Original Paper

Tsc1 is a Critical Regulator of Macrophage Survival and Function

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Key Words

Tsc1 • Macrophage • mTOR

Abstract

Background/Aims: Tuberous sclerosis complex 1 (Tsc1) has been shown to regulate M1/M2 polarization of macrophages, but the precise roles of Tsc1 in the function and stability of macrophages are not fully understood. Here we show that Tsc1 is required for regulating the survival, migration and phagocytosis of macrophages. **Methods:** Mice with Tsc1 homozygous deletion in myeloid cells (LysMCreTsc1^{flox/flox}; Tsc1 KO) were obtained by crossing Tsc1^{flox/flox} mice with mice expressing Cre recombinase under the control of Lysozyme promoter (LysMCre). The apoptosis and growth of macrophages were determined by flow cytometry and Real-time PCR (RT-PCR). The phagocytosis was determined using a Vybrant™ phagocytosis assay kit. The migration of macrophages was determined using transwell migration assay. **Results:** Peritoneal macrophages of Tsc1 KO mice exhibited increased apoptosis and enlarged cell size. Both M1 and M2 phenotypes in Tsc1-deficient macrophages were elevated in steady-state as well as in inflammatory conditions. Tsc1-deficient macrophages demonstrated impaired migration and reduced expression of chemokine receptors including CCR2 and CCR5. Phagocytosis activity and ROS production were enhanced in Tsc1-deficient macrophages. Furthermore, pharmacological inhibition of the mammalian target of rapamycin complex 1 (mTORC1) partially reversed the aberrance of Tsc1-deficient macrophages. **Conclusion:** Tsc1 plays a critical role in regulating macrophage survival, function and polarization via inhibition of mTORC1 activity.

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Introduction

Tuberous sclerosis complex 1 (TSC1) and Tuberous sclerosis complex 2 (TSC2) form a complex to regulate metabolism and energy homeostasis via inhibition of mammalian target of rapamycin complex 1 (mTORC1) [1]. Loss-of-function mutations in TSC1 or TSC2 result in elevated activity of mTORC1, which leads to increased cell growth and proliferation. mTORC1 controls activation of p70 S6 kinase and eukaryotic initiation factor 4E-binding protein 1 to orchestrate transcriptional expression and translation of critical proteins involved in metabolic processes [2].

Recent studies have highlighted an important function of Tsc-mTOR signaling pathway in regulation of innate and adaptive immunity [3-5]. Macrophages play an essential role in the initiation and resolution of both innate and adaptive immunity, and are critical for protective immunity and immune-mediated pathology [6, 7]. Macrophages exert different biological function through phagocytosis, pathogens clearance as well as secretion of a bunch of cytokines and chemokines. Activated macrophages include three phenotypes: M1 that is involved in host defense, M2 that is involved in immuno-suppression and promotes wound healing, and regulatory macrophages that is involved in immunoregulation [8]. M1 macrophages are induced by Toll-like receptor (TLR) agonists or Th1 cytokines to produce pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF- α), interleukin-6 (IL-6), IL-12 and IL-23 [9]. M2 macrophages are induced by Th2 cytokines IL-4 and/or IL-13 and display anti-inflammatory properties by producing anti-inflammatory cytokines and substances such as arginase, epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and tumor growth factor beta (TGF- β) [10, 11].

CCR2 and CCR5 are chemokine receptors that mediate trafficking of monocytes/macrophages. Mice deficient of CCR2 exhibits an impaired ability to recruit monocytes/macrophages to the sites of inflammation [12, 13]. CCR2 binds multiple ligands including CCL2, CCL8, CCL7, CCL13. CCR5 binds CCL4, CCL5, CCL3, CCL8. In addition to expressing CCR2 and CCR5, macrophages secrete a set of chemokines which affect angiogenesis and wound healing. It was reported that Rapamycin (Rapa, an inhibitor of mTORC1) suppressed the expression of CCL2, CXCL8, CCL5, CCL8 and CCL4 in the THP-1 cells induced by LPS and human primary monocytes [14]. The stimulated production of reactive oxygen species (ROS) by phagocytic cells is necessary for the bactericidal action of phagocytes [15]. In response to viral infection, ROS mediate innate immune signaling or generate danger signals to activate immune cells to elicit defending responses against pathogens.

Recent studies have demonstrated that Tsc1-deficient macrophages are refractory to M2 polarization induced by IL-4, but are sensitive to pro-inflammatory stimuli [16, 17]. Nevertheless, the roles of Tsc1 in controlling the survival, migration, and phagocytosis of macrophages are still not well defined so far.

Here we demonstrate that mice with Tsc1 deletion in myeloid cells showed decreased absolute number and enlarged cell size of primary peritoneal macrophages. Intriguingly, although previous studies have demonstrated that Tsc1-deficient macrophages are refractory to IL-4 induced polarization [17]. We found Tsc1-deficient macrophages exhibited enhanced polarization of both M1 and M2 phenotypes in steady-state condition and after treatment with LPS or IL-4. Moreover, Tsc1-deficient macrophages showed impaired migration, potentially due to reduced expression of chemokine receptors. Phagocytosis and ROS production were enhanced in Tsc1-deficient macrophages. Activating effect of Tsc1-deficient macrophages on CD4⁺ T cell was impaired. Inhibition of mTORC1 partly reversed the aberrance of Tsc1-deficient macrophages. Thus our study revealed that Tsc1 played a key role in controlling the survival, migration and phagocytic function of macrophage, and mTORC1 was involved in Tsc1 signalling.

