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The Role of Tuberous Sclerosis Complex 1 in Regulating Innate Immunity

Hongjie Pan,* Thomas F. O’Brien,*† Ping Zhang,* and Xiao-Ping Zhong*†

The mechanisms that control TLR-induced responses, including endotoxin tolerance, have been not well understood. The tuberous sclerosis complex 1 (TSC1) is a tumor suppressor that inhibits the mammalian target of rapamycin (mTOR). We show in this study that deficiency of TSC1 results in enhanced activation of not only mTOR complex 1 (mTORC1), but also JNK1/2, following LPS stimulation in macrophages. TSC1-deficient macrophages produce elevated proinflammatory cytokines and NO in response to multiple TLR ligands. Such enhanced TLR-induced responses can be inhibited by reducing mTORC1 and JNK1/2 activities with chemical inhibitors or small hairpin RNA, suggesting that TSC1 negatively controls TLR responses through both mTORC1 and JNK1/2. The impact of TSC1 deficiency appeared not limited to TLRs, as NOD- and RIG-I/MDA-5-induced innate responses were also altered in TSC1-deficient macrophages. Furthermore, TSC1 deficiency appears to cause impaired induction of endotoxin tolerance in vitro and in vivo, which is correlated with increased JNK1/2 activation and can be reversed by JNK1/2 inhibition. Our results reveal a critical role of TSC1 in regulating innate immunity by negative control of mTORC1 and JNK1/2 activation. The Journal of Immunology, 2012, 188: 3658–3666.

Toll-like receptors recognize pathogen-associated molecular patterns and are critical for not only innate immunity, but also adaptive immune responses against microbial infection (1–3). TLR-mediated responses are mediated by the MyD88-dependent and Toll/IL-1R domain-containing adapter inducing IFN-β–dependent pathways. The MyD88-dependent pathway is used by all TLRs, except TLR3, whereas the Toll/IL-1R domain-containing adapter inducing IFN-β–dependent pathway is used by TLR3 and TLR4. These pathways eventually lead to the activation of IkB kinases (IKKs) and MAPKs JNK1/2, p38, and ERK1/2. Activated IKKs phosphorylate IkB, resulting in its degradation and the nuclear translocation of NF-κB. These MAPKs activate multiple transcription factors such as AP1, which, together with NF-κB, promote transcription of cytokines and costimulatory molecules (4–9). Whereas TLR-mediated responses are important for host defense against microbial infection, TLR-mediated responses have to be tightly regulated. Dysregulated TLR-induced inflammatory responses can contribute to the pathogenesis of diseases (10).

The mammalian target of rapamycin (mTOR) is a central regulator of cell growth and proliferation by integrating signals from nutrients, energy status, and growth factors. It has been implicated in numerous processes, such as autophagy, transcription, protein synthesis, ribosome biogenesis, and cell survival (11–13). mTOR forms two signaling complexes, mTORC1 and mTORC2, with distinct signaling properties. mTORC1 phosphorylates pS6K1 and 4E-BP1 to promote cell growth and proliferation and is sensitive to rapamycin inhibition (14). mTORC2 phosphorylates Akt on serine 473 and protein kinase Cα to regulate cell survival and actin polymerization, respectively (15, 16). Recently, mTORC2 has been found to be able to phosphorylate protein kinase Cδ to promote Th2 immune responses in T cells (17).

Mounting evidence indicates that mTOR-mediated signaling regulates both adaptive and innate immune cell development and functions (12, 18). In T cells, mTOR can be activated by the TCR in a RasGRP1-dependent manner, and is negatively controlled by diacylglycerol kinases α and ζ (19). mTOR signaling regulates effector T cell differentiation, regulatory T cell generation and function, memory T cell responses, and T cell trafficking (20–23). Similarly, LPS and several other TLR ligands have been shown to be able to induce mTORC1 and mTORC2 activation. Both positive and negative functions of mTOR in TLR-induced innate immune responses have been reported in various innate cell lineages using rapamycin as a mTOR inhibitor (24–31). Whereas mTOR deficiency in innate immune cells has not been reported, defects of effector molecules downstream of mTOR have profound impacts on innate immunity. Deficiency of pS6K1/2 drastically decreases TLR- or virus-induced IFN-α production by plasmacytoid dendritic cell (DC) (32). In contrast, deficiency of 4E-BP1/2 results in enhanced IFN-α and IFN-β production and resistance to viral infection in vitro and in vivo due to increased IFN regulatory factor-7 translation (33). Although these observations have suggested critical roles of mTOR signaling in innate immune response, the mechanisms and the importance of mTOR regulation in innate immunity are not well understood.

