RESEARCH ARTICLE

TSC1 controls IL-1 β expression in macrophages via mTORC1-dependent C/EBP β pathway

Tao Yang^{1*}, Linnan Zhu^{1*}, Yanhua Zhai², Qingjie Zhao³, Jianxia Peng¹, Hongbing Zhang⁴, Zhongzhou Yang⁵, Lianfeng Zhang⁶, Wenjun Ding³ and Yong Zhao¹

The tuberous sclerosis complex 1 (TSC1) is a tumor suppressor that inhibits the mammalian target of rapamycin (mTOR), which serves as a key regulator of inflammatory responses after bacterial stimulation in monocytes, macrophages, and primary dendritic cells. Previous studies have shown that TSC1 knockout (KO) macrophages produced increased inflammatory responses including tumor necrosis factor- α (TNF- α) and IL-12 to pro-inflammatory stimuli, but whether and how TSC1 regulates pro-IL-1 β expression remains unclear. Here using a mouse model in which myeloid lineage-specific deletion of TSC1 leads to constitutive mTORC1 activation, we found that TSC1 deficiency resulted in impaired expression of pro-IL-1 β in macrophages following lipopolysaccharide stimulation. Such decreased pro-IL-1 β expression in TSC1 KO macrophages was rescued by reducing mTORC1 activity with rapamycin or deletion of mTOR. Rictor deficiency has no detectable effect on pro-IL-1 β synthesis, suggesting that TSC1 positively controls pro-IL-1 β expression through mTORC1 pathway. Moreover, mechanism studies suggest that mTORC1-mediated downregulation of the CCAAT enhancer-binding protein (C/EBP β) critically contributes to the defective pro-IL-1 β expression. Overall, these findings highlight a critical role of TSC1 in regulating innate immunity by control of the mTOR1-C/EBP β pathway.

Cellular & Molecular Immunology (2016) 13, 640–650;doi:10.1038/cmi.2015.43;published online 25 May 2015

Keywords: IL-1; inflammation; innate immunity; macrophages; mTOR, TSC1

INTRODUCTION

Macrophages are migratory phagocytic cells and play a key role in the innate immune response and adaptive immune response¹. Macrophages are polarized to the classic M1 macrophages by exposure to IFN- γ and GM-CSF, or in the presence of bacterial products such as lipopolysaccharide (LPS). M1 macrophages produce high levels of pro-inflammatory cytokines including tumor necrosis factor- α (TNF- α), interleukin 1 β (IL-1 β), IL-6, IL-12, and IL-23, and increased levels of reactive oxygen species^{2–6}. IL-1 β , an important pro-inflammatory cytokine, belongs to the IL-1 family of cytokines. The significant pro-inflammatory function of cytokine IL-1 β is to

recruit inflammatory and immunocompetent cells infiltrating from the circulation into the extravascular space and then into the site of injury or infection^{7–9}. IL-1 β is also an angiogenic factor and functions in tumor angiogenesis, invasiveness, and metastasis^{10,11}. Moreover, IL-1 β has long been linked to the pathogenesis of atherosclerosis and rheumatoid arthritis diseases^{12–14}. The cytokine IL-1 β is mostly produced by activated monocytes, macrophages, and primary dendritic cells (DCs) mainly through a NF- κ B-dependent pathway^{15,16}. The expression of IL-1 is regulated at the levels of transcription, mRNA stabilization, and post-translational proteolytic processing¹⁴. IL-1 β is first translated as an inactive precursor protein,

¹State Key Laboratory of Biomembrane and Membrane Biotechnology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China; ²Division of Molecular Embryonic Development, State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China; ³School of Life Science, University of Chinese Academy of Sciences, Beijing, China; ⁴Department of Physiology and Pathophysiology, National Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences and School of Basic Medicine, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China; ⁵MOE Key Laboratory of Model Animal for Disease Study, Model Animal Research Center, Nanjing University, Nanjing, China; and ⁶Key Laboratory of Human Diseases Comparative Medicine, Ministry of Health; Institute of Laboratory Animal Science, CAMS & PUMC, Beijing, China

*Tao Yang and Linnan Zhu contributed equally to this work as co-first authors.

Correspondence: Dr Yong Zhao, Transplantation Biology Research Division, State Key Laboratory of Biomembrane and Membrane Biotechnology, Institute of Zoology, Chinese Academy of Sciences, Beichen West Road 1-5, Chaoyang District, Beijing 100101, China.

E-mail: zhaoy@ioz.ac.cn

Dr Wenjun Ding, College of Life Sciences, Graduate University of the Chinese Academy of Sciences, No. 19A Yuquan Road, Beijing 100049, China.

E-mail: dingwj@gucas.ac.cn

Received: 3 August 2014; Revised: 23 April 2015; Accepted: 23 April 2015

pro-IL-1 β , which is then proteolytically cleaved at Asp116 into its mature form by an inflammasome¹⁷.

The mammalian target of rapamycin (mTOR) is a conserved serine–threonine kinase that is essential in broad aspects of cellular activity, including cell growth, survival and aging. mTORC1 can be activated by growth factors through the tuberous sclerosis complex (TSC), which consists of TSC tumor suppressors, TSC1 and TSC2, and Tre2-TBC1D7¹⁸. TSC1/2 complex negatively regulates mTORC1 through the GTPase activation property of TSC2 to RheB, a small GTPase protein that promotes mTORC1 activation¹⁹. Some studies showed that activation of mTOR downregulated IL-12p70 and IL-23 production in human macrophages^{20,21}. Moreover, IL-12 was enhanced, whereas IL-10 was blocked after mTOR inhibition in mouse bone marrow-derived macrophages (BMDMs) and mDCs²². These findings indicate that activated mTOR may limit pro-inflammatory responses. On the contrary, other studies indicated that TSC1-deficient macrophages produced elevated pro-inflammatory cytokines including TNF- α , IL-12p40, and IL-6 in response to multiple TLR ligands^{19,23}. Moreover, TSC1-deficient BMDMs treated by LPS secreted more of the pro-inflammatory cytokines such as IL-6 and TNF- α but less of the anti-inflammatory cytokine IL-10²⁴.

The involvement of mTOR pathway in the regulation of and IL-1 β expression has been studied in mouse and human macrophages^{22,25,26}. In the present study, using TSC1-deficient macrophages and macrophage cell lines, we studied the role of TSC1 on pro-inflammatory cytokine IL-1 β expression and explored the associated molecular mechanism. We found that LPS-induced pro-IL-1 β synthesis was significantly downregulated at both the mRNA and protein levels in TSC1-deficient macrophages, and prolonged rapamycin (Rapa) treatment or mTOR deletion significantly rescued this phenotype. mTORC1, but not mTORC2, was involved in the suppression of pro-IL-1 β transcription by selectively inhibiting the CCAAT enhancer-binding protein (C/EBP β) expression. Moreover, overexpression of C/EBP β significantly reversed the defect of pro-IL-1 β expression in TSC1 knockout (KO) BMDMs.