Materials and Methods

Animals

Mice with Tsc1 homozygous deletion in myeloid cells (LysMCreTsc1^{flox/flox}; Tsc1 KO) were obtained by crossing Tsc1^{flox/flox} mice (The Jackson Laboratory) with mice (The Jackson Laboratory) expressing Cre recombinase under the control of Lysozyme promoter (LysMCre). LysMCre-negative, Tsc1^{flox/flox} mice were served as control. All mice were maintained in a specific pathogen-free facility and were used in accordance with protocols approved by Animals Care and User Committee at the Institute of Hematology of Peking Union Medical College.

Cell culture

Peritoneal macrophages were obtained from the peritoneal exudates of 5-8 weeks old mice with PBS containing 2% FBS. CD11b⁺F4/80⁺ macrophages were sorted using a BD FACS Aria III flow sorter. The sorted population were >90% pure.

Western blot assay

Peritoneal macrophages were lysed in RIPA buffer with protease and phosphatase inhibitor cocktails for 15 min on ice. Protein concentration was determined by bicinchoninic acid (BCA) assay. Protein samples were analysed on SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto Nitrocellulose filter membranes (Millipore). Each membrane was blocked with TBST (with 5% not-fat dried milk) for 1 h, then incubated with primary antibodies including endogenous control gene (GAPDH (Cell Signalling Technology) or Tsc1 (Cell Signalling Technology) overnight on a shaker at 4 °C. The appropriate HRP-coupled secondary antibody was then added, and was detected through western Bright™ ECL (advanta). GAPDH was used as control.

Isolation of splenocytes and lymph node cells

5-8 weeks old mice (male and female) were sacrificed and the spleens, peripheral lymph nodes (pLNs) and mesenteric LNs (mLNs) were resected and transferred into PBS containing 1% FBS. The splenocytes and lymph node cells were obtained by filtering the spleens or LNs through a 48 micron nylon mesh.

Flow cytometry

Cells were stained in staining buffer (PBS containing 1% FBS) with antibodies for 40 min at 4 °C for staining of surface markers, then washed with staining buffer, and analyzed by LSR II (BD Falcon). Data were analyzed by FlowJo software (Treestar). ROS was measured by incubation with CM-H2DCFDA (10 uM; Invitrogen) at 37 °C after staining of surface markers with anti-mCD11b-APC and anti-mF4/80-PE. Annexin V Cell Apoptosis Analysis Kit (Tianjin Sungene Biotech) was used to determine apoptosis of cells positive for CD11b and F4/80. After staining with anti-mCD4-FITC, anti-mCD8-PE-cy7, Foxp3 intracellular staining was conducted by a Mouse Regulatory T Cell Staining Kit (ebioscience). Anti-mCD209-FITC, anti-mF4/80-PE, anti-mCD71-FITC, anti-mFoxp3-PE, anti-mCD44-PerCP-Cy5.5, anti-mCD62L-APC-eFluor®780, anti-mCD98-PE, anti-mLy6C-PerCP-Cy5.5, anti-mCD11b-APC were purchased from eBioscience. Anti-mCD86-PerCP-Cy5.5, anti-mCD206-APC, anti-mCD25-APC, anti-mCD8-PE-cy7, anti-mCD4-FITC, anti-mCD80-FITC, anti-mMHCII-APC were purchased from Biolegend.

Rapa treatment

Rapa (Cayman Chemical) was dissolved in PBS containing 5% DMSO and mice were injected intraperitoneally with Rapa (1.5 mg/kg) every day for 3 days.

RT-PCR

Total cellular RNA were isolated with Trizol reagent (Invitrogen). Reverse transcription was performed with M-MLV reverse transcriptase (TransGen Biotech) according to the manufacturer's instructions. RT-PCR was determined using SYBR Green Master Mix (Life Technologies™). The primers used in the present study are listed in Table 1. The cycling threshold value of the GAPDH was subtracted from the cycling threshold (ΔCT). The relative expression of each target gene is expressed as the fold change relative to that of WT

samples ($2^{-\Delta\Delta CT}$) as described [18]. The M1-like phenotype was induced by 100 ng/ml LPS (Sigma) for 6 h *in vitro*. The M2-like phenotype was induced by 10 ng/ml IL-4 (Peprotech) for 24 h *in vitro*.

Arginase activity assay

Arginase activity of primary macrophages was determined as previously described [19]. Briefly, 1×10^5 cells per sample were harvested and washed with PBS. The cells were lysed for 10 min in 100 μ l Tris-HCL (100 mM, pH 7.4) containing 1 μ M pepstatin A, 1 μ M leupeptin, and 0.4% Triton X-100. The lysate was centrifuged and supernatant was then added to a 96-well flat-bottomed plate with appropriate blank controls. Samples were mixed with 10 μ l of the substrate buffer and incubated at 37 °C for 2 h. Urea reagent was then added to the individual wells to stop the arginase reaction. The absorbance was measured at 430 nm and enzyme activity (in IU) was calculated according to the kit instructions after incubating at room temperature for another 60 min.