The tuberous sclerosis complex (TSC) 1 associates with TSC2 to form a heterodimer. TSC1 stabilizes TSC2 and prevents its ubiquitin-mediated degradation (34). TSC1/2 complex negatively regulates mTORC1 through the GTPase activation property of TSC2 to RheB, a small GTPase protein that promotes mTORC1 activation (35). Loss-of-function mutations in TSC1 or TSC2 result in tumorigenesis correlated with elevated mTORC1 signaling.

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Abbreviations used in this article: BMM, bone marrow-derived macrophage; DC, dendritic cell; IKK, IkB kinase; iNOS, inducible NO synthase; MDP, muramyl dipeptide; MEF, murine embryonic fibroblast; mTOR, mammalian target of rapamycin; shRNA, small hairpin RNA; TSC, tuberous sclerosis complex; WT, wild type.

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(34). The role of TSC1 and the importance of mTOR regulation in the immune system have been poorly understood. Recent reports have demonstrated that TSC1 plays important roles in hematopoietic stem cells for the generation of multiple hematopoietic cell lineages (36, 37). In this study, we demonstrate that TSC1 deficiency results in increased expression of proinflammatory cytokines and NO in macrophages in response to TLR stimulation due to increased activation of mTORC1 and JNK1/2. Furthermore, TSC1 deficiency causes impairment of endotoxin tolerance in vitro and in vivo. Our data establish that TSC1 is an important negative regulator of TLR-induced innate immunity by inhibiting both mTORC1 and JNK1/2 activation.

Materials and Methods

Mice and reagents

TSC1f/f mice and ErCre mice were described previously (38, 39). TSC1f/f, ErCre or TSC1f/f ErCre mice were i.p. injected with 200 μl 10 mg/ml tamoxifen (Sigma-Aldrich, St. Louis, MO) on days 1, 2, and 5. Mice were used for experiments at 7–9 weeks of age. Tamoxifen treatment was effective, as determined by PCR analysis. EMD Biosciences (San Diego, CA) provided Poly(I:C), Pam3CSK4, C12-iE-DAP, muramyl dipeptide (MDP), and LyoVec were purchased from Invivogen (San Diego, CA). Rapamycin, SP600125, JNK inhibitor VIII, and SB203580 were purchased from EMD Biosciences (San Diego, CA).

Generation of bone marrow-derived macrophages

Bone marrow cells from fetuses and tibias were flushed and plated into Petri dishes containing RPMI 10 (RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 1000 U/ml streptomycin, and 20 mM l-glutamine) containing 15% L929 cell conditioned medium, as previously described (40). After 2–3 d of culture at 37°C in a CO2 incubator, nonadherent cells were transferred to new plates with fresh medium for another 3–5 d before they were used for experiments. More than 95% of cells were CD11b+ by flow cytometry analysis.

Phagocytosis

Listeria monocytogenes strain was grown overnight in brain heart infusion broth at 37°C with shaking. Approximately 1 × 10⁶ L. monocytogenes were added to BMMs, washed twice with PBS, and suspended in 1 ml RPMI 1640 medium. A total of 1 × 10⁶ bone marrow-derived macrophages (BMMs) in 1.0 ml medium was added to each well in a 12-well plate. After overnight incubation, 1 × 10⁶ CFSE-labeled L. monocytogenes were added to BMMs. The cells were incubated at 37°C for 0, 20, 40, and 60 min. After removal of culture medium, adherent BMMs were washed twice with 2.0 ml PBS and then incubated with PBS containing 1% paraformaldehyde for 30 min at room temperature. Cells were scraped off for flow cytometry analysis.

