p65 down-regulates *DEPTOR* expression in response to LPS stimulation in hepatocytes

Xiaoling Yu¹, Dan Jin¹, An Yu^{1,2}, Jun Sun¹, Xiaodong Chen¹, Zaiqing Yang^{1*}

¹ Key Laboratory of Agricultural Animal Genetics, Breeding and Reproduction of Ministry of Education, College of Life Science and Technology, Huazhong Agricultural University, Wuhan 430070, China

² Huffington Center on Aging, Baylor College of Medicine, Houston, Texas 77030, USA

* Corresponding author, address: College of Life Science and Technology, Huazhong Agricultural University, Wuhan 430070, China. Tel: +86 27 87282669; fax: +86 27 87287376.

E-mail addresses: yuy07@webmail.hzau.edu.cn (Xiaoling Yu), yangzq@mail.hzau.edu.cn (Zaiqing Yang)

Abstract

DEPTOR, a novel endogenous inhibitor of mTOR, plays an important role in regulating the inflammatory response in vascular endothelial cells (ECs) and in mouse skeletal muscle. However, the regulatory mechanism of *DEPTOR* transcription and its effects on liver inflammation are unknown presently. Here we reported the role of DEPTOR in regulating inflammatory response in mouse liver-derived Hepa1-6 cells and in a mouse model with LPS-induced hepatic inflammation. The results revealed that DEPTOR over-expression in Hepa1-6 liver cells increased the mRNA levels of the pro-inflammatory cytokines interleukin-6 (*IL-6*) and monocyte chemotactic protein-1 (*MCP-1*). Contrasting results were observed in Hepa1-6 cells with DEPTOR interference. Treatment Hepa1-6 cells with rapamycin, a specific inhibitor of mTORC1, increased *MCP-1* mRNA, but have no significant effect on *IL-6* mRNA. DEPTOR expression was down-regulated in Hepa1-6 cells with the treatment of inflammatory stimuli LPS or the over-expression of p65/NF- κ B, a key inflammatory transcription factor. NF- κ B antagonist

(PDTC) and inhibitor ($\text{I}\kappa\text{B}\alpha$) blocked the effect of LPS on DEPTOR expression. The study *in vivo* showed that *DEPTOR* mRNA and protein were significantly reduced in a mouse model with LPS-induced hepatic inflammation, which was accompanied by a concurrent activation of the mTOR signaling pathway. Further, the transcriptional regulation of *DEPTOR* was explored, which revealed that *DEPTOR* promoter activity was significantly down-regulated by NF- κ B. The progressive deletions and mutations demonstrated that the NF- κ B binding motif situated at -145/-127 region is an essential component required for the *DEPTOR* promoter activity. Chromatin immunoprecipitation(ChIP) assays determined that p65 can directly interact with the *DEPTOR* promoter DNA. Those results indicate DEPTOR regulates liver inflammation at least partially via mTORC1 pathway, and is down-regulated by LPS through p65.

Keywords: mTOR, NF- κ B, promoter, transcription, inflammation

Abbreviations

DEPTOR, DEP domain containing MTOR-interacting protein; ECs, vascular endothelial cells; NF- κ B, nuclear factor for kappa B; IL-6, cytokine interleukin-6; MCP-1, monocyte chemotactic protein-1; LPS, lipopolysaccharide

1. Introduction

Approximately 80% of the hepatic blood supply is from the gastrointestinal tract via the portal vein, so the liver is continually exposed to dietary and microbial antigens such as lipopolysaccharide (LPS) which stimulates Toll-like receptors (TLR), and causes inflammatory

responses(Nemeth et al., 2009; Miyake and Yamamoto, 2013). The liver is comprised of 60–80% hepatocytes with the rest of the cell population consisting of endothelial cells, Kupffer cells, lymphocytes, biliary cells and stellate cells(Racanelli and Rehmann, 2006). Hepatocytes can directly clear LPS(Treon et al., 1993; Mimura et al., 1995; Scott and Billiar, 2008), indicating their important role in inflammatory responses.

Inflammation, a common and important pathological process, is associated with homeostasis(Kotas and Medzhitov, 2015), metabolic diseases(Hotamisligil, 2006; Baker et al., 2011), cancer(Lisa M. Coussens, 2002; Mantovani et al., 2008; Grivennikov et al., 2010) and other diseases. Nuclear factor for kappa B (NF- κ B), a heterodimer with p50/p52 and p65 subunits, is critical for inflammatory regulation(Baker et al., 2011). The mammalian target of rapamycin (mTOR), an evolutionarily conserved serine/threonine protein kinase, forms two distinct multiprotein complexes named mTORC1 and mTORC2 that share the mTOR, mLST8/G β L, and DEPTOR proteins, and differ with raptor and PRAS40 in mTORC1 and rictor, mSin1 in mTORC2(Laplane and Sabatini, 2012). mTORC1 directly phosphorylates the translational regulators eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) and S6 kinase 1 to promote protein synthesis through different pathway, and directly phosphorylates and suppresses ULK/Atg13/FIP200 (unc-51-like kinase 1/mammalian autophagy-related gene 13/focal adhesion kinase family-interactiong protein of 200 kDa) to inhibit autophagy. mTORC2 controls Akt, serum- and glucocorticoid-induced protein kinase 1 (SGK1), and protein kinase C- α (PKC- α) to regulate cell survival/metabolism and cytoskeletal organization. mTORC1 is sensitive to rapamycin, while mTORC2 is insensitive to rapamycin. The effects of rapamycin on mTORC2 signaling are much more complex as long term treatment in different types of cells(Sarbassov et al., 2006; Laplane and Sabatini, 2012). Recently, studies

indicated that mTORC1 can control NF- κ B activity (Dan et al., 2008; Fielhaber et al., 2012; Gao et al., 2015; Yin et al., 2015), as well as can be activated by pro-inflammatory cytokines (Lee et al., 2007). These evidences suggest that mTORC1 is involved in inflammatory responses.

