Macrophage-induced tumor angiogenesis is regulated by the TSC2-mTOR pathway

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Running title: TSC2-mTOR regulates macrophage-induced tumor angiogenesis

Word count of text: 4688(excluding references)

Number of figures: 6
Abstract

Tumor-associated macrophages (TAM) have multifaceted roles in tumor development but they have been associated particularly closely with tumor angiogenesis. However, while the accumulation of TAM (M2 phenotype) promotes tumor angiogenesis the mechanism through which monocytes differentiate to generate TAM is unclear. Here we report that the mTOR pathway is a critical element in the regulation of monocyte differentiation to TAM. In human peripheral monocytes stimulated by lipopolysaccharide (LPS), mTOR was inhibited by rapamycin or activated by RNAi-mediated knockdown of the mTOR repressor TSC2. Rapamycin caused the monocytes to differentiate into M1 macrophages releasing more IL-12 and less IL-10, whereas TSC2 knockdown caused the monocytes to differentiate into M2 macrophages releasing less IL-12 and more IL-10. In parallel fashion, angiogenic properties were promoted or reduced in HUVEC cells co-cultured with TSC2-deficient monocytes or rapamycin-treated monocytes, respectively. Furthermore, tumor angiogenesis and growth in murine xenografts were promoted or reduced by infusion of hosts with TSC2-deficient or TSC2-overexpressing monocytes, respectively. Lastly, in vivo depletion of macrophages was sufficient to block the anti-angiogenesis effects of rapamycin on tumors. Our results define the TSC2-mTOR pathway as a key determinant in the differentiation of monocytes into M2 phenotype TAM that promote angiogenesis.

Keywords: tumor-associated macrophages, TAM; mammalian target of rapamycin, mTOR; signal transducer and activator of transcription 3, STAT3; angiogenesis; cancer.
Abbreviations

4E-BP1, initiation factor 4E-binding protein 1; DEN, diethylnitrosamine; GdCl₃, gadolinium chloride; HUVEC, human umbilical vein endothelial cell; IL: interleukin; KCs, Kupffer cells; MCP-1, monocyte chemotactic protein-1; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor kappa B; p70S6K, ribosomal p70S6 kinase; PBMCs, peripheral blood mononuclear cells; TAM, tumor associated macrophage; TNF-α, tumor necrosis factor-alpha; TSC2, tuberous sclerosis complex 2; STAT3, signal transducer and activator of transcription 3; SFM, serum free medium; VEGF, vascular endothelial growth factor.
Introduction

Inflammation and cancer are connected, as cancers arise at chronic inflammatory sites (1, 2), and inflammatory cells participate in processes of tumor progression such as neoangiogenesis. Tumor-associated macrophages (TAMs) play a prominent role in tumor invasion by promoting tumor angiogenesis in contrast to the antitumor effects of unactivated macrophages (3-6). Depleting macrophages in tumors reduces tumor angiogenesis (7, 8), though the molecular mechanisms governing macrophages and angiogenesis are unknown.

The mammalian target of rapamycin (mTOR) is a central regulator of cell growth that phosphorylates ribosomal p70S6 kinase (p70S6K) and initiation factor 4E-binding protein 1(4E-BP1) to control the synthesis of translation components (9). The tuberous sclerosis complex 2 (TSC2) is an upstream negative regulator of mTOR (10, 11). The TSC2-mTOR pathway may alter cytokine secretion to regulate innate immune responses such the monocyte macrophage system (12-14). However, the involvement of the TSC2-mTOR pathway in macrophage-induced angiogenesis is unclear.

The signal transducer and activator of transcription 3 (STAT3) controls an important inflammation-related signaling pathway in cancer development, and it is activated by tyrosine phosphorylation in both immune and tumor cells (15). STAT3 inhibits macrophage-derived antitumor immune responses (16) and is involved in macrophages differentiation and development of the tumor microenvironment (17-20). mTOR, which regulates STAT3 activation in cells including immune cells, might promote tumor angiogenesis (12, 21).
Rapamycin down regulates microvessel density (MVD) \textit{in vivo} (22, 23). Together, these findings suggest that TSC2-mTOR may promote angiogenesis via macrophages and that STAT3 may be a downstream effector.

