of NF-κB in the transcription of many inflammatory cytokines, we next assessed
whether the phosphorylated and DNA binding levels of NF-κB were affected in rictor-deficient cells stimulated with LPS. Analysis of NF-κB p65 phosphorylation demonstrated that the phosphorylation levels of NF-κB p65 were not discernibly affected in rictor-deficient MEFs stimulated with LPS, as compared with wild type cells stimulated with LPS (Fig. 3D).

Moreover, both the basal and LPS-induced DNA binding levels of NF-κB p65 in wild type and rictor-deficient cells were similar at all time points tested (Fig. 3E). In summary, whereas GSK3β and mTORC1 are critical downstream mediators of TLR4 signaling, their function remains unaffected in rictor-deficient cells stimulated with LPS.
Rictor-deficient Cells Exhibit Defective SGK-1 and PKC-\(\alpha\) Phosphorylation when Stimulated with LPS—SGK-1 and PKC-\(\alpha\) belong to the AGC kinase family along with Akt and share many downstream targets with Akt. As with Akt, the activation of SGK-1 and PKC-\(\alpha\) requires mTORC2. The phosphorylation of SGK-1 and PKC-\(\alpha\) can be negatively affected in rictor-deficient cells (9, 30). Thus, we determined whether rictor-deficient MEFs were unable to phosphorylate SGK-1 or PKC-\(\alpha\) when stimulated with LPS. Wild type cells exhibited increased levels of phosphorylated SGK-1 (serine 422) and PKC-\(\alpha\) (serine 657) when stimulated with LPS, as compared with nonstimulated control levels (Fig. 4, A and B). In sharp contrast, no detectable levels of phosphorylated SGK-1 (serine 422) or PKC-\(\alpha\) (serine 657) were observed in rictor-deficient MEFs stimulated with LPS (Fig. 4, A and B). Because we observed that rictor-deficient cells were unable to phosphorylate SGK-1 or PKC-\(\alpha\), we tested whether inhibition of SGK-1 or PKC-\(\alpha\) mimicked the hyperinflammatory phenotype observed in rictor-deficient cells stimulated with LPS. For these studies, wild type MEFs were pretreated with an SGK or PKC inhibitor and stimulated with LPS, and the production of IL-6 was measured. Inhibition of either SGK (Fig. 4C) or PKC (Fig. 4D) in LPS-stimulated cells significantly reduced the levels of inflammatory cytokines produced by LPS-stimulated cells (\(p < 0.01\)). Although phosphorylation levels of SGK-1 and PKC-\(\alpha\) are attenuated in LPS-stimulated, rictor-deficient MEFs, the inhibition of SGK or PKC in wild type MEFs did not mimic the hyperinflammatory phenotype of rictor-deficient cells. In contrast, these kinases promoted the TLR4 inflammatory response. Thus, defective PKC or SGK activity is not responsible for the hyperinflammation observed in rictor-deficient cells. Although SGK-1 inhibition does not result in hyperinflammation similar to rictor-deficient MEFs, it does illustrate that SGK-1 activity promotes the TLR4 inflammatory response, which is the first time SGK-1 has been reported to do so.

Rictor Affects the Phosphorylation and Cytoplasm/Nuclear Localization of FoxO1—The phosphorylation of FoxO proteins can be mediated by Akt, and the phosphorylation of FoxO proteins has been reported to be negatively affected in rictor-deficient cells.
pared with control cells ($p < 0.001$) (Fig. 5D). Utilizing a luciferase transcriptional reporter assay, we observed that FoxO1 transcriptional activity was significantly higher in rictor-deficient MEFs as compared with wild type cells stimulated with LPS ($p < 0.01$) (Fig. 5E). In addition, FoxO1 transcriptional activity was elevated in unstimulated, rictor-deficient MEFs relative to wild type cells (Fig. 5E). These data demonstrate that rictor plays a fundamental role in the phosphorylation, nuclear localization, and transcriptional activity of FoxO1. However, these findings are not the result of an influence of rictor on total FoxO1 levels, because total cellular levels of FoxO1 are similar in wild type and rictor-deficient MEFs (Fig. 5A).