DEPTOR interacts with mTOR and inhibits mTORC1/2 pathways (Peterson et al., 2009). It plays an important role in proliferation (Zhang et al., 2013b), apoptosis (Zhang et al., 2013a), regulation pluripotency of embryonic stem cells (Agrawal et al., 2014), and adipogenesis (Laplane et al., 2012). Recent studies reveal that DEPTOR is a critical upstream regulator of vascular endothelial cells (ECs) activation and is involved in the inflammatory response of ECs (Bruneau et al., 2013). DEPTOR is also a target of pro-inflammatory cytokine *TNF- α* and participates in skeletal muscle inflammation in obese mice (Meng et al., 2014). These data demonstrated the roles of DEPTOR in ECs and skeletal muscle inflammation through its interaction with mTOR, however, it is unclear presently whether DEPTOR is involved in regulating hepatic inflammatory responses.

In the present study, our results suggest that DEPTOR over-expression significantly up-regulated the mRNA levels of the pro-inflammatory cytokines interleukin-6 (*IL-6*) and monocyte chemotactic protein-1 (*MCP-1*) in Hepa1-6 liver cells, while its own expression was obviously down-regulated in Hepa1-6 cells treated with inflammatory stimuli LPS or p65/NF- κ B over-expression. DEPTOR expression was also significantly inhibited in a mouse model with LPS-induced liver inflammation, which was accompanied by a concurrent activation of mTOR signaling pathway. mTORC1 inhibitor rapamycin increased *MCP-1* mRNA, but have no significant effect on *IL-6* mRNA. The progressive deletions and mutations demonstrated that the NF- κ B binding motif situated at -145/-127 region is an essential component required for the *DEPTOR* promoter activity. Chromatin immunoprecipitation (ChIP) assays determined that p65

can directly interact with the *DEPTOR* promoter DNA. Our data together suggested that DEPTOR, transcriptionally regulated by p65/NF- κ B, plays an important role in regulating liver inflammatory response at least partially via mTORC1 signaling pathway.

Materials and methods

1.1. Animal experiments

C57BL/6 mice (7 weeks-of-age) were purchased from the animal experiment center of Wuhan University. Mice were housed in individual plastic cages under a 12/12 h light-dark cycle with free access to water and food at room temperature. They acclimated for one week before the study, after which they were treated with or without LPS (L2880, Sigma, 10mg/kg, ip) for 12 h, and then sacrificed. Liver portions were removed and fixed with 4% paraformaldehyde solution. Some liver tissue and muscle and visceral fat were placed in liquid nitrogen and stored in -70 °C. All procedures were in accordance with the Hubei Province Committee on Laboratory Animal Care.

1.2. Plasmids

The complete coding sequences of murine *DEPTOR* were obtained from the NCBI gene database. The gene fragment was amplified by PCR and inserted into pcDNA3.1 vector. The target sequences (CAAGGAAGACATTACGAT) for DEPTOR interference from the published literature [17] were ligated to the pSilencer4.1 CMV vector. Murine *DEPTOR*

promoter vectors were constructed according to bioinformatic analysis. A series of murine *DEPTOR* promoter fragments were amplified by PCR and inserted into the pGL3-basic vector. Primer sequences are shown in Table 1.

1.3. Cell culture

Hepa1-6 cells were obtained from the Cell Bank of the Chinese Academy of Science, and cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone) supplemented with 10% fetal bovine serum (FBS, Sijiqing) under 5% CO₂ and 37°C. HEK293 cells were purchased from the Wuhan Cell Bank of Wuhan University, and cultured as Hepa1-6 cells.

1.4. Cell transient transfection

Cells were cultured on 12-well culture plates to 70–80% confluency in 10% FBS/DMEM at the time of transfection. Plasmids (2 µg) were transfected into cells with 2 µl Lipofectamine 2000 reagent (Invitrogen) in serum-free medium Opti-MEM (Gibco). The medium was replaced with 10% FBS/DMEM after 6 h. pEGFP vector was used to monitor the transfection efficiency. In over-expression experiments, cells were collected 24 h later. In shRNA knockdown experiments, cells were collected 48 h later. For transfection with LPS, LPS was added to the medium 12 h prior to cell collection.

1.5. Nuclear acid isolation and quantitative real time PCR (qRT-PCR)

Total RNA was extracted from tissue or cells using TRIzol reagent (Takara) according to the manufacturer's protocol. RNA (2-3 μ g) was reverse transcribed into cDNA in 30 μ L reaction solution using a Reverse Transcription Kit (Takara). qRT-PCR was performed in a 20 μ L reaction volume containing appropriate primers (Table 2) using SYBR Green PCR Mix (Bio-Rad) with a CFX96TM real time PCR system. Murine β -actin was used as an internal reference to normalize relative gene expression. qRT-PCR conditions were 95 °C for 3 min, followed by 45 cycles of 95 °C for 12 sec, 60 °C for 12 sec, and 72 °C for 12 sec.