Here, we used both \textit{in vivo} and \textit{in vitro} assays to investigate the role of the TSC2-mTOR pathway in regulating macrophages to induce tumor angiogenesis. Our findings suggest that activating mTOR promoted macrophage-induced angiogenesis through STAT3 while inhibiting mTOR promoted macrophage-mediated anti-tumor effect.

\textbf{Materials & Methods}

\textbf{Cell isolation and culture}

Human or mouse peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (12) and the monocytes were isolated from the respective PBMCs by magnet-mediated cell separation (CD14 magnetic beads, Miltenyi Biotec, Bergisch Gladbach, Germany). The PE-conjugated antibody against CD14 was used to analyze the purity of PBMCs. The purity of human and mouse monocytes was about 95\% (Supplemental Figures S1A and S1B). The isolated monocytes were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10\% fetal bovine serum (FBS; Invitrogen), 100 U/ml penicillin, and 100 \mu g/ml streptomycin. Five mice were killed for every reinfusion to have sufficient mouse monocytes.

Kupffer cells (KCs) were isolated from rat livers. The nonparenchymal cells were separated
with an 18% Nycodenz gradient (Nycomed Pharma A/S, Oslo, Norway), and then KCs were further separated with counterflow centrifugal elutriation in a J2-MC centrifuge (Beckman-Coulter, Brea, CA USA). The purity of the KCs was determined with CD68 immunofluorescence and latex beads-mediated phagocytosis (Sigma Aldrich, St Louis, MO USA).

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical veins, and cultured in M199 medium (Invitrogen, Carlsbad, CA USA) containing 20% FBS, 15 µg/ml endothelial cell growth supplement, 2 nM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin.

The Huh-7 hepatocarcinoma cell line (Japanese Collection of Research Bioresources, Sennan-shi, Osaka, Japan) was cultured similar to HUVECs. We did not do test and authentication for this cell line after we purchased from the company.

**Cell transfection**

Monocytes were transfected with scrambled siRNA (5’ to 3’ : GATCATCCTTGATCTTATA) or siRNA for TSC2 or STAT3 (Santa Cruz Biotechnology, CA, USA) with Lipofectamine-2000 (Invitrogen) for 48 hours before use. Mouse monocytes were similarly transfected with the TSC2 plasmid (pEGFP-c2-TSC2) or the TSC2 siRNA plasmid (pEGFP-c2-TSC2 siRNA) before being reintroduced to the mice.

**Determination of cytokine concentrations**
A total of $1 \times 10^6$ TSC2 siRNA-transfected human monocytes or $2 \times 10^5$ KCs were pretreated with 20 nM rapamycin (Sigma Aldrich) for 90 minutes while plated in 24-well plates, and then stimulated by 100 ng/ml LPS (Sigma Aldrich). After 48 hours, cell-free supernatants were collected. The concentrations of IL-12p40, IL-12p70, IL-6, TNF-a, IL-10, VEGF, IL-1, and monocyte chemotactic protein-1 (MCP-1) were measured by ELISA using kits (R&D Company, Minneapolis, MN, USA).

**Immunoblot analysis**

A total of $1 \times 10^7$ human monocytes were stimulated as indicated after 24 hours of starvation in serum-free medium. Cellular lysates (40μg) were analyzed by standard Western blot techniques. Immunoreactive bands were developed by enhanced chemiluminescence (ECL, GE Healthcare, United Kingdom) and visualized by autoradiography (Kodak, Rochester, NY USA). Relative levels of total and phosphorylated proteins were determined with these antibodies: anti-phospho-STAT3 (Tyr705) and anti-STAT3 (Santa Cruz Biotechnology, Santa Cruz, CA USA); anti-phospho-TSC2 (Ser939), anti-TSC2, anti-phospho-mTOR (Ser2448), anti-mTOR, anti-phospho-p70S6k (Thr389), anti-p70S6K, anti-phospho-4EBP1(Thr37/46), anti-4EBP1, mouse anti-phospho-NK-kB, and mouse anti-phospho-p38 MAPK (Cell Signaling); and anti-GAPDH (Shanghai Kangcheng, Shanghai, China).

