Tsc1 expression by dendritic cells is required to preserve T-cell homeostasis and response

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Dendritic cells (DCs) are pivotal to the induction of adaptive T-cell immune responses. Recent evidence highlights a critical role of tuberous sclerosis complex 1 (Tsc1), a primarily upstream negative regulator of mammalian target of rapamycin (mTOR), in DC development, but whether and how Tsc1 directly regulate mature DC function in vivo remains elusive. Here we show that selective disruption of Tsc1 in DCs results in a lymphoproliferative disorder with the spontaneous activation of T cells. Tsc1 deficiency results in the activation of mTORC1-PPARγ pathway, which leads to the upregulation of neuropilin-1 (Nrp1) expression on DCs to stimulate naive T-cell proliferation. However, Tsc1-deficient DCs have defects in the ability to induce antigen-specific T-cell responses in vitro and in vivo owing to impaired survival during antigen transportation and presentation. Indeed, Tsc1 promotes DC survival through restraining independent mTORC1 and ROS-Bim pathways. Our study identifies Tsc1 as a crucial signaling checkpoint in DCs essential for preserving T-cell homeostasis and response.

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Dendritic cells (DCs) are specialized sentinels that induce adaptive immune responses according to environmental stimuli. Under steady-state conditions, DCs contribute to immunological tolerance against self-antigens. During overt immunization or infection, foreign antigens activate DCs to upregulate the expression of major histocompatibility complex (MHC) molecules, co-stimulatory molecules and cytokines to trigger adaptive T-cell responses. How DCs shape an efficient immune response to peripheral cues while avoiding immune activation under steady-state conditions remains incompletely understood.

Mammalian target of rapamycin (mTOR) is a central integrator of immune responses, and its activity is repressed by the upstream tuberous sclerosis complex 1 (Tsc1)–Tsc2 complex. Several studies indicated that mTOR signaling was a particularly critical regulator of DC differentiation, maturation and function. Three recent studies investigated the roles of Tsc1 in DC development and activation. Pan et al. reported that Tsc1 increased IRF4/CIITA/MHC II expression in bone marrow (BM)-derived DCs to enhance OT-II T-cell priming without affecting DC development in response to GM-CSF. But this study was limited by its basis in the in vitro-cultured BM-derived DC system, which may be different from DCs in vivo. Wang et al. demonstrated that Tsc1-deficient DC precursors were inclined to differentiate into macrophages and neutrophils, exhibited impaired survival and IL-12 production and therefore arrested DC-mediated Th1 immune responses, although it had increased expression of CD40, CD80 and CD86. This study suggested that Tsc1 ablation impaired DC development and consequently attenuated DC T-cell response. Further, Sathaliyawala et al. identified that Tsc1 loss did not affect DC terminal differentiation or maintenance. These results collectively support an important but complex role of Tsc1 in DC development. However, the precise role and underlying molecular mechanism of Tsc1 in mature DC function in vivo are still not well defined.

Here we investigate the direct role of Tsc1 in mature DC function and the potential molecular basis using a mouse line with Tsc1 specifically deleted in CD11c+ DCs (CD11cCreTsc1f/f mice). Distinct from the results observed in BM-derived DCs, we did not detect significantly altered expression of MHC II, co-stimulatory molecules CD40, CD80 and CD86, cytokine IL-12 or abnormal differentiation in DCs from secondary lymphoid organs in CD11cCreTsc1f/f mice. Instead, we found that specific depletion of Tsc1 in DCs resulted in the development of splenomegaly and lymphadenopathy, increased serum immunoglobulin levels and body weight loss in mice, which were characteristics of spontaneous autoimmunity. The mTORC1–PPARγ axis-dependent upregulation of neuropilin 1 (Nrp1) in Tsc1-deficient DCs drove naive T-cell proliferation. In contrast, Tsc1-deficient DCs showed a defective ability to induce antigen-specific responses in vitro and in vivo as a result of severely reduced number of DCs and hesitated to drive Th2 and Th17 immune response in asthma model. Mechanistically, mTORC1- and ROS-Bim-induced excessive apoptosis of Tsc1-deficient DCs during antigen presentation is responsible for the observed phenotypes.
transportation and presentation then prevented the efficient priming of antigen-specific T-cell responses. Thus our data define Tsc1 as a critical regulator in mature DCs to ensure T-cell homeostasis and immune response.