Stimulation of BMMs

A total of 200,000 BMMs from both TSC1f/f ErCre+ (TSC1KO) and TSC1f/f ErCre wild type (WT) were equally seeded in 24-well plates in 0.5 ml RPMI 10 in triplicates. After overnight incubation at 37°C in a CO2 incubator, BMMs were treated with 10 ng/ml LPS, Poly(I:C), Pam3CSK4, C12-EI-DAP (C12-EI), MDP, and Poly(I:C)-LyoVec for the indicated times. At each time point, supernatants were harvested for cytokine analyses and cells were used for making RNA using TRizol reagent. For biochemical analysis, 0.5 × 10⁶ BMMs were plated in 60-cm petri dishes and cultured at 37°C overnight. BMMs cells were then starved in serum-free medium for 2–3 h and treated with 10 ng/ml LPS for 0, 15, 30, and 45 min. Cells were washed once with cold PBS, lysed in Nonidet P-40 in Trition X-100 lysis buffer with freshly added protease and phosphatase inhibitor mixtures (Sigma-Aldrich), and then subjected for Western blot analysis. For induction of endotoxin tolerance, BMMs were treated with 1 ng/ml LPS prior to high dose of LPS restimulation (10 ng/ml). For the in vivo assay, tamoxifen-treated TSC1f/f ErCre and TSC1f/f ErCre mice were i.p. injected with 1 and 2 mg/kg LPS on days −3 and −2 and with 40 and 90 mg/kg LPS on days 0 and 2 in 200 μl PBS, respectively.

Cytokine and NO concentration

Cytokine levels in culture supernatant or in sera were determined using commercial ELISA kit for TNF-α, IL-12p40, and IL-6 (BioLegend, San Diego, CA), according to the manufacturer’s instruction. NO was measured using Griess assay with NaNO2 as standard. Each value represents the mean of triplicate values.

RNA interference with small hairpin RNAs

plKOPuro-ShRaptor plasmid was purchased from Addgene (Cambridge, MA). The complementary oligonucleotides to generate the small hairpin RNA (shRNA) against mouse JNK1 were designed using BLOCK-it RNAi Designer from Invitrogen (Carlsbad, CA). Oligo sequences that we used were as follows: ShJNK1-RNA, 5'-GGATGCAATCTTGTGCAGTG-3'. Double-stranded oligos were subcloned into plKOPuro. plKOPuro-ShLuc was used as a control. plKOPuro-ShLuc and plKOPuro plasmids were generous gifts of J. Yang and R.A. Weinberg (Whitehead Institute for Biomedical Research, Cambridge, MA). plKOPuro-ShLuc– and plKOPuro-targeted ShJNK1 were cotransfected with pCMV-VSVG and pH’8.Δ2Δ (Weinberg) into 293T cells. After 48 h, culture media were collected and centrifuged at 2000 rpm for 10 min at room temperature. Supernatants containing lentiviruses were used for transducing BMMs. To transduce BMMs, nonadherent cells on day 3 of vitro BMMs culture were transferred into a 6-well plate (1 × 10⁶ cells/well in 2 ml BMMs culture medium), followed by addition of 500 μl viral supernantant together with polybrene at a final concentration of 1 μg/ml. The mixture of cells and viruses was spun at 500 rpm for 90 min at room temperature. After overnight incubation at 37°C in a CO2 incubator, culture medium was replaced with 2 ml fresh medium containing 1 μg/ml puromycin. With two additional replacements of medium containing puromycin on days 2 and 3 after transduction to remove dead cells, the selected cells were used for experiments on day 5 or 6 postinfection.

Real-time PCR

Total RNA was prepared by TRIzol reagent (Invitrogen). Reverse-transcription reaction was performed using random primers and SuperScript III reverse transcriptase (Invitrogen). SYBR Green real-time PCR was conducted using RealMasterMix on Realplex machine (Eppendorf), according to manufacturer’s protocol. The primer pairs used were as follows: β-actin, forward, 5'-TGGCTACCTTTGACAGTAGT-3' and reverse, 5'-AGCTGACCAAGTTCCTGAGGTA-3'; TNF-α, forward, 5'-GCCCAGACAGGTGAGTGTG-3' and reverse, 5'-CACCCTGGTGAGTTGTCG-3', TGCATACA-3'; IL-12p40, forward, 5'-TCTGAGGACTCATACACTCGTC-3' and reverse, 5'-TGTTGCTTCACACTCACGGG-3'; IL-6, forward, 5'-TCCATACGTTGCTTCTGAGG-3' and reverse, 5'-ACCCTTGCTACCTGAGTGC-3'; and IFN-β, forward, 5'-CCCTATGAGTACGAGCAAGA-3' and reverse, 5'-CTGCTGCTGTGAGGTC-3'.