In vitro chemotaxis assay

Primary macrophages (1×10^5 cells/well in DMEM without FBS) were loaded into the upper portion of a 6.5-mm Transwell with 8.0- μ m Pore Polycarbonate Membrane Insert (Corning). DMEM containing 10% FBS with or without 200 ng/ml CCL2 was placed in the lower chamber. After 6 h, cells were fixed in 4% Formalin for 10 min. Cells were then stained with 1 g/L crystal violet for 30 min and were counted.

In vitro phagocytosis assay

The phagocytosis of macrophages was determined using a Vybrant™ phagocytosis assay kit (Life Technologies™) according to the manufacturer's instructions. Briefly, 100 μ l of the pre-

Table 1. Primers used for Real-time PCR analysis

Genes	Primer sequence(5'-3')
CCL2	Forward primer: GTCCCTGTCATGCTTCTGG Reverse Primer: GCTCTCCAGCCTACTCATTG
CCL3	Forward primer: GATTCCACGCCAATTTCATCG Reverse Primer: TTCAGTTCCAGGTCAGTGATG
CCL5	Forward primer: CAAGTGCTCCAATCTTGACAG Reverse Primer: GAAGCGTATACAGGGTCAGAATC
CCL4	Forward primer: TGACCAAAAGAGGCAGACAG Reverse primer: GTGAGAAGCATCAGGGCTG
CCL7	Forward primer: GAAAACCCCAACTCCAAAGC Reverse primer: CCTTAGGACCGTGATCAACAC
CCL8	Forward primer: ATGTACTAAAGCTGAAGATCCCC Reverse primer: GTACTACTGACCCACTTCTG
CCR2	Forward primer: GCTCTACATTCACTCCTTCCAC Reverse primer: ACCACTGTCTTTGAGGCTTG
GAPDH	Forward primer: CTTTGTCAGCTCATTTTCCTGG Reverse primer: TCTTGCTCAGTGTCTTGC
CD206	Forward primer: TTGGACGGATAGATGGAGGG Reverse primer: CCAGGCAGTTGAGGAGGTTTC
TNF- α	Forward primer: GAGTGACAAGCCTGTAGCC Reverse primer: CTCCTGGTATGAGATAGCAAA
Arginase	Forward primer: CCAGAA GAATGGAAGAGTCAGTGT Reverse primer: GCAGATATGCA GGGAGTCACC
Fizz1	Forward primer: CTGCCCTGCTGGGATGACT Reverse primer: CATCA ATCAAAGCTGGG TTCTCC
IL-6	Forward primer: CAAAGCCAGAGTCCTTCAGAG Reverse primer: GTCCTTAGCCACTCCTTCTG
IL-12p40	Forward primer: CACGGCAGCAGAATAAATA Reverse primer: CTTGAGGGAGAAGTAGGAATG
CD71	Forward primer: AGTGTCAGAAAACCAAGAGG Reverse primer: CGTTTCAGCCAGTTTCACAC
CD98	Forward primer: AGACCTTCTACAAAGTGCCAAG Reverse primer: TCCTGCAACCAAGACTCAG
CD80	Forward primer: GCTGATTGCTCTTTCACAAGTG Reverse primer: GCCAGTAGATTCCGTCTTCAG
CD86	Forward primer: CAGACTCCTGTAGACGTGTTC Reverse primer: GTCCCATTTGAAATAAGCTTGCG
IL-1 β	Forward primer: ACGGACCCCAAAAGATGAAG Reverse primer: TTCTCCACAGCCACATGAG
CD209a	Forward primer: GCAAGGAGTCTACATGGTACTG Reverse primer: TGTCAATTCAGCCGTCATC
CD209c	Forward primer: ATGGGAAGAAGACTGTGCTG Reverse primer: GGTCATTTTGCCAACAGGTG
CD209d	Forward primer: TCAAGAGTTTGGCAGAGTATACG Reverse primer: TTGTCTGAACTCTGAGCTG
CXCL9	Forward primer: AGTCCGCTGTTCTTTTCCTC Reverse primer: TGAGGTCTTTGAGGGATTGTAG
Hk2	Forward primer: TCAAAGAGAACAAGGGCGAG Reverse primer: AGGAAGCGGACATCACAATC
Ldha	Forward primer: GCTCCCCAGAACAAGATTACAG Reverse primer: TCGCCCTTGAGTTTGTCTTC
Tpil	Forward primer: AACTGGAAGATGAACGGGAG Reverse primer: GCAATTTTGGGATCCAGCTTC
Hif1a	Forward primer: GAACATCAAGTCAGCAACGTG Reverse primer: TTTGACGGATGAGGAATGGG
iNOS	Forward primer: CACCAAGCTGAACCTTGAGCG Reverse primer: CGTGGCTTTGGGCTCCTC

IL, interleukin; CCL, chemokine; CCR, chemokine receptor; TNF- α , tumor necrosis factor alpha; iNOS, inducible nitric oxide synthase

pared fluorescent Bioparticle suspension was added to the sorted primary macrophages (1×10^5) and the cell suspension was transferred to the 96-well flat-bottomed plate. After incubating (37 °C) for 2 h, the Bioparticle loading suspension was removed from the wells. The cells were washed twice with PBS and

then were transferred to the tubes. 10 μ l prepared Trypan Blue suspension was added into each tube and incubated for 10 min before being assayed using flow cytometry.