Results

Tsc1 in DCs prevents the development of lymphoproliferative disorder. To determine whether Tsc1 in DCs regulates T-cell homeostasis and response in vivo, we generated a mouse line with Tsc1 conditionally depleted in DCs by crossing Tsc1<sup>fl/+</sup> mice (mice with loxP-flanked alleles of the gene encoding mouse Tsc1<sup>15</sup>) with CD11c<sup>Cre</sup> mice (mice with transgenic expression of Cre recombinase under the control of the CD11c promoter<sup>16</sup>). Tsc1 protein was ablated in splenic DCs from CD11c<sup>Cre</sup>Tsc1<sup>fl/+</sup> mice (Figure 1a). Despite being outwardly healthy, CD11c<sup>Cre</sup>Tsc1<sup>fl/+</sup> mice developed a lymphoproliferative disorder characterized by considerable splenomegaly and lymphadenopathy with increased total cell numbers with age (Figure 1b). Further analysis revealed that a population expansion of B cells and myeloid cells accounted for the increased cellularity of secondary lymphoid organs in elder CD11c<sup>Cre</sup>Tsc1<sup>fl/+</sup> mice (Figure 1c). We examined the body weight and found that the elder CD11c<sup>Cre</sup>Tsc1<sup>fl/+</sup> mice displayed lower body weight than Tsc1<sup>fl/+</sup> mice (wild-type (WT) mice) (Figure 1d). Furthermore, serum levels of IgG1 and IgE in CD11c<sup>Cre</sup>Tsc1<sup>fl/+</sup> mice were higher than in WT mice (Figure 1e), indicating a role of Tsc1 in autoimmune disorders. However, we did not observe alterations in histopathology (data not shown) and specific markers of autoimmune disease in CD11c<sup>Cre</sup>Tsc1<sup>fl/+</sup> mice (Supplementary Figure S1). These findings indicate a role of Tsc1 in DCs for preserving immune homeostasis in steady-state conditions.

Tsc1 in DCs prevents spontaneous T-cell activation in vivo. The major function of DCs is controlling the homeostasis and function of T cells. We found a significantly higher frequency and number of CD4<sup>+</sup> and CD8<sup>+</sup> T cells that displayed the CD44<sup>high</sup> CD62L<sup>low</sup> activated/memory phenotype in CD11c<sup>Cre</sup>Tsc1<sup>fl/+</sup> mice (Figure 2a). In contrast, the naive CD4<sup>+</sup> and CD8<sup>+</sup> T cells were progressively lost with age (Figure 2b). T cells from CD11c<sup>Cre</sup>Tsc1<sup>fl/+</sup> mice also exhibited increased proliferation and higher production of the cytokines IFN-γ, IL-4, IL-5 and IL-17 (Figures 2c and d). These observations indicate that Tsc1-deficient DCs delivers strong signals to induce aberrant activation and proliferation of naive T cells without foreign antigen challenge.

Tsc1 represses Nrp1 in DCs to prevent naive T-cell proliferation. To investigate whether Tsc1-deficient DCs directly induce T-cell activation, we used the in vitro DC-naive T-cell co-culture system and found that Tsc1-deficient DCs induced more proliferation of naive T cells, in the absence of foreign antigen (Figure 3a).