MATERIALS AND METHODS

Animals

Myeloid-specific TSC1, mTOR, and Rictor conditional KO mice were obtained by crossing *TSC1^{loxp/loxp}*, *mTOR^{loxp/loxp}*, or *Rictor^{loxp/loxp}* mice with mice-expressing Cre recombinase under the control of the Lysozyme promoter (*LysMCre*)²³. *LysMCre^{+/-}TSC1^{loxp/loxp}*, *LysMCre^{+/-}mTOR^{loxp/loxp}*, and *LysMCre^{+/-}Rictor^{loxp/loxp}* were referred to as TSC1 KO, mTOR KO, and Rictor KO, respectively. *LysMCre*-negative littermates served as the control (referred to as wild-type (WT)). About 4- to 5-week-old mice were usually used for the *in vitro* experiments. *TSC1^{loxp/loxp}* mice were the generous gifts of Dr Hongbing Zhang (Institute of Basic Medical Sciences and School of Basic Medicine, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing, China)^{27,28}. *mTOR^{loxp/loxp}* mice were kindly provided

by Dr Zhongzhou Yang from the Center of Model Animal Research at Nanjing University, China²⁷. *LysMCre* mice were kindly offered by Dr Lianfeng Zhang from the Key Laboratory of Human Diseases Comparative Medicine, Ministry of Health; Institute of Laboratory Animal Science, CAMS & PUMC. All mice were maintained in a specific pathogen-free facility. All experimental manipulations were undertaken in accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals, Institute of Zoology (Beijing, China).

Reagents

Rapa (LC laboratory) was reconstituted in ethanol at 60 mg/mL and then diluted with DMSO (Sigma-Aldrich) to 60 μ g/mL. Bacterial LPSs (*E. coli* 055:B5) were purchased from Sigma-Aldrich (St Louis, MO, USA). The antibodies against TSC1, TSC2, CREB, H3, C/EBP β , Rictor, IL-1 β , ASC, p-S6 (Ser240/244), p-JNK (Thr183/Tyr185), p-P38 (Thr180/Tyr182), p-IKK α/β (Ser176/180), p-I κ B α (Ser32/36), and p-P65 (Ser536) were obtained from Cell Signaling Technology. Anti-c-Fos and anti-c-Jun antibodies were purchased from Enogene. Anti-NLRP3 and anti-caspase-1 (mouse) antibodies were from Enzo Life Sciences. The above-mentioned antibodies were diluted at 1:1000 in 5% bovine serum albumin (BSA), except for anti-p-S6 (Thr421/Ser424; 1:4000) and anti-H3 (1:3000). Anti- β -Actin mAb (diluted at 1:50 000) was purchased from Sigma-Aldrich. PD98059 and SP600125 were from Selleck Chemicals. Mouse TLR1-9 agonist kit was obtained from InvivoGen.

Cell preparation

Bone marrow cells were cultured with DMEM containing 10% (V/V) FBS and 10 ng/ml of mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) for 7–12 days to obtain BMDMs²⁹. The non-adherent cells and loosely attached cells were washed by swirling the plates on day 3, 5, 7, and 9^{30,31}. The purity of BMDMs was more than 95% (Supplementary Figure 1A). Primary mouse peritoneal macrophages were obtained from the peritoneal exudates of 4-week-old mice. The peritoneal exudate cells were washed twice with PBS solution, adjusted to 1×10^6 cells/mL in DMEM, and then cultured for 3–4 h at 37 °C and 5% CO₂. The non-adherent cells were removed by washing with warm PBS. The purification rate of macrophage was analyzed by FCM (Beckman, CA) using the mouse macrophage marker F4/80. F4/80⁺ macrophages constituted more than 90% of the adherent cells.

Quantitative PCR analysis

Total RNA was isolated with TRIzol (Invitrogen), and reverse transcription was performed with M-MLV superscript reverse transcriptase according to the manufacturer's instructions³². Real-time PCR kit (SYBR Premix Ex Taq, DRR041A) was purchased from Takara Bio Inc. PCR was performed on CFX96 (Bio-Rad). All mRNA expression levels were normalized to that of HPRT. Each sample was run at least in triplicate. Primers used for the amplification are summarized in Table 1.

Table 1 Primers used for the real-time PCR

pro-IL-1 β	TGAAATGCCACCTTTTGACAG	CACGGGAAAGACACAGGTAG
pro-IL-1 β pre-mRNA	GGGCCTCAAAGGAAAGAATC	AAAGGCAGAGTCTTCGGTGA
ASC	CAGAACAGGACACTTTGTGGACC	CCTCATCTTGTCTTGGCTGGTG
NLRP3	ATCAACAGGCGAGACCTCTG	GTCTCCTGGCATACCATAGA
pro-caspase 1	ACAAGGCACGGGACCTATG	TCCCAGTCAGTCCTGGAAATG
c-Jun	CCTTCTACGACGATGCCCTC	GGTTCAAGGTCATGCTCTGTTT
c-Fos	TCCTTACGGACTCCCCAC	CTCCGTTTCTCTTCTCTTCAG
SP1	CCTAACACCCATTGCCCTCAG	CACCAGATCCATGAAGACCAAG
PU.1	ATCGGATGACTTGGTTACTTAC	GGTTCTCAGGGAAGTTCTCAA
HPRT	AGTACAGCCCCAAAATGGTTAAG	CTTAGGCTTTGTATTGGCTTTTC

Western blot assay

Cells were cultured in DMEM medium with 10% FCS in 12-well plate. Cells were then treated with LPS (1 μ g/mL) for different durations. After stimulation, the cells were washed once in cold PBS, lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA) with protease and phosphatase inhibitor cocktails (Sigma) for 10 min on a rocker at 4 °C. Cells were scraped, centrifuged at 12 000 rpm for 10 min at 4 °C and the supernatants were mixed with $\times 5$ protein loading buffer. Protein concentration was determined using a BCA assay. Protein samples were analyzed on SDS-PAGE and transferred onto PVDF membrane (Millipore). PVDF membrane was blocked with TBST (100 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20) with 5% non-fat dried milk for 1 h, then incubated with primary antibodies overnight on a shaker at 4 °C. The appropriate HRP-coupled secondary antibody was then added and was detected through chemiluminescence (Millipore). β -Actin was used as a protein-loading control.

shRNA sequences, lentiviral preparation, and cell infection

Constitutively active Rheb (Rheb QL64) was amplified by PCR method and cloned into lentiviral overexpression vector pWPXLd-puro. The scrambled shRNA (non-target shRNA vector) and shRNA for TSC1 and mTOR vectors were purchased from Santa Cruz Biotechnology. TSC2 and Rictor shRNA were generated by cloning the target oligonucleotides into the BamHI and EcoRI sites of pShRNA lentiviral plasmid. The target sequences of shRNAs used in this study were: TSC2, 5'-CCTCTCTCTTAACTTGATAT; and Rictor, 5'-CGGTTTCATACAAGAGTTATTT. Lentiviral plasmids were co-transfected with the lentiviral-packaging plasmids pSPAX2 and pMD2.G into HEK293T cells for virus production. RAW264.7 cells were infected with lentivirus supernatant with 8 μ g/mL polybrene (Invitrogen). Stable shRNA or overexpressing cells were selected with puromycin (3 μ g/mL) for two weeks.