In vitro T cell activation and proliferation assay

To detect the effect of Tsc1 deficient macrophages on T cell proliferation, lymph node cells from WT mice were incubated at 10^7 /ml with 2 μ M carboxyfluorescein succinimidyl ester (CFSE, Invitrogen) for 10 min at room temperature. After being washed twice with PBS containing 10% FBS, 2.5×10^5 lymph node cells were cocultured with 5×10^4 sorted macrophages from WT or Tsc1-KO mice in DMEM containing 10% FBS in 96-well flat-bottomed plate. After 3 days, T cells proliferation were determined using flow cytometry. At the same time, T cell activation was determined by analyzing FSC-A and expression of CD44 and CD62.

Statistical analysis

Unpaired student t-test was used to statistically evaluate the difference of sample means among two groups. Differences were considered significant when $P < 0.05$. Data are presented as mean $\pm$ SEM.

Results

Tsc1 promotes macrophage survival

To address the role of Tsc1 in macrophage, we generated mice with Tsc1 deletion specifically in myeloid cells as previously reported [20]. After several rounds of crossing, we obtained Tsc1 KO mice. Effective deletion of Tsc1 by LysMCre-mediated recombination in macrophages was confirmed by western blot assays (Fig. 1a).

Peritoneal exudate cells were obtained from 5-8 weeks old mice. The percentage of CD11b⁺F4/80⁺ macrophages in the total peritoneal exudate cells was significantly reduced in Tsc1 KO mice compared with WT mice (Fig. 1b). Tsc1 KO mice showed decreased absolute cell numbers of peritoneal macrophages compared with WT mice (Fig. 1c). To determine the effect of Tsc1 on macrophage survival, we tested the apoptosis of peritoneal macrophages

Fig. 1. Tsc1 promotes macrophage survival. (a) Tsc1 protein levels in WT and Tsc1-deficient peritoneal macrophages were determined by western blot. (b) The percentages of macrophages in peritoneal exudate cells from WT and Tsc1 KO mice assayed by flow cytometry. (c) The absolute number of macrophages in peritoneal exudates from WT and Tsc1 KO mice (6 mice per group). (d) The apoptosis of peritoneal macrophages in WT, Tsc1 KO and Rapa pretreated Tsc1 KO mice. (e) The absolute number of CD11b⁺F4/80⁺Ly6C⁺ macrophages in the spleens and LNs of WT and Tsc1 KO mice (6 mice per group). Data are shown as mean $\pm$ SEM. * $P < 0.05$ compared with WT control mice (Student's t-tests). Data are representative of three to five independent experiments.

Fig. 2. Tsc1 restrains macrophage growth. (a) The cell size of WT and Tsc1-deficient peritoneal macrophages determined by flow cytometry. (b) The expression levels of CD98 and CD71 on WT and Tsc1-deficient macrophages. (c) The cell size of peritoneal macrophages isolated from Rapa pretreated or untreated WT and Tsc1 KO mice. (d) mRNA expression levels of CD71 and CD98 of macrophages isolated from WT, Tsc1 KO and Rapa pretreated Tsc1 KO mice. (e) mRNA expression levels of glycolysis-related genes of WT and Tsc1-deficient macrophages. Data are shown as mean $\pm$ SEM. * P < 0.05 compared with WT mice (Student's *t*-tests). Data are representative of two or three independent experiments.

isolated from WT and Tsc1 KO mice. Tsc1-deficient macrophages showed significantly increased apoptosis as indicated by the loss of the live Annexin V-PI population (Fig. 1d). These data indicated an important role of Tsc1 in promoting survival of macrophages.

To further determine whether the increased apoptosis of Tsc1-deficient macrophages was dependent on mTORC1, the mice were treated with Rapa *in vivo* for 3 days. The macrophages isolated from Tsc1 KO mice pretreated with Rapa rescued the increased apoptosis compared with the macrophages isolated from untreated Tsc1 KO mice (Fig. 1d). These results indicated a direct role of Tsc1 in promoting macrophage survival via mTORC1-dependent pathways.

Tsc1 KO mice showed less absolute numbers of macrophages in the spleens compared with WT mice (Fig. 1e). However, the absolute numbers of macrophages in LNs did not show difference in Tsc1 KO mice compared with those in WT mice (Fig. 1e).

Tsc1 restrains macrophage growth

We next noticed that macrophages developed in the absence of Tsc1 exhibited increased cell size which indicated that Tsc1-deficient macrophages underwent more cell growth (Fig. 2a). Cell growth is dependent on the regulated expression of amino acid transporters such as CD98 and transferrin receptor such as CD71 [4]. Flow cytometry analysis revealed elevated levels of CD98 and CD71 on macrophages upon loss of Tsc1 (Fig. 2b). Consistently, Tsc1-deficient macrophages expressed higher mRNA expression levels of CD98 and CD71 compared with WT macrophages (Fig. 2d).

The mice were treated with Rapa to further determine the increased cell size of Tsc1-deficient macrophages was dependent on mTORC1 or not. Pretreatment with Rapa partially reversed the elevated size of the macrophages isolated from Tsc1 KO mice (Fig. 2c). The mRNA expression of CD71 and CD98 was also decreased in the Rapa pretreated Tsc1-deficient macrophages (Fig. 2d). These results indicated Tsc1 played a role in restraining macrophage growth via inhibiting activation of mTORC1 which may be ascribed to the increased expression of CD98 and CD71.