**Complementation of Rictor-deficient Cells with Constitutively Active Akt Restores FoxO1 Phosphorylation and Promotes FoxO1 Export**—The findings of the current study demonstrate that the hyperinflammatory response by LPS-stimulated, rictor-deficient cells was accompanied by a loss of Akt phosphorylation and was attenuated upon complementation with a constitutively active Akt (Fig. 2H). Because we also found that rictor-deficient cells exhibited defective FoxO1 phosphorylation and increased nuclear localization, we sought to determine whether complementation of rictor-deficient cells with a constitutively active Akt affected the phosphorylation of FoxO1. LPS stimulation of rictor-deficient MEFs transfected with an empty vector control plasmid exhibited an inability to phosphorylate FoxO1 (Fig. 6A). However, both unstimulated and LPS-stimulated rictor-deficient cells transfected with a constitutively active Akt exhibited highly elevated levels of phosphorylated FoxO1, as compared with rictor-deficient cells transfected with an empty vector control plasmid (Fig. 6A). Moreover, analysis of nuclear FoxO1 levels revealed that rictor-deficient MEFs transfected with a constitutively active Akt exhibited a significant decrease in nuclear FoxO1 levels, as compared with rictor-deficient cells transfected with an empty vector control plasmid in both the unstimulated and LPS-simulated groups (Fig. 6B).

**Loss of FoxO1 Abrogates Inflammatory Response in LPS-stimulated Rictor-deficient Cells**—To determine whether the dysregulation of FoxO1 in rictor-deficient cells was responsible for their hyperinflammatory phenotype, we knocked down FoxO1 in rictor-deficient MEFs using siRNA and assessed their ability to produce IL-6 when stimulated with LPS. As shown in Fig. 6C, siRNA targeting FoxO1 in rictor-deficient MEFs decreased the total cellular levels of FoxO1, as compared with control cells. As compared with rictor-deficient cells transfected with a control siRNA plasmid, rictor-deficient cells transfected with siRNA targeting FoxO1 produced significantly less IL-6 when stimulated with LPS ($p < 0.001$) (Fig. 6C). To confirm the role of FoxO1 in LPS-mediated inflammation, we utilized Cre/loxP mice in which FoxO1 was deleted upon expression of the DC marker CD11c (Fig. 6D). After generation of DC from bone marrow, we investigated whether the poinflammatory cytokine profile of FoxO1 KO DC was suppressed in a similar manner observed in FoxO1 knockout MEFs stimulated with LPS. Indeed, FoxO1 KO DC exhibited diminished IL-6 and IL-12 upon LPS stimulation compared with wild type DC (Fig. 6, D and E). In contrast, IL-10 levels were elevated in FoxO1 KO DC.
mTORC2 Regulates FoxO1

A. Plasmid

|          | LPS | Total Akt | Total FoxO1 | p-Foxo1(S256) | β-actin |
|----------|-----|-----------|-------------|---------------|---------|
| Ctrl     |    +| -         | -           | -             | -       |
| Myr-Akt  |    +| -         | -           | -             | -       |

B. LPS and Myr-Akt

|          | Ctrl | Myr-Akt |
|----------|------|---------|
| Time (hrs) | 0 | 6       | - | + |
| Nuclear FoxO1 | 0 | 6       | 0 | 6 |
| Histone H3    |     |         |   |     |

C. Total-FoxO1 and β-actin

D. T-FoxO1 and β-actin

E. IL-6 levels

F. mIL-12p40 levels

G. mIL-6 levels

H. mIL-10 levels

I. mIL-10 levels
upon LPS stimulation when compared with wild type DC (Fig. 6f).

To determine whether FoxO1 is a critical mediator of LPS-induced inflammation in rictor-deficient cells, we next utilized siRNA-mediated knockdown of rictor in FoxO1 KO DC. After rictor was knocked down, LPS-stimulated FoxO1 KO DC failed to up-regulate IL-6 or TNF-α compared with rictor knockdown, WT DC stimulated with LPS (Fig. 6, G and H). In addition, whereas rictor knockdown diminished IL-10 production in LPS stimulated WT DC, rictor knockdown had no effect on IL-10 production in FoxO1 KO DC (Fig. 6f).