DC activation and functional maturation have always been associated with increased expression of MHC class II, co-stimulatory molecules and cytokines. We found that Tsc1-deficient DCs were bigger in cell size and had slightly increased expression of CD80 but not of MHC or other common co-stimulatory molecules (Supplementary Figure S2a). Cytokine production was comparable between WT and Tsc1-deficient DCs (Supplementary Figure S2b). These results suggest that loss of Tsc1 does not lead to overt activation of DCs. As the number of DCs was decreased in
secondary lymphoid organs, elevated naive T-cell proliferation could not be attributed to decreased numbers of DCs (Supplementary Figure S2c).

Nrp1 is a membrane molecule that is known to be essential for driving naive T-cell proliferation by DCs. We hypothesized that the increased proliferation of naive T cells is partly dependent upon Nrp1. Indeed, although barely expressed in WT DCs, a large amount of Nrp1 was expressed in Tsc1-deficient DCs (Figure 3b). We further explored whether the increased Nrp1 expression contributed to the naive T-cell proliferation. Blocking Nrp1, with a neutralizing antibody, partly dampened the enhanced priming of naive CD4+ T cells by Tsc1-deficient DCs (Figure 3c). Thus the aberrant upregulation of Nrp1 in Tsc1-deficient DCs accounts, at least partially, for the T-cell activation/proliferative phenotype in CD11cCre Tsc1f/f mice.

Tsc1 represses Nrp1 expression through inhibiting mTORC1-PPAR-γ pathway. We next explored the signaling pathway alterations in Tsc1-deficient DCs. Owing to the rarity of the DCs in vivo, we used phospho-flow cytometry to examine the activation of the major signaling pathways, including AKT, Erk, JNK, p38MAPK, NF-κB, S6K and PPAR-γ, in Tsc1-deficient DCs. We found that the mTORC2 substrate Akt (Ser473), p38MAPK and NF-κB activation were barely altered, and Erk and JNK activation were slightly increased, whereas S6K and PPAR-γ activation were significantly increased in Tsc1-deficient DCs compared with that in WT cells (Figures 4a and b). Although Myc has been reported to be elevated in Tsc1-deficient BM-derived DCs, we did not find altered expression of Myc in splenic Tsc1-deficient DCs (data not shown). We utilized a panel of chemical inhibitors to determine which signaling alteration caused the upregulation of Nrp1 in Tsc1-deficient DCs. Both in vivo administration of the mTORC1 inhibitor rapamycin (RAPA) and in vitro treatment with the mTORC1 inhibitor everolimus downregulated the expression of Nrp1 in DCs (Figure 4c). These results suggest that mTORC1-activated transcription factors might regulate Nrp1 gene expression. The known mTORC1-regulated transcription factors include PPAR-γ, STAT3, and HIF. Indeed, inhibition of PPAR-γ, but not STAT3 and HIF, reduced the expression of Nrp1 in Tsc1-deficient DCs.

In addition to mTORC1, Tsc1 might also act through other signaling pathways. To precisely determine the effects of mTORC1 hyperactivation on the biological function of Tsc1-deficient DCs, we generated CD11cCre Tsc1f/f Raptorf/+ mice to deplete Tsc1 and one allele of Raptor, which is an obligatory component of mTORC1, in which the hyperactivation of mTORC1 was diminished (Figure 4d). The increased expression of Nrp1 in DCs and the T-cell activation phenotype were largely rescued in CD11cCre Tsc1f/+ Raptorf/+ mice (Figures 4e and f). Taken together, Tsc1 represses Nrp1 expression in DCs through suppressing the mTORC1-PPAR-γ signaling pathway to prevent spontaneous T-cell activation in steady-state conditions.
DCs need Tsc1 to promote antigen-specific T-cell responses. To test whether Tsc1 in DCs is important for foreign antigen-driven T-cell immune responses, we first co-cultured WT or Tsc1-deficient DCs with CD4+ T cells from OT-II mice or CD8+ T cells from OT-I mice. Surprisingly, splenic Tsc1-deficient DCs showed a considerable defect in the ability to drive antigen-specific T-cell proliferation in vitro (Figure 5a). In vivo, Tsc1-deficient DCs also had an apparent impairment in inducing proliferation of OT-II and OT-I T cells, which were adoptively transferred into CD11cCreTsc1f/f mice immunized with ovalbumin (OVA) (Figure 5b).