Overexpression of C/EBP β in BMDMs using adenoviral vectors

An adenoviral construct encoding C/EBP β was prepared with a ViraPower adenovirus expression system (Invitrogen) according to the manufacturer's instructions. cDNA that encodes for the C/EBP β gene and Ubc promoter-GFP fragment were sub-

cloned into the pENTR vector. As a control, only Ubc promoter-GFP fragment was subcloned into the pENTR vector. These constructs were then recombined with pAd/CMV/V5-DEST, using LR Clonase II (Invitrogen) according to the manufacturer's protocol, to generate the pAd plasmids. The plasmids were linearized using PacI and then transfected into 293A cells with Lipofectamine 2000. After 10 days, cells from wells showing 100% cytopathic effect were collected by scraping, lysed with three rounds of freeze/thaw and centrifuged to collect the supernatant. One more round of infection with the supernatant was performed to enhance the viral titer. BMDMs were prepared as described above and infected with the freshly prepared viral supernatant (50% viral supernatant containing empty vector or C/EBP β , supplied with 50% DMEM complete media) for 24 h. The cells were then washed and stimulated with LPS for an additional 6 h.

In vivo LPS challenge

WT and TSC1 KO mice were intraperitoneally injected with LPS (5 mg/kg body weight). The serum was collected after 2 h and IL-1 β was measured by ELISA according to the manufacturer's instructions (Invitrogen).

Detection of IL-1 β by ELISA assay

Mature IL-1 β protein levels in cultured supernatant samples or sera were measured using an IL-1 β Mouse ELISA Kit according to the manufacturer's instructions (Invitrogen).

Statistical analysis

All data are presented as the mean $\pm$ SD. Student's unpaired *t*-test for comparison of means was used to compare groups. A *P*-value less than 0.05 was considered to be statistically significant.

RESULTS**Decreased pro-IL-1 β expression in TSC1 KO macrophages**

First we measured the efficiency of TSC1 deletion in macrophages. Immunoblotting assays demonstrated that TSC1 was absent in the TSC1 KO peritoneal macrophages and BMDMs, in which phosphorylation of the mTORC1 downstream targets ribosomal S6 was enhanced (Figure 1A and B). Following LPS stimulation for different durations, freshly isolated TSC1 KO peritoneal macrophages produced less pro-inflammatory cytokines pro-IL-1 β at the mRNA level than WT cells (*P* < 0.01,

Figure 1 pro-IL-1 β expression is inhibited in TSC1 KO macrophages. Immunoblot analysis of WT and TSC1 KO peritoneal macrophages (**A**) or BMDMs (**B**) is shown. Actin was used as a loading control. (**C**) pro-IL-1 β mRNA synthesis was inhibited in TSC1 KO macrophages. Gene expression of pro-IL-1 β in WT and TSC1 KO peritoneal macrophages after treatment with LPS (1 μ g/mL) for 3–24 h was indicated by quantitative real-time PCR. Data presented are mean $\pm$ SD ($N = 4$). (**D**) Expression of pro-IL-1 β in WT and TSC1 KO BMDMs treated with LPS (1 μ g/mL) for 3 h by quantitative real-time PCR. Data presented are mean $\pm$ SD ($N = 4$). (**E–F**) Immunoblot analysis of WT and TSC1 KO peritoneal macrophages (**E**) or BMDMs (**F**) after treatment with LPS (1 μ g/mL) for 6 h is shown. (**G**) Immunoblot analysis of scramble and TSC1 shRNA RAW264.7 cells treated with LPS for 1 h. (**H**) Gene expression of pro-IL-1 β in scramble and TSC1 shRNA RAW264.7 cells after treatment with LPS (1 μ g/mL) for 1–24 h as indicated by quantitative real-time PCR. Data presented are mean $\pm$ SD ($N = 4$). (**I**) pro-IL-1 β protein level was detected using immunoblot analysis of scramble and TSC1 shRNA RAW264.7 cells after treatment with LPS (1 μ g/mL) for the indicated time. (**J**) RAW264.7 cells after treatment with different concentration of LPS (0, 0.01, 0.1, and 1 μ g/mL) for 6 h. pro-IL-1 β protein level was detected using immunoblot analysis of scramble and TSC1 shRNA. (**K**) WT and TSC1 KO macrophages were stimulated with LPS for 6 h followed by treatment with 5 mM ATP for 30 min. Secreted IL-1 β in the supernatant was determined by ELISA. (**L**) WT and TSC1 KO mice were challenged intraperitoneally with LPS (5 mg/kg body weight). IL-1 β concentration in the sera of WT and TSC1 KO mice was measured 2 h after LPS injection by ELISA. One representative of at least three experiments with similar results was shown. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with the control.

Figure 1C). Moreover, the mRNA expression level of pro-IL-1 β was noticeably impaired in TSC1 KO BMDMs relative to WT BMDMs ($P < 0.001$, Figure 1D). To test the protein level of pro-IL-1 β , the WT and TSC1 KO peritoneal macrophages were stimulated with LPS for 6 h. We found that the expression level of pro-IL-1 β protein significantly decreased in TSC1 KO peritoneal macrophages and BMDMs (Figure 1E and F).

To further investigate the role of TSC1 on pro-inflammatory cytokine IL-1 β expression, we generated a cell line that stably expressed short hairpin RNA against TSC1 (TSC1 shRNA) in RAW264.7 cells, which was a mouse macrophage cell line. As

shown in Figure 1G, TSC1 protein was severely decreased in TSC1 shRNA cells compared with scramble cells. TSC1 knock-down also reduced TSC2 protein level (Figure 1G), which is consistent with previous reports that TSC1 is required for TSC2 stability¹⁹. In addition, LPS stimulation induced rapid phosphorylation of S6, and p-S6 level was higher in TSC1 shRNA cells than in scramble cells, indicating that mTORC1 activation was enhanced (Figure 1G). Next, quantitative real-time PCR analysis was performed to assess mature mRNA expression of pro-IL-1 β in scramble and TSC1 shRNA cells after time-varying exposure to LPS (0, 1, 3, 6, 12, 18, and 24 h). We found that

mRNA expression of pro-IL-1 β increased rapidly after time-varying exposure to LPS and began to decrease after 3 h. Less mRNA synthesis of pro-IL-1 β was observed in TSC1 shRNA cells compared with scramble cells at each time point (Figure 1H). Notably, suppression of pro-IL-1 β transcription in TSC1 shRNA cells had already occurred as early as 1 h after LPS stimulation ($P < 0.01$, Figure 1H). Immunoblotting analysis demonstrated that pro-IL-1 β protein expression peaked after 6 h exposure to LPS and TSC1 shRNA cells produced less pro-IL-1 β protein levels than scramble cells at each time point (Figure 1I). Moreover, concentration gradient experiment showed that pro-IL-1 β protein expressions were decreased at each concentration of LPS in TSC1 shRNA cells compared with scramble cells, although pro-IL-1 β protein was barely detected in TSC1 shRNA cells treated with low concentration of LPS (0.01 or 0.1 $\mu\text{g/mL}$) (Figure 1J). In addition, TLR1-9 ligands were used to induce IL-1 β synthesis in scramble and TSC1 shRNA cells. Although TLR1-3 ligand only induced low level of pro-IL-1 β protein comparing with LPS, TSC1 shRNA also inhibited pro-IL-1 β expression after stimulation with these ligands. We did not detect pro-IL-1 β protein expression in both scramble and TSC1 shRNA RAW264.7 cells treated with TLR5, 6, 7, or 9 ligand (Supplementary Figure 2).