Western blot and Abs

Protein concentrations in cell lysates were determined using the Bio-Rad protein assay at OD 595 nm. Equivalent amounts of protein for each sample were subjected to SDS-PAGE and transferred onto nitrocellulose membrane (Bio-Rad). After blocking with 5% nonfat dry milk/PBS, the membranes were incubated with a primary Ab overnight at 4°C, washed with TBST (100 mM Tris [pH 7.5], 0.9% NaCl, 0.1% Tween-20) three times, reacted with a secondary Ab for 45 min, and washed with TBST three times. Protein bands were visualized by ECL (PerkinElmer Life Sciences). The blots were stripped and reprobed for loading control. Anti- phospho-ERK1/2, anti-phospho-p70S6K, anti-phospho–4E-BP1 Thr14/Ser46, anti–phospho-Akt S473 (193H12), anti–phospho-IκBα, anti–phospho-p38, anti–phospho-JNK1/2 (JIS8Y135), anti-TSC1, anti-TSC2, anti-pERK1/2, anti-p70S6K, anti–4E-BP1, anti-AKT, anti-IEBx, anti-JNK1/2, and anti-p38 Abs were purchased from Cell Signaling Technology. PE-conjugated anti-mouse F4/80, CD11b, and IgG control Abs were obtained from BioLegend (San Diego, CA). Anti–NF-κBp65 and anti–β-actin were purchased from Santa Cruz Biotechnology and Sigma-Aldrich, respectively.

Immunofluorescence staining and confocal microscopy

WT and TSC1 knockout BMMs were grown on chamber slides (BD Discovery Labware, Bedford, MA) in RPMI 1640 supplemented with 15% L cell conditioned medium for 24 h at 37°C. The cells were treated with or without LPS at 10 ng/ml to 30 and 60 min. Immediately after LPS treatment, the cells were washed with ice-cold PBS twice, and then were fixed in 4% paraformaldehyde/PBS for 15 min, permeabilized with 0.2% Triton X-100/0.1% Tween 20/PBS for 5 min, and blocked in 1% BSA/PBS for 30 min. The slides were then incubated with anti-mouse NF-κBp65 Ab that was diluted at 1/100 in blocking solution for 2 h, washed three times with PBS, and incubated with FITC-conjugated anti-mouse IgG Ab (Jackson Laboratory, Cambridge, MA). Anti–phospho-JNK, anti–phospho-p38, and anti–phospho–4E-BP1 Abs were obtained from Cell Signaling Technology.
ImmunoResearch Laboratories) for 1 h. After additional washes, the slides were incubated with DAPI for 3 min and mounted with antifade solution. Cells were examined under the Zeiss LSM510 inverted confocal microscope, and images were acquired and analyzed with LSM image software.

Statistical analysis
For statistical analysis, the two-tail Student t test was performed for all data, except those from the in vivo endotoxin tolerance experiment. Endotoxin tolerance data were analyzed by the Fisher’s exact test (*p < 0.05, **p < 0.01).

Results
TSC1 and TSC2 expression in macrophages
To determine the role of TSC1 and the importance of tight control of mTOR signaling in innate immune responses, we first examined TSC1 and TSC2 expression in macrophages. Both TSC1 and TSC2 were detected in BMMφ, and their expression was upregulated following LPS (TLR4 ligand) stimulation for 24 h (Fig. 1A). To investigate the role of TSC1 in innate immunity, we crossed TSC1 conditional knockout mice (TSC1f/f) with the ERCre mice and generated TSC1f/f-ERCre mice to allow deletion of TSC1 following tamoxifen treatment. TSC1 was barely detectable in BMMφ from tamoxifen-treated TSC1f/f-ERCre (TSC1KO) mice (Fig. 1B), confirming effective deletion of the gene. TSC2 protein was severely decreased in TSC1KO BMMφ, suggesting that TSC1 is required for TSC2 stability in macrophages, which is consistent with the reported role of TSC1 in other cells (41). Almost all in vitro differentiated TSC1f/f (WT) and TSC1KO BMMφ were CD11b and F4/80 positive, and TSC1KO BMMφ expressed similar levels of CD11b and F4/80 to WT BMMφ, suggesting that in vitro macrophage differentiation is not inhibited by TSC1 deficiency (Fig. 1C). However, TSC1 deficiency obviously impacted on BMMφ size. Although similar to controls on day 3, TSC1KO BMMφ progressively increased their size after 7 d of in vitro differentiation (Fig. 1D, 1E), indicating that TSC1 inhibits the growth of BMMφ. Macrophages express MHC and costimulatory molecules that can be induced by LPS stimulation to regulate adaptive immune responses. TSC1KO BMMφ expressed similar levels of MHC class II I-Aβ, but elevated CD80 and CD86 co-