**Matrigel angiogenesis assay in vitro**

A total of $2 \times 10^4$ primary HUVEC cells were plated on 24-well plates coated with matrigel
(BD Bioscience, Franklin Lakes, NJ USA), incubated one hour at 37°C with 5% CO₂, co-cultured with monocytes for 12 hours, and subsequently the development of capillary structures and tubular networks was analyzed by light microscopy (Leica Microsystems, Exton, PA, USA).

**Subcutaneous xenograft of Huh-7 cells in nude mice**

Male nude mice that were 5 weeks old weighing 18 to 20 g (Shanghai Experimental Animal Institute, Shanghai, China) were housed in a pathogen-free room. The experimental animals were handled in compliance with the guidelines of the Animal Ethics Committee of Zhejiang University. After Huh-7 cells were harvested, 10⁶ cells were resuspended in 100 μl saline solution and inoculated into one flank of each mouse. After three weeks, the tumors were 100 mm³ and the mice were divided into three treatment groups.

In **Experiment A**, 35 tumor-bearing mice were divided into four groups: control group (n=5), rapamycin group (5 mice intraperitoneally administered with 1 mg/kg rapamycin daily), GdCl₃ group (5 mice injected with 10mg/kg GdCl₃ once a week through the caudal vein), and combined group (20 mice injected with both GdCl₃ and rapamycin). After two weeks, five mice from each group were killed, and then the xenografts were removed and saved for additional research. The remaining 15 mice from the combined group were injected with GdCl₃ again, and then divided into three groups: rapamycin group (5 mice administered 1 mg/kg rapamycin daily), rapamycin plus monocyte group (5 mice administered 1 mg/kg rapamycin daily and daily infusion of 5×10⁶ mouse monocytes), and monocyte groups (5
mice had only $5 \times 10^6$ mouse monocytes reintroduced the next day). Injected monocytes were not pretreated with rapamycin or LPS. The different experimental monocytes were directly injected intravenously into mice. After one week, the mice were sacrificed by cervical dislocation, and the xenografts were removed for analysis.

In **Experiment B**, 20 male nude mice were treated with subcutaneous xenografts of Huh-7 cells established as above and divided into four groups: control group (5 mice injected with vehicle daily), GdCl$_3$ group (5 mice injected with 10 mg/kg GdCl$_3$ weekly), TSC2 siRNA group (5 mice treated with $5 \times 10^6$ monocytes transfected with the TSC2 siRNA), and TSC2 group (5 mice treated with $5 \times 10^6$ monocytes transfected with the TSC2).

In **Experiment C**, 20 additional tumor-bearing nude mice were divided into four groups: control group (5 mice injected with vehicle daily), NSC 74859 group (5 mice treated with 5mg/kg NSC 74859 (Merck KgaA, Darmstradt, Germany) intraperitoneally every other day), GdCl$_3$ group (5 mice injected with 10mg/kg GdCl$_3$ weekly), and combined group (5 mice injected with both 10mg/kg GdCl$_3$ and 5 mg/kg NSC 74859 as described above). All the mice were killed by cervical dislocation 2 weeks later. The xenografts were removed and stored for further examination.

**Rat tumor induction**

A total of 20 male Sprague-Dawley rats with body weights of 160 to 180 g and aged from 6 to 7 weeks (Shanghai Experimental Animal Institute, Shanghai, China) had hepatomas induced by continuously administering 0.01% diethylnitrosamine (DEN) via drinking water.
for 12 weeks.

Two weeks after tumor inoculation, the 10 rats in the rapamycin and 10 in the control group had 2 mg/kg rapamycin (Hangzhou Huadong Medicine Group, Zhejiang, China) or saline solution, respectively administered intragastrically. All rats were sacrificed by cervical dislocation 4 weeks later, and the livers were removed, photographed, and examined by immunohistochemistry for CD31 and CD68 expression.