Fig. 3. Tsc1-deficient macrophages displayed an altered M1 and M2-associated markers in steady-state and inflammatory conditions. (a) The arginase activity of WT and Tsc1-deficient peritoneal macrophages. (b) The mRNA expression levels of CD209 and CD206 on WT and Tsc1-deficient macrophages. (c) mRNA expression levels of M2 and M1-associated genes of macrophages isolated from Rapa treated or untreated WT and Tsc1 KO mice. (d) mRNA expression levels of M2 and M1-associated genes after treatment with 10 ng/ml IL-4 for 24 h or 100 ng/ml LPS for 6 h *in vitro*. Data are shown as mean $\pm$ SEM. * P < 0.05 compared with WT control mice (Student's t-tests). Data are representative of two or three independent experiments.

Glycolysis plays an important role in cell metabolism, but the mRNA expression levels of transcription factors including Hk2, Ldha, Tpi1 and Hif1a that regulate glucose metabolism did not show significant differences between Tsc1-deficient and WT macrophages (Fig. 2e).

Tsc1 suppresses both M1 and M2 phenotypes of macrophage in steady-state and inflammatory conditions

A hallmark of M2 macrophages is an increase in Arginase-1 gene expression and activity to convert L-arginine to L-ornithine to promote polyamine synthesis and tissue repair [21]. Tsc1-deficient macrophages showed higher arginase activity than WT macrophages under steady-state condition (Fig. 3a). M2 macrophages are characterized by the upregulation of CD209 and CD206 [22]. Expression of CD209 and CD206 was elevated on Tsc1-deficient macrophages (Fig. 3b). Furthermore, the expression of CD206 on macrophages isolated from Rapa pretreated Tsc1 KO mice was significantly decreased compared with that on macrophages isolated from untreated Tsc1 KO mice (Fig. 3b).

Tsc1-deficient macrophages expressed significantly higher mRNA levels of M2-associated genes including CD206, Arg1, Fizz1, CD209a, CD209d, CD209c than WT macrophages (Fig. 3c). Tsc1-deficient macrophages expressed reduced levels of IL-6 and IL-12p40, elevated level of IL-1 β , similar level of TNF- α compared with WT macrophages

Fig. 4. Tsc1 promotes migration and expression of chemokine receptors of macrophage. (a) The migration of WT and Tsc1-deficient peritoneal macrophages induced by DMEM containing 10% FBS or DMEM containing 10% FBS and 200 ng/ml CCL2. (b) mRNA expression levels of CCR2 and CCR5 on macrophages

isolated from Rapa pretreated or untreated WT and Tsc1 KO mice. (c) mRNA expression levels of chemokines of macrophages isolated from Rapa pretreated or untreated WT and Tsc1 KO mice. Data are shown as mean $\pm$ SEM. * P < 0.05 compared with WT control mice (Student's t -tests). Data are representative of two or three independent experiments.

Fig. 5. Tsc1 regulates phagocytosis and ROS production in macrophage. (a) The phagocytosis of WT and Tsc1-deficient peritoneal macrophages. (b) The ROS production in WT and Tsc1-deficient macrophages. Data are representative of three independent experiments.

(Fig. 3c). We also found that Tsc1-deficient macrophages expressed increased CXCL9 (Fig. 3c), a inflammatory chemokine typically expressed in M1 macrophages [23].

To determine the aberrant expression of M1 or M2-associated mRNA levels in Tsc1-deficient macrophages were dependent on mTORC1 or not, the mice were treated with Rapa. The mRNA expression levels of CD206, arginase, Fizz1, CD209a, CD209d, CD209c were lower in macrophages isolated from Rapa pretreated WT and Tsc1 KO mice compared with those in macrophages isolated from untreated WT and Tsc1 KO mice (Fig. 3c). Tsc1-deficient macrophages expressed increased levels of IL-6, TNF- α , IL-1 β , CXCL9, decreased levels of IL-12p40 and IL-1 α after treatment with Rapa (Fig. 3c). Our results indicated that Tsc1 in macrophage suppressed the expression of M1 and M2-associated mRNA under steady-state condition via mTORC1-dependent manners.

Fig. 6. Tsc1-deficient macrophages impaired function of T cell activation. (a) The expression levels of CD80, CD86, MHC-II on WT and Tsc1-deficient peritoneal macrophages. (b) The activation of CD4⁺ T cells cocultured with WT or Tsc1-deficient macrophages. Data are representative of two or three independent experiments.

We further determined the polarization of WT macrophages and Tsc1-deficient macrophages after treatment with LPS or IL-4. The mRNA expression levels of M2-associated genes including CD206, CD209a, Fizz1 were much higher in Tsc1-deficient macrophages than those in WT macrophages after treatment with IL-4 (Fig. 3d). Furthermore, Tsc1-deficient macrophages expressed higher mRNA levels of TNF- α and iNOS than WT macrophages to LPS stimulation (Fig. 3d). However, Tsc1-deficient macrophages expressed lower level of IL-6 than WT macrophages after treatment with LPS (Fig. 3d). These results revealed that the M2 polarization of Tsc1-deficient macrophages was increased after treatment with IL-4 and the M1 polarization of Tsc1-deficient macrophages was also increased after treatment with LPS (Fig. 3d).

Tsc1 promotes expression of chemokine receptors and migration of macrophage

Migration of Tsc1-deficient macrophages induced by CCL2 was impaired compared with WT macrophages (Fig. 4a). Tsc1-deficient macrophages expressed decreased expression of CCR2 and CCR5 (Fig. 4b). Tsc1-deficient macrophages expressed higher mRNA levels of chemokines including CCL2, CCL3, CCL4, CCL5, CCL7, CCL8 compared with WT macrophages (Fig. 4c).