1.6. Western Blotting

Cells collected and centrifuged in PBS or tissue ground with liquid nitrogen were lysed in RIPA lysis buffer P0013 (Beyotime) with PMSF. Before lysates were added to 5X native loading buffer, protein concentrations were measured with a BCA kit (Invitrogen). Samples were boiled at 100 °C for 15 min, and subjected to SDS-PAGE, and then transferred to PVDF membranes. Membranes were blocked with 1XTBST buffer (20 mM Tris, PH 7.5, 150 mM NaCl, 50 mM KCl, and 0.5% Tween-20) containing 5% non-fat dry milk (R&D) on an orbital shaker for 1 h or overnight at 4 °C. Individual protein was incubated with primary antibody at the appropriate dilution with constant rocking for 1 h or overnight at 4 °C. HRP-conjugated secondary antibody (Dingguo) was incubated with the membrane (1: 5000) for 1 h on an orbital shaker at room temperature. Signals were measured with an ECL chemiluminescence detection system (Beyotime). The following antibodies were used for Western blot: p-Akt (S473, #4060, Cell Signaling Technology), p-S6K (T389, #9205, Cell Signaling Technology), Akt (bs-6951R,

Bioss), S6K (14485-1-AP, Proteintech), DEPTOR (20985-1-AP, Proteintech), p65 (sc-8008, Santa Cruz Biotechnology), β -actin(sc-81178, Santa Cruz Biotechnology).

1.7. Luciferase reporter assays

Cells were lysed and assayed for promoter activity with a dual-luciferase reporter assay kit (Promega) after 24 h of transfection. Transfection efficiency was normalized by co-transfection with *Renilla luciferase* reporter plasmid.

1.8. Chromatin immunoprecipitation (ChIP) assays

Hepa1-6 cells were cultivated in a 10-cm culture dishes to 90% confluence and then treated with or without LPS (1 μ g/ml) for 12 h. Next, ChIP assays were performed using a ChIP assay kit (Millipore) according to the manufacturer's protocol. Finally, three pairs of primers (Table 1) were prepared to amplify regions (-2303/-2289, -1780/-1771, -145/-127). I κ B α was used as a positive control, and its primers were obtained from the literature(Doig et al., 2014). ChIP-qPCR data was normalized with a fold enrichment method.

1.9. ELISA

ELISA was performed according to TNF α ELISA Kit (CSB-E04741m, Cusabio) or MCP-1 ELISA Kit (CSB-E07430m, Cusabio) protocols. A microplate reader was used to measure absorbance at 450nm and data were analyzed with Curve Expert 1.3 software.

1.10. H&E straining

Liver tissues were fixed with 4% paraformaldehyde solution for at least 24 h, and processed for routine paraffin histology. Paraffin sections were stained with hematoxylin and eosin using routine procedures and samples were visualized under a Motic AE2000 microscope.

1.11. Statistical

Data are presented as means $\pm$ standard deviation (SD) or the standard error of the mean (SEM). Statistical analysis was performed using the unpaired two-tailed *t* test for two groups and analysis of variance for multiple groups. *P* values < 0.05 were considered statistically significant.

2. Results

2.1. Effects of DEPTOR on inflammatory cytokines in mouse liver-derived Hepa1-6 cells

pcDNA3.1 or pcDNA3.1-*DEPTOR* vectors were transfected to mouse liver-derived Hepa1-6 cells, and after 12 h, the cells were treated with/without LPS (1 μ g/ml) for 12 h. qRT-PCR was used to measure expression of inflammatory cytokines, including *TNF- α* , *IL-1 β* , *IL-6*, *MCP-1*, and *p65*. DEPTOR over-expression significantly promoted *IL-6* and *MCP-1* mRNA expression and strengthened LPS-induced these cytokines expression, and did not obviously up-regulated the mRNA levels of *TNF- α* , *IL-1 β* or *p65* (Figure 1A). In contrast, when Hepa1-6 cells were

transfected with sh-control or sh-*DEPTOR* vectors, and after 36 h, treated with/without LPS (1 µg/ml) for 12 h, DEPTOR knock-down reduced *MCP-1* and *p65* mRNA expression, but had little effect on these cytokines in LPS stimulation, and had no obviously down-regulated the mRNA levels of *TNF-α*, *IL-1β*, *IL-6* (Figure 1B). Western blot showed that DEPTOR protein expression was reduced to 18% in sh-*DEPTOR* group compared to controls (Figure 1B).

Since many DEPTOR functions are associated with inhibition of mTOR activity. Rapamycin, a specific inhibitor of mTORC1, was used to assess the effect of mTORC1 on inflammation. We pretreated Hepa1-6 cells with DMSO or rapamycin (100 nM for 30 min), and then added LPS (1 µg/ml for 8 h). Figure 1C shows that rapamycin inhibited the mRNA levels of SREBP1c and C/EBPa, which are two downstream gene of mTORC1, suggesting mTORC1 activity was inhibited by rapamycin. Rapamycin up-regulated *MCP-1* mRNA level, but had no significant effects on *TNF-α*, *IL-1β*, *IL-6*, *p65* or *DEPTOR* mRNA levels (Figure 1C and 1D). Thus, these data indicate that DEPTOR regulates inflammatory cytokines expression in hepatocytes at least partially via mTORC1 pathway.

2.2. LPS down-regulates DEPTOR expression in Hepa1-6 cells

To study whether LPS stimulation affects DEPTOR expression, the mRNA level of DEPTOR was measured in Hepa1-6 cells treated with LPS (0, 0.5, 1, 5 µg/ml) for 12 h. *DEPTOR* mRNA was decreased after LPS stimulation compared to control, but this was not dose

dependent (Figure 2A). Next, we treated Hepa1-6 cells with LPS (1 $\mu\text{g/ml}$) for different time periods (0, 0.5, 1, 2, 4, 8, 12, 24 h). *TNF- α* mRNA was rapidly increased and peaked at 1 h (Figure 2B) and *DEPTOR* mRNA was slightly decreased during 0 to 2 h, and significantly reduced to 46.0% at 4 h and then stabilized (Figure 2C). Hepa1-6 cells were treated with LPS(1 $\mu\text{g/ml}$) for different time periods (0, 15 m, 30 m, 1, 2, 4, 8, 12, 24 h), and mTORC1/2 signaling pathways and DEPTOR protein was assayed. Akt phosphorylation was increased during 15 m to 1 h and declined to baseline at 2 h, and S6K1 phosphorylation was increased over a longer duration from 30 m to 8 h (Figure 2D). p65 protein was increased in a time-dependent manner and peaked at 4 h, but DEPTOR protein was decreased in a time-dependent manner (Figure 2E). Thus, DEPTOR is down-regulated by LPS in a time-dependent manner, and mTORC1/2 signaling pathways are activated in LPS-induced inflammation in Hepa1-6 cells.