Pro-IL-1 β protein is cleaved into the active form by an inflammasome and then secretes into the supernatant. WT and TSC1 KO peritoneal macrophages were stimulated with LPS for 6 h followed by ATP treatment. We found dramatically decreased mature IL-1 β secretion in the supernatant of TSC1 KO macrophages ($P < 0.001$, Figure 1K). Similarly, IL-1 β level in the sera of TSC1KO mice were also decreased compared with WT mice after treatment with LPS for 2 h ($P < 0.001$, Figure 1L). Further research showed that TSC1 KO in macrophages also dramatically suppressed caspase-1 activation (Supplementary Figure 3A) but did not affect the expression of inflammasome components at both the mRNA and protein level (Supplementary Figure 3B and C). Thus, pro-IL-1 β expression was inhibited at both the mRNA and post-translational levels in TSC1 KO macrophages.

The decreased pro-IL-1 β expression in TSC1 KO macrophages is due to prolonged mTORC1 hyperactivity

It is well known that the TSC complex serves as a negative regulator of mTORC1 and enhanced mTORC1 activity is detected in TSC1 KO macrophages. It is possible that the effect of TSC1 deficiency on pro-IL-1 β production is due to over-activated mTOR signaling pathway. To test our hypothesis, we generated a cell line that stably expressed short hairpin RNA against TSC2 (TSC2 shRNA) in RAW264.7. The basal and LPS-induced p-S6 increased in TSC2 shRNA cells, suggesting enhanced mTORC1 activity (Figure 2A). As expected, the pro-IL-1 β expression was also decreased in the TSC2 shRNA cells compared with the scramble cells at both mRNA and protein levels after stimulation with LPS ($P < 0.001$, Figure 2A and B). Moreover, overexpression of constitutively active Rheb-Q64L, a direct activator of mTORC1, inhibited pro-IL-1 β expression in RAW264.7 cells similarly

(Figure 2C). Thus, our data suggest that active mTORC1 signaling pathway may contribute to the low pro-IL-1 β expression in TSC1/2-deficient macrophages.

To further demonstrate the role of over activated mTORC1 on pro-IL-1 β expression, we pretreated scramble and TSC1 shRNA cells with the well-known mTOR-specific inhibitor Rapa followed by LPS stimulation. Though pre-treatment with Rapa drastically decreased basal and LPS-induced p-S6 as determined by immunoblotting in RAW264.7 cells (Figure 2D), Rapa pre-treatment for a short period of time (1 h) failed to rescue the poor pro-IL-1 β expression at both mRNA and protein levels in TSC1 shRNA cells (Figure 2E and F). The same phenomenon was also observed in freshly isolated TSC1 KO peritoneal macrophages (Figure 2G). Our results are consistent with the previous study showing that intracellular pro-IL-1 β production after LPS stimulation are not influenced in BMDMs pretreated with Rapa for 1 h²². Next, we prolonged Rapa pre-treatment (48 h) for chronic mTOR inhibition followed by stimulation with LPS for 6 h. The results showed that pro-IL-1 β mRNA expression was significantly rescued by Rapa treatment for long periods of time in TSC1 KO BMDMs ($P < 0.001$, Figure 2H). The pro-IL-1 β protein level was similar between WT and TSC1 KO peritoneal macrophages after Rapa treatment for 48 h (Figure 2I). Moreover, extended (48 h) Rapa treatment also rescued pro-IL-1 β expression at both mRNA and protein levels in TSC1 shRNA RAW264.7 cells compared with scramble cells (Figure 2J, Supplementary Figure 4). To further confirm the ability of mTOR to suppress pro-IL-1 β expression, we knocked down mTOR in WT and TSC1 shRNA RAW264.7 cells. Consistent with the results mentioned above, the decreased pro-IL-1 β levels in TSC1 shRNA RAW264.7 cells was significantly rescued in TSC1/mTOR double knockdown cells (Figure 2K). The studies using mTOR KO and TSC1/mTOR KO BMDMs also demonstrated that deletion of mTOR recovered pro-IL-1 β expression in TSC1 KO macrophages (Figure 2L). In summary, our results demonstrate that mTORC1 hyperactivity inhibited pro-IL-1 β expression.

mTORC2 has no detectable effect on pro-IL-1 β expression in macrophages

mTOR forms two functionally distinct complexes in mammalian cells: mTORC1 and mTORC2. mTORC1 is highly sensitive to Rapa, but mTORC2 is relatively insensitive to Rapa. Rapa acutely and specifically inhibits mTORC1, whereas prolonged Rapa treatment leads to disruption of mTORC2 assembly^{33,34}. Based on our data that prolonged Rapa treatment or mTOR KO can rescue pro-IL-1 β expression in TSC1 KO macrophages and TSC1 shRNA RAW264.7 cells, we wondered whether mTORC2 complex also participated in regulating pro-IL-1 β synthesis. To test this hypothesis, the short hairpin RNA against Rictor, the unique component in mTORC2, was used to dissect mTORC2 involvement in pro-IL-1 β synthesis. As shown in Figure 3, Rictor knockdown in TSC1 shRNA cells failed to reverse decreased pro-IL-1 β synthesis in TSC1 shRNA cells (Figure 3A and B). Moreover, the mRNA and protein levels of pro-IL-1 β were similar between WT and

Figure 2 Prolonged mTOR deficiency regulates pro-IL-1 β expression in macrophages. **(A)** Immunoblot analysis of scramble and TSC2 shRNA RAW264.7 cells treated with LPS (1 μ g/mL) for 6 h. **(B)** Gene expression of pro-IL-1 β in scramble and TSC2 shRNA RAW264.7 cells after treatment with LPS (1 μ g/mL) for 3 h by quantitative real-time PCR. Data presented are mean $\pm$ SD ($N = 4$). **(C)** Immunoblot analysis of empty vector and Rheb Q64L overexpressing RAW264.7 cells treated with LPS (1 μ g/mL) for 1 h or 6 h. **(D)** Immunoblot analysis of RAW264.7 cells after treatment with LPS (1 μ g/mL) for 20 min in the absence or presence of Rapa (100 nM, pretreatment for 15–45 min as indicated). **(E)** mRNA expression of pro-IL-1 β in scramble and TSC1 shRNA RAW264.7 cells pretreated with or without Rapa (100 nM) for 1 h followed by stimulation with LPS (1 μ g/mL) for 3 h by quantitative real-time PCR. Data presented are mean $\pm$ SD ($N = 4$). **(F)** Immunoblot analysis of scramble and TSC1 shRNA RAW264.7 cells treated with or without Rapa (100 nM) for 1 h followed by stimulation with LPS (1 μ g/mL) for 6 h. **(G)** Immunoblot analysis of WT and TSC1 KO macrophages treated as in **F**. **(H)** mRNA expression of pro-IL-1 β in WT and TSC1 KO BMDMs pretreated with or without Rapa (100 nM) for 48 h followed by stimulation with LPS (1 μ g/mL) for 3 h by quantitative real-time PCR. Data presented are mean $\pm$ SD ($N = 4$). **(I)** Immunoblot analysis of WT and TSC1 KO peritoneal macrophages pretreated with or without Rapa (100 nM) for 48 h followed by stimulation with LPS (1 μ g/mL) for 6 h. **(J)** Immunoblot analysis of lysates from scramble and TSC1 shRNA cells treated as in **I**. **(K)** Immunoblot analysis of scramble, TSC1 shRNA, mTOR shRNA, and TSC1/mTOR shRNA RAW264.7 cells after treatment with LPS (1 μ g/mL) for 6 h. **(L)** Immunoblot analysis of WT, TSC1 KO, mTOR KO, and TSC1/mTOR KO BMDMs treated as in **K**. One representative of at least three experiments with similar results is shown. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with the control or the indicated group.