FIGURE 1. Effects of TSC1 deficiency on macrophage morphology and expression of costimulatory molecules. (A) Increased TSC1 and TSC2 proteins in BMMφ following LPS stimulation. WT BMMφ were left unstimulated or stimulated with LPS for 24 h. TSC1 and TSC2 protein in cell lysates were detected by immunoblotting analysis with anti-TSC1 and anti-TSC2 Abs as well as an anti–β-actin Ab for loading control. (B) Diminished TSC2 proteins in TSC1KO BMMφ. TSC1KO and WT BMMφ lysates were subjected to immunoblotting analysis, as in (A). (C) TSC1 deficiency does not inhibit BMMφ differentiation in vitro. WT and TSC1KO BMMφ were generated in vitro with M-CSF. CD11b and F4/80 expression on these BMMφ on day 7 during in vitro differentiation were determined by flow cytometry. (D and E) Increased sizes of TSC1KO BMMφ during in vitro differentiation. (D) Bar figures show BMMφ sizes (mean ± SD) on indicated days from three experiments. *p < 0.05, **p < 0.01. (E) Typical microscopic morphology of WT and TSC1KO BMMφ on day 8 during in vitro differentiation. (F) Increased costimulatory molecule expression in TSC1KO BMMφ. WT and TSC1KO BMMφ were left unstimulated or stimulated with LPS for 24 h. MHC class II, CD80, and CD86 expression on CD11b+ BMMφ were determined by flow cytometry. Data shown are representative of at least three experiments. (G) Phagocytosis of L. monocytogenes by WT and TSC1KO BMMφ. Macrophages were incubated with CFSE-labeled L. monocytogenes for 0, 20, 40, and 60 min. After being washed with PBS and fixed with 1% paraformaldehyde, cells were subjected to flow cytometry analysis to assess CFSE intensity. Data shown are representative of at least three (A–F) and two experiments (G).
stimulatory molecules before and after LPS stimulation compared with WT BMMφ (Fig. 1F). However, we could not rule out a potential contribution of enlarged sizes of TSC1KO BMMφ to the increased detection of CD80 and CD86 levels in these cells.

To further determine whether TSC1KO BMMφ maintain macrophage properties, we examined their ability to phagocytose bacteria by incubating the cells with CFSE-labeled L. monocytogenes for the indicated times, followed by FACS analysis. As shown in Fig. 1G, TSC1KO BMMφ uptake similar amount of L. monocytogenes to WT BMMφ, suggesting that TSC1 deficiency did not obviously affect BMMφ phagocytosis. Together, these observations reveal that TSC1 and TSC2 expression is regulated by LPS stimulation, that TSC1 is critical for maintaining normal expression of TSC2, that TSC1 is not required for in vitro macrophage differentiation, and that TSC1 may inhibit costimulatory molecule expression in macrophages.

Altered proinflammatory cytokine production in the absence of TSC1

To investigate how the absence of TSC1 may affect TLR-mediated innate immune responses, we first measured TLR-induced cytokine production. Following LPS stimulation for different times, TSC1KO BMMφ produced more proinflammatory cytokines TNF-α, IL-12p40, and IL-6 at both the protein and mRNA levels than WT BMMφ (Fig. 2A, 2B). Thus, TSC1 functions at least at the transcription level to inhibit the production of these cytokines. To test whether such enhanced proinflammatory responses are specific to TLR4, we stimulated WT and TSC1KO BMMφ with Pam3CSK4 and Poly(I:C), ligands for TLR2 and TLR3, respectively. TSC1KO BMMφ produced higher levels of TNF-α than WT BMMφ (Fig. 2C), suggesting that TSC1 negatively controls cytokine production from multiple TLRs in macrophages.

NOD1, NOD2, RIG-I, and MDA-5 are important intracellular microbial pattern recognition molecules. To test whether TSC1 also plays a role in innate responses induced by these receptors, we stimulated WT and TSC1KO BMMφ with C12-iE-DAP and MDP to activate NOD1 and NOD2, respectively. As shown in Fig. 2D, TNF-α mRNA levels induced by these ligands were much lower in TSC1KO BMMφ than in WT control. When stimulated with Poly(I:C)/LyoVec to activate RIG-I/MDA-5, TSC1KO BMMφ produced higher levels of TNF-α than WT BMMφ (Fig. 2E), suggesting that TSC1 negatively controls cytokine production from multiple TLRs in macrophages.