2.3. NF- κ B down-regulates DEPTOR expression in Hepa1-6 cells after LPS stimulation

Because NF- κ B contributes to inflammation and is activated after LPS stimulation, we speculated that NF- κ B may affect DEPTOR expression. Thus, Hepa1-6 cells were transfected with pcDNA3.1/pcDNA3.1-*p65* vectors for 12 h and treated with/without LPS (1 $\mu\text{g/ml}$) for 12 h. Data show that *DEPTOR* mRNA and protein was significantly decreased in the pcDNA3.1-*p65* group compared to controls (Figure 3A, B). DEPTOR protein was declined more in *p65* over-expressing cells after LPS stimulation (Figure 3B). NF- κ B is inactive when it's physical

association with its inhibitor I κ B α . We next evaluated *DEPTOR* mRNA expression in Hepa1-6 cells transfected with pcDNA3.1/pcDNA3.1-I κ B α vectors for 12 h and treated with/without LPS (1 μ g/ml) for 12 h. I κ B α over-expression increased the mRNA level of *DEPTOR* and resisted the effects of LPS (Figure 3C). Furthermore, PDTC, an NF- κ B inhibitor, was applied to the cell culture and reduced LPS effects (Figure 3D).

2.4. p65 binds to the *DEPTOR* promoter to reduce its activity

NF- κ B is a transcription factor so we investigated whether *DEPTOR* is down-regulated via a transcriptional mechanism. With bioinformatic analysis, both promoters of *Murine* and *Homo* *DEPTOR* had several NF- κ B binding sites (Figure 4A). CHIP analysis with Hepa1-6 cells treated with/without LPS stimulation allowed assessment of NF- κ B binding to three putative response sites (-2303/-2289, -1780/-1771, -145/-127) in the *DEPTOR* promoter. DNA gel images revealed that the -145/-127 region was enriched with p65 binding compared to control IgG (Figure 4B). Quantitative analysis of CHIP samples with real time PCR confirmed that LPS stimulation promoted p65 binding to the *DEPTOR* promoter (Figure 4C).

To learn whether p65 regulates *DEPTOR* promoter activity, luciferase activity analysis was performed. First, we measured activity of different lengths of *DEPTOR* promoters (-2391/+14, -2274/+14, -294/+14) (Figure 4D). Then, HEK293 cells co-transfected with pGL3-*DEPTOR* (-2391/+14) /(-2274/+14) /(-294/+14) /mut (-294/+14) vectors and pcDNA3.1 or pcDNA3.1-p65 vectors were measured for luciferase activity. Data show that p65 had no visible effect on

luciferase activity of pGL3-*DEPTOR* (-2391/+14), and significantly inhibited luciferase activity of pGL3-*DEPTOR* (-2274/+14)/(-294/+14), but not the pGL3-*DEPTOR* (-294/+14) mutation (Figure 4E). Taken together, p65 down-regulates *DEPTOR* expression by binding to the *DEPTOR* promoter at the -145/-127 region and this is enhanced with LPS stimulation.

2.5.LPS inhibits *DEPTOR* expression and activates the mTOR signaling pathway in mouse livers

To assess *DEPTOR* participation in inflammation in vivo, we established an inflammatory C57BL/6 mouse model with LPS (10mg/kg, ip), in which hepatic Kupffer cells were obviously increased and serum TNF- α level was higher, compared with controls (Figure 5A, B). Inflammatory cytokines *TNF- α* , *IL-1 β* , *IL-6*, and *IL-10* mRNA expression were increased or on the up trend in LPS groups (Figure S1). Thus, the inflammatory model was successfully established. Using this model, we measured *DEPTOR* mRNA level in different tissues and noted that it was significantly less in the liver and visceral fat in LPS groups. It was also less in muscle but there was no significant difference between LPS groups and controls (Figure 5C). Western blot analysis showed that hepatic *DEPTOR* protein was significantly decreased, which was accompanied by a concurrent phosphorylation increase of S6K1 and Akt implying mTORC1/2 signaling pathways activation (Figure 5D and 5E).

3. Discussion

The liver is continuously exposed to pathogens, toxins and toxicants, and dietary antigens from the intestine which can trigger inflammation and disease (Racanelli and Rehermann, 2006; Miyake and Yamamoto, 2013). Inflammation is a complex defence mechanism in which pro-inflammation and antiinflammation exists to return pathological organ to homeostasis. Insufficient inflammatory response results in immunodeficiency, while excessive inflammatory response cause morbidity and mortality (Tracey, 2002). DEPTOR is firstly reported to be associated with inflammation in vascular endothelial cells. DEPTOR is down-regulated by the inflammatory cytokine TNF- α in human microvascular endothelial cells (Bruneau et al., 2013). Meng's group reported that DEPTOR is down-regulated in skeletal muscle in obesity. Further study demonstrated that DEPTOR is inhibited by TNF- α but not IL-6 or myostatin (Mstn) through the ERK pathway in C2C12 myotubes (Meng et al., 2014). Consistent with these data, we observed DEPTOR was down-regulated by inflammatory stimuli LPS in hepatocytes *in vitro* and *in vivo*. *In vitro*, we confirmed that both *DEPTOR* mRNA and protein levels were reduced in LPS treated Hepa1-6 cells compared to controls. *In vivo*, with an LPS-induced inflammatory mouse model to simulate liver insult, we noted that DEPTOR expression was significantly decreased in mouse livers and visceral fat and less in mouse muscle after LPS treatment 12 h. However, over-expression of DEPTOR in Hepa1-6 cells increased *IL-6* and *MCP-1* mRNA expression and enhanced LPS-induced these cytokines expression. DEPTOR knockdown in Hepa1-6 cells reduced *MCP-1* mRNA but failed to reverse LPS stimulation, suggesting that DEPTOR may be one of LPS target genes. Furthermore, we also treated macrophages (RAW264.7 cells) with LPS and noted that *DEPTOR* mRNA was down-regulated by LPS (data

not shown). Thus, our data suggest that DEPTOR participates in LPS-induced inflammation in liver, visceral fat and muscle.