We further tested the ability of DCs to mount antigen-specific immune responses to OVA in an asthma model. Here we showed that CD11cCreTsc1f/f mice had a profound impairment in adaptive T-cell immunity in this model (Figure 5c). Systemic antibody production and Th2 and Th17 responses were significantly reduced in CD11cCreTsc1f/f mice (Figures 5d and e). Together, these findings suggest that CD11cCreTsc1f/f mice have a profound defect in eliciting antigen-specific T-cell responses both in vitro and in vivo.

Tsc1 maintains sufficient numbers of antigen-pulsed DCs in foreign antigen-specific T-cell responses. A previous study demonstrated Tsc1-deficient BM-derived DCs have an impaired capacity to induce an antigen-specific T-cell response owing to diminished IRF4/CIITA/MHCII expression.14 Another work indicated that induced deletion of Tsc1 in the early stage of hematopoietic cells caused abnormal development, which resulted in an impaired Th1 response in the mice.15 However, in our CD11cCreTsc1f/f mice system, we did not observe altered expression of MHC in Tsc1-deficient DCs (Supplementary Figure S1a) or abnormal accumulation of F4/80 macrophage or Ly6G neutrophil differentiation in vivo (data not shown). In addition, Tsc1-deficient DCs did not display reduced expression of co-stimulatory molecules (Supplementary Figure S1a) or decreased production of Th helper cell cytokines (Supplementary Figure S1b). To examine the ability of DC antigen presenting, a 33 amino-acid peptide derived from the histocompatibility factor H2-Eα was administered, and then the specific Y-Ae antibody was used to detect the presented I-Aβ-Eα52-68 peptide-MHCII complex.21 We demonstrated that Tsc1-deficient DCs had increased antigen processing (Figure 6a). Previous studies showed that autophagy was an important source of antigens for CD4+ T cells,22 and mTORC1 inhibited autophagy.23 In our study, the autophagy was comparable between WT and Tsc1-deficient DCs and could be further promoted by rapamycin, which suggest that other pathways are involved in the autophagy in DCs (Figure 6b). In addition, the expression of migration-associated molecules chemokine (C-C motif) receptor 3 (CCR3), CCR5, CCR6 and CCR7 were comparable between WT DCs and Tsc1-deficient DCs (Figure 6c). Thus, the impaired function of Tsc1-deficient DCs could not be attributed to impaired antigen processing, autophagy or migration.

We detected a considerably higher rate of apoptosis in ex vivo Tsc1-deficient DCs than in WT DCs (Figure 6d), although the proliferation was enhanced (Figure 6e), suggesting that increased apoptosis was the causative reason for the loss of DCs in vivo. In the DC-OT-II T cell co-culture system, the apoptosis of Tsc1-deficient DCs was remarkably higher than that of the WT DCs (Figure 6f), suggesting that insufficient DCs would be one of the reasons for impaired antigen-specific T-cell responses. The magnitude and quality of T-cell response is directly proportional to the number of antigen-pulsed DCs that arrived at the draining lymph nodes (LN).24 The defective induction of antigen-specific T-cell responses by Tsc1-deficient DCs may be due to the decreased number of antigen-carrying DCs from the site of the challenge to the draining LNs. To test this, we used a model of fluorescein isothiocyanate (FITC) skin painting to track the transportation of DCs from the skin to the LNs. FITC-painted CD11cCreTsc1f/f mice contained less FITC+ DCs in the draining LNs at 48 h (Figure 6g). Furthermore, B6.SJL mice were injected i.v. with the same number of carboxylfluorescein diacetate succinimidyl ester (CFSE)-labeled WT or Tsc1-deficient DCs. After 3 days, we found less CFSE+ Tsc1-deficient DCs in the recipients’ spleen and LNs (Figure 6h). These results proved that the declined numbers of antigen-pulsed Tsc1-deficient DCs arriving at draining LNs led to impaired function of Tsc1-deficient DCs in inducing antigen-specific T-cell responses in vivo.
**Tsc1 inhibit DCs apoptosis via repressing mTORC1 and ROS-Bim pathways.** We further explored the molecular mechanisms by which Tsc1 prevents DCs from undergoing apoptosis. Partially reduced activity of mTORC1 can rectify the reduction in the DCs’ number and increased apoptosis of Tsc1-deficient DCs (Figures 7a and b). Administration of the mTORC1 suppressor RAPA or S6K inhibitor in vivo also partially restored cell survival (Figures 7c and d). These results indicate that Tsc1 promotes DC survival partly via mTORC1.