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Altered innate immune responses in TSC1KO mice

To further assess the role of TSC1 in LPS-induced responses in vivo, we compared serum cytokine concentrations in tamoxifen-treated TSC1f/f-ERcre and TSC1f/f control mice following LPS injection. As shown in Fig. 2F, higher serum concentrations of IL-10, TNF-α, and IL-6 were detected in TSC1f/f-ERcre mice than in TSC1f/f mice. Together, these data demonstrate that TSC1 functions as a negative regulator of TLR-induced proinflammatory cytokine production in vitro and in vivo.
**Increased NO production in TSC1-deficient macrophages**

NO is a highly reactive molecule involved in innate immunity that can be induced by TLR stimulation. Increased NO levels were detected in TSC1KO BMM\(\phi\) following LPS stimulation compared with WT BMM\(\phi\) (Fig. 2G). In WT BMM\(\phi\), the inducible NO synthase (\(i\)NOS, the enzyme responsible for NO production) can be induced 6 h after LPS stimulation. In TSC1KO BMM\(\phi\), LPS-induced \(i\)NOS protein expression was elevated (Fig. 2H). \(i\)NOS mRNA could be detected 3 h and further upregulated 6 and 24 h after LPS stimulation. In TSC1KO BMM\(\phi\), much higher levels of \(i\)NOS mRNA were detected during the course of LPS stimulation (Fig. 2I), suggesting that TSC1 also inhibits \(i\)NOS production at least at the transcription level.

**Enhanced JNK1/2 activation in TSC1-deficient macrophages**

Enhanced JNK1/2 activation in TSC1-deficient macrophages

To explore the mechanisms by which TSC1 inhibits TLR-induced innate immune responses, we examined how TSC1 deficiency may affect TLR signaling. A critical event in TLR signaling is the activation of IKKs. IKKs phosphorylate I\(\kappa\)B, leading to its degradation and subsequent NF-\(\kappa\)B nuclear translocation to activate gene transcription. I\(\kappa\)B phosphorylation and total I\(\kappa\)B protein level were not drastically altered in TSC1KO BMM\(\phi\) compared with WT BMM\(\phi\) (Fig. 3A). Furthermore, NF-\(\kappa\)B translocation from the cytosol to the nucleus following LPS stimulation was similar between WT and TSC1KO BMM\(\phi\) (Fig. 3B). Thus, absence of TSC1 does not obviously alter the activation of the IKK/NF-\(\kappa\)B pathway.

TLR-induced activation of MAPKs also contributes to inflammatory responses. LPS-induced ERK1/2 phosphorylation in TSC1KO BMM\(\phi\) was slightly higher than WT BMM\(\phi\) (Fig. 3A). The p38 phosphorylation in TSC1KO BMM\(\phi\) exhibited delayed kinetics compared with WT BMM\(\phi\). However, JNK1/2 phosphorylation was significantly enhanced in TSC1KO BMM\(\phi\) following LPS stimulation, suggesting that TSC1 negatively controls TLR4-induced JNK1/2 activation in macrophages.

**FIGURE 3.** TSC1 inhibits JNK1/2 and mTORC1, but not IKK/NF-\(\kappa\)B activation in macrophage. (A) Enhanced TLR4-induced JNK1/2 activation in the absence of TSC1. Cell lysates from unstimulated or LPS (10 ng/ml)-stimulated WT (W) and TSC1KO (K) BMM\(\phi\) were subjected to immunoblotting analysis with the indicated Abs. (B) Nuclear translocation of NF-\(\kappa\)B p65 in WT and TSC1KO BMM\(\phi\) following LPS stimulation. WT and TSC1KO BMM\(\phi\), grown on chamber slides, were treated with or without LPS at 10 ng/ml for 30 and 60 min. After fixation and permeabilization, cells were stained with an anti-mouse p65 Ab, followed by FITC-labeled anti-mouse secondary Ab. Nuclei were counterstained with DAPI. Cells were examined under the Zeiss LSM510 inverted confocal microscope. Original magnification ×440. (C) Enhanced mTORC1, but decreased mTORC2 activation in the absence of TSC1. Cell lysates from unstimulated or LPS (10 ng/ml)-stimulated WT and TSC1KO BMM\(\phi\) were subjected to immunoblotting analysis with the indicated Abs. Data shown are representative of three experiments.