mTOR, as an evolutionarily conserved serine/threonine protein kinase, regulates multiple biological processes involved in mRNA translation, ribosome biogenesis, cell growth and proliferation, cell survival, and autophagy(Laplane and Sabatini, 2012), as well as influences inflammation(Dan et al., 2008; Fielhaber et al., 2012; Jiang et al., 2014; Gao et al., 2015; Yin et al., 2015; Song, 2010). DEPTOR is an intrinsic inhibitor of mTOR, and involves in inflammation, so we speculate that DEPTOR influences inflammation via mTOR pathways. After treating Hepa1-6 cells with/without rapamycin or LPS, we observed that rapamycin increased *MCP-1* expression but did not change *IL-6* expression. Rapamycin is a common inhibitor of mTORC1, but it can not block all aspects of mTORC1 functions(Choo et al., 2008; Feldman et al., 2009; Alam et al., 2015). Therefore, our data indicate that DEPTOR promotes inflammation at least partially via mTORC1 signaling pathways.

Although DEPTOR expression is reported to be regulated by mTOR signals and the ERK pathway at translational/post-translational levels, its regulatory mechanism of transcription is poorly studied presently(Peterson et al., 2009; Duan et al., 2011; Gao et al., 2011; Zhao et al., 2011; Meng et al., 2014). NF- κ B can be activated by LPS or inflammatory cytokine such as TNF- α or IL-1. It plays an important role in inflammation vis promoting transcription of inflammatory factors(Baldwin, 1996; Tak and Firestein, 2001). p65, an active subunit of NF- κ B, reduced the levels of DEPTOR mRNA and protein in the current study. Both of NF- κ B inhibitors I κ B α and PDTC can block DEPTOR down-regulation by LPS. Bioinformatic analysis revealed the presence of several conserved NF- κ B binding sites in *murine* and *Homo DEPTOR* promoter. Luciferase activity analysis indicated that *murine DEPTOR* promoter activity is controlled by

p65. ChIP analysis revealed that p65 directly bound to the murine *DEPTOR* promoter at the -145/-127 region and this combination was enhanced under LPS stimulation condition. In general, DEPTOR is down-regulated by LPS through the NF- κ B pathway at the transcriptional level. In addition, we observed that the decline of DEPTOR protein prior to the reduction of DEPTOR mRNA, so speculated that LPS also regulates DEPTOR at post-transcriptional level.

4. Conclusion

LPS down-regulates DEPTOR expression via p65 binding to DEPTOR promoter at the -145/-127 region. Whereas, DEPTOR over-expression promotes *IL-6* and *MCP-1* mRNA expression. mTOR special inhibitor rapamycin increases *MCP-1* mRNA expression, suggesting that DEPTOR plays an important role in regulating liver inflammatory response at least partially via mTOR signaling pathway.

Acknowledgements

This work was supported by the grants from National key Basic Research Program of China (2012CB124702), 948 Program of China (2015-Z33), Specialized Research Fund for the Doctoral Program of Higher Education (20110146130002), Program of National Natural Science Foundation of China (31172093, 31572382, 31372418), the National Science Foundation for Fostering Talents in Basic Research (J1103510), the Fundamental Research Funds for the Central Universities (2013PY005, 2013PY131).