ROS contributes to apoptotic cell death.26 We found that the level of ROS was increased in Tsc1-deficient splenic DCs (Figure 8a), which is consistent with the reported role of Tsc1 on ROS production in BM-DCs.15 The ROS scavenger 8-acetylcysteine (NAC) alleviated the abnormal cell apoptosis (Figure 8b). NAPDH oxidases (NOXs) and mitochondria are major source of endogenous ROS,27 and we found that both the NOX inhibitor (apocynin) and mitochondria respiration inhibitor (decylubiquinone) can rescue the apoptosis (Figure 8c). These results suggest that increased ROS production from NOX system and mitochondria may promote the apoptosis of Tsc1-deficient DCs. Surprisingly, administration of the mTOR suppressor RAPA or the S6K inhibitor in vivo did not suppress ROS levels in Tsc1-deficient DCs (Figure 8d) but partially restored cell survival (Figures 7c and d), which were also confirmed in CD11c<sup>Cre</sup>/Tsc1<sup>f/f</sup>Raptor<sup>+/+</sup> conditional mice (Figure 8e). These results indicate that mTORC1 signaling does not regulate ROS production in Tsc1-deleted DCs. Intracellular staining of the Bcl family of proteins revealed that the levels of pro-apoptotic protein Bim were elevated in Tsc1-deficient DCs, but the levels of antiapoptotic Bcl-xl and Bcl-2 were unaltered (Figure 8f). The ROS/JNK signaling pathway has been shown to upregulate Bim and lead to Bax-mediated apoptosis.28 Indeed, NAC reduced ROS production (Figure 8b) and Bim expression (Figure 8g), supporting the hypothesis that the ROS-Bim pathway is one of the major
cause of apoptosis in Tsc1-deficient DCs. Taken together, Tsc1 promotes DC survival via the independently repression of the mTORC1 and ROS-Bim pathways.

Discussion

The molecular mechanisms by which DCs regulate T-cell homeostasis in steady-state conditions and antigen-specific T-cell response remain incompletely understood. Recent publications demonstrate that Tsc1 regulates DC development and activation through mTORC1/Myc-dependent and -independent signals. In contrast, our study highlights the role of Tsc1 in mature DCs that maintains naive T-cell quiescence in steady-state conditions but promotes antigen-specific T-cell responses.

In this paper, we demonstrated for the first time that Tsc1-deficient DCs resulted in the development of lymphoproliferative disorder, disturbed serum immune globulin and limited weight gain in mice, which indicates the role of Tsc1 in DCs in preserving immune tolerance. Selective loss of Tsc1 in DCs perturbs T-cell homeostasis and causes spontaneous activation of naive T cells, which were not observed previously. Proinflammatory cytokines promote naive T-cell proliferation. Pan et al. indicated that Tsc1 deficiency increased the expression of TNF and IL-6 in BM-derived DC, which was not detected in splenic DCs in our study. Nrp1 expression by DCs has been reported to be essential for activation of naive T cells and thus the initiation of primary immune responses. Meanwhile, the expression of co-stimulatory molecules CD40, CD80 and CD86 was not significantly altered in splenic DCs, which was increased in BM-derived DCs. We found that Tsc1 deficiency led to mTORC1-PPARγ-dependent upregulation of the expression of Nrp1 on DCs, which led to the aberrant proliferation of naive T cells. Therefore, in steady-state conditions Tsc1 functions as a tolerant factor repressing Nrp1 expression by DCs, thus avoiding aberrant immune