To further determine whether the changes of chemokine receptors and chemokines were dependent on mTORC1 or not, the mice were treated with Rapa for 3 days. The mRNA expression levels of CCR2 and CCR5 in macrophages isolated from Rapa pretreated WT and Tsc1 KO mice were increased compared with those in macrophages isolated from untreated WT and Tsc1 KO mice (Fig. 4b). The mRNA expression levels of CCL3, CCL4, CCL7 and CCL8 were all reduced after Rapa treatment in both WT and Tsc1-deficient macrophages (Fig. 4c). The mRNA expression levels of CCL2 and CCL5 were increased in both WT and KO macrophages after Rapa treatment (Fig. 4c). These data indicated that Tsc1 regulates the expression of CCR2, CCR5, CCL3, CCL4, CCL7, CCL8 via mTORC1-dependent manner, and regulates the expression of CCL2 and CCL5 via mTORC1-independent manner.

Tsc1 regulates phagocytosis and ROS production in macrophage

Tsc1-deficient macrophages showed elevated phagocytosis determined using flow cytometry compared with that of WT macrophages (Fig. 5a). Furthermore, Tsc1-deficient macrophages produced much higher level of ROS than WT macrophages did (Fig. 5b).

Fig. 7. Tsc1 KO mice develop lymphoproliferative disorder spontaneously. (a) The body weights of WT and Tsc1 KO mice. (b) The spleens and LNs of WT and Tsc1 KO mice. (c) CD4⁺CD25⁺Fxp3⁺ regulatory T cells in the spleens and LNs of WT and Tsc1 KO mice. (d) The memory/activated CD4⁺CD62⁺CD44^{high} T cells, CD8⁺CD62⁺CD44^{high} activated T cells and CD8⁺CD62⁺CD44^{high} memory T cells in the spleens and LNs of WT and Tsc1 KO mice. Data are shown as mean±SEM. **P*<0.05 compared with WT control mice (Student's *t*-tests). Data are representative of three to five independent experiments.

Tsc1 promotes T cell activation

We examined the expression of various surface markers associated with macrophage maturation including CD80, CD86 and MHC-II. Tsc1-deficient macrophages showed higher levels of CD80, CD86 and MHC-II than WT macrophages (Fig. 6a). This results revealed that loss of Tsc1 resulted in macrophage spontaneous activation and maturation.

The sorted Tsc1-deficient and WT macrophages were cocultured with lymph node cells respectively for 3 days and it was indicated that CD4⁺ T cells cocultured with Tsc1-deficient macrophages showed impaired activation and proliferation, judged from the reduced expression of CD44, impaired CFSE dilution and decreased FSC-A (Fig. 6b). These data indicated that Tsc1-deficient macrophages though showed elevated maturation than WT macrophages, it inhibited CD4⁺ T cell activation *in vitro*.

Myeloid expression of Tsc1 is required for preventing lymphoproliferative disorder

The body weights of 6-week-old male and female mice between WT and Tsc1 KO mice showed no significant difference (Fig. 7a). The spleen, pLNs, mLNs were larger in Tsc1 KO mice than those in WT mice (Fig. 7b). The percentage of CD4⁺CD25⁺Fxp3⁺ regulatory T cells in the spleen of Tsc1 KO mice was higher than that in WT mice. The percentage of CD4⁺CD25⁺Fxp3⁺ regulatory T cells was of no significant difference in the LNs of Tsc1 KO mice and WT mice (Fig. 7c). Memory/activated CD4⁺CD62⁺CD44^{high} T cells, CD8⁺CD62⁺CD44^{high}

activated T cells and CD8⁺CD62⁺CD44^{high} memory T cells were all elevated in the spleen and LNs of Tsc1 KO mice than those in WT mice (Fig. 7d). Thus Tsc1 KO mice spontaneously develop lymphoproliferative disorder.

Discussion

In this report, a model of myeloid cell-specific Tsc1 conditional knockout mice was generated to elucidate the function of Tsc1 in regulating the survival, function and stability of macrophage. We found that the percentage of peritoneal macrophages in Tsc1 KO mice was significantly decreased compared with that in WT mice. Moreover, Tsc1 inhibited macrophages apoptosis and growth via suppressing mTORC1. Upregulated expression of CD98 and CD71 may get involved in the enhanced growth of Tsc1-deficient macrophages. These results were consistent with the effects of Tsc1 on dendritic cell (DC) which were reported by Wang Y, etc [4]. They found that Tsc1 promotes DC survival, coordinates DC growth and Tsc1-deficient DCs showed elevated expression of CD98 and CD71. WT and Tsc1-deficient macrophages expressed comparable levels of glycolysis-related genes. However, DCs developed from Tsc1-deficient bone marrow cells revealed elevated mRNA expression of glycolytic enzymes, including HK2, Ldha, Tpi1 and Hif1a [4].