Reference

- Agrawal, P., Reynolds, J., Chew, S., Lamba, D.A. and Hughes, R.E., 2014. DEPTOR is a stemness factor that regulates pluripotency of embryonic stem cells. *J Biol Chem* 289, 31818-26.
- Alam, M., Khan, M., Veledar, E., Pongprutthipan, M., Flores, A., Dubina, M., Nodzenski, M. and Yoo, S.S., 2015. Correlation of Inflammation in Frozen Sections With Site of Nonmelanoma Skin Cancer. *JAMA Dermatol*, 1-4.
- Baker, R.G., Hayden, M.S. and Ghosh, S., 2011. NF-kappaB, inflammation, and metabolic disease. *Cell Metab* 13, 11-22.
- Baldwin, A.S., Jr., 1996. The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu Rev Immunol* 14, 649-83.
- Bruneau, S., Nakayama, H., Woda, C.B., Flynn, E.A. and Briscoe, D.M., 2013. DEPTOR regulates vascular endothelial cell activation and pro-inflammatory and angiogenic responses. *Blood* 122, 1833-42.
- Choo, A.Y., Yoon, S.O., Kim, S.G., Roux, P.P. and Blenis, J., 2008. Rapamycin differentially inhibits S6Ks and 4E-BP1 to mediate cell-type-specific repression of mRNA translation. *Proc Natl Acad Sci U S A* 105, 17414-9.
- Dan, H.C., Cooper, M.J., Cogswell, P.C., Duncan, J.A., Ting, J.P. and Baldwin, A.S., 2008. Akt-dependent regulation of NF- κ B is controlled by mTOR and Raptor in association with IKK. *Genes Dev* 22, 1490-500.
- Doig, C.L., Bashir, J., Zielinska, A.E., Cooper, M.S., Stewart, P.M. and Lavery, G.G., 2014. TNF α -mediated Hsd11b1 binding of NF-kappaB p65 is associated with suppression of 11 β -HSD1 in muscle. *J Endocrinol* 220, 389-96.
- Duan, S., Skaar, J.R., Kuchay, S., Toschi, A., Kanarek, N., Ben-Neriah, Y. and Pagano, M., 2011. mTOR generates an auto-amplification loop by triggering the betaTrCP- and CK1 α -dependent degradation of DEPTOR. *Mol Cell* 44, 317-24.
- Feldman, M.E., Apsel, B., Uotila, A., Loewith, R., Knight, Z.A., Ruggero, D. and Shokat, K.M., 2009. Active-site inhibitors of mTOR target rapamycin-resistant outputs of mTORC1 and mTORC2. *PLoS Biol* 7, e38.
- Fielhaber, J.A., Carroll, S.F., Dydensborg, A.B., Shourian, M., Triantafillopoulos, A., Harel, S., Hussain, S.N., Bouchard, M., Qureshi, S.T. and Kristof, A.S., 2012. Inhibition of mammalian target of rapamycin augments lipopolysaccharide-induced lung injury and apoptosis. *J Immunol* 188, 4535-42.
- Gao, D., Inuzuka, H., Tan, M.K., Fukushima, H., Locasale, J.W., Liu, P., Wan, L., Zhai, B., Chin, Y.R., Shaik, S., Lyssiotis, C.A., Gygi, S.P., Toker, A., Cantley, L.C., Asara, J.M., Harper, J.W. and Wei, W., 2011. mTOR drives its own activation via SCF(betaTrCP)-dependent degradation of the mTOR inhibitor DEPTOR. *Mol Cell* 44, 290-303.
- Gao, S.S., Liu, W.M., Zhuo, X.D., Wang, L.J., Wang, G., Sun, T., Zhao, Z., Liu, J.H., Tian, Y.L., Zhou, J., Yuan, Z.Y. and Wu, Y., 2015. The activation of mTOR is required for monocyte pro-inflammatory response in patients with coronary artery disease. *Clinical Science* 128, 517-526.
- Grivennikov, S.I., Greten, F.R. and Karin, M., 2010. Immunity, inflammation, and cancer. *Cell* 140, 883-99.
- Hotamisligil, G.S., 2006. Inflammation and metabolic disorders. *Nature* 444, 860-7.

-
- Jiang, H., Westerterp, M., Wang, C., Zhu, Y. and Ai, D., 2014. Macrophage mTORC1 disruption reduces inflammation and insulin resistance in obese mice. *Diabetologia* 57, 2393-404.
- Kotas, M.E. and Medzhitov, R., 2015. Homeostasis, inflammation, and disease susceptibility. *Cell* 160, 816-27.
- Laplante, M., Horvat, S., Festuccia, W.T., Birsoy, K., Prevorsek, Z., Efeyan, A. and Sabatini, D.M., 2012. DEPTOR cell-autonomously promotes adipogenesis, and its expression is associated with obesity. *Cell Metab* 16, 202-12.
- Laplante, M. and Sabatini, D.M., 2012. mTOR signaling in growth control and disease. *Cell* 149, 274-93.
- Lee, D.F., Kuo, H.P., Chen, C.T., Hsu, J.M., Chou, C.K., Wei, Y., Sun, H.L., Li, L.Y., Ping, B., Huang, W.C., He, X., Hung, J.Y., Lai, C.C., Ding, Q., Su, J.L., Yang, J.Y., Sahin, A.A., Hortobagyi, G.N., Tsai, F.J., Tsai, C.H. and Hung, M.C., 2007. IKK beta suppression of TSC1 links inflammation and tumor angiogenesis via the mTOR pathway. *Cell* 130, 440-55.
- Lisa M. Coussens, Z.W., 2002. Inflammation and cancer. *Nature* 420, 860-867.
- Mantovani, A., Allavena, P., Sica, A. and Balkwill, F., 2008. Cancer-related inflammation. *Nature* 454, 436-44.
- Meng, Z.X., Wang, L., Xiao, Y. and Lin, J.D., 2014. The Baf60c/Deptor pathway links skeletal muscle inflammation to glucose homeostasis in obesity. *Diabetes* 63, 1533-45.
- Mimura, Y., Sakisaka, S., Harada, M., Sata, M. and Tanikawa, K., 1995. Role of hepatocytes in direct clearance of lipopolysaccharide in rats. *Gastroenterology* 109, 1969-76.
- Miyake, Y. and Yamamoto, K., 2013. Role of gut microbiota in liver diseases. *Hepatol Res* 43, 139-46.
- Nemeth, E., Baird, A.W. and O'Farrelly, C., 2009. Microanatomy of the liver immune system. *Semin Immunopathol* 31, 333-43.
- Peterson, T.R., Laplante, M., Thoreen, C.C., Sancak, Y., Kang, S.A., Kuehl, W.M., Gray, N.S. and Sabatini, D.M., 2009. DEPTOR is an mTOR inhibitor frequently overexpressed in multiple myeloma cells and required for their survival. *Cell* 137, 873-86.
- Racanelli, V. and Rehmann, B., 2006. The liver as an immunological organ. *Hepatology* 43, S54-62.
- Sarbassov, D.D., Ali, S.M., Sengupta, S., Sheen, J.H., Hsu, P.P., Bagley, A.F., Markhard, A.L. and Sabatini, D.M., 2006. Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Mol Cell* 22, 159-68.
- Scott, M.J. and Billiar, T.R., 2008. Beta2-integrin-induced p38 MAPK activation is a key mediator in the CD14/TLR4/MD2-dependent uptake of lipopolysaccharide by hepatocytes. *J Biol Chem* 283, 29433-46.
- Song, X., Kusakari, Y., Xiao, C., Kinsella, S.D., Rosenberg, M.A., Crosbie, M.S., Hara, K., X., Kusakari, Y., Xiao, C., Kinsella, S.D., Rosenberg, M.A., Crosbie, M.S., Hara, K., 2010. mTOR attenuates the inflammatory response in cardiomyocytes and prevents cardiac dysfunction in pathological hypertrophy. *Am J Physiol Cell Physiol* 299, C1256-C1266.
- Tak, P.P. and Firestein, G.S., 2001. NF-kappaB: a key role in inflammatory diseases. *J Clin Invest* 107, 7-11.
- Tracey, K.J., 2002. The inflammatory reflex. *Nature* 420, 853-9.
- Treon, S.P., Thomas, P. and Broitman, S.A., 1993. Lipopolysaccharide (LPS) processing by Kupffer cells releases a modified LPS with increased hepatocyte binding and decreased tumor necrosis factor-alpha stimulatory capacity. *Proc Soc Exp Biol Med* 202, 153-8.