Figure 5 DCs need Tsc1 to promote antigen-specific T-cell responses. (a) Proliferation of CFSE-labeled purified OT-II/OT-I T cells, after being co-cultured with splenic DCs for 72 h, which were treated with OVA323-339/OVA257-264 for 2 h before co-culture. (b) CFSE-labeled OT-II/OT-I cells (CD45.1) were i.v. injected into WT or CD11c<sup>cre</sup>Tsc<sup>fl</sup> mice (CD45.2). After 1 day, recipient mice were challenged by subcutaneous injection of 10 μg OVA protein with 100 μl complete Freund’s adjuvant in each flank, and 3 days later, the spleen was removed and analyzed by flow cytometry. (c) The cell percentages and numbers of bronchoalveolar lavage fluid (BALF) in the asthma model (n = 6). (d) Concentration of serum OVA-IgG1 in the asthma model (n = 6). (e) Intracellular staining of cytokines in CD4<sup>+</sup>Foxp3<sup>-</sup> and CD8<sup>+</sup>Foxp3<sup>-</sup> T cells in the lung and BALF from WT and CD11c<sup>cre</sup>Tsc<sup>fl</sup> mice, stimulated with phorbol myristate acetate and ionomycin for 4 h. All mice analyzed were 6 week old. *P < 0.05, **P < 0.01; error bars represent S.D.; all data are representative of at least three independent experiments.
activation. Several studies have suggested that Tsc1 mutation in the patients with human tuberous sclerosis complex have caused immune activation, which might further contribute to the development of the disease. The spontaneous T-cell activation and lymphoproliferative disease in mice lacking Tsc1 in DCs implies that disruption of Tsc1-dependent DC function may be a mechanism underlying the immune activation phenotype in human tuberous sclerosis complex patients. Nevertheless, future studies are needed to address whether Tsc1 deficiency may cause susceptibility to autoimmune disorders or immunodeficiency disorders in tuberous sclerosis complex patients.

Despite their effect on promoting naive T-cell proliferation, Tsc1-deficient DCs failed to mount an efficient antigen-specific OT-I/OT-II T-cell immune response in vivo and in vitro. In contrast, Tsc1-deficient BM-derived DCs seemed to have a normal ability to activate MHC-I-restricted OT-I T cells in vitro. Efficient presentation of antigen to T cells results in activated antigen-specific T-cell response. Previous studies demonstrated that autophagy was an important
source of antigens for CD4+ T cells and mTORC1 function as a transcriptional regulator of autophagy. Here we showed that the ability of antigen presentation was elevated and the autophagy was unchanged in Tsc1-deficient DCs, ruling out their roles in impaired antigen-specific T-cell immune response. Although mTORC1 signaling was activated in Tsc1-deficient DCs, the autophagy was unaltered, indicating that other signaling pathways regulate the autophagy in DCs. Distinct from the previous study with in vitro-cultured BM-derived DCs, Tsc1-deficient splenic DCs did not display significantly decreased expression of IRF4/CiITA/MHC II and derived DCs, Tsc1-deficient splenic DCs did not display in vitro Tsc1-deficient DCs, the autophagy was unaltered, indicating that the ability of antigen presentation was elevated and the function of DCs, ensuring normal immune homeostasis and function.

Thus the immune system utilizes the Tsc1 enzyme to restrain mTORC1 activity and to preserve survival and function of DCs, ensuring normal immune homeostasis and function.