**DISCUSSION**

The findings of the present study demonstrate that rictor plays a critical role in controlling the innate inflammatory response. The genetic deletion or siRNA-mediated knockdown of rictor resulted in the enhanced production of proinflammatory cytokines by LPS-stimulated MEFs or DC. The hyperinflammatory phenotype exhibited by rictor-deficient MEFs or DC was due to a defective Akt signaling axis, because rictor-deficient MEFs or rictor knockdown DC exhibited attenuated Akt phosphorylation and complementation with a constitutively active Akt abrogated the hyperinflammatory response. Analysis of downstream targets of Akt revealed that the phosphorylation of FoxO1 was impaired in rictor-deficient MEFs and rictor siRNA-treated dendritic cells. In contrast to wild type cells, rictor-deficient cells exhibited highly elevated levels of nuclear FoxO1 and an inability to export FoxO1 into the cytoplasm when stimulated with LPS, an effect that was restored upon complementation with a constitutively active Akt. A knockdown in the cellular levels of FoxO1 attenuated the hyperinflammatory phenotype exhibited by rictor-deficient MEFs stimulated with LPS. Furthermore, genetic deletion of FoxO1 in DC resulted in an abrogated inflammatory response to LPS. Interestingly, FoxO1 KO DC exhibited elevated IL-10 production when LPS stimulation, suggesting that, in addition to enhancing proinflammatory cytokine production, FoxO1 activity promotes suppression of IL-10 production. In support of this finding, rictor knockdown in WT DC results in suppression of IL-10. We were unable to confirm this observation with rictor-deficient cells because MEFs do not produce detectable IL-10 (data not shown).

The PI3K pathway and its ability to activate Akt has been shown to be a critical regulatory mechanism controlling the inflammatory response to LPS (19–21, 23, 28, 33). Stimulation of innate immune cells with LPS has been demonstrated to induce the phosphorylation of Akt on both threonine 308 and serine 473, in which the blockade of PI3K attenuates this site-specific phosphorylation (19, 21, 28, 34). The functional relevance of Akt in affecting the LPS-induced inflammatory response was demonstrated by the findings that the direct inhibition of Akt in monocytes resulted in the elevated production of proinflammatory cytokines and exhibited an inflammatory phenotype similar to that observed with PI3K inhibition (28). Recently the isoform of Akt involved in regulating the inflammatory response downstream of PI3K was identified by Androulidaki et al. (23) using mice deficient for Akt1 or Akt2. These studies showed that Akt1-deficient mice exhibited a hyperinflammatory response to LPS. Our current findings also demonstrated that defective Akt1 phosphorylation in rictor-deficient cells was accompanied by an elevated inflammatory response to LPS. We also provide evidence that the hyperinflammatory properties of rictor-deficient cells were attenuated upon complementation with a constitutively active Akt. Taken together, these findings highlight rictor and its ability to affect Akt signaling as a fundamental process in controlling the LPS inflammatory response.

We have shown that genetic deletion of rictor or siRNA-mediated knockdown of rictor expression resulted in the loss of Akt phosphorylation. Upon PI3K–Akt activation, Akt can phosphorylate and inactivate the tuberous sclerosis complex 2 (TSC2)–Rheb protein complex that impedes mTORC1 activity. Interestingly, studies using the mTORC1 inhibitor rapamycin have reported that the inhibition of mTORC1 augments the levels of IL-12 and TNF produced by LPS-stimulated cells (15). These findings, in conjunction with our present observations, demonstrate that both mTORC1 and mTORC2 are involved in controlling the inflammatory response to LPS. However, the underlying molecular mechanisms responsible for the ability of mTORC1 and mTORC2 to regulate the LPS inflammatory response differ. For example, studies by Weichhart et al. (15) have also shown that mTORC1 inhibition potently increased NF-κB activity, leading to the enhanced production of IL-12 in LPS-stimulated cells. In the present study, rictor-deficient MEFs stimulated with LPS displayed no difference in NF-κB p65 phosphorylation or promoter binding compared with wild type cells, illustrating a key difference between mTORC1 and mTORC2-mediated regulation of the immune response. Another downstream Akt target known to regulate TLR4 responses is GSK3-β (16, 21, 28). Inhibition of GSK3-β leads to diminished inflammatory cytokine production by innate immune cells in response to LPS along with increased in IL-10 production. However, an assessment of the phosphorylation levels of GSK3-β in rictor-deficient MEFs did not reveal any discernible differences when compared with wild type cells. These results add to the evidence that a loss of mTORC2 activ-