-
- Yin, J., Gu, L., Wang, Y., Fan, N., Ma, Y. and Peng, Y., 2015. Rapamycin improves palmitate-induced ER stress/NF kappa B pathways associated with stimulating autophagy in adipocytes. *Mediators Inflamm* 2015, 272313.
- Zhang, H., Chen, J., Zeng, Z., Que, W. and Zhou, L., 2013a. Knockdown of DEPTOR induces apoptosis, increases chemosensitivity to doxorubicin and suppresses autophagy in RPMI-8226 human multiple myeloma cells in vitro. *Int J Mol Med* 31, 1127-34.
- Zhang, H.R., Chen, J.M., Zeng, Z.Y. and Que, W.Z., 2013b. Knockdown of DEPTOR inhibits cell proliferation and increases chemosensitivity to melphalan in human multiple myeloma RPMI-8226 cells via inhibiting PI3K/AKT activity. *J Int Med Res* 41, 584-95.
- Zhao, Y., Xiong, X. and Sun, Y., 2011. DEPTOR, an mTOR inhibitor, is a physiological substrate of SCF(betaTrCP) E3 ubiquitin ligase and regulates survival and autophagy. *Mol Cell* 44, 304-16.

Captions of figures

Fig.1 DEPTOR increases the expression of inflammatory cytokine in hepa1-6 cells.

- (A) Hepa1-6 cells were transfected with pcDNA3.1/pcDNA3.1-*DEPTOR*, after 12 h, treated with/without LPS for 12 h. The cells were collected for qRT-PCR analysis.
- (B) Hepa1-6 cells were transfected with pSilencer4.1 CMV/ pSilencer4.1 CMV-*DEPTOR* (sh-control/sh-*DEPTOR*) vectors, after 36 h, treated with/without LPS for 12 h. Inflammatory cytokines were measured by qRT-PCR. Western blot of total protein lysates from Hepa1-6 cells transfected with sh-control/sh-*DEPTOR* vectors for 48 h.
- (C-D) Hepa1-6 cells pre-incubated with/without rapamycin (mTOR inhibitor, 100 nM), and after 30 min, treated with /without LPS (1 µg/ml) for 8 h. mRNA was measured by qRT-PCR.

Data are presented as means±SEM. Values with different letters are significantly different ($P < 0.05$). Data represent at least three independent experiments.

Fig. 2 Downregulation of DEPTOR by LPS stimulation in Hepa1-6 cells.

- (A) *DEPTOR* mRNA level was measured by qRT-PCR in Hepa1-6 cells treated with LPS (0, 0.5, 1, 5 µg/ml) for 12 h.
- (B-C) *DEPTOR* and *TNA-α* mRNA were measured by qRT-PCR in Hepa1-6 cells treated with LPS (1 µg/ml) for different time periods (0, 0.5, 1, 2, 4, 8, 12, 24 h).
- (D-E) Western blot of total protein lysates from Hepa1-6 cells treated with LPS (1 µg/ml) for different time periods (0, 15m, 30m, 1, 2, 4, 8, 12, 24 h). The fold change in p65 and *DEPTOR* following LPS treatment relative to control determined by density analysis is shown below the panels.

Data are presented as means $\pm$ SEM. Values with different letters are significantly different ($P < 0.05$), * $P < 0.05$, ** $P < 0.01$.*** $P < 0.0001$. All data represent at least three independent experiments.

Fig. 3 Downregulation of DEPTOR by LPS through NF- κ B in Hepa1-6 cells.

- (A) *DEPTOR* mRNA level was measured by qRT-PCR in Hepa1-6 cells transfected with pcDNA3.1/pcDNA3.1-*p65* vectors for 24 h.
- (B) Western blot of total protein lysates from Hepa1-6 cells transfected with pcDNA3.1/pcDNA3.1-*p65* vectors for 12 h, and then treated with or without LPS (1 μ g/ml) for 12 h. The fold change of DEPTOR in groups transfected with pcDNA3.1/pcDNA3.1-*p65* vectors following LPS treatment relative to control determined by density analysis is shown below the panels.
- (C) Hepa1-6 cells were transfected with pcDNA3.1/pcDNA3.1- *I κ B α* vectors for 12-h, and then treated with or without LPS (1 μ g/ml) for 12 h. *DEPTOR* mRNA level was measured by qRT-PCR.
- (D) Hepa1-6 cells pre-incubation with or without PDTC (inhibitor of NF- κ B, 10 μ M), after 30 min, treated with or without LPS (1 μ g/ml) for 2 h or 4 h, and *DEPTOR* mRNA were measured by qRT-PCR.

Data are presented as means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$.*** $P < 0.0001$. All data represent at least three independent experiments.