Materials and Methods

Mice. All animals were maintained in specific pathogen-free barrier facilities and were used in accordance with protocols approved by the Institutional Animal Care and User Committee at the Institute of Hematology, Chinese Academy of Medical Sciences. C57BL/6 (CD45.2) and B6.SJL (CD45.1) were provided by the Animal Centre of the Institute of Hematology and Hospital of Blood Diseases, Chinese Academy of Medical Sciences and Peking Union Medical College (Tianjin, China). CD11ccre, Tsc1ff, Raptorff, OT-I and OT-II mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). All used mice have been backcrossed with C57BL/6 mice for at least five generations. Tsc1ff mice were crossed with CD11cCre transgenic mice to generate CD11cCreTsc1ff and Tsc1ff mice (WT mice). CD11cCreTsc1ff mice were crossed with Raptorf mice to generate CD11cCreTsc1ffRaptorff mice.

Antibodies and reagents. The following antibodies for flow cytometry were from BD Biosciences (Heidelberg, Germany), eBioscience (San Diego, CA, USA); PerCP-cy5.5-anti-CD4, PerCP-cy5.5-anti-IFN-γ, PerCP-cy5.5-anti-MHC II, Alexa Fluor 700-anti-CD44, PE-anti-CD8, PE-anti-CD209, PE-anti-Nrp1, PE-anti-B7-DC, PE-anti-B7-H1, PE-anti-B7-H2, PE-anti-B7-H3, PE-anti-Ox40L, PE-anti-Bim, PE-anti-Bcl-xL, PE-anti-Bcl2, PE-anti-Foxp3, PE-anti-CCR3, PE-anti-CCR5, PE-cy7-anti-MHC II, PE-cy7-anti-CCR6, PE-cy7-anti-CCR7, FITC-anti-IL-4, APC-anti-CD11c, APC-anti-CD19, APC-anti-F4/80, APC-anti-IL-5, APC-anti-IL-17, APC-anti-CD45.1, APC-anti-CD62L, APC-cy7-anti-CD45.2, APC-cy7-anti-Ly6c, FITC-anti-Ly6g, FITC-anti-Y-Ae, FITC-anti-Annexin V, FITC-anti-K67, PE-cy7-anti-CD40, PE-cy7-anti-80, and PE-cy7-anti-CD86. Intracellular staining antibodies of phospho-Erk1/2 (Thr 202/Tyr 204), phospho-Akt (Ser 473), phospho-JNK (Tyr 185), phospho-p38 MAPK (Thr 180/Tyr182), phospho-NF-kB p65 (Ser 536) and phospho-S6K (Ser 235/Ser 236) were obtained from Cell Signaling Technology (Beverly, MA, USA). Propidium iodide.
solution (PI) and NAC were obtained from Sigma-Aldrich (St. Louis, MO, USA). RAPA, apocynin, decylubiquinone, mTORC1 inhibitor everolimus, PPARγ inhibitor T0070907, STAT3 inhibitor S3I-201, HIF inhibitor 2-methoxyestradiol, ERK inhibitor FR 182024, JNK inhibitor IX and S6K inhibitor PF-4708671 were obtained from Selleck (Houston, TX, USA).

**Cell purification, flow cytometry and intracellular staining.** Single-cell suspensions were prepared from spleen and peripheral LNs for staining or cell purification. CD11c+ DCs were purified with CD11c MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). CD44+CD8α+ T cells were purified from the spleen and LNs of OT-II/OT-I transgenic mice by Dynabeads Untouched Mouse CD4/CD8 Cells Kits (Invitrogen). Cell sorting, flow cytometry and intracellular staining were performed as described.35 Flow cytometric data were acquired with LSR II (BD Biosciences). Surface antigens on cells were stained, cells were fixed with 2.0% formalin and permeabilized with methanol and phosphorylated proteins in cells were stained and permeabilized with methanol and phosphorylated proteins in cells were stained for 1 h. ROS production was determined by CM-H2DCFDA (C-6827, Thermo Fisher Scientific, Billerica, MA, USA).