Rictor KO peritoneal macrophages after LPS stimulation *in vitro* (Figure 3C and D). Therefore, these data suggest that decreased pro-IL-1 β synthesis in TSC1 KO macrophages was mainly mediated by mTORC1 pathway but not mTORC2.

TSC1 deficiency does not inhibit activation of MAPKs and NF- κ B in macrophages

We have demonstrated that the mRNA expression of pro-IL-1 β was impaired in TSC1-deficient macrophages. Next, we investigated whether the synthesis of unspliced IL-1 β mRNA, pre-mRNA, was also inhibited in TSC1 KO macrophages. As shown

in Figure 4, pro-IL-1 β pre-mRNA, was evidently inhibited in TSC1 KO peritoneal macrophages and TSC1 shRNA cells ($P < 0.001$, Figure 4A and B), indicating that the regulation of TSC1 on IL-1 β mRNA expression occurred at the level of transcription.

LPS is known to signal through the TLR4 signaling pathway, which plays a role in the production of inflammatory cytokines including pro-IL-1 β through activating the NF- κ B and MAPK pathway^{35–37}. We therefore investigated the activation of the TLR4 signaling pathway in LPS-stimulated macrophages. Scramble and TSC1 shRNA cells were stimulated with LPS

for different durations (0, 0.5, 1, 3, 6, 12, and 24 h) and cell lysates were immunoblotted with antibodies recognizing activated forms of the proteins involved in the NF- κ B and MAPK pathways. LPS induced rapid IKK α/β , I κ B α , p65, p38, ERK, and JNK activation by phosphorylation in both scramble and TSC1 shRNA cells. However, identical phosphorylation levels of these proteins were found after LPS stimulation between scramble and TSC1 shRNA cells except p-ERK (Figure 4C).

We have demonstrated that increased p-ERK in TSC1 KO macrophages was dependent on the loss of TSC2 GTPase-activating protein (GAP) activity²³. However, blocking ERK and JNK activity (PD98059 and SP600125, respectively) significantly inhibited pro-IL-1 β expression in WT and TSC1 KO macrophages (Supplementary Figure 5). Thus, increased p-ERK was not responsible for the impaired pro-IL-1 β expression in TSC1 KO macrophages.

Given that only prolonged Rapa treatment or mTOR deletion can rescue pro-IL-1 β expression in TSC1 KO macrophages and TSC1 shRNA RAW264.7 cells, this raised the possibility that enhanced mTORC1 activity inhibited certain related transcriptional factor expression, which regulates pro-IL-1 β synthesis in macrophages. To test this, we assayed the mRNA and protein levels of SP1, PU.1, CREB, c-Jun, c-fos by quantitative real-time PCR, but no obvious difference was found between scramble and TSC1 shRNA cells (Figure 4C and D). These data were further confirmed in WT and TSC1 KO BMDMs (Figure 4E). To further explore whether the decreased pro-

IL-1 β expression in TSC1 shRNA cells was due to altered transcriptional factor nuclear translocation, including P65, c-fos, and CREB, scramble and TSC1 shRNA RAW264.7 cells were stimulated with LPS for 1 h and then nuclear extracts were prepared. The results showed that LPS-stimulated rapid nuclear translocation of P65, c-fos, and CREB. However, TSC1 deficiency did not impair the nuclear translocation of these transcriptional factors (Figure 4F). Moreover, the same profile of p65 nuclear translocation was observed in WT and TSC1 KO peritoneal macrophage (Figure 4G). These results indicate that TSC1 deficiency did not significantly affect the activity of P65, c-fos, and CREB in macrophages during LPS stimulation.

TSC1 controls pro-IL-1 β expression via mTORC1-dependent C/EBP β

Previous studies showed that C/EBP β protein, which could be induced by LPS (Supplementary Figure 6)^{14, 38}, is required for the activation of mouse and human IL-1 β gene expression^{39–43}. We found that TSC1 KO peritoneal macrophages had markedly reduced C/EBP β protein expression compared with WT macrophages (Figure 5A), whereas its expression was not affected in Rictor KO macrophages (Figure 5B). In addition, prolonged Rapa treatment for 48 h, but not short period Rapa treatment (1 h), markedly reversed the impaired C/EBP β expression in TSC1 KO macrophages (Figure 5C). Similarly, reduced C/EBP β expression was observed in TSC1 shRNA cells and its expression increased after treatment with Rapa for 24 h

Figure 3 mTORC2 pathway has no detectable effect on pro-IL-1 β expression in macrophages. Immunoblot analysis of scramble, TSC1 shRNA, and TSC1/Rictor shRNA cells (**A**) and these cells treated with LPS (1 μ g/mL) for 6 h (**B**). (**C**) mRNA expression of pro-IL-1 β in WT and Rictor KO peritoneal macrophages after treatment with LPS (1 μ g/mL) for 3 h by quantitative real-time PCR. Data presented are mean $\pm$ SD ($N = 4$). (**D**) Immunoblot analysis of WT and Rictor KO peritoneal macrophages stimulated with LPS (1 μ g/mL) for 6 h. One representative of at least three experiments with similar results is shown.

Figure 4 TSC1 deficiency does not inhibit activation of MAPKs and NF- κ B in macrophages. **(A)** Scramble and TSC1 shRNA RAW264.7 cells were treated with LPS (1 μ g/mL) for 3 h. Expression of unspliced pro-IL-1 β pre-mRNA was detected by quantitative real-time PCR. Data presented are mean $\pm$ SD ($N = 4$). **(B)** Expression of unspliced pro-IL-1 β pre-mRNA was detected in BMDMs treated with LPS (1 μ g/mL) for 3 h. Data presented are mean $\pm$ SD ($N = 4$). **(C)** Immunoblot analysis of scramble, TSC1 shRNA cells after treatment with LPS (1 μ g/mL) for different durations as indicated. **(D)** mRNA expression of transcriptional factors in scramble and TSC1 shRNA cells by quantitative real-time PCR. Data presented are mean $\pm$ SD ($N = 4$). **(E)** Immunoblot analysis of CREB, c-fos, and c-Jun expression in WT and TSC1 KO macrophages after treatment with LPS (1 μ g/mL) for 6 h. **(F)** Immunoblot analysis of p65, c-fos, and CREB in nuclear extracts of scramble and TSC1 shRNA cells after treatment with LPS (1 μ g/mL) for 1 h. H3 was used as a loading control. **(G)** Immunoblot analysis of p65 in nuclear extracts of WT and TSC1 KO peritoneal macrophages stimulated as in **F**. One representative of at least three experiments with similar results is shown. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with the control or the indicated group.

and was rescued completely after 48 h in TSC1 shRNA cells compared with scramble cells (Supplementary Figure 7). Notably, deletion of mTOR significantly reversed the decreased C/EBP β expression in TSC1 KO peritoneal macrophages as determined by immunoblotting (Figure 5D). To test whether the decreased C/EBP β expression was responsible for the decreased pro-IL-1 β expression in TSC1 KO macrophages, we overexpressed C/EBP β in WT and TSC1 KO BMDMs using adenovirus (Figure 5E, Supplementary Figure 8). Overexpression of C/EBP β significantly rescued the decreased pro-IL-1 β expression in TSC1 KO macrophages as determined by immunoblotting (Figure 5E). The present results collectively reveal that the decreased C/EBP β expression was induced by the over-activated mTOR activity and was involved in impaired pro-IL-1 β expression in TSC1 KO macrophages.