Macrophages can be classified into M1 and M2. The polarization of M1 and M2 have been indicated in several disease conditions as sepsis, tumors and obesity [24-26]. It was reported that in response to IL-4 stimulation, Tsc1-deficient macrophages express much lower levels of M2-associated mRNA including Arg1, Fizz1 and YM1 than WT macrophages. The defective response to IL-4 of Tsc1-deficient macrophages is mediated by mTOR [17]. Intriguingly, our data revealed that Tsc1-deficient macrophages showed significantly enhanced M2 phenotype in steady-state condition and after treatment with IL-4 as compared with WT macrophages, pointing out a role of Tsc1 in suppressing M2 polarization. We also showed that Tsc1 inhibits M2 polarization on mTOR-dependent pathway. It was reported that mice with myeloid-specific deletion of Tsc1 exhibits enhanced M1 response and develop M1-related inflammatory disorders spontaneously. Tsc1-deficient macrophages express higher levels of TNF- α , IL-12p40 and iNOS to LPS or LPS plus IFN- γ stimulation. The hypersensitivity of Tsc1-deficient macrophages to LPS stimulation was mTOR-independent [17]. Our results showed that Tsc1-deficient macrophages expressed elevated mRNA levels of TNF- α , iNOS and decreased IL-6 to LPS stimulation. We concluded that Tsc1 inhibits M1 polarization mainly on mTOR-independent manners, and mTOR may also play a role in Tsc1 regulating M1 polarization of macrophage.

The chemokine receptors such as CCR2 and CCR5 are important in the trafficking of monocytes/macrophages [27, 28]. CCR2 and CCR5 effect function of monocytes/macrophages which are involved in the pathogenesis of animal models of transplant rejection, atherosclerosis, Alzheimer's disease and rheumatoid arthritis. In our study, Tsc1-deficient macrophages displayed decreased expression of CCR2 and CCR5, which may lead to the impaired migration of Tsc1-deficient macrophages induced by CCL2. Chemokines play important roles in the chemoattraction of cells of the immune system, altered expression of chemokines can be served as useful diagnostic or prognostic biomarkers during specific disease states [29]. The results revealed that Tsc1-deficient macrophages showed increased expression of chemokines including CCL2, CCL3, CCL4, CCL5, CCL7, CCL8 on both mTORC1-dependent and independent pathways. These data were consistent with a previous study showing that disruption of mTORC1 in macrophages decreased the expression of CCL2, CCL6, CCL3 [30]. It was also reported that G-protein-coupled bile acid receptor 1 (TGR5) reduces macrophage migration and chemokines expression via mTORC1-dependent pathway [31].

Macrophage is an important component of innate cellular immunity and is involved in host defense and immunity against foreign microorganisms including bacteria, viruses, fungi and parasites [32-34]. To further determine the phagocytosis of Tsc1-deficient macrophages, we examined their ability to phagocytize bacteria. It turned out that Tsc1-

deficient macrophages showed elevated phagocytosis. M1 macrophages produce ROS with which they can kill bacteria. Tsc1-deficient macrophages produced elevated level of ROS. These results suggested that Tsc1 is a key regulator of macrophage.

Though Tsc1-deficient macrophages showed elevated maturation than WT macrophages, it exhibited strong inhibitory effects on CD4⁺ T cells activation. This may be due to the increased apoptosis and elevated M2 polarization of Tsc1-deficient macrophages.

Though 5-12 weeks old Tsc1 KO mice showed no survival deficiencies and no loss of weight compared with WT mice, the spleen, pLNs and mLNs were larger in Tsc1 KO mice than in WT mice. More activating CD4⁺ and CD8⁺ T effector cells were detected in the spleens of Tsc1 KO mice than in the spleens of WT mice. Therefore, we infer that Tsc1 of macrophage is a key player in regulating homeostasis of organism.

In summary, Tsc1 plays an essential role in controlling macrophage survival, growth, polarization and phagocytosis, majorly via mTORC1-dependent pathways under steady-state condition. Although loss of Tsc1 in myeloid cells does not cause fatal autoimmune diseases in our study, Tsc1 is essential for maintaining stability of macrophage to protect spontaneous lymphoproliferative disorder in physiological conditions. The precise mechanism of Tsc1 in regulating the macrophage survival, function and stability need to be further researched and Tsc1 may be served as a target in the treatment of some immune diseases in the future.

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Disclosure Statement

The authors declare that they have no competing financial interests.

References

- 1 Orlova KA, Crino PB: The tuberous sclerosis complex. *Ann N Y Acad Sci* 2012;1184:87-105.
- 2 Harkewicz R, Hartvigsen K, Almazan F: Cholesteryl ester hydroperoxides are biologically active components of minimally oxidized low density lipoprotein. *J Biol Chem* 2008;283:10241-10251.
- 3 Weichhart T, Seemann MD: The multiple facts of mTOR in immunity. *Trends Immunol* 2009;30:218-226.
- 4 Wang Y, Huang G, Zeng H, Yang K, Lamb RF, Chi H: Tuberous Sclerosis 1(Tsc1)-dependent metabolic checkpoint controls development of dendritic cells. *Proc Natl Acad Sci USA* 2013;110:E4894-E4903.
- 5 Xie DL, Wu J, Lou YL, Zhong XP: Tumor suppressor Tsc1 is critical for T-cell anergy. *Proc Natl Acad Sci USA* 2012;109:14152-14157.
- 6 Gallis SJ, Borregaard N, Wynn TA: Phenotypic and functional plasticity of cells of innate immunity: macrophages, mast cells and neutrophils. *Nat Immunol* 2011;12:1035-1044.
- 7 Murray PJ, Wynn TA: Protective and pathogenic functions of macrophage subset. *Nat Rev Immunol* 2011;11:723-737.
- 8 Mosser DM, Edwards JP: Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* 2008;8:958-969.
- 9 Sica A, Mantovani A: Macrophage plasticity and polarization: in vivo veritas. *J Clin Invest* 2012;122:787-795.
- 10 Mills CD, Ley K: M1 and M2 macrophages: the chicken and the egg of immunity. *J Innate Immun* 2014;6:716-726.