Fig.4 NF- κ B binds to *DEPTOR* promoter to reduce its activity.

-
- (A) Consensus sequences for the putative binding sites of NF- κ B are black boxes.
- (B) CHIP assays were conducted in Hepa1-6 cells treated with/without LPS for 12 h. Putative binding sites of NF- κ B in the DEPTOR promoter were amplified by PCR using pairs of aimed primers. I κ B α was used as a positive control.
- (C) CHIP samples of A were performed qRT-PCR and analysed with a fold enrichment method.
- (D) Different lengths of *DEPTOR* promoters (-2391/+14, -2274/+14, -294/+14) were ligated to pGL3Basic vectors, which were co-transfected with *Renilla luciferase* reporter vectors into HEK293 cells. Twenty-four hours later, luciferase reporter activity was measured. *Renilla luciferase* reporter vector was used as an internal control to correct the transfection efficiency.
- (E) pGL3-*DEPTOR* (-2391/+14) / (-2274/+14) / (-294/+14) / (-294/+14) -mut vectors and *Renilla luciferase* reporter vectors were co-transfected with pcDNA3.1/pcDNA3.1-p65 into HEK293 cells. Twenty-four hours later, luciferase reporter activity was measured. pGL3-*DEPTOR* (-294/+14) -mut is the *DEPTOR* promoter mutated as indicated. pcDNA3.1 vector was used as a vector control.

Data are presented as means $\pm$ SEM from at least three independent experiments. * $P < 0.05$, ** $P < 0.01$.

Fig.5 LPS inhibits DEPTOR expression and activates the mTOR signaling pathway in mouse livers. C57BL/6J mice were intraperitoneally injected LPS (10g/kg) or normal saline (control) for 12-h, and subjected to histological and gene expression analysis.

(A) H&E analysis liver sections. Right panels are 40x and left panels are 400x. Red boxes (right panels) are enlarged in the left panels. Arrows indicate macrophages recruitment.

(B) Serum TNA α was determined by ELISA (n=7-8).

(C) *DEPTOR* mRNA was measured by qRT-PCR in liver, visceral fat, and muscle (n=3-4).

(D) Western blot of total protein lysates from liver (n=5).

(E) Quantification of p-S6K1/S6K, p-Akt/Akt, DEPTOR/ β -action, p65/ β -action protein levels in D (n=5).

Data are presented as means $\pm$ SD. * $P < 0.05$, ** $P < 0.01$.

Table 1. Primers used to perform PCR amplifications in this study.

<i>Gene Symbol</i>	<i>Product Size (bp)</i>	<i>Forward primer(5'-3') & Reverse primer (5'-3')</i>	<i>Accession number</i>
<i>Mus DEPTOR</i>	1230	F: ATGGAAGAGGGCAGCAG R: TCAGCAGTCCAACTCCTCC	NM_145470.2
<i>Mus DEPTOR (-2391/+14) Promoter</i>	2405	F: AGTTTGAGGCCAGCTTTG R: TCCTGACCGCGCGGTTGC	Gene ID: 97998
<i>Mus DEPTOR (-2274/+14) promoter</i>	2288	F: TTTAGCCTTACATTGCAG R: TCCTGACCGCGCGGTTGC	
<i>Mus DEPTOR (-294/+14) promoter</i>	308	F: GAAAGGCTCAACCTCCTG R: TCCTGACCGCGCGGTTGC	
<i>ChIP DEPTOR (-2303/-2289)</i>	161	F: GTGGAGGCAAGAGAATCAG R: AAATTACTGCAATGTAAGGC	Gene ID: 18036
<i>ChIP DEPTOR (-1780/-1771)</i>	153	F: CTGCACAGAGAAACCTTACC R: ATGGAGAGAGGTTTCAGTTC	
<i>ChIP DEPTOR (-145/-127)</i>	213	F: AAGCCGAAGTTCAGACC R: CTGCCCTCTTCCATGTTCTGAC	
<i>ChIP IκBα</i>	290	F: TAGCCAGCGTTTCCACTCTT R: GGTTCATGCACAGGGAACCTT	

Table 2. Primers used to perform qRT-PCR amplifications in this study.

<i>Gene Symbol</i>	<i>Product Size (bp)</i>	<i>Forward primer(5'-3') & Reverse primer (5'-3')</i>	<i>Accession number</i>
<i>Mus β-action</i>	245	F: GTGACGTTGACATCCGTAAAGA R: GCCGGAATCATCGTACTCC	NM_007393
<i>Mus DEPTOR</i>	190	F: GTACGCAAGGAAGACATTACG R: CACGGTGCGGTAGTCTACATTC	NM_145470.2
<i>Mus TNFα</i>	252	F: AGTGACAAGCCTGTAGCCC R: GAGGTTGACTTTCTCCTGGTAT	NM_013693.3
<i>Mus IL-1β</i>	124	F: CTGGTACATCAGCACCTCAC R: AGAAACAGTCCAGCCCATAC	NM_008361.3
<i>Mus IL-6</i>	197	F: TGTATGAACAACGATGATGCACTT R: ACTCTGGCTTTGTCTTTCTTGTTATCT	NM_031168.1
<i>Mus IL-10</i>	145	F: GCCAGAGCCACATGCTCCTA R: GATAAGGCTTGGAACCCCAAGTAA	NM_010548.2
<i>Mus p65</i>	156	F: CCAGACACAGATGATCGCCAC R: GACAGAAGTTGAGTTTCGGGTAGG	NM_009045.4
<i>Mus MCP-1</i>	173	F: GATGCAGTTAACGCCCCACT R: ACCCATTCCTTCTTGGGGTC	NM_011333.3

Figure(s)

Figure(s)

Figure(s)

Figure(s)

Figure(s)

Figure(s)