Enhanced mTORC1 but decreased mTORC2 activation in TSC1-deficient macrophages

We asked further how TSC1 deficiency may affect TLR-induced mTOR signaling in BMM\(\phi\). As shown in Fig. 3C, TSC1KO BMM\(\phi\) displayed elevated phosphorylation of p70S6K1 and 4E-BP1 before and/or after LPS stimulation compared with WT BMM\(\phi\), indicating that TSC1 inhibits TLR-induced mTORC1 activation. In contrast, Akt phosphorylation at S473, a mTORC2-dependent event, was decreased in TSC1KO BMM\(\phi\) compared with WT cells, suggesting that TSC1 is required for TLR-induced mTORC2 activation. TSC1/2 may promote mTORC2 signaling by directly participating in mTORC2 activation or by preventing mTORC1-mediated inhibitory mechanisms, as demonstrated in other cell types. Together, these observations indicate that TSC1 inhibits mTORC1 activation, but promotes mTORC2 activation following TLR4 stimulation in BMM\(\phi\).
contributed to the enhanced TLR-induced responses in the absence of TSC1.

**Contribution of JNK1/2 activity to the elevated LPS-induced innate immune responses in TSC1KO BMMφ**

Because JNK1/2 activation was elevated in TSC1KO BMMφ, we further examined whether the elevated JNK1/2 activation might contribute to the enhanced LPS-induced response in TSC1KO BMMφ. Inhibition of JNK1/2 with SP600125 (SP; JNK inhibitor VIII, VIII) and/or rapamycin further reduced NO production in TSC1KO BMMφ treated with either ShJNK1 or ShLuc. JNK1 protein levels were determined by Western blot (F). TNF-α protein (G) and mRNA (H) in WT and TSC1KO BMMφ transduced with the indicated shRNA following LPS (10 ng/ml) stimulation for 6 h were determined by ELISA and real-time quantitative PCR, respectively. $^{*}p < 0.05$, $^{**}p < 0.01$.

Together, these data suggest that JNK1/2 activity is important for the elevated proinflammatory responses in TSC1KO BMMφ and that increased mTORC1 activity partially contributes to the enhanced JNK1/2 activation in these cells.

**Effects of TSC1 deficiency on the induction of endotoxin tolerance**

TLR-induced tolerance is important in preventing uncontrolled proinflammatory responses detrimental to the host (42). The inhibitory role of TSC1 in TLR-mediated proinflammatory responses prompted us to examine how TSC1 deficiency may affect endotoxin tolerance. LPS-pretreated WT BMMφ failed to produce TNF-α and IL-12p40 following secondary high-dose LPS stimu-
luation. However, LPS-pretreated TSC1KO BMMφ still produced high levels of these cytokines following secondary LPS stimulation (Fig. 6A, 6B). Of note, LPS-pretreated TSC1KO BMMφ produced less cytokines than the same cells without LPS pretreatment following secondary LPS stimulation, suggesting that TSC1 deficiency only partially impaired the induction of LPS tolerance in vitro. To determine whether TSC1 deficiency affected the induction of LPS tolerance in vivo, we injected two low doses of LPS into tamoxifen-pretreated TSC1<sup>fl/fl</sup> and TSC1<sup>fl/fl-ER<sub>Cre</sub></sup> mice, followed by two high doses of LPS challenge. All TSC1<sup>fl/fl</sup> mice survived high-dose LPS challenges. In contrast, all TSC1<sup>fl/fl-ER<sub>Cre</sub></sup> mice succumbed to high-dose LPS challenges, correlating with elevated serum IL-6 and IL-12p40 levels (Fig. 6C, 6D). Together, these observations suggest that TSC1 is important for the induction of endotoxin tolerance in vitro and in vivo, although we cannot rule out that dysregulated proinflammatory cytokine production caused by TSC1 deficiency may contribute to the impaired induction of endotoxin tolerance in TSC1 deficiency mice.

Role of JNK1/2 activity for altered endotoxin tolerance in TSC1-deficient BMMφ

To investigate the mechanisms by which TSC1 contribute to endotoxin tolerance, we analyzed LPS-induced signaling in macrophages that had already been pretreated with a low dose of LPS. We first examined mTORC1 signaling in LPS-tolerated condition in WT BMMφ. As shown in Fig. 6E, phosphorylation of S6K was not reduced and phosphorylation of 4E-BP1 was obviously increased in LPS-pretreated WT BMMφ before and after secondary LPS stimulation. Because LPS stimulation for 24 h upregulates TSC1/2 expression, this result suggests that mechanisms in addition to TSC1/2 may be involved in regulating mTORC1 signaling in LPS-tolerated macrophages. In TSC1KO BMMφ, pretreatment with LPS did not obviously alter the hyperphosphorylation status of S6K and 4E-BP1 in these cells. Phosphorylation of these proteins in LPS-pretreated TSC1KO BMMφ remained high following secondary LPS stimulation.