Histology and immunohistochemistry

Formalin-fixed tumor sections were stained with hematoxylin and eosin (H&E). Both CD31 and CD68 were immunohistochemically examined on the paraffin sections using primary antibodies diluted 1:75 (Santa Cruz Biotechnology). The mean vessel density was quantified in sections stained for CD31 by capturing 10 random fields (0.159 mm²) at 100x magnification.

Statistical analysis

Data are presented as mean with standard deviation (SD). Independent two-sample t-tests compares differences between two groups, and one-way ANOVA with the least significant difference (LSD) test for post-host comparisons compared differences between three or four groups. A $P$-value below 0.05 indicated statistical significance. Statistics were analyzed with SPSS 15.0 software (SPSS Inc, Chicago, IL, USA).

Results
Manipulating the TSC2-mTOR pathway modulated cytokine secretion and vessel formation

To investigate the impact of mTOR activity on macrophages, cytokine secretion was assessed in human monocytes (Fig 1). Monocytes in Rapa+LPS group secreted more IL-12-p40, IL-12-p70, IL-6, TNF-α, and IL-1 and secreted significantly less IL-10, MCP-1 and VEGF compared with the LPS group (All \( P<0.05 \), Fig 1A) and the majority of cells in the population shifted to expressing IL-12p40 (Supplemental Figure S1C). LPS treatment significantly increased the release of these cytokines compared with non-LPS treated control cells. Treatment with LPS increased the phosphorylation of mTOR compared to control on Ser2448 (a frequent site of mTOR phosphorylation (24), 4EBP1, and STAT3 of the downstream mTOR pathway (Fig 1B). Treatment with rapamycin reduced mTOR phosphorylation although the phosphorylation was higher than the untreated control. CD206 (mannose receptor), a marker of M2 macrophages was not detected in the LPS+Rapamycin treatment group (Supplemental Figure S1D) but it was highly expressed on TSC2 siRNA-treated cell surfaces (Supplemental Figure S1E). To assess whether rapamycin may affect the phosphorylation of other LPS-stimulated pathways, we evaluated the phosphorylation NF-κB and p38 MAPK following rapamycin treatment. Rapamycin did not affect the phosphorylation of p38 but did increase the phosphorylation of NF-κB (Supplemental Fig S2A-B).

The effect of reduced TSC2 on cytokine secretion and protein expression was examined by transfection of cells with TSC2 siRNA. Compared with monocytes transfected with
unspecific siRNA, monocytes transfected with TSC2 siRNA secreted significantly less IL12-p40, IL12-p70, IL-6, TNF-α and IL-1 and secreted more IL-10, MCP-1 and VEGF (all \(P<0.05\), Fig 1C). Increased phosphorylation of mTOR, p70S6K, 4EBP1, and STAT3 was detected in monocytes transfected with TSC2 siRNA (Fig 1D). These results suggest that monocytes treated with rapamycin and subsequently stimulated by LPS developed the M1-like macrophage phenotype while monocytes transfected with TSC2 siRNA developed the M2-like macrophage phenotype.

To define the potential role of mTOR in monocyte-induced angiogenesis, vessel formation by human monocytes with different treatments was assayed in vitro (Fig 2). Rapamycin treatment significantly reduced LPS-induced vessel formation (\(P = 0.004\); Fig 2A,B). Conversely, TSC2 siRNA treatment significantly increased vessel formation (\(P = 0.005\); Fig 2C, D). However, adding of VEGF Ab did not significantly block TSC2 siRNA-induced angiogenesis (see Supplemental Figure S3 A and B).