DISCUSSION

The role of the TSC–mTOR pathway in TLR-induced pro-inflammatory cytokine production was controversial. In

TSC2-deficient MEFs, pro-inflammatory responses are decreased due to impaired IKK activation and NF- κ B translocation to the nuclei⁴⁴. However, other studies reported that Rapa treatment enhances IL-12 production in myeloid DCs by promoting NF- κ B activation but inhibits IL-12 production in monocyte-derived DCs and bone marrow-derived DCs^{20,45}. In addition, TSC1-deficient macrophages in response to LPS stimulation produce elevated pro-inflammatory cytokines, such as TNF- α , IL-12, and IL-6^{19,23,24}. The reasons for this inconsistency are unclear, possibly due to the different cell types and duration of mTOR inhibition with Rapa or mTOR deletion used in these studies. So far, how TSC1 regulates pro-inflammatory cytokine IL-1 β expression in macrophages remains unclear. Our results show that pro-IL-1 β production was significantly inhibited at mRNA and protein levels in TSC1 KO primary macrophages and TSC1 shRNA RAW264.7 cells (Figure 1). In addition, our results are similar with previous report that over-activated mTOR suppressed caspase-1 activation then active IL-1 β production (Supplementary Figure 3)²².

Figure 5 The defective expression of pro-IL-1 β in TSC1 KO macrophages is mainly mediated by mTOR-C/EBP β pathway. **(A)** C/EBP β expression was determined by immunoblot analysis of WT and TSC1 KO peritoneal macrophages. **(B)** Immunoblot analysis of C/EBP β in WT and Rictor KO peritoneal macrophages. **(C)** Prolonged Rapa treatment efficiently rescued the C/EBP β expression in TSC1 KO macrophages. WT and TSC1 KO peritoneal macrophages were pre-treated with Rapa (100 nM) for 1 h or 48 h, respectively. The C/EBP β expression was determined by immunoblot. **(D)** Deletion of mTOR reversed the decreased C/EBP β expression in TSC1 KO peritoneal macrophages as determined by immunoblot. **(E)** Overexpression of C/EBP β significantly rescued pro-IL-1 β expression in TSC1 KO macrophages. G-CSF-induced BMDMs were used to overexpress C/EBP β using adenoviral supernatant, followed by stimulation with LPS for 6 h. One representative of at least three experiments with similar results is shown.

Thus, TSC1 is required for the synthesis and maturation of IL-1 β in macrophages^{19,20,24,44,45}.

It is well known that the NF- κ B and MAPK signaling pathway are important in the activation of inflammatory cytokines including pro-IL-1 β expression⁴⁶. Previous research showed that JNK activity¹⁹, but not P38 or ERK, was enhanced in TSC1 KO peritoneal macrophages. However, TSC1 shRNA cells showed similar phosphorylation levels of P38 and JNK relative to scramble cells after stimulation with LPS (Figure 4C). Interestingly, increased p-ERK was observed in TSC1 KO macrophages due to loss of TSC2 GTPase-activating protein (GAP) activity²³. However, pro-IL-1 β expression was upregulated by ERK and JNK in macrophages (Supplementary Figure 5). NF- κ B signaling is also critical for IL-1 β transcription and is involved in activation of mTOR pathway^{47–51}. In addition, mTOR pathway is also relative to NF- κ B pathway activation. Ghosh *et al.* reported that NF- κ B signaling is attenuated in TSC2-deficient MEFs and several human tumor cell lines stimulated by DNA damage⁵². It was also reported that inhibition of mTORC2 signaling abrogates NF- κ B activity and also decreases the NF- κ B DNA-binding activity in U87/EGFRvIII cells⁵³. In our experiments, we have not observed obvious defect of IKK α / β and I κ B α as well as NF- κ B p65 nuclear translocation in TSC1-deficient macrophages compared with scramble cells (Figure 4C, F, and G). But our data is generally in agreement with previous studies in macrophage cells¹⁹. Our results are different from those observed in TSC2-deficient MEFs or tumor cells lines and such differences could be attributed to the different cells examined⁵³.

Molecular mechanism studies showed that the length and timing of Rapa treatment might have different effects on

LPS-stimulated IL-1 β expression. Our data showed that pre-treatment with Rapa for 1 h followed by stimulation with LPS for 6 h did not rescue pro-IL-1 β expression in TSC1 KO macrophages (Figure 2F and G). This is consistent with a previous report that Rapa treatment (1 h) has no effect on intracellular pro-IL-1 β expression in macrophages²². However, it was reported that activation of autophagy with Rapa promotes the degradation of pro-IL-1 β after its translation and blocks secretion of the mature cytokine in immortalized BMDMs and bone marrow-derived DCs²⁶. The reason for this inconsistency is possibly due to the different cell types used. Previous study showed that long-term treatment with Rapa (1 h then followed by LPS stimulation for another 24 h) promotes peripheral blood mononuclear cells to produce more IL-1 β in culture supernatants²⁵. We also observed the inhibition of mTORC1 by Rapa (48 h) or deletion of mTOR almost completely rescued the pro-IL-1 β expression at the mRNA and protein levels in TSC1 KO macrophages, inferring that mTORC1 inhibited pro-IL-1 β production indirectly and blocking mTORC1 pathway facilitated its synthesis. Given that mTOR is a conserved serine/threonine kinase that controls diverse processes including protein synthesis, ribogenesis, and mitochondrial capacity⁵⁴, we asked whether over-activated mTORC1 in TSC1 KO macrophages altered specific transcription factor expression important for pro-IL-1 β production. Unfortunately, all of the detected transcription factors were unimpaired in TSC1 KO macrophages relative to WT cells except for the C/EBP β (Figure 4D and E and Figure 5A), which were essential for LPS-induced pro-IL-1 β expression^{39–42}. The C/EBP β gene can produce several N-terminally truncated isoforms, LAP*, LAP, and LIP, by alternative translation initiation at

Figure 6 Proposed model for how mTORC1 activity controls pro-IL-1 β expression. Physiological activation of the Akt-mTORC1, MAPK, and NF- κ B signaling pathways by LPS stimulation allows for transient and inducible IL-1 β expression in macrophages. Activated mTORC1 inhibits C/EBP β expression, then brakes IL-1 β expression later. Constitutive or aberrant activation of mTORC1 impairs the ability of macrophages to synthesize IL-1 β in response to LPS stimuli. A critical mediator of this process is C/EBP β , whose expression is downregulated by increased mTORC1 activity.

downstream AUG codons⁵⁵. It was reported that C/EBP β isoform production is regulated by mTORC1 pathway: activated mTORC1 enhances generation of LIP while inhibition of mTOR promotes LAP expression^{56,57}. In our studies, we found that TSC1 KO macrophages displayed enhanced mTOR activity and decreased C/EBP β protein level. We have treated the scramble and TSC1 shRNA cells with Rapa for different durations. Brief treatment with Rapa had no effect on C/EBP β expression (treated for 6 h and 12 h), or slightly rescued its expression in TSC1 shRNA cells (treated for 24 h). Only prolonged treatment (48 h) with Rapa or deletion of the mTOR gene could completely rescue C/EBP β expression in TSC1-deficient cells (Figure 5C and Supplementary Figure 7). Moreover, overexpression of C/EBP β significantly reversed the defect of pro-IL-1 β expression in TSC1 KO macrophages. These results indicate that the pro-IL-1 β expression deficiency in TSC1 KO macrophages is, at least partially, mediated by the mTOR-C/EBP β pathway.