Different from mTORC1 signaling, JNK1/2 phosphorylation was decreased in LPS-pretreated WT BMMφ following secondary LPS stimulation (Fig. 6E). However, JNK1/2 phosphorylation remained increased in LPS-pretreated TSC1KO BMMφ following secondary LPS stimulation. Treatment of TSC1KO BMMφ with SP600125 significantly reduced NO and TNF-α production in the cells induced by secondary LPS stimulation (Fig. 6F), suggesting that elevated JNK1/2 activation in TSC1KO BMMφ may contribute to their resistance to endotoxin tolerance.

Discussion

The role of the TSC–mTOR pathway in TLR-induced innate immune responses has been controversial. Most published observations were based on in vitro and in vivo administration
of rapamycin. Rapamycin has been found to be able to inhibit or increase TLR-induced proinflammatory cytokine production (24, 25, 27, 28, 30). Such differences could be attributed to the different cells examined. For example, rapamycin was found to be able to enhance IL-12 production in murine and human myeloid DCs by promoting NF-κB activation, but inhibit IL-12 production in monocoyte-derived DCs and bone marrow-derived DCs (24, 27, 28). In addition, the length and timing of rapamycin treatment may have an impact on its effects on TLR-induced innate immune responses. Our study has provided genetic evidence that TSC1 inhibits TLR-induced proinflammatory innate immune responses. Because enhanced proinflammatory responses in TSC1KO BMMs in response to LPS can be inhibited by rapamycin, our data support that mTORC1 positively regulates TLR-induced proinflammatory cytokine production. The PI3K/Akt pathway has been reported to be able to inhibit TLR-induced cytokine production (43–45). At present, we cannot rule out that decreased mTORC2 signaling in TSC1-deficient macrophages may also contribute to the elevated cytokine production in these cells. In addition to regulating TLR-mediated innate immunity, we have also shown that TSC1 differentially controls NOD1/2- and RIG-I/MDA5-induced innate immunity. TSC1 deficiency caused decreased TNF-α production, but increased IFN-β expression in BMMs after NOD1/2 and RIG-I stimulation, respectively.

Our results are different from those observed in immortalized TSC2-deficient murine embryonic fibroblasts (MEFs) with compound p53 deficiency. In TSC2-deficient MEFS, TLR-induced mTORC1 signaling is enhanced, but proinflammatory responses are decreased, as correlated with impaired IKK activation and NF-κB transcription to the nuclei (28). We have not observed obvious defect of IKK/NF-κB activation in TSC1-deficient macrophages. LPS-induced IκB phosphorylation and degradation as well as NF-κB p65 nuclear translocation are similar between WT and TSC1KO BMMs. The differences between these studies could be resulted from different cell types examined. Furthermore, the compound deficiency of both p53 and TSC2 in MEF cells could impact on the phenotype observed. Although the role of TSC1 in other cell lineages needs to be examined in the future, our data support that TSC1 is a negative regulator of TLR-induced response at least for BMMs and in vivo.

In addition to regulating mTOR signaling, our data reveal that TSC1 inhibits TLR-induced JNK1/2 activation and that enhanced JNK1/2 activation contributes to the enhanced proinflammatory response and resistance to endotoxin tolerance when TSC1 is deficient. Because enhanced JNK1/2 activation in TSC1-deficient BMMs can only be partially inhibited by acute rapamycin treatment, it suggests that mTORC1 functions as an upstream regulator for JNK1/2 activation during TLR signaling and that TSC1 may also control JNK1/2 activation through mTORC1-independent mechanisms. It has also been reported that TLR3-induced JNK1/2 activation in keratinocytes is inhibited by rapamycin (29). Thus, mTORC1 may function as a positive regulator of JNK1/2 activation for multiple TLRs.

In summary, we have demonstrated that TSC1 plays important roles in innate immune responses. TSC1 exerts differential roles for LPS-induced mTORC1 and mTORC2 signaling by inhibiting mTORC1, but promoting mTORC2 activation in macrophages. In addition, TSC1 functions as a negative regulator for LPS-induced JNK1/2 activation, and uncontrolled mTORC1 and JNK1/2 activation contributes to enhanced proinflammatory responses and impaired endotoxin tolerance caused by TSC1 deficiency.