**Macrophages mediate the anti-tumor effects of rapamycin in vivo**

Since rapamycin inhibits tumor angiogenesis in vivo, nude mice were inoculated with Huh-7 hepatocarcinoma cells to form subcutaneous tumors, and subsequently treated with GdCl3 to deplete monocytes or rapamycin to inhibit mTOR, or both (Fig 3, Experiment A). The monocytes/macrophages in GdCl3-treated tumor tissue were significantly lower than the untreated group (0.17% vs. 9.4%, Supplemental Fig S4). The GdCl3-treated mice trended toward lower gross tumor volume and relative lower ratio of tumor weight to body weight...
(15.4 mg/g vs. 39.6 mg/g, P =0.0022). Interestingly, the mice treated with both GdCl₃ and rapamycin had larger tumors than rapamycin alone (Fig 3A, P<0.05). The rapamycin-treated mice had the smallest tumors and a lower ratio of tumor weight to body weight (4.6 mg/g) than control mice (P = 0.0001, right panel).

The re-introduction of exogenous monocytes into GdCl₃-treated mice rescued tumor growth (Fig 3B) and the addition of exogenous monocytes plus rapamycin significantly inhibited tumor growth (P < 0.0001). Further analysis of microvessel density found rapamycin significantly inhibited exogenous monocytes-induced microvessel density formation (Fig 3C).

These findings suggest that rapamycin induced monocytes to differentiate into the anti-tumor M1 phenotypes.

**TSC2-mTOR signal pathway controls angiogenesis induced by macrophages *in vivo***

In human and mouse monocytes, down-regulation of TSC2 by TSC2 siRNA transfection significantly increased the ratio of phosphorylated mTOR/total mTOR in a time dependent pattern while over-expression of TSC2 by TSC2 cDNA transfection significantly decreased mTOR phosphorylation (Fig 4A and 4B).

To investigate the role of mTOR in the xenograft tumor growth induced by macrophages *in vivo*, we manipulated TSC2 expression in monocytes that had been reintroduced into mice (Fig. 4C, Experiment B). Mice treated with monocytes over-expressing TSC2 had the smallest tumor size. Tumors from mice treated with TSC2 siRNA monocytes were larger than mice treated with GdCl₃ (P = 0.0003) or control mice (P < 0.05). Microvessel density assay indicated that over-expression of TSC2 significantly inhibited exogenous monocytes-induced
blood vessel formation while TSC2 siRNA-treated monocytes dramatically induced
angiogenesis (Fig 4D).

**STAT3 mediates the effects of rapamycin on cytokine secretion of monocytes**

In order to elucidate the downstream target of mTOR, we investigated the effect of rapamycin
on STAT3, which is required for IL-10 secretion. NSC 74859 treatment or knocking down
STAT3 significant increased the concentration of IL12-p40 and reduced the concentration of
IL-10 (all \( P < 0.001 \), Fig 5A). NSC 74859 inhibits STAT3 activity by inhibiting STAT3
complex formation, DNA-binding, and transcriptional activities (25). NSC 74859 treatment
and STAT3 siRNA transfection decreased phosphorylation of STAT3 (Fig 5B) and inhibited
blood vessel formation (Fig 5C). *In vivo* xenografts indicated that tumors from mice treated
with NSC 74859 were smaller than those from control mice (\( P < 0.0001 \)), but mice treated
with GdCl\(_3\) or with GdCl\(_3\) and NSC 74859 had statistically similar tumor sizes (Fig 5D;
Experiment C). We further found that treatment with NSC74859 blocked TSC2
siRNA-induced blood vessel formation, indicating that STAT3 is necessary for
mTOR-induced angiogenesis (Supplemental Figure S5 A and B).

**The cytokine secretion of Kupffer cells and hepatocarcinogenesis induced by DEN are
affected by mTOR**

The KCs cells were identified by CD68 immunofluorescence staining (Fig 6A). Treating the
KCs with rapamycin *in vitro* increased the secretion of IL-12p40 (\( P = 0.011 \)), TNF-\( \alpha \) (\( P =
0.002 \)), and IL-6 (\( P < 0.001 \)) and decreased IL-10 compared to control (\( P = 0.002 \)) (Fig 6B).
Furthermore, large clumps of tumor were easily seen in the liver of the control rats but not in rapamycin-treated animals (Fig 6B). In addition, less CD31 staining was detected in the liver of rapamycin treated rats; however, CD68 staining did not alter the number of KCs (Fig 6C).