In summary, pro-IL-1 β expression is suppressed in TSC1-deficient macrophages at both the mRNA and protein levels, indicating that TSC1 is essential for pro-IL-1 β expression in macrophages. TSC1 regulates pro-IL-1 β expression through downregulation of mTOR and subsequently upregulation of C/EBP β pathway (Figure 6).

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

ACKNOWLEDGEMENTS

The authors wish to thank Dr Chenming Sun, Dr Lina Sun, and Mr Steven Shao for their kind review of the manuscript. This work was supported by grants from the National Basic Research Program of China (2011CB710903, 2010CB945301, Yong Zhao), the National Natural Science Foundation of China for General and Key Programs (C81130055, C81072396, Yong Zhao), and the CAS/SAFEA International Partnership Program for Creative Research Teams (Yong Zhao).

Supplementary Information accompanies the paper on *Cellular & Molecular Immunology's* website (<http://www.nature.com/cmi>).

- Fujiwara N, Kobayashi K. Macrophages in inflammation. *Curr Drug Targets Inflamm Allergy* 2005; **4**: 281–286.
- Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. *Nat Rev Immunol* 2005; **5**: 953–964.
- Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* 2008; **8**: 958–969.
- Sindrilariu A, Peters T, Wieschalka S, Baican C, Baican A, Peter H et al. An unrestrained proinflammatory M1 macrophage population induced by iron impairs wound healing in humans and mice. *J Clin Invest* 2011; **121**: 985–997.
- Hu X, Liu G, Hou Y, Shi J, Zhu L, Jin D et al. Induction of M2-like macrophages in recipient NOD-scid mice by allogeneic donor CD4(+)CD25(+) regulatory T cells. *Cell Mol Immunol* 2012; **9**: 464–472.
- Qin H, Holdbrooks AT, Liu Y, Reynolds SL, Yanagisawa LL, Benveniste EN. SOCS3 deficiency promotes M1 macrophage polarization and inflammation. *J Immunol* 2012; **189**: 3439–3448.
- Dinarello CA. Immunological and inflammatory functions of the interleukin-1 family. *Annu Rev Immunol* 2009; **27**: 519–550.
- Apte RN, Dotan S, Elkabets M, White MR, Reich E, Carmi Y et al. The involvement of IL-1 in tumorigenesis, tumor invasiveness, metastasis and tumor-host interactions. *Cancer Metastasis Rev* 2006; **25**: 387–408.
- Apte RN, Voronov E. Is interleukin-1 a good or bad 'guy' in tumor immunobiology and immunotherapy? *Immunol Rev* 2008; **222**: 222–241.
- Carmi Y, Voronov E, Dotan S, Lahat N, Rahat MA, Fogel M et al. The role of macrophage-derived IL-1 in induction and maintenance of angiogenesis. *J Immunol* 2009; **183**: 4705–4714.
- Voronov E, Shouval DS, Krelm Y, Cagnano E, Benharroch D, Iwakura Y et al. IL-1 is required for tumor invasiveness and angiogenesis. *Proc Natl Acad Sci U S A* 2003; **100**: 2645–2650.
- Sheedy FJ, Moore KJ. IL-1 signaling in atherosclerosis: sibling rivalry. *Nat Immunol* 2013; **14**: 1030–1032.
- Alghasham A, Rasheed Z. Therapeutic targets for rheumatoid arthritis: Progress and promises. *Autoimmunity* 2014; **47**: 77–94.
- Basak C, Pathak SK, Bhattacharyya A, Mandal D, Pathak S, Kundu M. NF-kappaB- and C/EBPbeta-driven interleukin-1beta gene expression and PAK1-mediated caspase-1 activation play essential roles in interleukin-1beta release from Helicobacter pylori lipopolysaccharide-stimulated macrophages. *J Biol Chem* 2005; **280**: 4279–4288.
- Chan C, Li L, McCall CE, Yoza BK. Endotoxin tolerance disrupts chromatin remodeling and NF-kappaB transactivation at the IL-1beta promoter. *J Immunol* 2005; **175**: 461–468.
- Zhang Y, Saccani S, Shin H, Nikolajczyk BS. Dynamic protein associations define two phases of IL-1beta transcriptional activation. *J Immunol* 2008; **181**: 503–512.
- Martinson F, Burns K, Tschoep J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell* 2002; **10**: 417–426.
- Dibble CC, Elis W, Menon S, Qin W, Klekota J, Asara JM et al. TBC1D7 is a third subunit of the TSC1-TSC2 complex upstream of mTORC1. *Mol Cell* 2012; **47**: 535–546.