**FIGURE 6.** **Complementation with constitutively active Akt or knockdown of FoxO1 abrogates the hyperinflammatory response of LPS-stimulated rictor-deficient cells.** A and B, LPS-stimulated, rictor-deficient MEFs have restored FoxO1 phosphorylation (Ser-256) (44) and nuclear export of FoxO1 (44, 53) when transfected with Myr-Akt compared with rictor-deficient MEFs transfected with a control plasmid and LPS. C, siRNA was utilized to knockdown FoxO1 in rictor-deficient MEFs. Knockdown of FoxO1 by siRNA in rictor-deficient MEFs resulted in decreased production of IL-6 upon LPS stimulation compared with LPS-stimulated, rictor-deficient MEFs treated with control siRNA as assessed by ELISA. D–F, WT and FoxO1 KO DC were stimulated with LPS for 20 h, and IL-6 (D), IL-12p40 (E), and IL-10 (F) levels were assessed by ELISA. G–I, rictor was knocked down by siRNA in both WT and FoxO1 KO DC. Three days after transfection, the DC were stimulated with LPS for 20 h. IL-6 (G), TNF-α (H), and IL-10 (I) levels were determined by ELISA. For A–I, the data are representative of three to five separate experiments. For C–I, the data represent the arithmetic means ± S.D. of five separate experiments. ** and *** indicate statistical significance at p < 0.01 and p < 0.001, respectively. Ctrl, control; NS, not significant.
mTORC2 Regulates FoxO1

The FoxO family of transcription factors regulates a wide range of signaling pathways involved in cellular differentiation, survival, and tumor suppression (35). Recently evidence has shown that FoxO proteins affect the host inflammatory response. Endothelial cells and fibroblasts with FoxO1 knockdown have reduced expression of inflammatory mediators (36, 37). Macrophages harvested from obese, diabetic mice exhibit defective FoxO1 export from the nucleus upon growth factor stimulation and also display a propensity toward a hyperinflammatory immune response (25). Deletion of FoxO1 consensus sequences within the IL-1β promoter abrogated the proinflammatory phenotype in these cells, demonstrating that a failure to export FoxO1 from the nucleus could promote inflammation. Indeed, we show that rictor-deficient cells exhibit a severe defect in their ability to properly export FoxO1. Also, FoxO1 promoter binding and transcriptional activities were elevated in rictor-deficient cells, as compared with wild type cells stimulated with LPS. However, a knockdown of FoxO1 in rictor-deficient MEFs or deletion of FoxO1 in DC was sufficient to reverse the inflammatory phenotype, demonstrating that mTORC2 controls the production of inflammatory cytokines through FoxO1 regulation. Furthermore, Su et al. (25) demonstrated that overexpression of FoxO1 in RAW264.7 cells resulted in diminished IL-10 production upon LPS stimulation. Similarly, our studies demonstrated that a loss of FoxO1 in DC leads to elevated IL-10 production. The ability of FoxO1 to regulate IL-10 production could stem from the ability of FoxO1 to bind, and potentially negatively regulate, the transcription factor STAT3, which is critical for IL-10 production (38). Recently, the role of FoxO1 in TLR4 signaling was reiterated by Olefsky and co-workers (39). Overexpression of FoxO1 in RAW264.7 led to elevated production of IL-1β and IL-6 upon LPS stimulation. Because FoxO1 deletion is embryonically lethal, these investigators generated mice deficient in a single FoxO1 allele (FoxO 1/−). Ex vivo stimulation of FoxO1 1/− bone marrow-derived macrophages resulted in suppressed levels of TNF-α, IL-6, and IL-1β. In our findings, Cre/Loxp deletion of FoxO1 (1/−) in DC resulted in suppressed production of IL-6 and TNF-α. Thus, our studies, along with those of Olefsky and co-workers, demonstrate that FoxO1 actively promotes inflammation in response to LPS in both macrophages and dendritic cells. Our study also provides a mechanism by which TLR4 regulates FoxO1 activity, i.e. through mTORC2.

In summary, our findings have shown that mTORC2 suppresses inflammation in innate immune cells through Akt-mediated regulation of FoxO1. Restoring Akt function in rictor-deficient cells attenuated their defects in FoxO1 phosphorylation and nuclear export, as well as abrogated their hyperinflammatory response to LPS. These findings demonstrate a critical and novel role for mTORC2 in the innate inflammatory response through its ability to regulate FoxO1.

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