**Discussion**

In current study, we found that inhibiting of mTOR by either rapamycin or overexpression of TSC2 or blocking STAT3 in monocytes/macrophages promoted the release cytokines of the M1 and inhibited tumor growth in vitro and in vivo. These effects were confirmed with decreased tumor angiogenesis and the requirement of monocytes. We also confirmed that activating the mTOR signalling pathway by treating monocytes/macrophages with TSC2 siRNA promoted release of cytokines of the M2 phenotype cytokines and promoted tumor growth. These findings were confirmed by the increased tumor and the requirement for monocytes.

Tumor associated macrophages (TAMs) are considered a polarized population of M2 macrophages, especially when the tumor begins to invade, vascularize, and develop (3, 4). The classification of polarized macrophages as either the M1 or M2 phenotype is mainly based on differential secretion of cytokines. The M1 phenotype secretes IL-12 and TNF, and later the M2 phenotype secretes IL-10. Our present study found that TAMs profoundly influenced the regulation of tumor angiogenesis, and that depleting macrophages reduced vascular density and delayed tumor growth.
Our present study shows that inhibiting mTOR increased IL-12 production and decreased IL-10 production in monocytes, in agreement with previous studies (12, 13). The opposing roles of IL-12 and IL-10 provide a new explanation of the antitumor effects of inhibiting mTOR activity. The fact that IL-12 p70 was only modestly up-regulated might suggest that excess IL-12 p40 may form homodimers, although we did not detect IL12 p40 homodimers in these experiments.

Since the antitumor effects of rapamycin are mainly attributed to inhibited angiogenesis (22, 23), it is exciting that the rapamycin effects were eliminated by depleting macrophages with GdCl₃, a known inhibitor of KC activation (26, 27). Reintroducing monocytes recovered the antitumor effects of rapamycin. Rapamycin enhanced and TSC2 siRNA attenuated the phagocytotic capability of macrophages (Supplemental Figure S6) which was reversed by reintroduction of macrophage infiltration into tumors (Supplemental Figure S7 A-C). These findings strongly suggested that the antitumor effects of rapamycin were mediated by induction of macrophages with the anti-tumor M1 phenotype.

Inhibiting mTOR altered the monocyte cytokines in vitro by increasing IL-12 and decreasing IL-10. This finding suggested that the mTOR signal may participate in polarizing macrophages, which would explain the essential role of monocytes in the ability of rapamycin to inhibit angiogenesis. Phosphorylation of mTOR at Ser-2448 is mediated by the p70S6 kinase (24) and our finding suggest that this may be controlled by inhibiting the phosphorlation of p70S6. We found that rapamycin did not inhibit the phosphorylation of p38,
but did enhance the phosphorylation of NF-κB. Releasing of IL-12 required activated NF-κB in macrophages (28,29). This indicated that the cytokine releasing by rapamycin-treated macrophages might be mediated through NF-κB pathway. In addition, although TSC2 siRNA resulted in an up-regulation of VEGF, adding of VEGF antibody did not significantly block TSC2 siRNA-induced angiogenesis.

Our findings indicated that mTOR regulates the ability of macrophages to induce angiogenesis. Rapamycin down-regulates secretion of IL-10, which promotes the production of VEGF in both immune and tumor cells resulting in strengthening of the macrophages TH1 response. Our experiments suggest that that the TSC2-mTOR pathway-regulated angiogenesis may be mediated by other growth factors, not only through VEGF. We also found that rapamycin enhanced and TSC2 siRNA attenuation of macrophage phagocytosis (Supplemental Figure S6). Although our rapamycin experiments support the idea that the mTOR pathway acts via monocytes to promote tumor growth, it is possible that the effect of rapamycin may affect monocytes through other mechanism. In an in vitro experiment, we found rapamycin mainly stimulated monocytes to release cytokines which had anti-angiogenesis effects and reduced the release of pro-angiogenesis cytokines (Supplemental Figure S8A-B).