- 11 Gordon S, Martinez FO: Alternative activation of macrophages: mechanism and functions. *Immunity* 2010;32:593-604.
- 12 Kurihara T, Warr G, Loy J, Bravo R: Defects in macrophage recruitment and host defense in mice lacking the CCR2 chemokine receptor. *J Exp Med* 1997;186:1757-1762.
- 13 Boring L, Gosling J, Chensue SW: Impaired monocyte migration and reduced type 1 (TH1) cytokine responses in C-C chemokine receptor 2 knockout mice. *J Clin Invest* 1997;100:2552-2561.
- 14 Lin H, Chang KT, Hung CC, Kuo CH, Hwang SJ, Chen HC, Hung CH, Lin SF: Effects of the mTOR inhibitor Rapamycin on monocyte-secreted chemokines. *BMC Immunol* 2014;15:37.
- 15 Babior BM, Kipnes RS, Curnutte JT: The Productions by leukocytes of superoxide: a potential bactericidal agent. *J Clin Invest* 1973;52:741-744.
- 16 Byles V, Covarrubias AJ, Ben-Sahra I, Lamming DW, Sabatini DM, Manning BD, Horng T: The TSC-mTOR pathway regulates macrophages polarization. *Nat Commun* 2013;4:2834.
- 17 Zhu L, Yang T, Li L, Hou Y, Hu X, Zhang L, Tian H, Zhao Q, Peng J, Zhang H, Wang R, Yang Z, Zhang L, Zhao Y: Tsc1 controls macrophages polarization to prevent inflammatory disease. *Nat Commun* 2014;5:4694.
- 18 Wang Y, Huang G, Vogel P, Neale G, Reizis B, Chi H: Transforming growthfactor beta-activated kinase (TAK1) dependent checkpoint in the survival of dendritic cells promotes immune homeostasis and function. *Proc Natl Acad Sci USA* 2012;109:E343-352.
- 19 Xiaoyu Hu, Yushan Zhou, Kui Dong: Programming of the Development of Tumor-Promoting Neutrophils by Mesenchymal Stromal Cells. *Cell Physiol Biochem* 2014;33:1802-1814.
- 20 El Kasmi KC, Qualls JE, Pesce JT: Toll-like receptor-induced arginase 1 in macrophages thwarts effective immunity against intracellular pathogens. *Nat Immunol* 2008;9:1399-1406.
- 21 Van den Bossche J, Lemers WH, Koehler ES: Pivotal Advance: Arginase-1-independent polyamine production stimulates the expression of IL-4-induced alternatively activated macrophage markers while inhibiting LPS-induced expression of inflammatory genes. *J Leukoc Biol* 2012; 91:685-699.
- 22 Martinez FO, Helming L, Gordon S: Alternative activation of macrophages :an immunologic functional perspective. *Annu Rev Immunol* 2009;27:451-483.
- 23 Martinez FO, Gordon S, Locati M, Mantovani A: Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. *J Immunol* 2006;177:7303-7311.
- 24 Duvel K, Yecies JL, Menon S, Raman P, Lipovsky AI: Activation of a metabolic gene regulatory network downstream of mTOR complex 1. *Mol Cell* 2010;39:171-183.
- 25 Lumeng CN, Bodzin JL, Saltiel AR: Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J Clin Invest* 2007;117:175-184.
- 26 Biswas SK, Sica A, Lewis CE: Plasticity of macrophages function during tumor progression: regulation by distinct molecular mechanisms. *J Immunol* 2008;180:2011-2017.
- 27 Charo IF, Peters W: Chemokine receptor 2 (CCR2) in atherosclerosis, infectious diseases, and regulation of T-cell polarization. *Microcirculation* 2003;10:259-264.
- 28 Ness TL, Kunkel SL, Hogaboam GM: CCR5 antagonists: the answer to inflammatory disease? *Expert Opin Ther Pat* 2006;16:1051-1065.
- 29 Bajetto A, Bonavia R, Barber S, Florio T: Chemokines and their receptors in the central nervous system. *Front Neuroendocrinol* 2001;22:147-184.
- 30 Ai D, Jiang H, Westerterp M: Disruption of Mammalian target of Rapamycin complex 1 in macrophages decreased chemokine gene expression and atherosclerosis. *Circ Res* 2014;114:1576-1584.
- 31 Perino A, Pols TW, Nomura M: TGR5 reduces macrophage migration through mTOR-induced C/EBPβ differential translation. *J Clin Invest* 2014;doi:10.1172/JCI76289.
- 32 Wynn TA, Chawla A, Pollard JW: Macrophage biology in development, homeostasis and disease. *Nature* 2013;496:445- 455.
- 33 Murray PJ, Wynn TA: Protective and pathogenic functions of macrophage subsets. *Nat Rev Immunol* 2011;11:723-737.
- 34 Gordon S: The macrophage: past, present and future. *Eur J Immunol* 2007;37:9-17.