**Immunoblot analysis.** Sorted DCs were lysed and SDS-PAGE was performed as described.35 Antibodies to PPARγ, Bim and Actin were from Cell Signaling Technology.

**Enzyme-linked immunosorbent assay (ELISA).** Concentrations of secreted cytokines, serum immunoglobulins and auto-reactive antibodies were measured by commercial ELISA kit according to the manufacturer’s instruction. Purified WT or Tsc1-deficient DCs (1 x 10^6) were plated into each well of 48-well plates and treated with LPS (10 ng/ml) for 24 h. The culture supernatants were harvested to detect IL-1α, IL-6, IL-12a p40, IL-12a p70, TNF-α, IL-4, IL-5 and TGF-β1 cytokines (R&D Systems, Minneapolis, MN, USA) or auto-reactive antibodies TSHR, ISR, ANA, ssDNA and C1q-A band UACA IgG (Mibio, Shanghai, China). Measurements were made at 405 nm with Synergy 2 microplate reader (BioTek, Winooski, VT, USA).
In vitro DC-mediated antigen-independent naïve T-cell proliferation.

Purified CD4-CD44-CD62L- naïve T cells were labeled with CFSE (2 μM; Thermo Fisher Scientific) for 5 min at 37 °C and then washed twice with phosphate-buffered saline (PBS). In all, 5 × 10^5 WT or Tsc1-deficient DCs were co-cultured with 1 × 10^6 CFSE-stained T cells for 3 days in which the physiological T-cell survival factor IL-7 (100 ng/ml, PropeTec, Rocky Hill, NJ, USA) was added. CFSE profiles were examined by flow cytometry after culturing.

In vitro and in vivo DC-mediated antigen-specific T-cell proliferation.

Purified OVA/WT T cells were labeled with CFSE (2 μM) for 5 min at 37 °C and then washed twice with PBS. For in vitro studies, 3 × 10^5 T cells were transferred into WT or CD11c−/− Tsc1−/− mice by tail vein injection. After 1 day, mice were challenged by subcutaneous injection of 5 μg OVA protein (Sigma-Aldrich) with 100 μl complete Freund’s adjuvant in each flank. After 3 days, inguinal LNs were removed and analyzed by flow cytometry. For in vivo studies, 5 × 10^5 WT or Tsc1-deficient DCs were loaded with OVA263-309 or OVA257-266 (2 μg/ml, Sigma-Aldrich) overnight. Purified OVA/WT T cells (1 × 10^5) were labeled with CFSE and co-cultured with DCs in a 96-well plate for 3 days. CFSE profiles were examined by flow cytometry after culturing.

Asthma model.

Mice were sensitized as previously described. Six-to-eight-week-old mice were injected intraperitoneally on days 0, 7 and 14 with 50 μg OVA protein (Sigma-Aldrich) and 10 μg OVA/MHC class II complex formation assay. In all, 0, 25, or 100 μg/ml peptide (RLEEFAKFASFEAQGALANIAVDKANLDVMKKR) was added. CFSE profiles were examined by flow cytometry after culturing.

FITC painting.

The skin of the scapular region of WT and CD11c−/− Tsc1−/− mice was painted with 20 μl of 1% FITC in carrier solution (acetone: dibutyl phthalate, 1:1). After 48 h, draining LNs were collected, stained and analyzed by flow cytometry.

In vivo antigen transportation and presentation.

Purified WT or Tsc1-deficient DCs (1 × 10^5) were labeled with CFSE and injected i.v. into recipient B6. SJL (CD45.1) mice. After 3 days, splenocytes and LN cells from the recipients were stained and analyzed.

Statistics.

An unpaired two-tailed Student’s t-test (for two-group comparisons) or a two-way ANOVA (for more than two-group comparisons) was performed by Prism (GraphPad, San Diego, CA, USA) to calculate the statistical significance. P-values of < 0.05 were considered significant.

Conflict of Interest

The authors declare no conflict of interest.
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