Since STAT proteins regulate cytokine-dependent inflammation and immunity, they are central in determining whether cancer is promoted or inhibited by immune responses (30). The activation of STAT3 mediates such cancer promoting properties as neo-angiogenesis in
macrophages (31, 32), and secretion of IL-10 and IL-12 in TAMs (18, 24). We also found that inhibiting STAT3 mediated the up-regulation of IL-12 and the down-regulation of IL-10 in macrophages, resulting in neo-angiogenesis and inhibited tumor growth. The important role of macrophages in this process was demonstrated by the reduced antitumor effects of NSC 74859 when monocytes were depleted. Our results agreed with previous work showing that NSC 74859 inhibits tumor growth more in vivo than in vitro (33). Our study agreed with previous studies finding that STAT3 was the downstream target of mTOR in macrophages and in many other cell types (12, 21). We also found treatment with NSC74859 blocked TSC2 siRNA-induced blood vessel formation, indicating that STAT3 was necessary for mTOR-induced angiogenesis. The secretion of cytokines regulated by inhibiting mTOR also involves NK-κB, which regulates IL-12p40. Inhibiting mTOR led to activation of NK-κB, which augmented the secretion of IL-12p40, while inhibiting STAT3 only impaired the secretion of IL-10 (12). However, our present study found that, when mTOR was inhibited, STAT3 decreased the secretion of both IL-12p40 and IL-10. Thus, STAT3 mediates the effects of mTOR in macrophage induced neoangiogenesis, potentially providing new insight into us innate immune responses and new targets for cancer therapy.

We also found that rapamycin affects KCs by enhancing IL-12 and inhibiting IL-10 production, indicating that the mTOR pathway is involved in the function of KCs. Rapamycin reduced the angiogenesis and growth of tumors that were induced by DEN in rats. Additional experiments are required to further investigate how rapamycin affects tumor formation in the liver.
In summary, we have identified a critical role of the TSC2-mTOR pathway in regulating angiogenesis that is induced by phagocytic cells. When mTOR was activated in monocytes by knocking down TSC2 with siRNA, STAT3 was activated and IL-10 increased, while IL-12 decreased in a manner similar to TAM secretion and resulted in neoangiogenesis in vivo. Conversely, inhibiting mTOR had a reciprocal effect. These findings suggest that inhibiting TSC2-mTOR-STAT3 in the innate immune response may be a novel and effective therapeutic avenue.
Acknowledgment

Kathy Boltz of MedCom Asia provided professional English-language assistance for this manuscript.

Grant support

This study was supported by grants from the National Natural Science Funds for Distinguished Young Scholar (No. 30925033), the National Natural Science Fund of China (No. 30801101 and NO.81171884), The Innovation and High-Level Talent Training Program of Department of Health of Zhejiang.
References


Figure legends

Figure 1. The TSC2-mTOR pathway regulated cytokine secretion by human monocytes. (A) Expression of cytokines by human monocytes which stimulated with LPS, or incubated with rapamycin and then stimulated with LPS by ELISA assay. Data represent the mean ± SD, * indicates a significant difference compared to the control group, and † indicates a significant difference compared to the LPS group. (B) The effects of LPS stimulation and rapamycin treatment on the intracellular mTOR pathway were analyzed by Western blots. (C) Expression of cytokines by human monocytes which stimulated with TSC2 siRNA by ELISA. Data represent the mean ± SD, and * indicates a significant difference compared to negative siRNA group. (D) Western blots analyzed the changes in the proteins of the intracellular mTOR pathway after transfection with negative or TSC2 siRNA.

Figure 2. The TSC2-mTOR pathway regulated the formation of macrophage-induced vessels by HUVEC cells in vitro. The vessel formation of the HUVEC cells which were plated in 24-well plates coated with Matrigel and co-cultured with human monocytes which were pre-treated with LPS or/ and rapamycin (A&B) or TSC2 siRNA (C&D). Data represent mean ± SD, * indicates a significant difference compared to the control group, and † indicates a significant difference compared to the LPS group.