- 19 Pan H, O'Brien TF, Zhang P, Zhong XP. The role of tuberous sclerosis complex 1 in regulating innate immunity. *J Immunol* 2012; **188**: 3658–3666.
- 20 Weichhart T, Saemann MD. The multiple facets of mTOR in immunity. *Trends Immunol* 2009; **30**: 218–226.
- 21 Ohtani M, Nagai S, Kondo S, Mizuno S, Nakamura K, Tanabe M *et al*. Mammalian target of rapamycin and glycogen synthase kinase 3 differentially regulate lipopolysaccharide-induced interleukin-12 production in dendritic cells. *Blood* 2008; **112**: 635–643.
- 22 Schmitz F, Heit A, Dreher S, Eisenacher K, Mages J, Haas T *et al*. Mammalian target of rapamycin (mTOR) orchestrates the defense program of innate immune cells. *Eur J Immunol* 2008; **38**: 2981–2992.
- 23 Zhu L, Yang T, Li L, Sun L, Hou Y, Hu X *et al*. TSC1 controls macrophage polarization to prevent inflammatory disease. *Nat Commun* 2014; **5**: 4696.
- 24 Byles V, Covarrubias AJ, Ben-Sahra I, Lamming DW, Sabatini DM, Manning BD, Horng T. The TSC-mTOR pathway regulates macrophage polarization. *Nat Commun* 2013; **4**: 2834.
- 25 Weichhart T, Haidinger M, Katholnig K, Kopecky C, Poglitsch M, Lassnig C *et al*. Inhibition of mTOR blocks the anti-inflammatory effects of glucocorticoids in myeloid immune cells. *Blood* 2011; **117**: 4273–4283.
- 26 Harris J, Hartman M, Roche C, Zeng SG, O'Shea A, Sharp FA *et al*. Autophagy controls IL-1 β secretion by targeting pro-IL-1 β for degradation. *J Biol Chem* 2011; **286**: 9587–9597.
- 27 Chen H, Zhang L, Zhang H, Xiao Y, Shao L, Li H *et al*. Disruption of TSC1/2 signaling complex reveals a checkpoint governing thymic CD4⁺ CD25⁺ Foxp3⁺ regulatory T-cell development in mice. *Faseb J* 2013; **27**: 3979–3990.
- 28 Zhang L, Zhang H, Li L, Xiao Y, Rao E, Miao Z *et al*. TSC1/2 signaling complex is essential for peripheral naive CD8⁺ T cell survival and homeostasis in mice. *PLoS One* 2012; **7**: e30592.
- 29 Sun C, Sun L, Ma H, Peng J, Zhen Y, Duan K *et al*. The phenotype and functional alterations of macrophages in mice with hyperglycemia for long term. *J Cell Physiol* 2012; **227**: 1670–1679.
- 30 Inaba K, Inaba M, Romani N, Aya H, Deguchi M, Ikehara S *et al*. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* 1992; **176**: 1693–1702.
- 31 Fleetwood AJ, Dinh H, Cook AD, Hertzog PJ, Hamilton JA. GM-CSF- and M-CSF-dependent macrophage phenotypes display differential dependence on type I interferon signaling. *J Leukoc Biol* 2009; **86**: 411–421.
- 32 Wu T, Sun C, Chen Z, Zhen Y, Peng J, Qi Z *et al*. Smad3-deficient CD11b(+)Gr1(+) myeloid-derived suppressor cells prevent allograft rejection via the nitric oxide pathway. *J Immunol* 2012; **189**: 4989–5000.
- 33 Urbanska M, Gozdz A, Swiech LJ, Jaworski J. Mammalian target of rapamycin complex 1 (mTORC1) and 2 (mTORC2) control the dendritic arbor morphology of hippocampal neurons. *J Biol Chem* 2012; **287**: 30240–30256.
- 34 Julien LA, Carriere A, Moreau J, Roux PP. mTORC1-activated S6K1 phosphorylates Rictor on threonine 1135 and regulates mTORC2 signaling. *Mol Cell Biol* 2010; **30**: 908–921.
- 35 Ishida T, Mizushima S, Azuma S, Kobayashi N, Tojo T, Suzuki K *et al*. Identification of TRAF6, a novel tumor necrosis factor receptor-associated factor protein that mediates signaling from an amino-terminal domain of the CD40 cytoplasmic region. *J Biol Chem* 1996; **271**: 28745–28748.
- 36 Wesche H, Henzel WJ, Shillington W, Li S, Cao Z. MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. *Immunity* 1997; **7**: 837–847.
- 37 Muzio M, Ni J, Feng P, Dixit VM. IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. *Science* 1997; **278**: 1612–1615.
- 38 Lai TY, Wu SD, Tsai MH, Chuang EY, Chuang LL, Hsu LC, Lai LC. Transcription of Tnfrsf10 is regulated by NF- κ B and p38 via C/EBP β in activated macrophages. *PLoS One* 2013; **8**: e73153.
- 39 Yang Z, Wara-Aswapati N, Chen C, Tsukada J, Auron PE. NF-IL6 (C/EBP β) vigorously activates il1b gene expression via a Spi-1 (PU.1) protein-protein tether. *J Biol Chem* 2000; **275**: 21272–21277.
- 40 Cogswell JP, Godlevski MM, Wisely GB, Clay WC, Leesnitzer LM, Ways JP, Gray JG. NF- κ B regulates IL-1 β transcription through a consensus NF- κ B binding site and a nonconsensus CRE-like site. *J Immunol* 1994; **153**: 712–723.
- 41 Godambe SA, Chaplin DD, Takova T, Bellone CJ. Upstream NFIL-6-like site located within a DNase I hypersensitivity region mediates LPS-induced transcription of the murine interleukin-1 β gene. *J Immunol* 1994; **153**: 143–152.
- 42 Godambe SA, Chaplin DD, Takova T, Bellone CJ. An NFIL-6 sequence near the transcriptional initiation site is necessary for the lipopolysaccharide induction of murine interleukin-1 β . *DNA Cell Biol* 1994; **13**: 561–569.
- 43 Tengku-Muhammad TS, Hughes TR, Ranki H, Cryer A, Ramji DP. Differential regulation of macrophage CCAAT-enhancer binding protein isoforms by lipopolysaccharide and cytokines. *Cytokine* 2000; **12**: 1430–1436.
- 44 Weichhart T, Costantino G, Poglitsch M, Rosner M, Zeyda M, Stuhlmeier KM *et al*. The TSC-mTOR signaling pathway regulates the innate inflammatory response. *Immunity* 2008; **29**: 565–577.
- 45 Haidinger M, Poglitsch M, Geyeregger R, Kasturi S, Zeyda M, Zlabinger GJ *et al*. A versatile role of mammalian target of rapamycin in human dendritic cell function and differentiation. *J Immunol* 2010; **185**: 3919–3931.
- 46 Bauernfeind FG, Horvath G, Stutz A, Alnemri ES, MacDonald K, Speert D *et al*. Cutting edge: NF- κ B activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *J Immunol* 2009; **183**: 787–791.
- 47 Dan HC, Adli M, Baldwin AS. Regulation of mammalian target of rapamycin activity in PTEN-inactive prostate cancer cells by I κ B kinase α . *Cancer Res* 2007; **67**: 6263–6269.
- 48 Dan HC, Baldwin AS. Differential involvement of I κ B kinases α and β in cytokine- and insulin-induced mammalian target of rapamycin activation determined by Akt. *J Immunol* 2008; **180**: 7582–7589.
- 49 Lee DF, Kuo HP, Chen CT, Hsu JM, Chou CK, Wei Y *et al*. IKK β suppression of TSC1 links inflammation and tumor angiogenesis via the mTOR pathway. *Cell* 2007; **130**: 440–455.
- 50 Ermolaeva MA, Michallet MC, Papadopoulou N, Utermohlen O, Kranidioti K, Kollias G *et al*. Function of TRADD in tumor necrosis factor receptor 1 signaling and in TRIF-dependent inflammatory responses. *Nat Immunol* 2008; **9**: 1037–1046.
- 51 Pobezinskaya YL, Kim YS, Choksi S, Morgan MJ, Li T, Liu C, Liu Z. The function of TRADD in signaling through tumor necrosis factor receptor 1 and TRIF-dependent Toll-like receptors. *Nat Immunol* 2008; **9**: 1047–1054.
- 52 Ghosh S, Teragankar V, Rothlin CV, Correa RG, Bottero V, Bist P *et al*. Essential role of tuberous sclerosis genes TSC1 and TSC2 in NF- κ B activation and cell survival. *Cancer Cell* 2006; **10**: 215–226.
- 53 Tanaka K, Babic I, Nathanson D, Akhavan D, Guo D, Gini B *et al*. Oncogenic EGFR signaling activates an mTORC2-NF- κ B pathway that promotes chemotherapy resistance. *Cancer Discov* 2011; **1**: 524–538.
- 54 O'Hayre M, Degese MS, Gutkind JS. Novel insights into G protein and G protein-coupled receptor signaling in cancer. *Curr Opin Cell Biol* 2014; **27**: 126–135.
- 55 Welm AL, Timchenko NA, Darlington GJ. C/EBP α regulates generation of C/EBP β isoforms through activation of specific proteolytic cleavage. *Mol Cell Biol* 1999; **19**: 1695–1704.
- 56 Luedde T, Duderstadt M, Streetz KL, Tacke F, Kubicka S, Manns MP, Trautwein C. C/EBP β isoforms LIP and LAP modulate progression of the cell cycle in the regenerating mouse liver. *Hepatology* 2004; **40**: 356–365.
- 57 Jundt F, Raetzl N, Muller C, Calkhoven CF, Kley K, Mathas S *et al*. A rapamycin derivative (everolimus) controls proliferation through down-regulation of truncated CCAAT enhancer binding protein (β) and NF- κ B activity in Hodgkin and anaplastic large cell lymphomas. *Blood* 2005; **106**: 1801–1807.