Figure 3. The macrophages mediated the antitumor effects of rapamycin in vivo. Nude mice were treated with rapamycin in the presence or absence of monocytes as described in
Experiment A in the Experimental Procedures. (A) Their tumors were imaged (left), and the ratios of the weight of their tumors to their body weights were quantified (right).*: P<0.05 in comparison to Control; #:P<0.05 in comparison to GdCl3; &:P<0.05 in comparison to Rapa. (B) The effects of rapamycin on tumor growth with or without reintroduction of exogenous monocytes into GdCl3-treated mice were detected. Their tumors, after receiving the various treatments, were imaged (left), and the ratios of the weight of their tumors to their body weights were quantified (right). *:P<0.05 in comparison to the GdCl3 and rapamycin group. (C) The effects of rapamycin on blood vessel formation in the tumor from (B) were investigated. †:P<0.05 in comparison to GdCl3 and monocytes group. Data represent mean ± SD (n = 5).

Figure 4. Effects of TSC2 on mTOR and tumor formation. (A) Phosphorylation of mTOR and total mTOR in human monocytes were detected after transfected with TSC2 cDNA or TSC2 siRNA for different time (6h, 24h and 72h) in vitro. *:P<0.05 in comparison to Control, †:P<0.05 in comparison to 6h , ‡:P<0.05 in comparison to 24h. (B) Effects of over-expression or knockdown TSC2 on phosphorylation of mTOR and total mTOR in mouse monocytes were reversed in vitro. Data represent three separate experiments, * P<0.05 in comparison to the control group, and † P<0.05 in comparison to the TSC2 group. (C) Tumor formation in nude mice as described in Experiment B in the Experimental Procedures. * P<0.05 in comparison to the control group, † P<0.05 in comparison to the GdCl3 group, and ‡ P<0.05 in comparison to the TSC2 group. (D) The effects of TSC2 on blood vessel formation in the tumor from (C) were investigated. *
P<0.05 in comparison to the control group, † P<0.05 in comparison to the GdCl₃ group, and ‡ P<0.05 in comparison to the TSC2 group. Data represent mean ± SD (n = 5).

Figure 5. Inhibiting STAT3 blocked the angiogenesis induced by macrophages both in vitro and in vivo in a manner similar to rapamycin. (A) The production of IL-12-p40, TNF-α, and IL-10 by monocytes with different treatments. Data represent the mean ± SD, and * indicates a significant difference compared to the control group (P<0.05). (B) Western blots analyzed the expression of STAT3 after the treatments shown in (A). (C) Vessel-like structure formation by HUVEC cells co-cultured with human monocytes with different treatments in 24-well plates coated with Matrigel. * indicates a significant difference compared to the control group (P<0.05). (D) Tumor formation in nude mice as described in Experiment C in the Experimental Procedures under STAT3 inhibitor (NSC 74859, 5mg/kg) treatment. * indicates a significant difference compared to the control group (P<0.05).

Figure 6. Rapamycin regulated the secretion of cytokines by Kupffer cells from rats and prevented the induction of hepatocarcinogenesis by DEN in rats. (A) The Kupffer cells isolated from the rats were identified by phagocytosis that was mediated by latex particle (top) and by CD68 immunofluorescence (bottom). (B) The production of IL-12p40, TNF-α, IL-10, and IL-6 by Kupffer cells with different treatment. Data represent mean ± SD, and * indicates a significant difference compared to the control group. (C) Representative images for rat livers from the rats treated with the control and with
rapamycin after DEN treatment. Gross liver appearance were shown on the left; immunohistochemistry examined the expression of CD31 to detect angiogenesis and CD68 to detect Kupffer cells were shown on the right.
Figure 6

A

B

C

Morphology

CD31

CD68

Control

Rapamycin
Macrophage-induced tumor angiogenesis is regulated by the TSC2-mTOR pathway

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Cancer Res  Published OnlineFirst January 27, 2012.

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Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-11-2684

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